

Augmenting Pulmonary Rehabilitation in Chronic Obstructive Pulmonary Disease: Studies of ACE-inhibition and Nitrate Supplementation

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Declaration of originality

The data present in this thesis are the result of my original work. Where appropriate the contribution made by other persons has been appropriately acknowledged.

Statement of contribution

Contributions were made by Kawah Li and Professor Hugh Montgomery at University College London, who performed the ACE genotyping. In addition, Magda Minnion and Professor Martin Feelisch at the University of Southampton performed the measurement of the plasma nitrate and nitrite levels. Juliet Polkey provided assistance with the isotime data analysis in the nitrate supplementation work. Victoria Meyrick and Bhavin Mehta led the pulmonary rehabilitation programme at the Royal Brompton Hospital, and the team at Harefield Hospital are also acknowledged for their assistance in this respect.

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Abstract

This thesis addresses two approaches to enhancing exercise capacity in chronic obstructive pulmonary disease (COPD). The first is by manipulation of the renin-angiotensin system through angiotensin-converting enzyme (ACE) inhibition to establish if this can augment the response to pulmonary rehabilitation. The second is nitrate supplementation, assessed for its effects on endurance exercise parameters.

In a cross-sectional study of 78 patients with at least moderate severity COPD I found no association between ACE genotype and exercise parameters during incremental cycle ergometry, in contrast to previous work from a Chinese group. The role of the renin-angiotensin pathway in skeletal muscle impairment in COPD is likely to be highly complex, and there are both potential beneficial and adverse effects of angiotensin II, and potentially conflicting effects on strength and endurance exercise capacity. The absence of differences in a cross-sectional study do not preclude the possibility that the ACE genotype may influence the response to both training and detraining through physical inactivity, and this remains an area of possible future research.

I went on to test the effect of the ACE-inhibitor enalapril on the response to pulmonary rehabilitation, focussing on the change in peak exercise capacity. I undertook a double-blind randomised controlled trial of 80 COPD patients with at least moderate airflow obstruction referred for pulmonary rehabilitation. There was evidence of adequate suppression of ACE activity through both the suppression of serum ACE levels and alteration in blood pressure parameters in the enalapril treated arm. Contrary to expectations the peak power achieved on incremental cycle ergometry increased more in the placebo arm of the study than the ACE-inhibitor treated arm. No significant differences were noted in computed tomography measures of muscle bulk, quadriceps strength or health-related quality of life. Thus, in subjects without a pre-existing clinical indication for ACE-inhibition, use of the ACE-inhibitor enalapril reduced the response to exercise training in COPD.

I also conducted a pilot study to investigate the role of acute nitrate supplementation on endurance exercise characteristics and oxygen consumption during endurance exercise in COPD. I recruited 25 subjects into a double-blind, placebo-controlled, single-dose cross-over study. Nitrate supplementation, in the form of beetroot juice at a dose of 12.9 mmol, significantly lowered resting diastolic blood pressure and isotime pulmonary oxygen consumption. This did not translate

into an improvement in endurance time. This preliminary work will provide the basis for further studies, including the potential role of nitrate supplementation in enhancing the response to pulmonary rehabilitation and the role of nitrate supplementation on exercise capacity in individuals with exercise induced hypoxaemia.

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List of Abbreviations

ACE	Angiotensin converting enzyme
ACE-I	Angiotensin converting enzyme inhibitor
ADP	Adenosine diphosphate
AKT	Protein kinase B
ANT	Adenine nucleotide translocase
AtI	Angiotensin I
AtII	Angiotensin II
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
ATP	Adenosine triphosphate
AUC	Area under the curve
BF	Breathing frequency
BK	Bradykinin
BK2R	Bradykinin type 2 receptor
BMI	Body mass index
BNP	Brain natriuretic peptide
BP	Blood pressure
BPF	Bradykinin-potentiating factor
CAT	COPD assessment test
cGMP	Cyclic guanosine monophosphate
cGTP	Cyclic guanosine triphosphate
CO	Carbon monoxide
COPD	Chronic obstructive pulmonary disease
CPEX	Cardiopulmonary exercise test
CRP	C-reactive protein
CSA	Cross-sectional area
CT	Computed tomography
D	ACE deletion allele
dbP	Diastolic blood pressure
DEXA	Dual energy X-ray absorptiometry
eGFR	Estimated glomerular filtration rate
ECSC	European coal and steel community
FEV ₁	Forced expiratory volume in 1 second

FFM	Fat free mass
FFMI	Fat free mass index
FM	Fat mass
FoxO	Forkhead box O
FVC	Forced vital capacity
GDF	Growth differentiation factor
GFR	Glomerular filtration rate
GH	Growth hormone
GLUT4	Glucose transporter 4
GOLD	Global initiative for chronic obstructive lung disease
HPLC	High performance liquid chromatography
HR	Heart rate
I	ACE insertion allele
IC	Inspiratory capacity
ICS	Inhaled corticosteroid
IFN	Interferon
IGF	Insulin-like growth factor
IHD	Ischaemic heart disease
IL	Interleukin
IRS-1	Insulin receptor substrate-1
ISWT	Incremental shuttle walk test
LABA	Long-acting beta agonist
LAMA	Long-acting muscarinic antagonist
LTOT	Long term oxygen therapy
MAP	Mean arterial pressure
MHC	Myosin heavy chain
mRNA	Messenger ribonucleic acid
MRC	Medical Research Council
MRS	Magnetic resonance spectroscopy
MTMCSA	Mid-thigh muscle cross-sectional area
mTOR	Mammalian target of rapamycin
MuRF-1	Muscle ring finger protein-1
MyoD	Myogenic differentiation factor D
6MWD	Six minute walk distance

NAC	N-acetylcysteine
NADPH	Nicotinamide-adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NHS	National Health Service
NIRS	Near infra-red spectroscopy
NMES	Neuromuscular electrical stimulation
NO	Nitric oxide
NO ₂ ⁻	Nitrite
NO ₃ ⁻	Nitrate
NOS	Nitric oxide synthase
OUES	Oxygen uptake efficiency slope
PA	Physical activity
PaCO ₂	Partial pressure of carbon dioxide in arterialised blood gas
PAD	Peripheral arterial disease
PAL	Physical activity level
PaO ₂	Partial pressure of oxygen in arterialised blood gas
PCr	Phosphocreatine
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PFTs	Pulmonary function tests
PGC-1α	PPAR-γ coactivator-1α
Pi	Inorganic phosphate
PI3K	Phosphatidylinositol 3-kinase
PO	Power Output
PPAR	Peroxisome-proliferator-activated receptor
PR	Pulmonary rehabilitation
PUFA	Polyunsaturated fatty acids
QMVC	Quadriceps maximum voluntary contraction
RAS	Renin angiotensin system
ROS	Reactive oxygen species
RV	Residual volume
sBP	Systolic blood pressure
SERCA	Sarcoplasmic reticulum calcium-ATPase
SGRQ-C	St. George's Respiratory Questionnaire for COPD

TGF- β	Transforming growth factor- β
TLC	Total lung capacity
TLCO _c	Corrected transfer factor for carbon monoxide
TNF- α	Tumour necrosis factor- α
TwQ	Twitch quadriceps response
VCO ₂	Pulmonary carbon dioxide output
VE	Minute ventilation
VO ₂	Pulmonary oxygen uptake
VT	Tidal volume
WHO	World Health Organisation
WR	Work rate
XO	Xanthine oxidase

Keywords

COPD, ACE-inhibitor, pulmonary rehabilitation, nitrate, muscle, exercise

Chapter 1: Introduction

1.1 Background to COPD

Chronic obstructive pulmonary disease (COPD) is a respiratory disorder characterised by progressive airflow obstruction, which is not fully reversible, and parenchymal destruction of the lungs leading to breathlessness and limitation of daily activities (Devereux 2006). Emphysema was described eloquently in the 19th century by the French pathologist and clinician Laënnec in his *Treatise on the Diseases of the Chest and on Mediate Auscultation* (Laënnec 1821), where he documented the hyperinflation and air trapping that characterise this disease.

“In opening the chest, it is not unusual to find that the lungs do not collapse, but that they fill up the cavity completely on each side of the heart. When examined their cells appear full of air... The branches of the trachea are often at the same time a good deal filled with the mucous fluid.”

In his pathological description Laënnec alluded to the concurrent presence of emphysema and excessive mucus in the airways. With the advent of light microscopy emphysema was defined in histological terms as abnormal permanently dilated airspaces with alveolar destruction, and the term chronic bronchitis was introduced into clinical practice to describe patients with chronic cough accompanied by sputum production due to airway inflammation. The term COPD was first introduced in the 1960s by William Briscoe (Briscoe and Nash 1965) to draw together on these separate descriptions and define the spectrum of airways disease, chronic bronchitis and emphysema that lead to the state of irreversible airflow obstruction.

In individuals with a compatible history and the presence of appropriate symptoms, spirometric measures are most commonly used to confirm the diagnosis of COPD, with the forced expiratory volume in one second (FEV₁) expressed as a ratio of the forced vital capacity (FVC), less than 70% indicative of airflow obstruction (table 1.1) (Rabe, Hurd et al. 2007). The severity of airflow obstruction as indicated by the degree of reduction in the FEV₁ is then commonly expressed as the GOLD stage (table 1.1.).

Stage	Lung function criteria
Stage I: mild	FEV ₁ /FVC <0.70 FEV ₁ ≥80% predicted
Stage II: moderate	FEV ₁ /FVC <0.70 50% ≤ FEV ₁ <80% predicted
Stage III: severe	FEV ₁ /FVC <0.70 30% ≤ FEV ₁ <50% predicted
Stage IV: very severe	FEV ₁ /FVC <0.70 FEV ₁ <30% predicted <i>or</i> FEV ₁ <50% predicted plus chronic respiratory failure

Table 1.1. Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification of disease severity (Rabe, Hurd et al. 2007).

Abbreviations: FEV₁ - forced expiratory volume in 1 second; FVC – forced vital capacity.

The major risk factor for the development of COPD remains exposure to noxious chemicals by inhalation. In the developed world this occurs predominantly via tobacco consumption, although airborne pollutants and occupational exposure to noxious gases and particles are contributors. Some genetic traits may predispose to the development of COPD, the most well recognised being alpha 1-antitrypsin deficiency, where a lack of alpha 1-antitrypsin enzyme activity allows neutrophil elastase to disrupt the connective tissue of the lungs.

The WHO Global Burden of Disease Project predicted in 1997 that COPD would become the third leading cause of death and fifth leading cause of disability worldwide by 2020 (Murray and Lopez 1997), and data recently published in 2013 in fact already ranks COPD as the third leading cause of death worldwide (Lozano, Naghavi et al. 2013), indicating its status as a significant healthcare burden. Within the United Kingdom COPD is a common healthcare concern responsible for considerable morbidity and mortality despite optimal management strategies, and remains significantly underdiagnosed. Using model-based prevalence estimates the true prevalence of COPD in England alone is estimated to be 1.5 million persons (Nacul, Soljak et al. 2011) with direct National Health Service (NHS) costs of approximately £1 billion per year. Thus, as well as a global problem, COPD remains an important NHS priority.

1.2 Beyond the lungs: Extrapulmonary manifestations of COPD

COPD is well recognised to have effects beyond that on pulmonary structure and function and may be appropriately considered a multisystem disorder; notable extrapulmonary manifestations including cardiovascular disease, skeletal muscle dysfunction, osteoporosis and systemic inflammation (Bolton, Ionescu et al. 2004, Sin, Anthonisen et al. 2006, Barnes and Celli 2009, Maltais, Decramer et al. 2014). Reduced exercise capacity is a major consequence of COPD, which is often attributed to ventilatory limitation and the effect of dyspnoea. Whilst this is clearly a major limiting factor, it has been consistently shown that patients have variable exercise capacity despite similar values for airflow obstruction, as assessed by the forced expiratory volume in one second (FEV₁). This suggests that the loss of pulmonary function is not the sole factor causing exercise limitation (Celli, Cote et al. 2004). Consistent with this, patients themselves frequently report muscle fatigue as a limiting factor during exercise (Killian, Leblanc et al. 1992, Man, Soliman et al. 2003), often preceding the sense of breathlessness.

It is thus recognised that the FEV₁ alone is inadequate in reflecting all of the systemic manifestations in COPD and allowing a comprehensive patient assessment (Celli, Cote et al. 2004) although it remains a readily utilised parameter on which to assess disease severity. Other factors beyond lung function may also influence exercise performance, and measures of airflow obstruction alone do not relay all impairment of lung function, which includes both static and dynamic hyperinflation and reduced gas transfer. Composite measures such as the BODE index incorporate measures of airflow obstruction with body mass index (BMI), dyspnoea scoring and exercise capacity. These provide a more effective means to account for the systemic manifestations of COPD and to effectively stratify patients (Celli, Cote et al. 2004).

1.3 Skeletal muscle dysfunction in COPD

Skeletal muscle dysfunction is a well-recognised and common consequence noted in both early and late disease, as characterised by the degree of airflow obstruction (Seymour, Spruit et al. 2010). Involvement of the musculoskeletal system includes both reduced strength (Gosselink, Troosters et al. 1996) and endurance capacity (Allaire, Maltais et al. 2004, Swallow, Gosker et al. 2007); muscle

strength being a measure of capacity to maintain maximal force, and endurance being a measure of the ability to sustain mechanical output during a series of loaded contractions. Muscular strength is dependent on muscle mass, motor unit recruitment and the contractile velocity of muscle fibres, whereas endurance properties rely on oxygen delivery and utilisation within the musculature including fibre type, capillarity, mitochondrial function and oxidative capacity.

The skeletal muscle dysfunction seen in COPD particularly affects the musculature of the lower limbs which is primarily responsible for locomotion. The quadriceps, and in particular the *vastus lateralis*, has been a well characterised muscle group in this respect (Man, Hopkinson et al. 2005), being readily accessible to test both strength and endurance capacity, as well as amenable to biopsy to assess both structural and molecular changes. Alterations to the function of the skeletal muscle of the upper limbs in COPD has been an area of conflicting research findings. Some group have noted reduced function (Gosselink, Troosters et al. 2000, Franssen, Broekhuizen et al. 2005), but others have indicated that muscular function of the upper limbs is preserved (Bernard, LeBlanc et al. 1998, Gosselink, Troosters et al. 2000, Man, Soliman et al. 2003).

Some of the discrepant findings in the upper limb musculature may be explained by the fact that different muscle groups in the upper limb have been isolated and studied by various groups, as well as variation within the patient populations studied. Furthermore, to compensate for the increasing ventilatory requirements due to the underlying lung disease, accessory muscles of respiration are recruited. This includes proximal muscles of the upper limbs and may provide a training stimulus, meaning these muscles are less prone to disuse atrophy than the lower limb musculature. In line with these findings, in a group of COPD subjects with markedly reduced exercise capacity handgrip strength was relatively preserved, and both histological and metabolic characteristics noted to be altered in the lower limb musculature were not seen in studies of the deltoid muscle (Gea, Pasto et al. 2001). This alludes to the fact that both systemic and local factors are likely to influence skeletal muscle function in COPD.

Quadriceps weakness is of significant clinical interest in COPD as it is a common complication, occurring in approximately 30% of moderate to severe disease as classified by the degree of airflow obstruction. The mean reduction in quadriceps strength, as measured by isometric maximal volitional contraction, is noted to be approximately 30% (Bernard, LeBlanc et al. 1998, Man, Soliman

et al. 2003, Man, Hopkinson et al. 2005). It is important to note that quadriceps weakness is not simply an epiphenomenon but is itself independently associated with impaired health status (Shrikrishna and Hopkinson 2012), increased healthcare utilisation (Decramer, Gosselink et al. 1997) and mortality (Swallow, Reyes et al. 2007). In fact, quadriceps weakness predicts transplant-free survival in COPD, which is known to be poorly predicted by indices of airflow obstruction such as FEV₁ (figure 1.1) (Swallow, Reyes et al. 2007). The skeletal muscle thus provides an interesting target for intervention to improve disability in this patient population at a point where airflow obstruction may be irreversible.

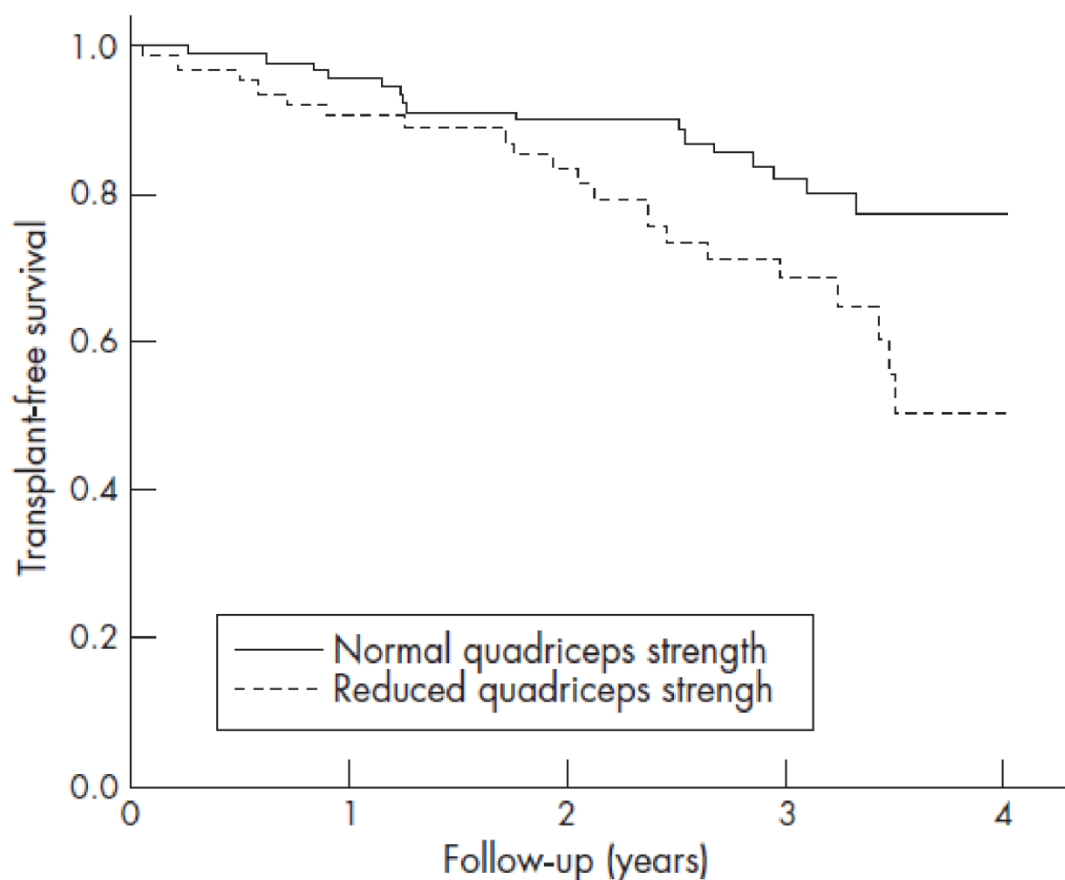


Figure 1.1: Transplant-free survival for patients with normal and reduced quadriceps strength, as assessed by maximal voluntary contraction >120% or <120% of the body mass index (BMI). The curves are significantly different; $p=0.017$ (Swallow, Reyes et al. 2007). Reproduced with permission.

1.3.1 Skeletal muscle structure and function

Skeletal muscle is responsible for voluntary motor function and is controlled by the somatic nervous system. It is composed of bundles of fascicles surrounded by a connective tissue sheath (the epimysium), which forms the tendons that anchor the muscle to its bony origin points. Each fascicle

is itself composed of bundles of myofibres surrounded by a layer of connective tissue known as the perimysium. In turn each myofibre is a large multinucleated cell encased in the sarcolemma, made up of individual myofibril units arranged in parallel. This creates the characteristic striated appearance of skeletal muscle, and it is the movement of these myofibrils that allows the generation of efficient contractile activity (figure 1.2).

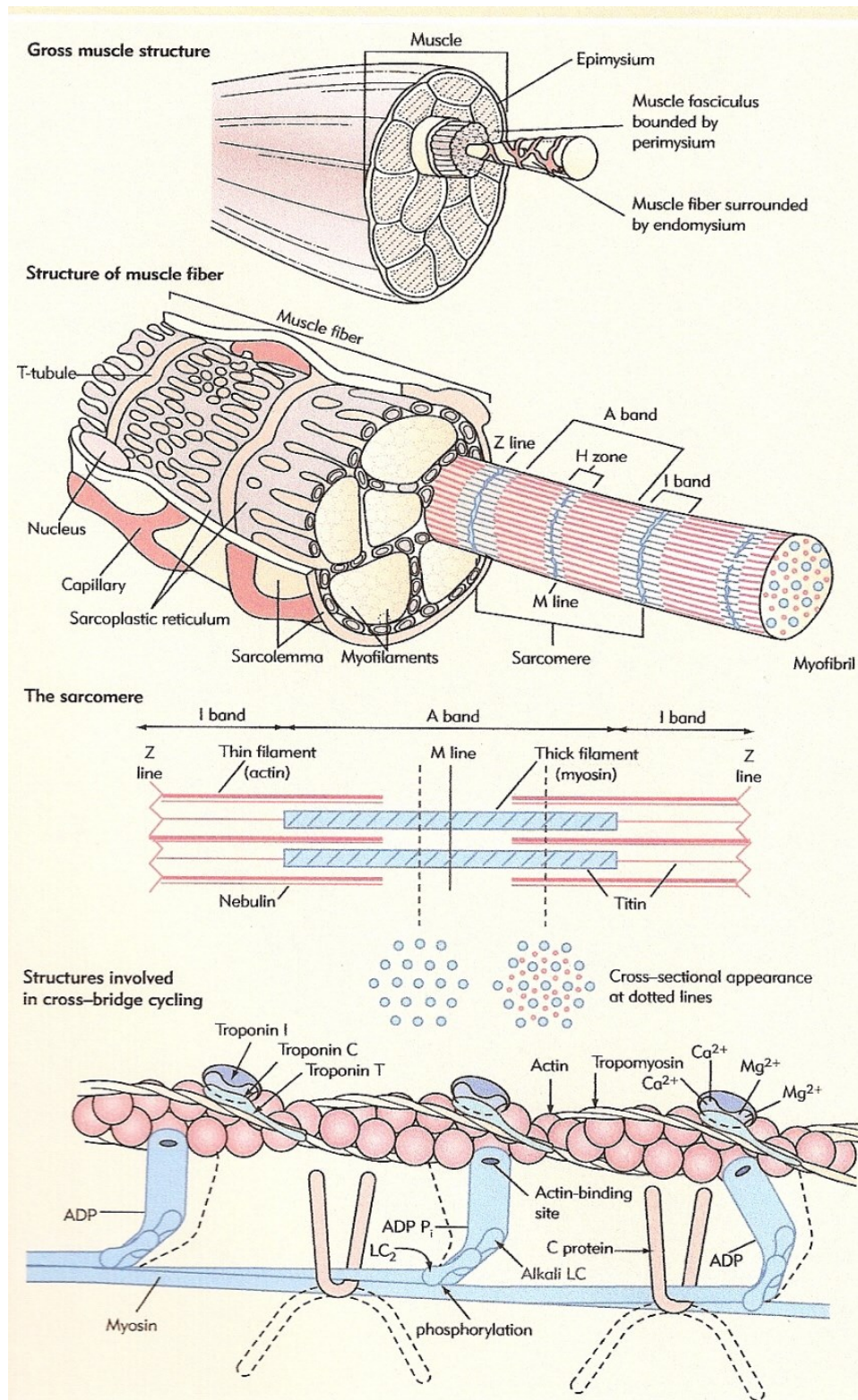


Figure 1.2: Structure of skeletal muscle. Taken from Hemmings and Hopkins (2006) with permission (Hemmings and Hopkins 2006).

Abbreviations: Ca^{2+} - calcium ion; Mg^{2+} - magnesium ion; ADP – adenine diphosphate.

The functional unit of the myofibril is the sarcomere made up of overlapping thick and thin filaments (figure 1.2), composed primarily of the proteins myosin and actin respectively. It is the interaction of these thick and thin filaments that is responsible for the shortening of myofibrils and thus coordinated contractile activity in the muscle. Muscle fibres are grouped into motor units, each innervated by a motor neurone. Neural activity in motor nerves in the form of action potentials is conducted down invaginations of the sarcolemma known as transverse tubules (T-tubules) to the sarcoplasm. This causes membrane depolarisation, which triggers the release of calcium ions from the sarcoplasmic reticulum where they are stored. This influx of calcium ions allows interaction between the actin and myosin filaments, eliciting contractile activity in the muscle.

Human skeletal muscle fibres are generally classified as type I (oxidative/slow) or type II (glycolytic/fast) dependent on the predominant myosin heavy chain (MHC) isoforms they contain. Type I fibres are rich in mitochondria, with high supplies of adenosine triphosphate (ATP), making them relatively fatigue resistant. Type IIx fibres have low levels of mitochondria and ATP, being more dependent on anaerobic metabolic pathways, and thus more susceptible to fatigue although able to generate more powerful and rapid contractions (Gollnick, Armstrong et al. 1972). Intermediate type IIa fibres utilise both oxidative and anaerobic metabolic pathways, and thus demonstrate mixed properties.

1.3.2 Skeletal muscle regulatory pathways

Skeletal muscle phenotype is dependent on a complex interplay between pathways of myogenesis, degradation and repair (figure 1.3). Much of our understanding of this field has come from animal models and through the study of other disease processes that affect skeletal muscle. In more recent years there has been an increasing body of evidence that sheds light on the potential molecular pathways implicated in COPD.

The growth hormone (GH)/insulin-like growth factor -1 (IGF-1) pathway is a key anabolic pathway in the control of muscle mass through muscle fibre repair and regeneration (Bark, McNurlan et al. 1998). Local IGF-1 is produced at the level of the muscle, controlled by the transcription factor myogenic differentiation factor D (MyoD). The production of IGF-1 occurs in response to growth hormone, testosterone and mechanical stretch, elicited by exercise. IGF-1 activates the

phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and once phosphorylated, AKT in turn activates the mammalian target of rapamycin (mTOR), leading to muscle anabolism (figure 1.3).

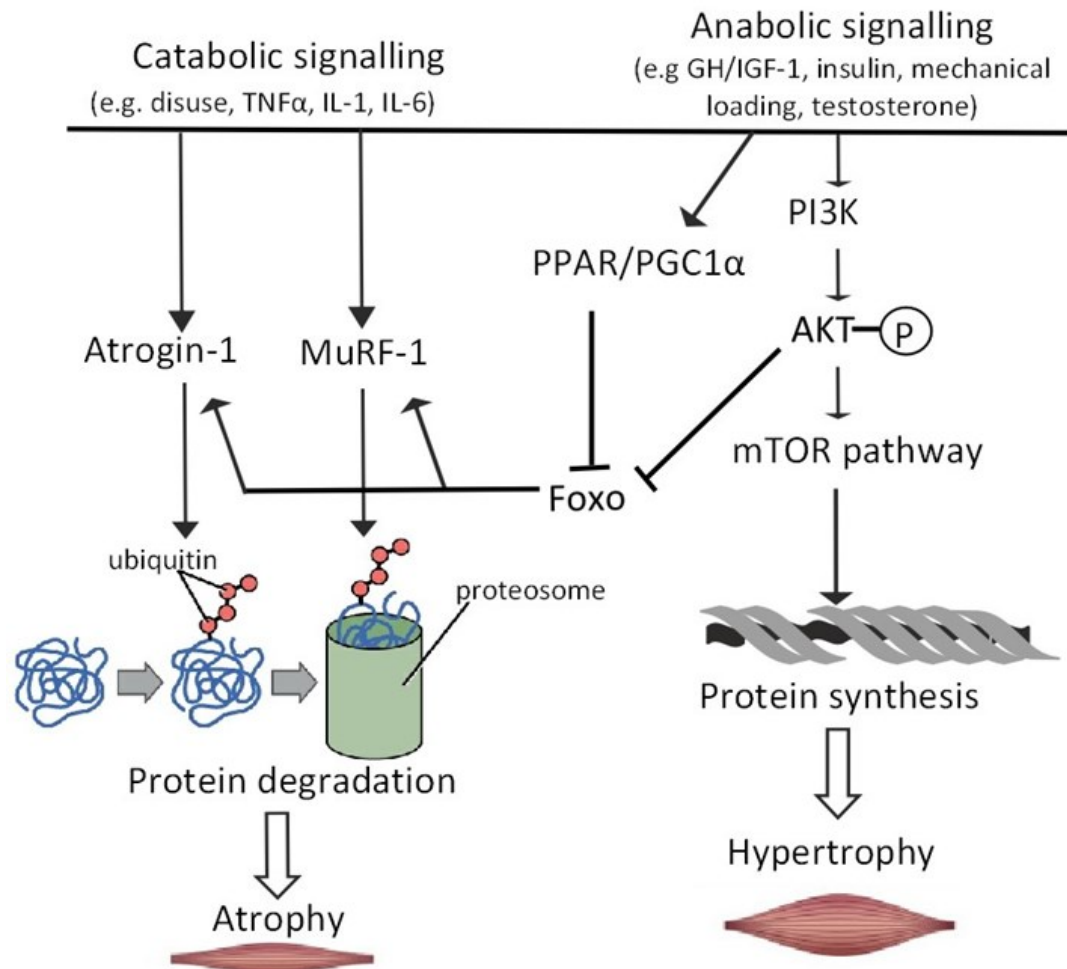


Figure 1.3: Schematic of muscle regulatory pathways.

⊥ Indicates an inhibitory pathway; → a stimulatory pathway.

Abbreviations: TNF - tumour necrosis factor; IL - interleukin; GH - growth hormone; IGF - insulin-like growth factor; MuRF-1 - muscle ring finger protein-1; PPAR - peroxisome proliferator activated receptor; PGC-1 α - peroxisome proliferator activated receptor gamma coactivator 1-alpha; PI3K - phosphatidylinositol 3-kinase; AKT - protein kinase B; mTOR - mammalian target of rapamycin; FoxO - forkhead box O.

Conversely the ubiquitin-proteasome pathway is a major route of protein degradation involved in muscle atrophy and catabolism (Lecker, Jagoe et al. 2004, Sandri, Sandri et al. 2004). Proteasomes are large multiunit complexes responsible for the degradation of intracellular proteins, marked by the attachment of molecules of ubiquitin via the action of the ubiquitin ligase enzyme family (figure 1.3). IGF-1 inactivates the FoxO (forkhead box O) transcription factors and inhibits the muscle-specific ubiquitin ligases, the atrogenes atrogin-1 and MuRF-1 (muscle ring finger protein-1), thus inhibiting muscle catabolism as well as promoting anabolism (Sandri, Lin et al. 2006).

The peroxisome proliferator activated receptors (PPARs) are a group of transcriptional cofactors which exists in three isoforms, α , β/δ and γ . PPAR- δ and PPAR- α isoforms are expressed in skeletal muscle along with PPAR gamma coactivator-1 α (PGC-1 α), a coactivator of PPAR transcriptional activity. These molecules are key to the regulation of muscle oxidative capacity (Luquet, Lopez-Soriano et al. 2003, Koves, Li et al. 2005, Arany 2008) through effects on fibre type, angiogenesis and mitochondrial biogenesis and activity. PGC-1 α expression and PPAR activity drive the formation of slow-twitch, oxidative muscle fibres (Lin, Wu et al. 2002) and promote resistance to atrophy through FoxO mediated pathways (Sandri, Lin et al. 2006) (figure 1.3).

1.3.3 Skeletal muscle atrophy

Weight loss is a common complication of COPD (Shoup, Dalsky et al. 1997) and was first documented by Fowler and Godlee in their seminal book Diseases of the Lungs (Fowler and Godlee 1898) where they made reference to the *“emaciated and withered appearance of the subjects of the disease”* and the consequences of this state, noting the *“capacity for exertion is limited owing to the feebleness of muscular power”*. Weight loss may include loss of fat free mass (FFM), fat mass (FM) or both, and this may be assessed by the use of bioimpedance techniques which provides greater detail on changes in body composition than through body weight alone.

Skeletal muscle, a key component of FFM, is commonly depleted in COPD (Hopkinson, Tennant et al. 2007). Furthermore, as well as bioimpedance indicating loss of FFM, radiological studies have demonstrated a reduction in the bulk of the locomotor muscles of the lower limb in COPD subjects (Bernard, LeBlanc et al. 1998, Seymour, Ward et al. 2009). Reduction in FFM is associated with reduced quadriceps strength (Bernard, LeBlanc et al. 1998, Hopkinson, Tennant et al. 2007), poor

exercise performance (Schols, Mostert et al. 1991, Gosselink, Troosters et al. 1996) and increased mortality (Schols, Broekhuizen et al. 2005). This state is seen most commonly in COPD patients with poor lung function and those who experience frequent pulmonary exacerbations (Hopkinson, Tennant et al. 2007) and is likely an important contributor to the mortality seen in these groups as well as a functional consequence of their disease burden.

In stable COPD patients reduction in FFM may be observed even in the presence of normal or even high body weight, and thus is not a state confined only to underweight patients (Schols, Soeters et al. 1993, Shoup, Dalsky et al. 1997, Mostert, Goris et al. 2000). When present, reduction in FFM is known to be associated with greater impairment in health-related quality of life and exercise capacity than loss of weight with preservation of FFM (Shoup, Dalsky et al. 1997, Mostert, Goris et al. 2000). Thus it is not the low body weight per se of these patients that contributes to poor outcomes, but the specific loss of muscle mass.

1.3.4 Structural changes in COPD

Measuring quadriceps strength and normalising for mid-thigh muscle cross-sectional area (MTMCSA) indicates that the quality of muscle contractile function is also altered, and thus weakness cannot just be accounted for solely by the loss of muscle bulk through atrophy (Seymour, Spruit et al. 2010). In fact quadriceps weakness has been demonstrated even in subjects who have preserved muscle mass (Franssen, Broekhuizen et al. 2005). It is thus possible that evidence of muscular weakness may precede the more obvious muscle atrophy and cachexia.

Histological studies in COPD have demonstrated a shift in skeletal muscle fibre type towards fast twitch fibres, which utilise anaerobic pathways of respiration (Jakobsson, Jorfeldt et al. 1995, Gosker, Zeegers et al. 2007) and reduced oxidative type I muscle fibres in the quadriceps muscle (Jakobsson, Jorfeldt et al. 1990, Maltais, Sullivan et al. 1999, Gosker, van Mameren et al. 2002, Couillard, Maltais et al. 2003). As well as alterations in skeletal muscle architecture, oxidative enzyme capacity is reduced (Jakobsson, Jorfeldt et al. 1995, Maltais, Simard et al. 1996). This provides contrast to the usual shift towards type I fibres, with greater oxidative capacity, as occurs during normal healthy human aging.

This loss of type I fibres has been positively correlated with increasing degree of airflow obstruction, indicating that it is associated with worsening measures of disease status (Gosker, Zeegers et al. 2007) (figure 1.4). Consistent with this fibre shift in the *vastus lateralis* has been shown to be a predictor of mortality from a retrospective analysis of a cohort of 392 COPD patients from four separate international sites (Patel, Natanek et al. 2014). In addition to loss of type I fibres there is an increase in the proportion of type IIx fibres (Gosker, van Mameren et al. 2002) which favour anaerobic respiration. Intermediary type IIa fibres, capable of both aerobic and anaerobic activity, also show reduced oxidative capacity in COPD. Thus there is an overall shift away from oxidative metabolism towards a muscle phenotype favouring early anaerobic metabolism.

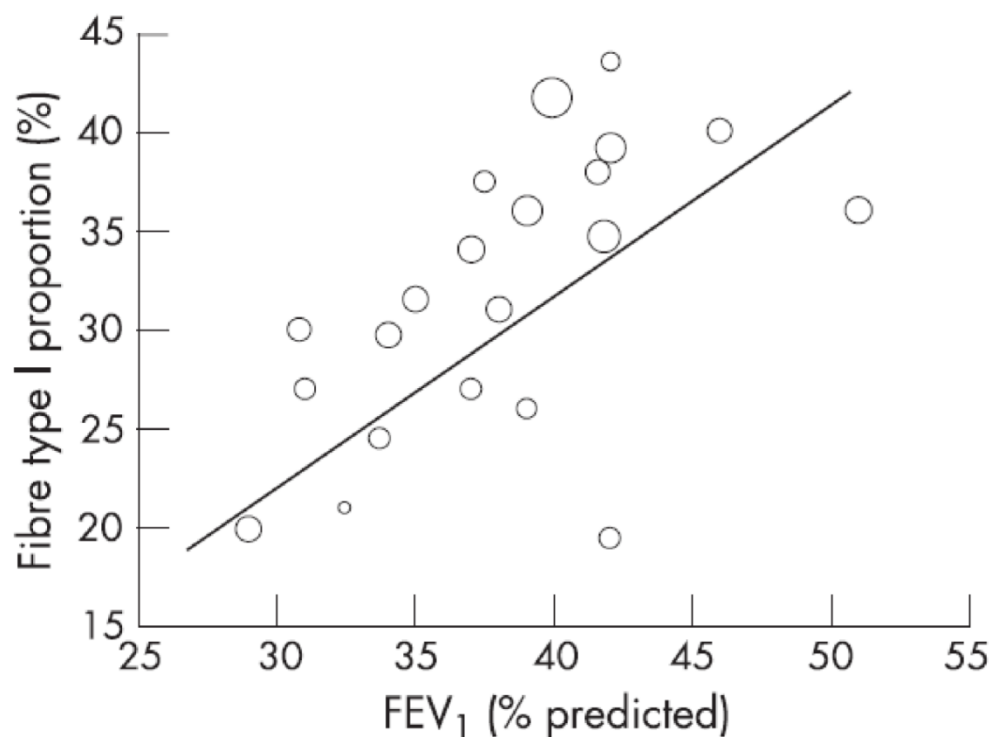


Figure 1.4: The relationship between *vastus lateralis* type I fibre proportion and FEV₁ collated and assessed in a meta-analysis. The size of the circle represents the cohort size of each individual study. Taken from Gosker et al. with permission (Gosker, Zeegers et al. 2007).

Whilst this may appear straightforward, there is marked heterogeneity in the changes in skeletal muscle structure seen in COPD and individual responses may not align to that seen within a group or cohort. It is thus important not to dismiss individual responses that may deviate from that seen within a larger body of data. In line with this, a large cohort study from our own group (studying 114 COPD patients of GOLD stage I-IV) has recently published data indicating isolated fibre type shift in 31%, isolated fibre atrophy in 20% and a combination of the two processes in 25% of the cohort studied (Natanek, Gosker et al. 2013). It also remains challenging to predict from physiological measures of skeletal muscle function alone which individuals will have ultrastructural alterations in the skeletal muscle and may thus benefit from targeted therapy (Natanek, Gosker et al. 2013).

Beyond changes in muscle fibre type, capillarity has been shown to be reduced to all fibre types (Jobin, Maltais et al. 1998) further impeding skeletal muscle function. Furthermore, at a mitochondrial level both the density of mitochondria (Gosker, Hesselink et al. 2007) and individual mitochondrial function (Rabinovich, Bastos et al. 2007) may be impaired in COPD subjects.

In line with these changes it is rather unsurprising that during exercise COPD patients experience functional consequences, with increased muscle lactate production and oxidative stress (Maltais, Simard et al. 1996, Rabinovich, Bastos et al. 2007). This contributes to early muscle fatigue and reduced endurance capacity (Man, Kemp et al. 2009). The development of systemic acidosis in response to muscle lactate production and hypercapnia during exercise may perpetuate further muscle dysfunction through inhibition of mitochondrial activity, thus further reducing oxidative metabolic capacity in a positive feedback manner (Vohwinkel, Lecuona et al. 2011). In addition, mechanisms to clear lactic acid rely on increased ventilatory rate to clear accumulated carbon dioxide. This is clearly more challenging in patients who are already compromised in terms of their respiratory function and experience ventilatory limitation during exertion. Of note the use of non-invasive ventilation during exercise is able to improve blood gases and diminish lactatemia (Polkey, Hawkins et al. 2000).

1.4 Aetiology of skeletal muscle dysfunction in COPD

Skeletal muscle dysfunction in COPD is a multifactorial process with contributions from multiple factors including disuse atrophy, systemic inflammation, oxidative and nitrosative stress, arterial

hypoxaemia, oral corticosteroid treatment and smoking (Choudhury, Rabinovich et al. 2014). The following narrative account provides information on the variety of factors that may contribute and is a personalised view of the data available and not a formal systematic review of the literature.

1.4.1 Aberrant muscle regulation

The balance of skeletal muscle atrophy-hypertrophy pathways in COPD is altered in favour of the promotion of atrophy. The ubiquitin-proteasome degradation pathway is reported to be upregulated in COPD patients, with increased expression of the atrogenes atrogin-1 and MuRF-1 in skeletal muscle (Doucet, Russell et al. 2007, Doucet, Dube et al. 2010). Rather surprisingly, these molecular markers of atrophy have not been shown to correlate to clinical measures of quadriceps function (Doucet, Russell et al. 2007). This provides some indication of the complexity of the processes occurring at the level of skeletal muscle and the challenges of delineating the exact molecular processes involved. It should also be acknowledged that other laboratories, including our own, have not been able to reproduce those findings (Natanek, Riddoch-Contreras et al. 2013).

As well as promotion of atrophy pathways there is evidence of reduced activity in muscle hypertrophy pathways. IGF-1 mRNA, noted previously as a key regulator of muscle anabolism, has been demonstrated to be reduced in the quadriceps of both hospitalised and stable patients with COPD when compared to healthy control subjects (Crul, Spruit et al. 2007). Thus patients may not be able to maintain the normal hypertrophy pathways that preserve muscle bulk or upregulate them in response to a stimulus, such as disuse, which promotes atrophy. This lends the balance of muscle regulation in favour of atrophy.

Growth differentiation factor-8 (GDF-8) or myostatin, a member of the TGF- β (transforming growth factor- β) superfamily, has been proposed as a significant contributor to muscle wasting seen in COPD. Myostatin is thought to act via multiple mechanisms, including downregulation of myoblast proliferation via reduced expression of essential myogenes for protein synthesis including MyoD (McFarlane, Plummer et al. 2006). Myostatin also reduces AKT phosphorylation and thus upregulates FoxO activity, promoting the ubiquitin-proteasome pathway and muscle breakdown (Thomas, Langley et al. 2000, McFarlane, Plummer et al. 2006). mRNA expression of myostatin in the *vastus lateralis* of weak COPD patients is 3-fold higher than those of healthy control patients

(Plant, Brooks et al. 2010), and demonstrates an inverse relationship with quadriceps strength (Man, Natanek et al. 2010), suggesting a potential role in the abnormal skeletal muscle phenotype seen in this group.

In addition to effects on the balance of atrophy-hypertrophy pathways there are also effects on skeletal muscle oxidative capacity. COPD patients have reduced levels of PGC-1 α and peroxisome proliferator activated receptors (PPARs) in skeletal muscle when comparison is made to healthy control patients with a similar smoking status (Remels, Schrauwen et al. 2007). As activity of the PPARs is essential in promoting aerobic metabolism, this change is likely to contribute to early muscle fatigue, although thus far these molecular changes have not been correlated to objective measures of muscle function.

1.4.2 Physical inactivity

For patients with ventilatory limitation exertional dyspnoea is a common occurrence, and this may discourage further exertion (O'Donnell 2006). Physical inactivity is a common feature of COPD (Watz, Waschki et al. 2009), and of great importance, having been the focus of a recent statement from a European Respiratory Society Task Force (Watz, Pitta et al. 2014). It is well recognised that, as opposed to exercise performance, habitual physical activity is influenced by many characteristics beyond physiological limitation, including behavioural, sociodemographic, environmental and cultural influences.

A large international study, including sites in both Europe and North America, demonstrated a significant reduction in physical activity in those with at least moderate disease (Troosters, Sciurba et al. 2010), and further studies have even demonstrated reduced physical activity in those with only early stage disease and mild airflow obstruction, when compared to healthy subjects (Shrikrishna, Patel et al. 2012). With increasing severity of COPD patients both spend less time engaged in physical activity and have fewer bouts of moderate or vigorous physical activity (Donaire-Gonzalez, Gimeno-Santos et al. 2013). Physical inactivity has been independently associated with evidence of quadriceps wasting suggestive of a possible mechanistic link between these two processes (Shrikrishna, Patel et al. 2012). As physical inactivity likely promotes skeletal muscle atrophy and

dysfunction, this is likely to perpetuate further inactivity and contribute to the commonly described 'spiral of decline' seen in this disease state (Jones 2009).

In addition to a more sedentary lifestyle during periods of disease stability, COPD patients often experience frequent acute pulmonary exacerbations, for which they may require hospitalisation. Both pulmonary exacerbations and hospitalisation are important recognised causes of further inactivity (Pitta, Troosters et al. 2006), and in turn low levels of physical activity are associated with both exacerbations and mortality (Gimeno-Santos, Frei et al. 2014). In fact, patients admitted for acute exacerbations show evidence of reduced quadriceps force and quadriceps muscle wasting even after only one week from the point of admission (Spruit, Gosselink et al. 2003, Pitta, Troosters et al. 2006). It is important to remember that this is not a problem that resolves itself at discharge, since even one month after discharge physical activity levels may not have returned to that noted in a stable COPD population (Pitta, Troosters et al. 2006). There is thus often a step-wise decline in physical activity promoted by these episodes, and many patients experience several exacerbations per year, making this an important cause of reduced physical activity.

Physical inactivity may significantly contribute to the extrapulmonary manifestations of COPD, and may thus be both a cause and consequence of skeletal muscle dysfunction. In part this may be through the promotion of oxidative stress and pro-inflammatory pathways as occurs during physical inactivity (Hopkinson and Polkey 2010). Additionally, inactivity has important consequences on the molecular pathways that govern muscle bulk and function, activating the ubiquitin-proteasome degradation pathway (Taillandier, Aurousseau et al. 1996, Reid 2005, Zhang, Chen et al. 2007) and reducing activity in hypertrophy pathways, with reduced IGF-1 and MyoD levels (Crul, Spruit et al. 2007). This promotes muscle atrophy over hypertrophy and may lead to further loss of muscle bulk, in turn making locomotion more challenging.

1.4.3 Systemic inflammation

COPD is a chronic inflammatory disorder of the airways but is also associated with systemic inflammation (Cazzola, Matera et al. 2007). It remains unclear as to whether this is related to the spillover of inflammatory mediators from the lung or a 'chronic systemic inflammatory syndrome' (Fabbri and Rabe 2007). This process of systemic inflammation is proposed as a contributor to the

extrapulmonary complications of COPD, including skeletal muscle dysfunction (Cazzola, Matera et al. 2007).

Higher systemic levels of circulating leucocytes (Gan, Man et al. 2004, Moermans, Heinen et al. 2011), C-reactive protein (CRP) (Spruit, Gosselink et al. 2003, Gan, Man et al. 2004), fibrinogen (Gan, Man et al. 2004), tumour necrosis factor- α (TNF α) (Di Francia, Barbier et al. 1994, Rabinovich, Figueras et al. 2003, Gan, Man et al. 2004), interleukin-6 (IL-6) and interleukin-8 (IL-8) (Spruit, Gosselink et al. 2003) have all been reported in stable COPD patients, even accounting for confounding factors such as smoking status and inhaled corticosteroid use. Serum CRP itself demonstrates a correlation to several important physiological parameters including arterial oxygen tension, 6-minute walking distance (6MWD) and FEV₁ (Gan, Man et al. 2005, de Torres, Cordoba-Lanus et al. 2006). This provides support for the role of systemic inflammation in both COPD and its extrapulmonary complications.

Muscle wasting and reduced strength have been associated with circulating markers of inflammation (Schols, Buurman et al. 1996) but the role of inflammatory cytokines at the level of the skeletal muscle remains unclear. One study focussing on severe COPD patients compared 19 patients with a control group of 7 healthy subjects. *Vastus lateralis* levels of the pro-inflammatory cytokines IL-6, interferon- γ (IFN- γ) and transforming growth factor- β (TGF- β) showed no significant difference between the groups, and in fact TNF- α protein levels were lower in the patient group and positively correlated with quadriceps force, the opposite relationship to that which would be expected if this cytokine was implicated in the aetiology of quadriceps weakness (Barreiro, Schols et al. 2008). However, this has not always been reproducible, and further studies have demonstrated elevated TNF- α mRNA in the muscle of COPD patients (Remels, Gosker et al. 2010), associated with low BMI and muscle wasting clinically, suggestive that it is playing an important role in skeletal muscle dysfunction. Additionally, using an in vitro model of cultured myotubes, stimulation with TNF- α led to diminished oxidative capacity (Remels, Gosker et al. 2010). Thus the role of inflammatory mediators within the milieu of the skeletal muscle remains a topic of active debate and investigation.

1.4.4 Oxidative and nitrosative stress

Research has suggested that oxidant molecules may work directly at the level of the skeletal myofilaments to reduce force production and contribute to premature fatigue (Reid 2001). Additionally, in the long-term oxidants have been shown to alter gene expression in muscle (Jackson 1999). In normal healthy muscle such oxidant molecules are buffered by antioxidant pathways, avoiding detrimental effects on the muscle itself. However, in COPD antioxidant pathways are aberrant, thus oxidative and nitrosative stress leads to the generation of reactive muscle oxygen and nitrogen species which cannot be dealt with effectively and contribute to pathological changes in muscle. Such reactive oxygen and nitrogen species can adversely affect normal muscle force generation and promote muscle atrophy (Reid 2001, Barreiro, Gea et al. 2003, Barreiro, Rabinovich et al. 2009).

Increased oxidative stress occurs in skeletal muscle as a consequence of inactivity (Powers, Kavazis et al. 2005) and, as noted previously, physical inactivity is a common complication of COPD. It may be that physically inactive patients have less antioxidant reserve and thus experience oxidative stress during exercise. Beyond the effects of inactivity, patients with COPD have an exaggerated increase in systemic markers of oxidative stress in response to an exercise challenge (Couillard, Maltais et al. 2003, Koechlin, Couillard et al. 2004). With reduced antioxidant capacity in COPD, this leads to a state where perversely exercise can thus increase oxidative stress which may then adversely affect skeletal muscle function. There is an important noted correlation between such markers of oxidative stress and both reduced quadriceps strength and endurance capacity (Couillard, Maltais et al. 2003, Allaire, Maltais et al. 2004, Barreiro, Schols et al. 2008). Thus aberrant oxidative stress pathways may have important functional consequences, contributing to premature muscle fatigue.

1.4.5 Hypoxia

Systemic hypoxia is a common finding in COPD due to the inherent parenchymal destruction noted in this disease, and impairment of normal ventilation-perfusion matching that allows efficient oxygenation of the circulating blood volume. Hypoxia is known to drive inflammatory pathways leading to inflammatory cytokine production by macrophages, including activation of the TNF- α system. This has been shown to be a particular problem in malnourished patients (Takabatake, Nakamura et al. 2000). In part then tissue hypoxia may promote systemic inflammation and thus affect skeletal muscle function.

1.4.5 Corticosteroid usage

Steroid-induced myopathy is a well-recognised complication of long-term therapy with corticosteroids (Dekhuijzen and Decramer 1992). An association between average daily dose of steroids and reduced quadriceps strength has been demonstrated in COPD, and was found to relate to generalised muscle fibre atrophy on biopsy appearances (Decramer, de Bock et al. 1996), although this association has not been replicated in other cohorts (Hopkinson, Nickol et al. 2004). A significant confounder in this situation is the use of oral corticosteroid therapy during acute exacerbations of the disease, meaning the ability to tease out the individual effects of the exacerbation itself and the effect of corticosteroid therapy is a more challenging problem to delineate. A study of short-term effects of oral prednisolone, in a stable COPD cohort with moderate to severe disease, did not show any alteration in quadriceps strength, whether assessed by volitional or non-volitional techniques (Hopkinson, Man et al. 2004). Thus short periods of corticosteroid use may not lead to deleterious effects on skeletal muscle as opposed to more long-term usage.

1.4.6 Smoking

Chronic tobacco smoke inhalation is likely to be an important driver of the skeletal muscle dysfunction seen in COPD, and certainly smoking cessation has been associated with a significant increase in fat free mass in a prospective study (Hopkinson, Tennant et al. 2007). In rodent models, smoking leads to reduced skeletal muscle fibre cross-sectional area and reduced type I fibre proportions (Nakatani, Nakashima et al. 2002), lending further support to this hypothesis.

At a molecular level, in a smoking mouse model, serum TNF- α was increased by cigarette consumption and such smoke-exposed mice also showed deregulation of PGC-1 α and upregulation of atrogin-1 and MuRF-1 mRNA levels (Tang, Wagner et al. 2010). Thus smoke inhalation drives factors that promote atrophy and skeletal muscle dysfunction including reduced oxidant capacity. This is in keeping with ex vivo experiments in myoblasts showing TNF- α administration reduced PGC-1 α mRNA levels (Tang, Wagner et al. 2010). This may provide one method by which cigarette smoke reduces oxidative muscle capacity, although this is likely to be a multifactorial process as smoking has also been demonstrated to affect both mitochondrial function and oxidative respiration in studies of human peripheral lymphocyte function (Cardellach, Alonso et al. 2003). The effects of

tobacco smoking on human skeletal muscle however remains a relatively under investigated area of research.

1.5 Exercise training in COPD

1.5.1 Effect on muscle regulatory pathways

Exercise training has been shown to reverse many of the detrimental changes seen in the skeletal muscle of COPD subjects, with improvements in mean fibre cross-sectional area, reduced proportion of anaerobic type IIb fibres and improved capillarity (Vogiatzis, Stratakos et al. 2007, Vogiatzis, Simoes et al. 2010). However, exercise training is unable to fully resolve all the abnormalities noted in this disease, particularly the fibre type shift (Whittom, Jobin et al. 1998, Vogiatzis, Stratakos et al. 2007).

At a molecular level rehabilitative exercise has been shown to lead to an increase factors responsible for muscle regeneration and hypertrophy, including IGF-1 and MyoD (Vogiatzis, Stratakos et al. 2007), explaining in part the development of muscle bulk in response to exercise training. Conversely factors leading to skeletal muscle degradation such as the myostatin and the ubiquitin-proteasome pathway are known to be activated in conditions of physical inactivity (Zhang, Chen et al. 2007), and thus reversal of this process provides another means by which muscle hypertrophy occurs in response to exercise.

Some COPD patients, however, show an aberrant response to training, particularly those with cachexia who fail to show the downregulation of myostatin and upregulation of MyoD following exercise training as is noted in healthy subjects (Vogiatzis, Simoes et al. 2010). This may be one reason why the magnitude of the response to rehabilitative exercise in COPD varies, and pharmacological manipulation may thus provide a means of improving sensitivity to the effects of exercise training.

Further work using serial skeletal muscle biopsies in COPD patients has demonstrated that such subjects may not show the magnitude of response at a molecular level to exercise interventions that is seen in healthy persons. Constantin et al. (2013) studied 59 COPD patients selected for significant

self-reported exercise limitation (MRC dyspnoea scale grade 3-5) compared to 21 age-matched healthy controls, all subjects undergoing 8 weeks of isokinetic knee extension resistance training with biopsies at baseline and then after 24 hours, 4 and 8 weeks of the training stimulus (Constantin, Menon et al. 2013). Despite a similar magnitude of improvement in thigh lean mass, as assessed by dual X-ray absorptiometry (DEXA), and strength in both COPD and control subjects, those with COPD showed blunted molecular responses to training, with the exception of markers of myogenesis (Constantin, Menon et al. 2013). These patients were perhaps not entirely typical of a COPD cohort selected for pulmonary rehabilitation, as despite reduced strength and endurance exercise capacity they had preserved muscle mass, but this work may allude to some of the variable responses seen following exercise training.

1.5.2 Reduction in systemic inflammation

Higher physical activity levels have been associated with reduced systemic markers of inflammation including C-reactive protein (CRP) in healthy elderly individuals (Das 2004). Exercise is known to lead to the release of cytokines including interleukin-6 (IL-6) that induce an anti-inflammatory environment through the production of anti-inflammatory cytokines, including interleukin-1 receptor agonist (IL-1ra) and IL-10, and reduction of pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α) (Starkie, Ostrowski et al. 2003, Petersen and Pedersen 2005). In part this may explain how exercise training helps to reverse the skeletal muscle dysfunction seen in COPD. Longitudinal studies are needed to fully explore the direction of causality between systemic inflammation and physical activity in COPD; whether this is due to the anti-inflammatory effects of regular physical activity or COPD-specific mechanisms of systemic inflammation influencing physical activity levels.

1.5.3 Reduction in oxidative stress

Therapy with antioxidants has been shown to reduce markers of oxidative stress and is associated with improved endurance exercise capacity (Koechlin, Couillard et al. 2004), indicating a possible causal relationship between oxidative stress pathways and skeletal muscle dysfunction in COPD. Endurance training interventions have been shown to increase the antioxidant buffering capacity of healthy subjects, but a similar effect is not noted in COPD subjects (Rabinovich, Ardite et al. 2001). Thus although exercise training is clearly beneficial in COPD this has not been shown to be

predominantly through reduced oxidative stress in the skeletal muscle, and again this maladaptive response to exercise may provide a potential target to improve the response to rehabilitation.

1.5.4 Pulmonary rehabilitation

Until the middle of the 20th century patients with COPD were often given advice to avoid activity and the inherent dyspnoea this provoked. The New York physician Barach recognised the value of physical training in this population stating, *“it may seem unusual perhaps to suggest exercise to these breathless people, but in fact it is one of the ways by which they can restore physical fitness”* (Noehren, Barach et al. 1964). Following on from this work the first outpatient structured programme of pulmonary rehabilitation was established in the 1960s under the pioneering guidance of the American physician Thomas Petty (Casaburi 2008).

Pulmonary rehabilitation consists of a structured intervention of supervised individually tailored exercise training and education with the aim to reverse the effects of skeletal muscle deconditioning. Furthermore, the value of pulmonary rehabilitation in alleviating the symptoms of COPD is now well established, with high grade evidence it improves exercise capacity, quality of life, muscle strength and frequency of hospital admissions, despite a lack of change in lung function variables (Lacasse, Goldstein et al. 2006, Nici, Donner et al. 2006, Dodd, Hogg et al. 2011, Bolton, Bevan-Smith et al. 2013). Rehabilitation during the recovery period following an acute exacerbation of COPD has also been shown to be beneficial in terms of reducing mortality and improving functional capacity and health status (Man, Polkey et al. 2004, Puhan, Gimeno-Santos et al. 2011).

Pulmonary rehabilitation is a high value, efficacious treatment modality, however, as with many therapeutic options including pharmacological agents, not without its limitations. Clinical trials and cohort studies show a significant proportion of patients may not improve their exercise capacity following a course of pulmonary rehabilitation, and even in those showing benefit such clinical improvements begin to decline towards baseline by 12-18 months (Griffiths, Burr et al. 2000, Troosters, Gosselink et al. 2000). In addition, inequalities exist in the provision and demand of pulmonary rehabilitation within the United Kingdom (Green and Morgan 2002). There is thus an unmet need to develop therapies to enhance and sustain the effect of pulmonary rehabilitation, ensuring patients gain the maximum benefit from this intervention. In addition, due to dysfunction

at the level of skeletal muscle patients may be responding suboptimally. Thus, pharmacological adjuncts that can enhance the benefits and sustain the effects of pulmonary rehabilitation are an important priority in research.

1.6 Possible agents to augment the effect of pulmonary rehabilitation

There is a great deal of research interest around agents to augment the effects of pulmonary rehabilitation. Such interventions may help ensure an improved response to whole-body exercise, and in some cases target the specific skeletal muscle dysfunction seen in COPD, with the aim of ensuring a maximal response to rehabilitation and maintenance of this response for as long as possible. Evidence suggests greater gain from exercising at higher intensity (Casaburi, Patessio et al. 1991), and thus any agents or interventions that allow this represent potential mechanisms to ensure greatest gains from an exercise intervention. The following provides a personalised opinion of the state of the literature at the current time and is not an extensive systematic review of the literature, although formal reviews have been conducted (Camillo, Osadnik et al. 2016). It is thus important to stress that whilst group data may fail to show a response this does not preclude that individual patients may benefit from certain interventions, alongside a tailored approach to exercise training.

1.6.1 Bronchodilator therapy

Appropriate maintenance bronchodilator therapy is essential to reduce the degree of ventilatory limitation, allowing the patient to exercise at higher intensity and thus gain greater benefit from training. Bronchodilation allows improvements in airflow and reduces both static and dynamic hyperinflation, and has been associated with improvements in endurance capacity and healthcare status both during and following completion of a pulmonary rehabilitation programme over a standard programme alone (Casaburi, Kukafka et al. 2005). It is thus essential to ensure any patients referred to pulmonary rehabilitation are established on effective maintenance bronchodilator therapy prior to initiation, and aware of the importance of appropriate inhaler technique and adherence (Bryant, McDonald et al. 2013).

1.6.2 Anabolic steroids

Anabolic steroids are known to lead to increases in muscular mass and have thus been viewed as potential agents to augment the response to pulmonary rehabilitation. Although studies in COPD have demonstrated the efficacy of testosterone analogues to increase muscular mass, this has not been shown to correlate with improved muscular strength (Yeh, DeGuzman et al. 2002) or endurance (Ferreira, Verreschi et al. 1998). Further research has investigated the potential synergistic effect of anabolic steroid administration with a high calorific diet. In a similar vein to the use of anabolic steroids alone, although this may lead to more favourable increases in muscular mass this has not been shown to correlate with improved measures of exercise capacity (Schols, Soeters et al. 1995). Thus as outcomes meaningful to patients have not shown improvement, and in the context of significant potential side effects, anabolic steroids have not received support for more widespread use in conjunction with resistance training.

1.6.3 Growth hormone

Growth hormone (GH) is a peptide hormone secreted by the anterior pituitary gland, known to lead to increases in muscular mass predominantly through hepatic production of IGF-1. In a similar manner to the use of anabolic steroid therapy, although administration of GH has been linked to increases in lean mass (Pape, Friedman et al. 1991), this has not corresponded to improved measures of strength or endurance even when combined with a rehabilitative intervention (Burdet, de Muralt et al. 1997).

1.6.4 Nutritional supplementation

Many COPD patients show evidence of nutritional depletion and thus interest has been stimulated in the use of nutritional supplements as an adjunct to pulmonary rehabilitation. Muscle creatine stores provide an important source of organic phosphate for the resynthesis of ATP during periods of high turnover, as occurs during whole-body exercise, and may be depleted in COPD patients with loss of fat free mass. In one small study creatine use alongside a standardised outpatient pulmonary rehabilitation programme was associated with an improvement in fat free mass and quadriceps strength beyond that seen in the placebo treated control group (Fuld, Kilduff et al. 2005). However, this finding was not reproducible in another cohort despite evidence of improved muscle creatine content (Deacon, Vincent et al. 2008). Both studies of creatine supplementation failed to elicit

improvements in whole-body exercise capacity (Fuld, Kilduff et al. 2005, Deacon, Vincent et al. 2008) and thus it remains an area of research interest only at the present time.

Carbohydrate supplementation has also not been shown to improve exercise performance beyond that achieved by pulmonary rehabilitation alone (Steiner, Barton et al. 2003) and neither has the use of combined protein/carbohydrate supplements (Constantin, Menon et al. 2013), showing no improvement at either a functional or molecular level. Some positive signal has been gained from research into the use of polyunsaturated fatty acids (PUFA), with an increase in both maximal and endurance exercise capacity as assessed by cycle ergometry following an 8 week rehabilitative intervention (Broekhuizen, Wouters et al. 2005). However, this finding clearly needs confirmation before its use could be more widely recommended. Thus at the current time the lack of substantial evidence means routine use of nutritional supplementation cannot be recommended for patients referred for pulmonary rehabilitation (van de Bool, Steiner et al. 2012, Bolton, Bevan-Smith et al. 2013).

1.6.5 Dietary antioxidants

The use of dietary anti-oxidants has also been explored as a possible intervention to negate muscle oxidative damage that may occur during exercise. Pressurised whey supplementation, which acts as a glutathione donor and has antioxidant properties, was shown in a small pilot study to potentiate the effects of exercise training with an improvement in endurance time over placebo (Laviolette, Lands et al. 2010). However, this occurred in the absence of any changes in blood markers of oxidative stress (Laviolette, Lands et al. 2010), raising questions about its validity and mechanism of action.

1.6.6 Oxygen

Patients already prescribed long-term oxygen therapy should use oxygen during rehabilitation and may need an increase in flow-rate to maintain adequate oxygenation (Spruit, Singh et al. 2013). The use of oxygen therapy in patients without resting hypoxaemia remains an area of controversy with some indication that it allows exercise at higher intensity and leads to improved performance in a laboratory-based training programme (Emtner, Porszasz et al. 2003). However, this finding has not always been reproducible and it remains unclear if this will translate from a laboratory to clinical setting (Garrod, Paul et al. 2000). At the current time use of supplemental oxygen therapy in

individuals without resting hypoxaemia should thus be assessed on a case-by-case basis and there is clear evidence that such patients can exercise safely without its provision. More recent work as part of a randomised controlled trial has suggested that those who experience hypoxaemia on exertion and have a positive acute response to ambulatory oxygen at baseline benefit from its provision during a programme of pulmonary rehabilitation (Dyer, Callaghan et al. 2012).

1.6.7 Non-invasive ventilation

Due to the alterations in the skeletal muscle seen in COPD these individuals experience a steeper rise in lactic acid during exercise than healthy individuals (Casaburi, Patessio et al. 1991, Maltais, Simard et al. 1996). This increases the load on the respiratory system by increasing the ventilatory requirement making it difficult for such patients to tolerate. Ventilatory support reduces dyspnoea and the work of breathing by offloading the respiratory muscles. It is reasonable then to study the use of ventilatory support in conjunction with exercise programmes to determine if this allows individuals with ventilatory limitation to tolerate a higher intensity of exercise, at which they may experience greater physiological benefit (Casaburi, Patessio et al. 1991), and exercise for a more prolonged period.

Early work by Hawkins et al. (2002) studied 19 individuals with severe COPD (mean FEV₁ 27% predicted) participating in a six week high intensity outpatient cycle exercise programme (Hawkins, Johnson et al. 2002); 10 randomised to receive ventilatory assistance via proportional assist ventilation (PAV) and 9 randomised to cycle unaided. After the period of training peak work rate achieved was 18% higher in those receiving ventilatory assistance, training intensity achieved was higher and lactate production at equivalent workload was significantly reduced in this group although not in the unassisted cohort, suggesting a true physiological effect from training.

This work and further studies have led to non-invasive positive pressure ventilation (NIPPV) being considered as an adjunct to pulmonary rehabilitation with a recent systematic review indicating augmentation of the effects of training, particularly in individuals with more severe disease (Corner and Garrod 2010). There remain, however, issues of patient acceptability, cost-effectiveness and feasibility, particularly outside of a hospital setting, meaning it is not widely implemented at present.

These issues are likely to be compounded when attempts are made to offer its use in a post exacerbation setting (Dyer, Flude et al. 2011).

1.6.8 Neuromuscular electrical stimulation

Passive stimulation of locomotor muscle groups through quadriceps femoral neuromuscular electrical stimulation (NMES) provides a possible therapeutic alternative or adjunct to pulmonary rehabilitation, with improvements in both maximal and endurance quadriceps strength as well as whole-body exercise capacity seen after a 6 week home-based programme (Neder, Sword et al. 2002). At the current time this intervention remains a research tool due to lack of sufficient evidence to promote its widespread use but may be a helpful tool in patients unable to attend a hospital or community-based pulmonary rehabilitation programme as it provides the opportunity for rehabilitation within a domiciliary setting. High quality evidence of its beneficial effects in individuals with muscle weakness in adults with advanced disease is, however, still lacking and further studies are needed to elucidate what role it may play as an adjunct to exercise training (Jones, Man et al. 2016).

In summary, whilst many options exist for possible augmentation of exercise training, relatively little evidence has been produced for their utility, even in meta-analysis studies (Camillo, Osadnik et al. 2016). In part some of this may be because of a failure to tailor the adjunct to the exercise training provided, and ensure that both are acting in a synergistic fashion, and this remains an area of further research potential.

1.7 Assessing the role of ACE-inhibition

1.7.1 Introduction to ACE-inhibitors

The renin-angiotensin system (RAS) plays a key role in circulatory homeostasis. In addition to involvement at a whole body level, local cellular (autocrine) and organ (paracrine) renin-angiotensin systems exist in many tissues and are known to be important in skeletal muscle (Jones and Woods 2003). Renin is responsible for the conversion of angiotensinogen to angiotensin I, and angiotensin converting enzyme (ACE), a zinc metallo-peptidase expressed in the lung capillary circulation and

present in the circulating plasma, catalyses the conversion of angiotensin I to the octapeptide angiotensin II (figure 1.5).

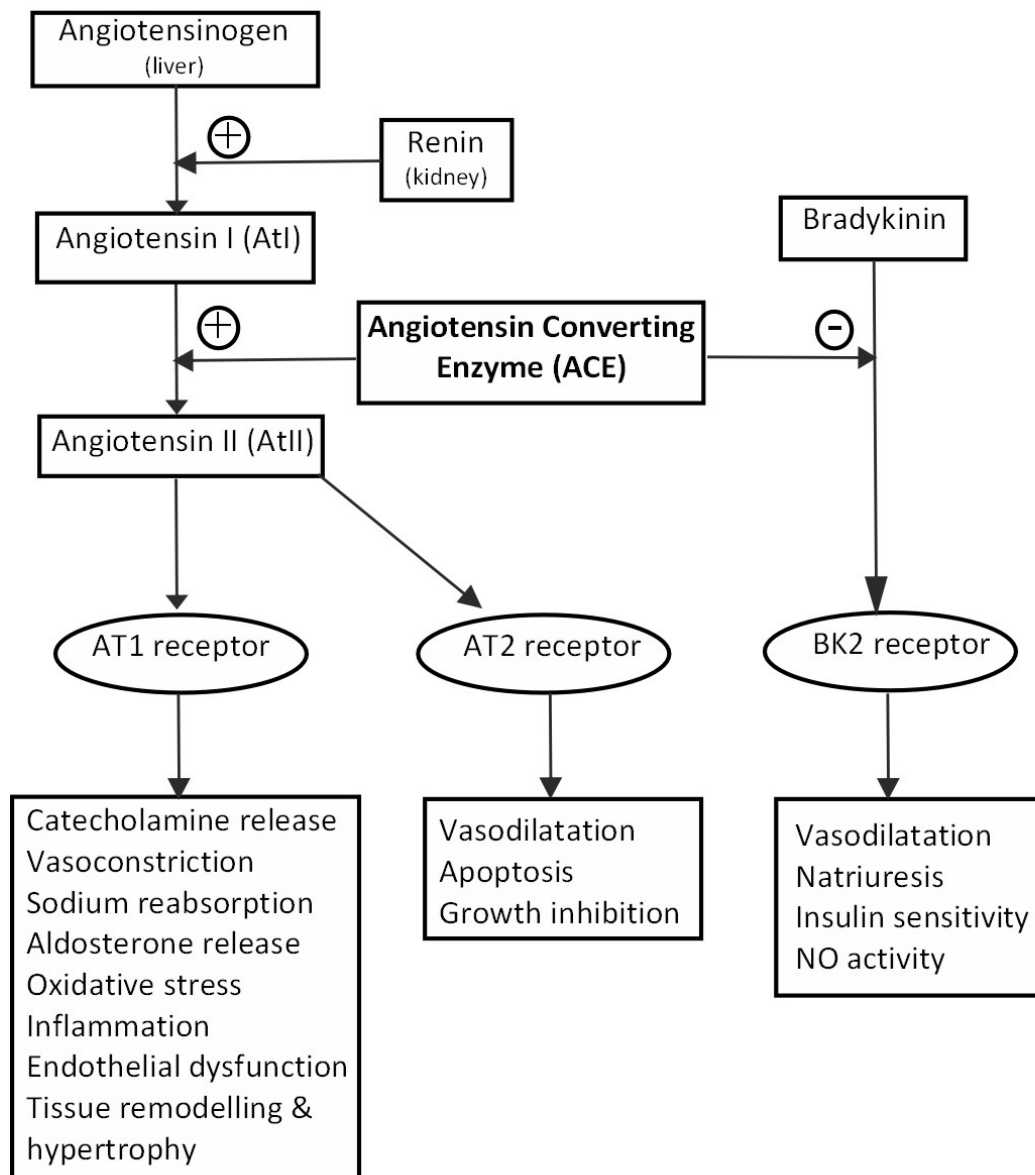


Figure 1.5: The Renin-Angiotensin System (RAS). Adapted from Carter et al. with permission (Carter, Onder et al. 2005).

Abbreviations: AT1 receptor - angiotensin II type 1 receptor; AT2 receptor - angiotensin II type 2 receptor; BK2 receptor - bradykinin type 2 receptor.

+ indicates a stimulatory effect on a pathway; - indicates an inhibitory effect.

Angiotensin II is a potent vasopressor through multiple mechanisms including the stimulation of sympathetic nerve terminals to release noradrenaline, enhanced sodium reabsorption by the renal tubule, direct action on smooth muscle cells and release of adrenal aldosterone driving renal salt and water retention. ACE also catalyses the breakdown of vasoactive kinins in particular bradykinin (Brown, Blais et al. 1998) which mediates the release of the vasodilator nitric oxide (NO). Thus ACE activity, which lowers bradykinin levels, inhibits its effects. The majority of the effects of angiotensin II are mediated through angiotensin II type 1 (AT1) receptors, some of which are negated by the activity of angiotensin II on type 2 (AT2) receptors. Both receptor subtypes are expressed in the lung whereas only the type 1 receptor is present in human skeletal muscle (Jones and Woods 2003).

Serum ACE specific activity has been shown to be elevated in COPD (Brice, Friedlander et al. 1995), and patients with hypoxaemia and hypercapnia show activation of the RAS and have measurably higher levels of plasma renin and aldosterone (Stewart, Waterhouse et al. 1994, Laghi, Adiguzel et al. 2009). Patients with COPD have elevated sympathetic activity (Heindl, Lehnert et al. 2001, Raupach, Bahr et al. 2008), which may in part be due to the association of the RAS with activation of the sympathetic nervous system. These patients are often described as showing 'neurohumoral activation' (Andreas, Anker et al. 2005) in a similar manner to that seen in chronic heart failure (Doehner, von Haehling et al. 2009).

ACE inhibitors are a group of pharmaceutical compounds developed following the discovery of the 'bradykinin-potentiating factor' (BPF) in the venom of *Bothrops jararaca*, a South American pit viper whose effects include profound hypotension. The research group lead by Nobel prize-winning Sir John Vane elucidated that the effects of BPF were mediated through the inhibition of ACE activity (Patlak 2004). Captopril, the first orally active ACE-inhibitor (ACE-I), was developed in 1975, and these compounds are now a well-established therapy in several conditions including hypertension, cardiac impairment and diabetes mellitus. Their good safety profile makes their use in other disease processes an attractive therapeutic option.

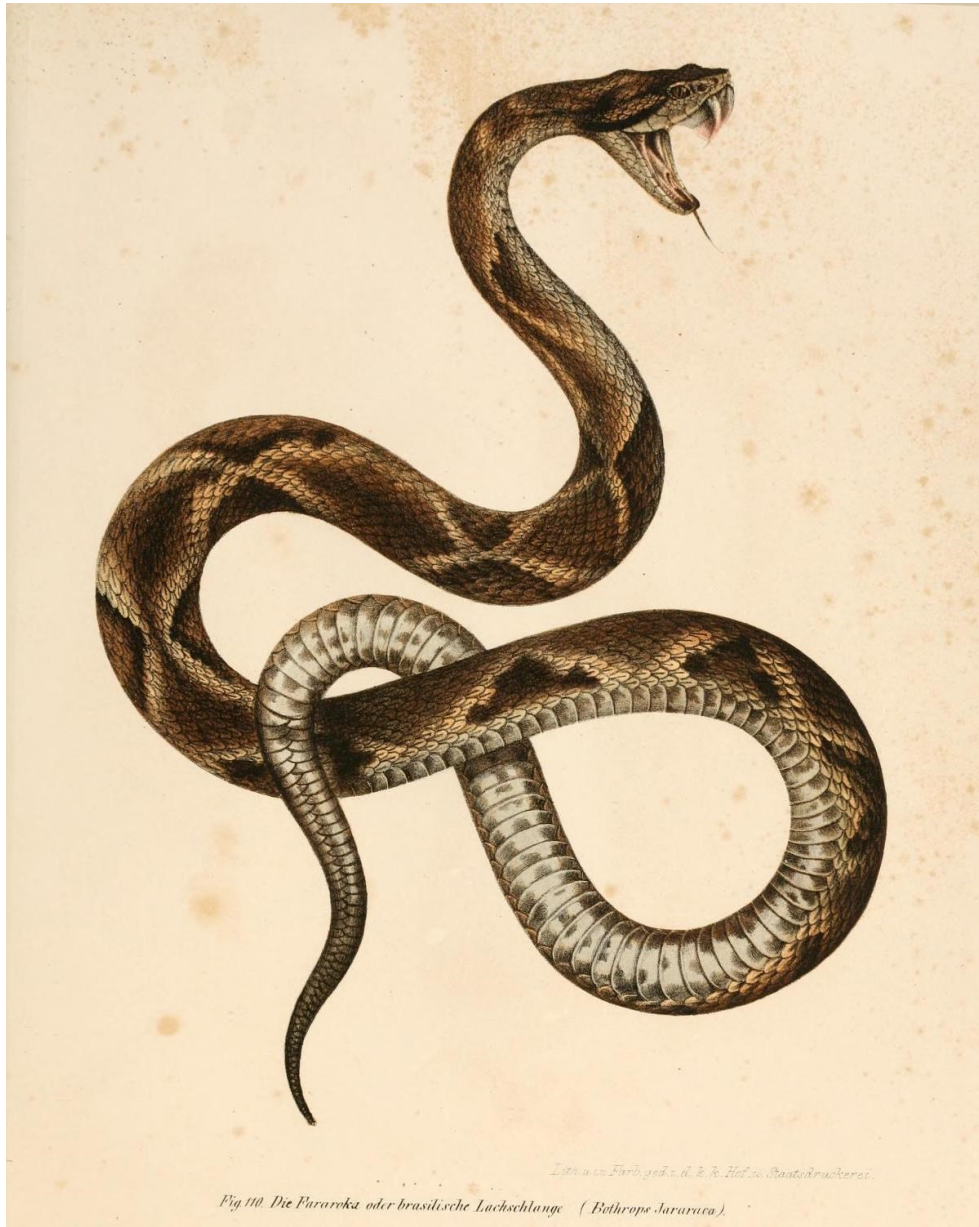


Figure 1.6: *Bothrops jararaca*, the South American pit viper. Taken from *Bilder-Atlas Zur Wissenschaftlich Popularen* by Leopold Joseph Fitzinger (Fitzinger 1867).

ACE inhibitors exert action at multiple levels that may help to ameliorate the skeletal muscle dysfunction seen in COPD. By inhibiting production of angiotensin II there is a reduction in pro-inflammatory pathways, improved insulin sensitivity, upregulation of the insulin-like growth factor (IGF-1) hypertrophy pathway and downregulation of the ubiquitin-proteasome atrophy pathway (Shrikrishna, Astin et al. 2012). Additionally bradykinin activity is enhanced leading to increased nitric oxide levels and effects on capillary vasodilatation and angiogenesis.

1.7.2 The renin-angiotensin system and skeletal muscle

1.7.2.1 Inflammatory pathways

Angiotensin II acting via the AT₁ receptor is known to promote activity in proinflammatory pathways through the production of reactive oxygen species (ROS), promotion of nuclear factor- κ B (NF- κ B) activity, reduced nicotinamide-adenine dinucleotide phosphate oxidase (NADPH) and attenuation of the anti-inflammatory effects of insulin (Zafari, Ushio-Fukai et al. 1998, Dandona, Dhindsa et al. 2007, Marchesi, Paradis et al. 2008). Angiotensin II increases adhesion molecules, cytokines and chemokines that are essential in the process of inflammation, in part via NF- κ B which mediates their transcription and gene expression (Russell, Wyke et al. 2006, Dandona, Dhindsa et al. 2007).

A correlation has been shown between ACE activity and serum high sensitivity CRP levels in a stable population of COPD patients which may be related to the pro-inflammatory effects of angiotensin II (Tkacova and Joppa 2007). In line with this, administration of ACE-inhibitors has also been shown to attenuate the pro-inflammatory effect of angiotensin II in peripheral human blood mononuclear cells (Constantinescu, Goodman et al. 1998), and lead to reduction in pro-inflammatory cytokines such as IL-6 in patients with chronic heart failure (Gullestad, Aukrust et al. 1999). This suggests reduction in ACE activity may dampen the inflammatory milieu of skeletal muscle seen in COPD, and is in line with animal work demonstrating that enalapril reduces angiotensin II dependent inflammatory myopathy in a dystrophic muscle model (Cozzoli, Nico et al. 2011).

1.7.2.2 Insulin sensitivity and glucose uptake

The RAS has an important influence over insulin sensitivity and skeletal muscle glucose metabolism (figure 1.7). Rat skeletal muscle studies have shown angiotensin II acting via AT₁ receptors inhibits insulin stimulated glucose transport via downregulation of GLUT-4 transporters, thus inducing insulin resistance (Diamond-Stanic and Henriksen 2010). This effect of angiotensin may be mediated, at least in part, by reactive oxygen species as it is reversed by superoxide dismutase (Diamond-Stanic and Henriksen 2010). Conversely bradykinin increases nitric oxide production and enhances glucose transport and uptake in part through upregulation and translocation of GLUT-4 transporters (Henriksen and Jacob 2003), knockout of the bradykinin receptor 2 gene being associated with insulin resistance in mice (Duka, Shenouda et al. 2001). Inhibition of ACE can thus modulate skeletal

muscle glucose metabolism via two main mechanisms, prevention of degradation of bradykinin which enhances glucose transport, and attenuation of angiotensin II mediated insulin resistance (Henriksen and Jacob 2003). In line with this ACE-inhibition has been demonstrated to improve whole body insulin sensitivity, insulin-mediated glucose transport in skeletal muscle and thus glucose tolerance (Henriksen and Jacob 2003).

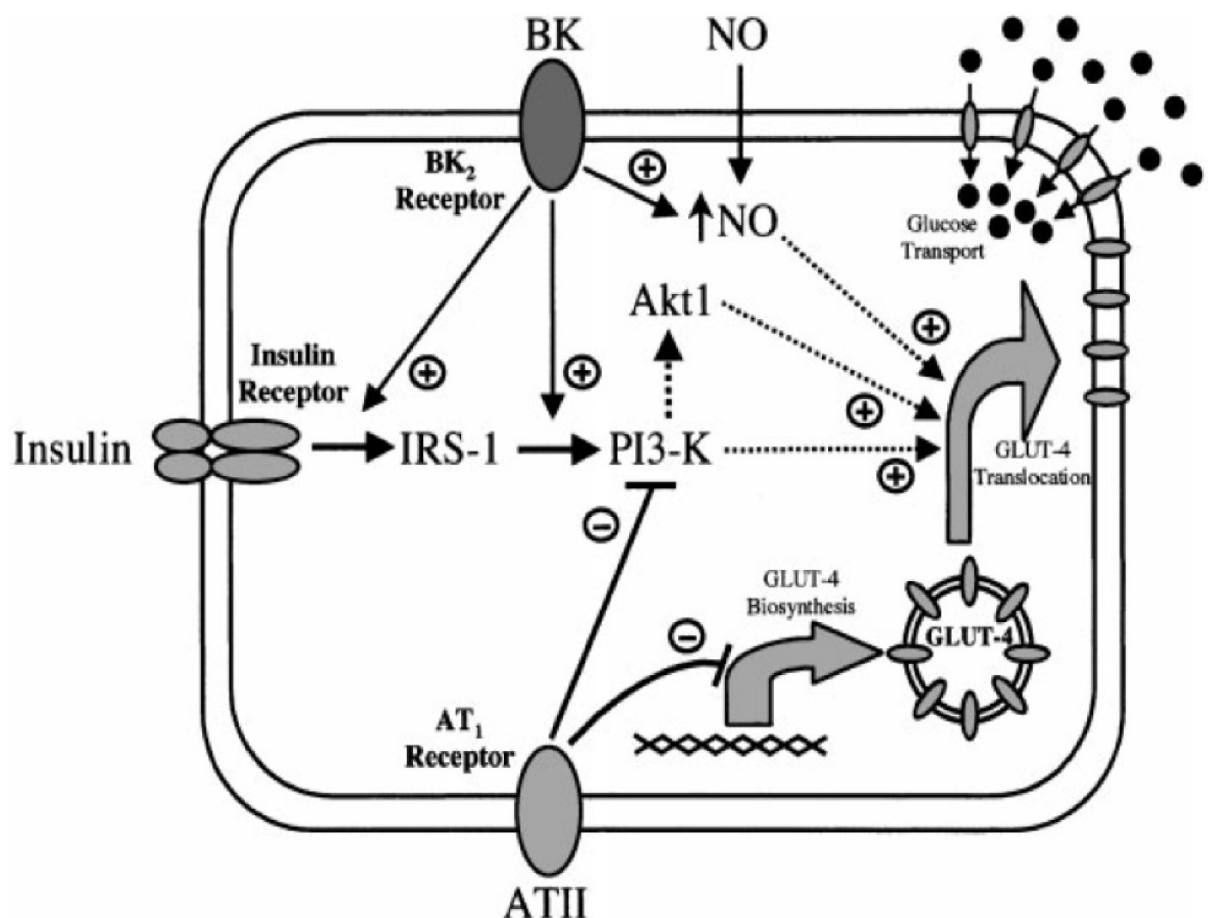


Figure 1.7: Summary of the potential sites for influence of the RAS on the insulin-mediated uptake of glucose in skeletal muscle. Bradykinin (BK) acting via BK₂Rs upregulates insulin signalling. Nitric oxide (NO) increases the translocation of GLUT-4 glucose transporter protein. Angiotensin II (ATII) acting via AT₁ receptors antagonises the action of insulin and reduces GLUT-4 translocation. Insulin acts via the insulin receptor substrate-1 (IRS-1) which interacts with phosphatidylinositol 3-kinase (PI3-kinase). Taken from Henriksen et al. with permission (Henriksen and Jacob 2003).

1.7.2.3 Muscle anabolic and catabolic pathways

Murine models have provided useful evidence of the effects of angiotensin II at a local level on skeletal muscle. Angiotensin II infusion leads to loss of muscle bulk, associated with reductions in both circulating and local muscle IGF-1 mRNA levels (Brink, Wellen et al. 1996, Song, Li et al. 2005). These effects of angiotensin II on muscle bulk and plasma IGF-1 levels can be blocked by the administration of an angiotensin II type 1 receptor antagonist in a rat model (Brink, Wellen et al. 1996).

Wild type mice infused with angiotensin II show increased mRNA levels of the ubiquitin ligases atrogen-1 and muscle ring finger-1 (MuRF-1) (Yoshida, Semprun-Prieto et al. 2010), suggesting that angiotensin II itself may influence the PI3K/AKT/atrogene pathway key in muscle atrophy through transcriptional regulation of the ubiquitin ligases. This effect can be blocked in muscle-specific IGF-1 transgenic mice, which overexpress IGF-1, providing support that the muscle wasting and weight loss induced by angiotensin II is causally related, at least in part, to a reduction in IGF-1 levels, an important signalling molecule in muscle hypertrophy pathways (Song, Li et al. 2005). Thus it can be reasonably expected that the administration of agents that block ACE activity and thus reduce angiotensin II levels alter the balance of atrophy-hypertrophy pathways in favour of improved muscle hypertrophy in response to stimuli such as exercise.

The InCHIANTI study conducted in 745 elderly (aged greater than 65 years) Italian subjects showed significantly higher levels of serum IGF-1 in those individuals taking ACE-inhibitors as therapy for hypertension as compared to the remaining study population, even after adjustment was made for multiple potential confounding factors (Maggio, Ceda et al. 2006). This relationship reached statistical significance for those taking ACE-inhibitors for less than 3 years. In those individuals whose use of ACE-inhibitors extended to 3-9 years there was a numerical increase in serum IGF-1 although this failed to reach statistical significance. This provides some observational evidence that the favourable effect of ACE-inhibitors on skeletal muscle may be in part through IGF-1 activity.

1.7.2.4 Bradykinin activity

Bradykinin is a vasoactive kinin synthesised in muscle during exercise whose breakdown is catalysed by ACE (Brown, Blais et al. 1998). Bradykinin has many positive effects on muscle metabolism

including vasodilatation, improving matching of oxygen supply to demand, production of nitric oxide and prostacyclin, effects on oxidative metabolism and alterations to glucose metabolism as discussed above. Fibre-specific nitric oxide also inhibits the effects of reactive oxygen species, protecting myofibres from oxidative damage as has been shown in both cultured myoblasts and in vivo murine models (Yu, Li et al. 2008).

The gene polymorphism associated with reduced activity at the bradykinin receptor (+9/+9 BK₂R) is associated with both reduced fat free mass and quadriceps strength in COPD patients (Hopkinson, Eleftheriou et al. 2006). This provides further support for the potential role of bradykinin in muscular strength in this population, indicating that the effect of ACE in reducing bradykinin activity is an additional mechanism by which the RAS may modulate skeletal muscle function.

1.7.2.5 PPAR pathways

ACE inhibitors may exert some of their protective effects through an influence on PPAR activity, maintaining mitochondrial biogenesis and the capacity of skeletal muscle to support oxidative metabolism. In human skeletal muscle cellular studies both ACE inhibitors and angiotensin receptor blockers show a high affinity for PPAR γ and may act agonistically with this molecule (Storka, Vojtassakova et al. 2008). In line with this, in a murine model, telmisartan, an angiotensin-II receptor blocker, has been shown to upregulate PPAR- δ in cultured myotubes. Telmisartan use in whole animal experiments has been associated with an increase in slow-twitch muscle fibres and endurance running capacity in a manner dependent on PPAR- δ activity (Feng, Luo et al. 2011), providing further evidence that this may be one mode of action of ACE-inhibitors on skeletal muscle.

1.7.2.6 TGF- β activity

The transforming growth factor beta (TGF- β) superfamily includes myostatin, a recognised negative regulator of muscle growth. The administration of losartan, an angiotensin type II receptor blocker, has been shown to normalise the muscle changes in structure and function induced by TGF- β in mouse models of myopathy (fibrillin-1 and dystrophin-deficient mice) (Cohn, van Erp et al. 2007). This suggests a potential role for TGF- β in mediating the deleterious effects of angiotensin II on skeletal muscle and thus a potential target for ACE-inhibition.

1.7.3 Epidemiological evidence

In epidemiological studies older patients taking ACE-inhibitors were noted to have greater muscle mass (Di Bari, van de Poll-Franse et al. 2004) and strength (Onder, Penninx et al. 2002) than those taking other therapies for hypertension even in the absence of cardiovascular disease. Onder et al. conducted a prospective observational study of 641 elderly hypertensive women participating in the Women's Health and Aging Study (Onder, Penninx et al. 2002), showing at 3 years those taking an ACE-inhibitor continuously had a lower decline in knee extensor muscle strength (-1.0 ± 1.1 vs. -3.7 ± 0.5 kg) and walking speed (-1.7 ± 4.1 vs. -15.7 ± 1.8 cm/s) in comparison to those taking other classes of anti-hypertensives. Similar differences were noted between those using ACE-inhibitors continuously and those who had never taken any therapy for hypertension. In addition, intermittent use of ACE-inhibitors was associated with a larger decline in walking speed than those taking the medication on a continuous basis. Although only observational in nature, this finding suggests a possible role for modulation of the RAS in influencing skeletal muscle function in aged individuals.

In support of this evidence the Health, Aging and Body Composition (Health ABC) study assessed cross-sectional data on lower limb extremity muscle mass, as assessed by dual X-ray absorptiometry (DEXA) imaging, in a cohort of 2431 hypertensive subjects (Di Bari, van de Poll-Franse et al. 2004). Preservation of muscle mass was found in those taking ACE-inhibitors rather than other anti-hypertensive therapy or no therapy for hypertension, with a trend towards this association being more pronounced in those with a longer duration of treatment defined according to a median split of 2 years (short-term users mean 16.3 ± 0.3 kg versus long-term users 17.4 ± 0.4 kg). These observational epidemiological studies have provided a basis for performing interventional studies involving manipulation of the RAS.

1.7.4 ACE gene polymorphism

Studies of the ACE gene polymorphism have helped to provide further support for a possible role of the skeletal muscle RAS. The ACE gene, located on chromosome 17q23, demonstrates a functional polymorphism dependent on the presence (insertion, I allele) or absence (deletion, D allele) of a 287-base pair non-coding DNA domain within intron 16. Thus subjects may be either homozygous (II or DD) or heterozygous (ID), the genotypes having an approximate distribution of 25%, 25% and 50% respectively in a Caucasian population (Jones and Woods 2003). The I allele leads to reduced tissue

(Costerousse, Allegrini et al. 1993) and serum (Brown, Blais et al. 1998) ACE activity (i.e. the endogenous equivalent of therapy with an ACE-inhibitor), leading to lower levels of angiotensin II and higher levels of bradykinin (Brown, Blais et al. 1998). Thus ACE activity is highest in those homozygous for the D allele (DD), intermediate in heterozygotes (ID) and lowest in those homozygous for the I allele (II). Several investigators have suggested a possible role for the ACE gene in the development of COPD although this has not been confirmed in European populations in meta-analysis studies, although the D allele remains a possible predisposing factor in Asian individuals (Li, Wei et al. 2012, Li, Lan et al. 2013).

The ACE genotype is an important contributor to determine endurance versus strength capacity. In health homozygosity for the I allele is associated with improved endurance performance (Montgomery, Marshall et al. 1998, Myerson, Hemingway et al. 1999, Zhang, Tanaka et al. 2003) and persons carrying the I allele demonstrate an increase in fatigue-resistant slow twitch type I muscle fibres (Zhang, Tanaka et al. 2003). Relatively little work has assessed exercise capacity as related to ACE genotype in COPD. In one Chinese study 61 COPD patients were compared to 57 healthy control subjects, demonstrating maximal workload and aerobic work efficiency greatest in those COPD patients possessing the II genotype, rather than ID or DD (Zhang, Wang et al. 2008). Such a response dependent on ACE genotype during CPEX testing was not evident in the control subjects, suggesting the role of the ACE pathway in the possible manipulation of the aerobic efficiency of skeletal muscle in COPD patients. As endogenous reduction in ACE activity through genetic tendency is associated with improved exercise capacity this suggests exogenous therapy with an ACE-inhibitor may perhaps lead to the same effect.

The D allele of the ACE gene has been linked to elite power-orientated athletic performance such as sprinting and short-distance swimming (Myerson, Hemingway et al. 1999, Woods, Hickman et al. 2001). In COPD subjects the presence of the D allele is also related to power activity, with a statistically significant association with quadriceps strength as assessed by maximal volitional contraction or by non-volitional methods (Hopkinson, Nickol et al. 2004, Hopkinson, Eleftheriou et al. 2006).

1.7.5 Animal work

As previously noted administration of angiotensin II type 1 receptor blockers, reducing angiotensin II activity, has been shown to normalise the changes in muscular structure and function in mouse models of myopathy (Cohn, van Erp et al. 2007). In further studies a potential synergistic effect of combining an exercise intervention with concomitant ACE-inhibition was demonstrated in an aged female rat model (Guo, Minami et al. 2010). Rats receiving both an exercise intervention and perindopril over a 6 month period showed a greater increase in capillary density in both soleus and gastrocnemius muscles, and a higher percentage of type I fibres in the gastrocnemius muscle, compared to exercise training alone (Guo, Minami et al. 2010). This did not, however, lead to a greater increase in exercise capacity in the group receiving both an exercise intervention with perindopril over the exercise intervention alone (Guo, Minami et al. 2010).

Work in spontaneously hypertensive rats has shown some support for these findings with an increase in untrained exercise capacity following 8 weeks therapy with perindopril but no increased response to exercise training over the exercise intervention alone (Minami, Li et al. 2007). In line with the work in aged female rats, however, capillary density and type I fibre proportions increased more in the group receiving both ACE-inhibition and exercise training over exercise training alone (Minami, Li et al. 2007). Thus adaptive changes in skeletal muscle in response to exercise may be promoted by concomitant ACE-inhibitor therapy, although animal models have yet to show that this translates to greater gains in exercise capacity.

1.7.6 Preliminary clinical studies

In the field of gerontology manipulation of the renin-angiotensin pathway has been associated with improved functional capacity. Use of perindopril over 20 weeks in a double-blind randomised controlled trial of 130 aged individuals (>65 years), with evidence of mobility or functional impairment but no evidence of cardiac failure, lead to a significant improvement in 6 minute walking distance in the perindopril treated group, with a mean between group difference of 31.4 metres (Sumukadas, Witham et al. 2007). This was also associated with maintained quality of life measures over the placebo group who declined over the same time period (Sumukadas, Witham et al. 2007). This research suggested a role for ACE-inhibition beyond its influence on cardiac impairment.

Further studies have examined alterations to angiotensin activity in COPD populations. Andreas et al. studied the administration of the angiotensin receptor blocker irbesartan for 4 months in a double-blind randomised controlled trial (Andreas, Herrmann-Lingen et al. 2006) in 60 patients with severe COPD ($FEV_1 < 50\%$ predicted). The therapy was well tolerated although the selected primary endpoint, maximum inspiratory strength, a measure of respiratory muscle strength, was not significantly altered in those receiving irbesartan over placebo. The treatment group, however, showed a 10% increase in quadriceps strength. Although this was not statistically significant it is worth noting that the study was underpowered to detect modest changes in quadriceps strength.

In a more recent study Di Marco et al. investigated the effects of 4 weeks therapy with the ACE-inhibitor enalapril in a cross-over study design of 18 patients with moderate to severe stable COPD and in the absence of cardiovascular disease (Di Marco, Guazzi et al. 2010). The study failed to show an improvement in the primary endpoint of improved ventilatory efficiency (VE/VCO_2 , minute ventilation/carbon dioxide production slope) in response to incremental cardiopulmonary exercise (CPEX) testing, but clearly demonstrated an increase in the peak work rate of the order of 7%. This study had several significant limitations, including the small sample size used and absence of any training intervention, however, it provides an interesting basis for further studies, particularly into whether use of an ACE-inhibitor in conjunction with a training intervention leads to an augmented effect.

In a selected group of weak COPD patients, using a cut-off of quadriceps strength associated with increased mortality in this condition (Swallow, Reyes et al. 2007), 3 months therapy with the ACE-inhibitor fosinopril did not lead to a significant change in quadriceps endurance or strength, or an improvement in functional exercise capacity as assessed by the incremental shuttle walking distance (Shrikrishna, Tanner et al. 2014). The subjects selected in this study being weak also unsurprisingly demonstrated low levels of physical activity, and it is possible this influenced the response to treatment. In addition this study was not stratified by ACE genotype which is known to influence the response to exercise training. Post-hoc analysis of the response to therapy by ACE genotype showed no difference between the groups, although was not powered prospectively to this end.

Animal studies have suggested a potential synergistic role for ACE-inhibition with a concurrent exercise stimulus (Guo, Minami et al. 2010), suggesting that ACE-inhibitors alongside pulmonary

rehabilitation may still provide functional benefit, and this remains an as yet unanswered research question. A small Canadian study of 21 COPD patients investigated the effects of the angiotensin receptor blocker losartan in conjunction with an aerobic exercise training programme (Marquis, Maltais et al. 2008). This study intended to look at blood pressure parameters and heart rate variability but was not powered to a specific endpoint and had a significant rate of non-completion, as well as poorly matched groups at baseline. It is thus impossible at present to draw any specific conclusions about how manipulation of the renin-angiotensin axis may augment the response to exercise training.

1.8 Nitrate supplementation and exercise capacity

1.8.1 Nitrate metabolism

Nitric oxide (NO) is well-recognised as an important physiological mediator in the body. It is produced endogenously via the action of the NO synthase (NOS) family of enzymes which catalyse the conversion of the amino acid L-arginine to NO and L-citrulline (Alderton, Cooper et al. 2001). NOS exists in three distinct isoforms; constitutively expressed endothelial NOS (eNOS) and neuronal NOS (nNOS), and the inducible form (iNOS). The action of NOS was long thought to be the only method of generation of NO, however, increasing knowledge has revealed that NO is also produced in a NOS-independent manner via the reduction of exogenous dietary nitrate (NO_3^-) and nitrite (NO_2^-) (figure 1.8). In fact where endogenous NO production is limited via the knock-out of the eNOS gene, the supplementation of nitrite (Bryan, Calvert et al. 2008) or nitrate (Carlstrom, Larsen et al. 2010) enhances plasma nitrite and nitrate levels towards those seen normally. This would suggest there is an important interplay between the endogenous and exogenous sources of NO production.

Nitrate is found at high levels in leafy green vegetables, including rocket and spinach, and some root vegetables, particularly beetroot. These sources account for 60-80% of our daily dietary nitrate intake (Ysart, Miller et al. 1999). Thus there is increasing interest in the 'nitrate-nitrite-NO' pathway in the production of NO, particularly with low oxygen availability as the action of NOS is dependent on the presence of molecular oxygen as a cofactor and thus functions poorly under such conditions.

Following enteral absorption into the bloodstream approximately 25% of ingested nitrate is actively taken up by the salivary glands via the enterosalivary circulation (Lundberg and Weitzberg 2010),

where concentrations may reach over 20-fold greater than in the plasma, the remainder undergoing renal excretion. Nitrate excreted in saliva is then reduced to nitrite by the action of the nitrate reductase enzyme produced by facultative anaerobic bacteria which reside in the oral cavity (Lundberg and Govoni 2004). As saliva is swallowed nitrite may enter the circulating blood volume directly (Lundberg and Govoni 2004), or undergo further reduction to NO in the acidic environment of the stomach (Benjamin, O'Driscoll et al. 1994) before absorption into the plasma. Following dietary ingestion of nitrate plasma concentrations peak after 1-2 hours followed by a peak in plasma nitrite at 2-3 hours, with both returning to their baseline value after approximately 24 hours (Wylie, Kelly et al. 2013). The further reduction of nitrite to NO is facilitated in conditions of low oxygen availability and low pH, as occurs in metabolically active tissues. Dietary nitrate supplementation thus provides a means of increasing circulating plasma nitrite levels, which provide an indicator of bioavailable NO.

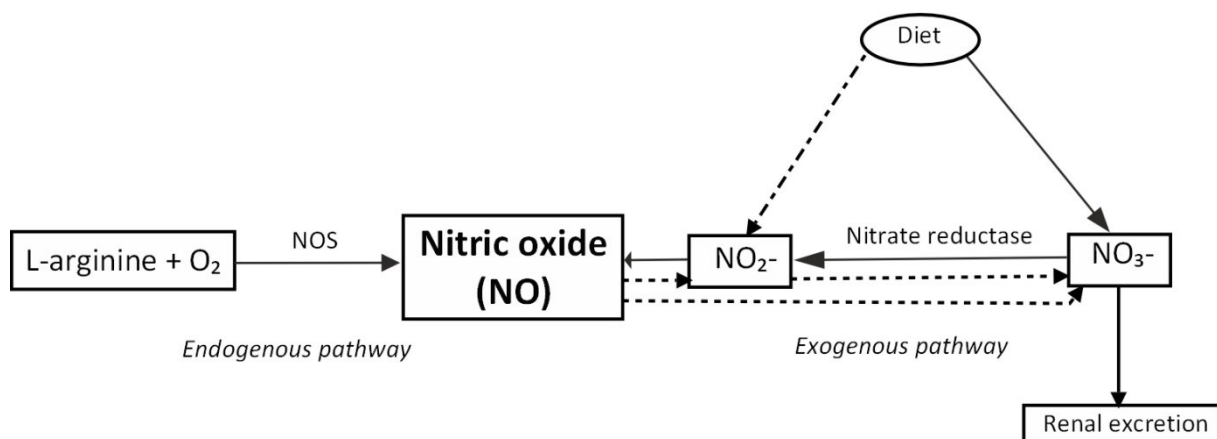


Figure 1.8: Nitrate-nitrite-NO pathway in humans. Plasma nitrate originates from the oxidation of endogenously produced NO and dietary sources. Nitrite reduction to NO is enhanced in acidic, hypoxic and reducing conditions. Adapted from Lundberg et al. with permission (Lundberg and Govoni 2004).

Abbreviations: O₂ – molecular oxygen; NOS – nitric oxide synthase; NO – nitric oxide; NO₂⁻ - nitrite; NO₃⁻ - nitrate.

1.8.2 Nitrate supplementation and exercise physiology

A link between plasma nitrite levels and exercise performance has been recognised providing early evidence for the possible role of the nitrate-nitrite-NO pathway in determining exercise capacity.

Dreißigacker et al. documented a correlation between plasma nitrite levels and performed work in a group of 22 healthy male subjects performing cycle ergometry (Dreissigacker, Wendt et al. 2010). In addition, exercise-induced upregulation of eNOS activity leads to increased endogenous NO production and a further rise in plasma nitrite levels, as the main product of NO oxidation. Post-exercise nitrite levels in 55 healthy individuals have also been correlated with achieved workload, suggesting that exercise induced changes in nitrite levels may influence exercise capacity (Rassaf, Lauer et al. 2007), and lending support to speculation that manipulation of nitrate metabolites may have an ergogenic effect.

Inhibition of NOS activity, and thus reduced endogenous basal NO production, has been demonstrated to increase oxygen consumption in conscious dogs during submaximal exercise, independent of any effects on vascular tone or cardiac output (Shen, Xu et al. 1994). This suggests endogenous NO production may have a potentially important role in the regulation of tissue metabolism and oxygen consumption, and inhibition of this process may increase the oxygen requirement of exercise.

Early experimental work has fuelled the study of the role of nitrate supplementation on human exercise performance, suggesting that NO may reduce the oxygen requirement of external work. The first study to investigate this in more detail was conducted by Larsen et al. in 2007 (Larsen, Weitzberg et al. 2007), using a double-blind randomised, placebo-controlled, cross-over trial design. 9 healthy well trained cyclists or tri-athletes (VO_2 peak 55 ± 4 ml/min/kg) were dosed over 3 days with sodium nitrite (0.1 mmol/kg/day) or placebo and underwent both submaximal and maximal workload cycle ergometry testing on the final day of dosing. Both resting systolic and diastolic blood pressure parameters were reduced by nitrite administration, and during submaximal workload exercise (at 45-80% VO_2 peak) the average pulmonary oxygen consumption reduced by 5% (2.98 ± 0.57 placebo vs. 2.82 ± 0.58 L/min nitrate-supplemented arm). This effect was seen in the absence of any change in heart rate, ventilation, lactate or respiratory exchange ratio. This is an impressive finding as the oxygen cost of exercise had previously been thought to be essentially fixed, and was known to be uninfluenced by such interventions as prior exercise (Burnley, Jones et al. 2000) and oxygen supplementation (Wilkerson, Berger et al. 2006). This finding suggested that exercise efficiency was improved, and thus working at a specific workload required a lower oxygen cost, and in turn may lead to improve exercise performance.

Since this original study, nitrate administration has been studied in a variety of forms, including pure sodium nitrate (Larsen, Weitzberg et al. 2010, Bescos, Rodriguez et al. 2011), beetroot juice rich in nitrate (Bailey, Winyard et al. 2009, Bailey, Fulford et al. 2010, Vanhatalo, Bailey et al. 2010, Lansley, Winyard et al. 2011, Lansley, Winyard et al. 2011, Christensen, Nyberg et al. 2013, Wilkerson, Hayward et al. 2012) and even whole beetroot preparations (Murphy, Eliot et al. 2012). Research predominantly from the field of sports medicine has demonstrated a reduction in the oxygen cost to perform at a submaximal workload following nitrate supplementation in healthy individuals (Larsen, Weitzberg et al. 2007, Bailey, Winyard et al. 2009, Bailey, Fulford et al. 2010, Vanhatalo, Bailey et al. 2010, Lansley, Winyard et al. 2011) as indicated by reduced steady state pulmonary oxygen uptake (VO_2) amplitude for a given low or moderate-intensity work rate (figure 1.9). This has in turn correlated with improved measures of exercise performance (Bailey, Winyard et al. 2009, Bailey, Fulford et al. 2010, Vanhatalo, Bailey et al. 2010, Lansley, Winyard et al. 2011, Murphy, Eliot et al. 2012) and has been tested in a variety of exercise modalities including running, cycling, rowing, repetitive leg extension and arm crank exercise.

Beetroot juice has been widely used as a nitrate donor due to the ease of administration, acceptability to subjects and ability to control the nitrate dose. It contains many compounds that could be hypothesised to influence exercise energetics either alone or in a synergistic manner, including nitrate, antioxidants such as betaine and the polyphenols such as quercetin. However, the effect of nitrate supplementation in the form of beetroot juice on exercise parameters have been shown to be dependent in large part on the nitrate content itself, the effect failing to be seen when using beetroot juice preparations specifically depleted of nitrate. This is achieved by passage through an ion exchange resin prior to pasteurisation, in a method developed by the University of Exeter (Lansley, Winyard et al. 2011).

Lansley et al. (Lansley, Winyard et al. 2011) compared dietary supplementation with nitrate-rich beetroot juice (providing 6.2 mmol nitrate/day) versus a nitrate-depleted beetroot juice, identical in appearance and taste, in a randomised cross-over design in 9 healthy, physically active males who undertook walking, moderate and severe-intensity running exercise on days 4-6 of supplementation. In this study nitrate-rich beetroot juice reduced the oxygen cost of all exercise modalities and extended the time to exhaustion during severe-intensity running by 15% (from 7.6 ± 1.5 to 8.7 ± 1.8

minutes). This provided strong support that it was the nitrate metabolites present in the beetroot juice preparation that were responsible for the noted effects on exercise performance.

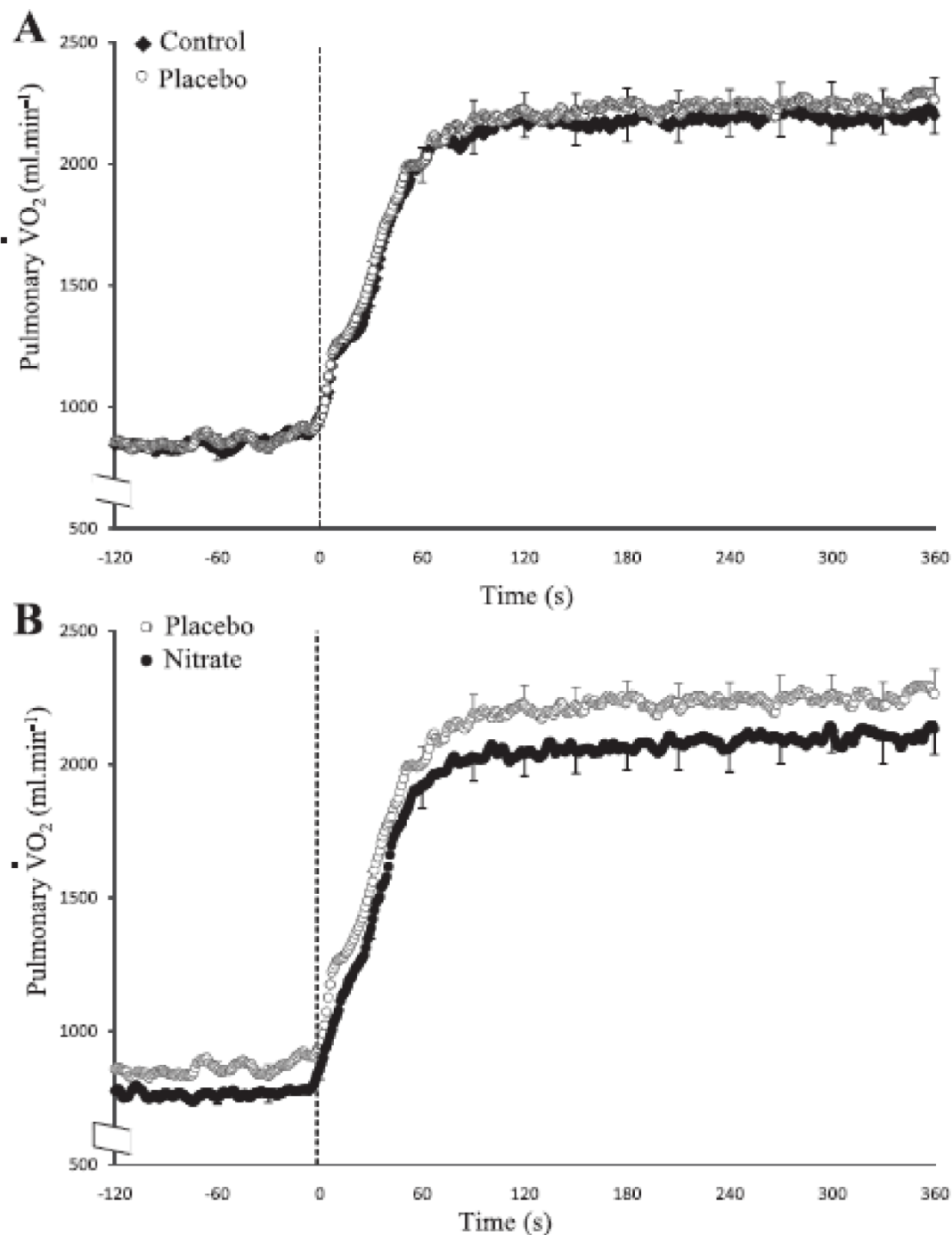


Figure 1.9: Pulmonary uptake ($\dot{V}O_2$) responses during step increment to a moderate intensity running speed from walking pace (represented by the dashed line) in 9 healthy subjects. There was no significant change comparing group mean responses for the non-supplemented control and placebo (nitrate-depleted beetroot juice) supplementation (upper panel, A). There was a significant reduction in the group mean $\dot{V}O_2$ when administered nitrate-rich beetroot juice supplementation versus placebo (lower panel, B). Taken from Lansley et al. with permission (Lansley, Winyard et al. 2011).

Similar effects have been demonstrated when exercising at a high intensity constant workload. Studies have shown a reduction in whole body oxygen uptake in combined arm and leg exercise (Larsen, Weitzberg et al. 2010), cycling (Bescos, Rodriguez et al. 2011), repetitive leg extension (Bailey, Fulford et al. 2010) and running (Lansley, Winyard et al. 2011) at high intensity following nitrate supplementation, indicating improved exercise efficiency and reduced oxygen cost as has been seen during submaximal exercise. This has been associated with extended time to exhaustion (Bailey, Winyard et al. 2009), improved time-trial performance (Lansley, Winyard et al. 2011) and improved power output (PO):VO₂ ratio (Lansley, Winyard et al. 2011) indicating improved overall exercise performance and economy when exercising at high intensity induced by nitrate supplementation.

Many studies have been conducted after around 3 days of nitrate supplementation, however, the beneficial effect of nitrate administration has been demonstrated following the administration of a single bolus dose, with effects 2 to 3 hours post administration, coincident with the rise in plasma nitrite levels (Webb, Patel et al. 2008, Vanhatalo, Bailey et al. 2010, Lansley, Winyard et al. 2011). Vanhatalo et al. studied eight healthy volunteers given 15 days of consecutive supplementation (5.2 mmol nitrate/day), this being the maximum duration of supplementation over which its effects on exercise performance have been studied. Exercise testing was conducted on day 1, 5 and 15 of administration using moderate-intensity step tests, demonstrating reduced VO₂ during submaximal exercise as compared to control subjects both acutely (2.5 hours post first dosing) and at 5 and 15 days of supplementation. Thus up to 15 days there has been persistence of effects on exercise energetics with no indication of the development of any nitrate tolerance (Vanhatalo, Bailey et al. 2010).

Although the majority of studies have focussed on young healthy recreationally active or athletic individuals the effects of nitrate supplementation have been recognised in older physically active adults. Kelly et al. (2013) studied a group of 60-70 year old healthy adults indicating that nitrate supplementation improved pulmonary oxygen uptake kinetics during treadmill walking, although this failed to translate to improved exercise capacity during the six minute field walking test (Kelly, Fulford et al. 2013). This raises important questions around the effects of ageing on response to nitrate that remain an area of active research and may be important when considering clinical

populations that are likely to be of greater age than most subjects participating as healthy volunteers.

1.8.3 Role of nitrate in hypoxic conditions

As COPD is frequently associated with tissue hypoxia which impairs exercise tolerance there is interest in how nitrate supplementation may affect exercise energetics under these conditions. As noted previously, NO production via NOS activity is dependent on the presence of molecular oxygen and thus may malfunction in hypoxic conditions, whereas the nitrate-nitrite-NO pathway is upregulated in such conditions and thus may be of increasing importance.

Masschelein et al. (Masschelein, Van Thienen et al. 2012) tested 15 healthy volunteers during cycle ergometry at a submaximal workload under hypoxic conditions (11% inspired oxygen concentration) after loading with either nitrate supplementation (0.07 mmol/kg nitrate) or placebo control. Oxygen consumption was notably lower at both rest and during submaximal exercise (at 45% peak VO_2) after nitrate supplementation than in the control condition. This was associated with improved tissue oxygenation index and lower rate of muscle oxygen extraction as assessed by near infrared spectroscopy (NIRS) (Masschelein, Van Thienen et al. 2012). Further work in healthy subjects performing repetitive leg extension exercise has demonstrated that nitrate supplementation can improve exercise tolerance when breathing a hypoxic inspirate (14.5% inspired oxygen concentration), normalising it such that the same work can be achieved as under normoxic conditions (Vanhatalo, Fulford et al. 2011). Acute nitrate administration can also ameliorate the reduction in endothelial function, as measured by flow mediated dilatation, induced by the hypoxia experienced at high altitude (Bakker, Engan et al. 2015).

Given the value of exercise training in intermittent hypoxia, investigation has thus been made of the use of nitrate supplementation to potentiate the effects of athletic training under such conditions. However, when tested in healthy volunteers performing high intensity interval training in hypoxic conditions, such supplementation has not translated to improved endurance exercise performance at sea level compared to placebo administration (Puype, Ramaekers et al. 2015, De Smet, Van Thienen et al. 2016). Although changes in exercise performance have not been noted, alterations at a histological level have been found. A five week exercise programme of sprint interval training in

hypoxic conditions alongside nitrate supplementation led to an increased proportion of type IIa skeletal muscle fibres in the *vastus lateralis* of healthy human volunteers not seen when placebo was administered (De Smet, Van Thienen et al. 2016).

1.8.4 Clinical trials of nitrate supplementation

Given the improved exercise efficiency following nitrate supplementation in healthy individuals, interest has been stimulated as to how this may translate to a variety of patient populations who experience exercise limitation. Clinical studies have been undertaken in patients with peripheral arterial disease where oxygen delivery is unable to meet the demands of metabolically active locomotor muscles during walking. This leads to the development of claudicant pain that then limits further exertion. Kenjale et al. (Kenjale, Ham et al. 2011) studied 9 patients with peripheral arterial disease in an open-label, randomised, cross-over trial of nitrate supplementation versus placebo. The supplementation of nitrate, in the form of beetroot juice, was shown to improve both walking distance and time before the onset of claudicant pain, by 18% and 17% respectively, in an incremental walking test (Kenjale, Ham et al. 2011). This was associated with lower gastrocnemius fractional oxygen extraction as assessed by NIRS (Kenjale, Ham et al. 2011) suggesting a reduction in the oxygen cost of exertion.

Acute dietary nitrate supplementation has been shown to increase exercise capacity as assessed by the incremental shuttle walking test in a small COPD population, with an increase of 39 metres over baseline in the nitrate treated group versus the placebo preparation (Kerley, Cahill et al. 2015). Further work has shown improved exercise capacity during cycle ergometry (Berry, Justus et al. 2015). However, both of these studies were heavily limited by the absence of a rigorous placebo arm, using blackcurrant and prune juice respectively, which calls into question the validity of the results obtained and the possible role of a 'placebo effect' as the preparations were clearly distinguishable. Additionally, the work by Kerley et al. (2015) included no measures of pulmonary VO_2 and although this measurement was recorded by Berry et al. (2015) there was no statistically significant change despite some large individual improvements in exercise capacity. In fact some of these responses were of a greater magnitude than that expected given a plausible biological mechanism of nitrate action, causing a skew in the data and calling into question its validity. These trials are a reminder of the need for true double-blinded studies in this field.

A small clinical study of patients with mild-moderate COPD (FEV₁ 30-80% predicted) using nitrate-depleted beetroot juice as a rigorous placebo preparation failed to elicit any alteration in blood pressure parameters, six minute walking distance or pulmonary oxygen uptake and exercise tolerance during submaximal cycling after nitrate supplementation (Shepherd, Wilkerson et al. 2015). This small study was possibly underpowered to detect these effects, and the dosing regimen (6.77 mmoles twice daily with the last dose 2.5 hours prior to testing) provided a lower acute dose than has been shown to elicit effects in younger adults (Wylie, Kelly et al. 2013). However, significantly raised plasma nitrate levels were still achieved and similar dosing regimens have elicited physiological effects in other studies of young healthy subjects. The lack of any change in blood pressure parameters may suggest the dose selected prior to testing was too low to elicit a biological effect in this particular population. Previous work has demonstrated that both changes in blood pressure parameters and VO₂ are dose-dependent (Wylie, Kelly et al. 2013) and thus it is important to identify a dose of nitrate in this specific population which is both safe and biologically active.

The most recently published study by an Australian group in patients with moderate stable COPD also used a twice daily nitrate dosing over three days, with a dose of 4.8 mmoles provided on the morning of testing, with comparison made to a nitrate-depleted beetroot juice placebo (Leong, Basham et al. 2015). Subjects completed endurance shuttle walk tests at 85% of their predicted VO₂max, and following nitrate dosing there was no significant difference between groups in either endurance walking distance or time. Although there were reported improvements in systolic blood pressure these measurements were taken in the open label 'safety phase' of the study, and although systolic blood pressure reduced from the sitting to standing position there were no clinically significant changes in one postural position and none during the blinded phase of the study compared to placebo. This again raises issues over whether a sufficient dose had been administered, particularly in the absence of any measures of plasma nitrate or nitrite in this study. In addition exercise testing was performed only one hour post dosing when nitrite levels may not yet have peaked, indicating a significant study limitation.

1.8.5 Proposed mechanisms of nitrate action on skeletal muscle

1.8.5.1 Skeletal muscle ATP generation

In order to perform work skeletal muscle requires a source of adenosine triphosphate (ATP). ATP may be produced via both glycolysis and oxidative phosphorylation, the substrates of which are

delivered by blood flow to the skeletal muscle, or ATP may be regenerated from adenosine diphosphate (ADP) through the use of muscle phosphocreatine (PCr) stores as a phosphate donor.

Several studies have used serum lactate as a surrogate measure of anaerobic metabolism (Larsen, Weitzberg et al. 2007, Bailey, Winyard et al. 2009), demonstrating a failure of alteration in blood lactate levels during exercise following nitrate administration as compared to placebo. This is a highly simplified and limited approach, and although suggestive that anaerobic pathways are not increasingly favoured following nitrate administration, does not effectively exclude that this may be contributing to its mode of action.

Phosphorus-31 magnetic resonance spectroscopy (^{31}P -MRS) is an experimental method that provides information on phosphate metabolism, and can be used to monitor muscle PCr, ATP, ADP and phosphate kinetics during both rest and exercise. Thus, measures obtained by ^{31}P -MRS provide an excellent surrogate of events at a cellular level and an insight into in vivo myocyte function. ^{31}P -MRS has provided support that nitrate supplementation may influence the whole-body oxygen cost of exercise, at least in part, via reduced ATP cost of muscle force production. Bailey et al. (Bailey, Fulford et al. 2010) demonstrated that following nitrate supplementation overall ATP turnover was reduced, with reduced ADP and phosphate accumulation. This occurred in the absence of an increased phosphocreatine breakdown or markers of anaerobic metabolism, and has been replicated in other studies (Lansley, Winyard et al. 2011). Whilst the overall ATP requirement was reduced subjects were able to maintain the same power output, implying improved muscle efficiency and reduced tendency to fatigue. The changes seen by MRS suggest this may occur through improved coupling between ATP hydrolysis and muscle force generation (Bailey, Fulford et al. 2010). These findings are consistent with known control factors for oxidative phosphorylation, given that ATP resynthesis is itself stimulated by ATP usage and the products of ATP hydrolysis (Brown 1992, Bose, French et al. 2003).

1.8.5.2 Oxidative phosphorylation efficiency

The generation of ATP by oxidative phosphorylation is dependent on the generation and maintenance of the electrochemical proton gradient across the inner mitochondrial membrane. The efficiency of this process can be estimated using the P/O ratio, the amount of oxygen consumed per

molecule of ATP generated (a higher ratio indicating more efficient oxidative phosphorylation (Hinkle 2005)). Not all of the membrane potential created by the hydrogen ion gradient across the inner mitochondrial membrane is eventually coupled to ATP production due to proton leakage both across the membrane, and passage through uncoupling proteins and the adenine nucleotide translocase (ANT). This dissipates the proton gradient before it can be coupled to ATP production and provides a site of inherent inefficiency in ATP production.

The most definitive mechanistic study thus far of nitrate action on skeletal muscle was conducted by Larsen et al. (Larsen, Schiffer et al. 2011). Following 3 days of nitrate supplementation (0.1 mmol/kg/day) or placebo in 14 healthy subjects skeletal muscle mitochondria were harvested following a muscle biopsy of the *vastus lateralis*. These mitochondria demonstrated a 19% increase in the P/O ratio and 23% increase in ATP production, and Western blot analysis showed significant downregulation of ANT expression. The change in the P/O ratio was correlated with the reduction in whole-body oxygen consumption during submaximal workload exercise in the same healthy subjects from which the skeletal muscle biopsies had been obtained (Larsen, Schiffer et al. 2011). These findings lend support to the hypothesis that improved oxidative phosphorylation capacity, in part through reduced dissipation of the hydrogen ion gradient, may explain the mode of action of nitrate on exercise efficiency.

In furtherance of this, in vitro studies of rat liver mitochondria have demonstrated reversible inhibition of cytochrome c oxidase, the terminal electron acceptor of the electron transport chain, by NO and the effect of NO in reducing proton leak across the inner mitochondrial membrane (Clerc, Rigoulet et al. 2007). In fact mitochondrial NOS is located in close proximity to cytochrome c oxidase and deletion of the domain that anchors NOS in the mitochondrial outer membrane leads to increased oxygen consumption (Gao, Chen et al. 2004). This provides further support that NO may be a physiological regulator of this enzyme, and thus exert control over the oxidative phosphorylation process.

1.8.5.3 Reduction in ATP requirements

Beyond effects on ATP generation efficiency, NO may also act by reducing the energy requirements of cellular processes in the myocyte, the most ATP consuming being the actin-myosin ATPase crucial

for cross-bridge cycling, and calcium transport by calcium-ATPases that maintain the calcium ion gradient across the sarcoplasmic reticulum. Physiological concentrations of NO have been demonstrated to alter striated muscle myosin cross-bridge kinetics from a high-speed low-force state, to a low-speed high-force generating state in an in vitro preparation (Evangelista, Rao et al. 2010). NO is also known to influence calcium handling, and in a rabbit femoral striated muscle preparation NO has also been shown to reduce sarcoplasmic reticulum calcium-ATPase (SERCA) activity (Ishii, Sunami et al. 1998). These studies have thus far been conducted in animal ex vivo preparations and have yet to be demonstrated using human myocytes.

1.8.5.4 Skeletal muscle blood flow

NO is an important vasodilator through activation of guanylate cyclase, which synthesises cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) leading to the relaxation of vascular smooth muscle. Thus not surprisingly the administration of nitrate has been correlated with both reduced systolic and diastolic blood pressure (Larsen, Ekblom et al. 2006, Webb, Patel et al. 2008). NO may thus improve blood flow to metabolically active skeletal muscle, ensuring more efficient matching of oxygen delivery to local metabolic demand, thus permitting more prolonged exercise.

In a rat model, use of beetroot juice as a nitrate donor over a period of 5 days has been associated with a reduction in exercising mean arterial blood pressure and increased hindlimb muscle perfusion during submaximal treadmill running. These changes were measured using radio-labelled microsphere infusions, with increases in hindlimb muscle blood flow and vascular conductance of 38% and 48% respectively over placebo (water) administration (Ferguson, Hirai et al. 2013). These changes were seen preferentially in muscles comprised of type II fast-twitch fibres. The improvement in skeletal muscle blood flow despite reductions in mean arterial pressure alluded to an important role of NO in regulating muscle perfusion and thus improving oxygen supply to exercising muscle. This has been confirmed in further studies of microvascular oxygen delivery in rats undergoing electrically induced twitch spinotrapezius muscular contractions where an improvement in oxygen delivery has been noted at the onset of muscular contraction (Ferguson, Hirai et al. 2013).

In human studies NIRS has been used to estimate muscle blood volume changes. This is, however, not equivalent to direct measures of skeletal muscle blood flow which can currently only be achieved through the use of animal models. Despite its limitations NIRS measurements have suggested nitrate may improve skeletal muscle blood flow during exercise (Kenjale, Ham et al. 2011, Masschelein, Van Thienen et al. 2012) and thus some of the effects of nitrate may be mediated through vasodilatory effects, with improvements in oxygen delivery, allowing increased tolerance of fatigue.

1.8.5.5 Other possible mechanisms

Following 3 consecutive days of nitrate supplementation in healthy humans Larsen et al. (Larsen, Schiffer et al. 2011) showed no alteration in either skeletal muscle mitochondrial biogenesis or density. However, given the influence on exercise energetics has been demonstrated up to 15 days of supplementation it is plausible that more prolonged periods of supplementation may influence total mitochondrial number as well as individual mitochondrial activity, although this has yet to be investigated.

It remains naïve to consider that a pluripotent mediator such as NO is only acting at a single level and it is likely its effects are mediated through several different mechanisms that may act synergistically. The complexity of the potential mechanisms is summarised in the following schematic diagram (figure 1.10).

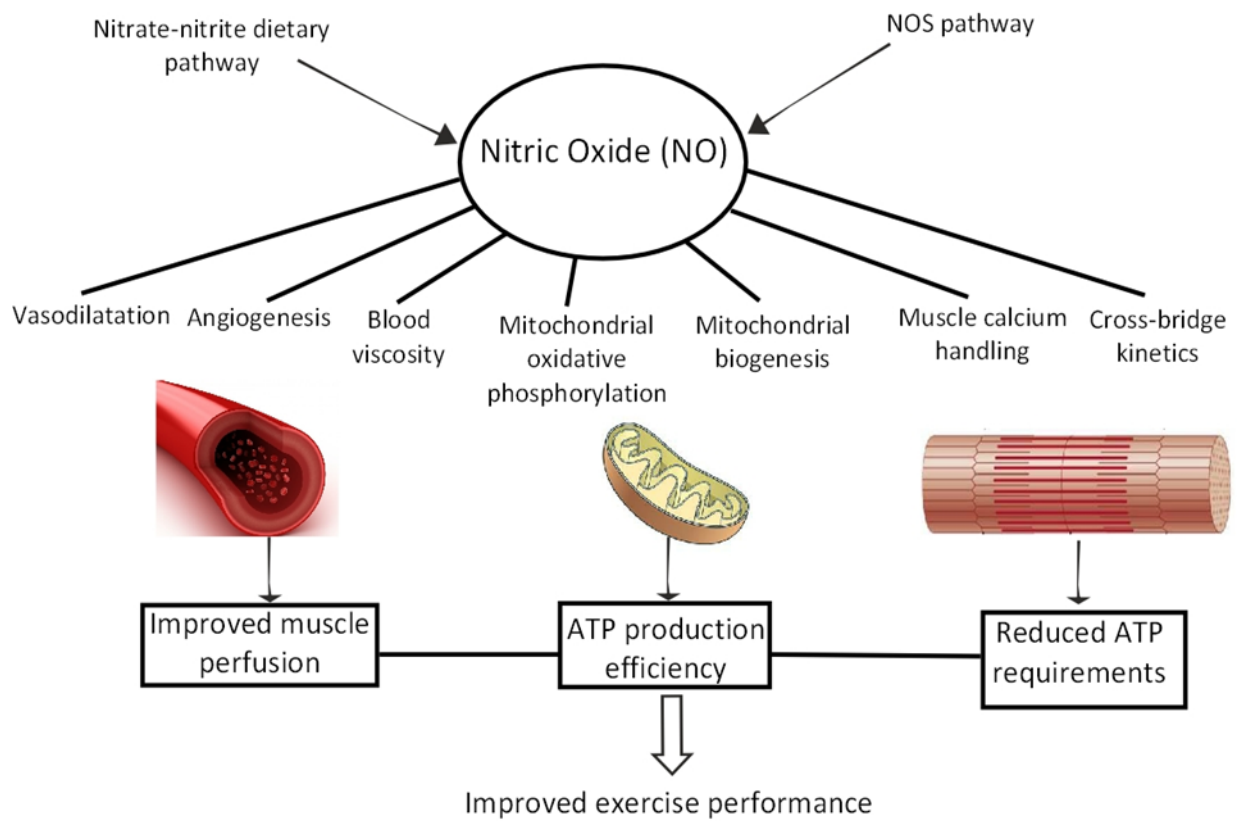


Figure 1.10: Possible mechanisms of action of nitrate supplementation in improving exercise performance.

1.9 Research questions and hypotheses

The aim of this thesis is to further evaluate potential strategies to reverse exercise limitation in COPD. An observational study was conducted to assess the role of the ACE genotype in the exercise characteristics of a UK based COPD population. A double-blind randomised controlled trial is then conducted to investigate if manipulation of the renin-angiotensin pathway through ACE-inhibition alongside a concurrent programme of pulmonary rehabilitation can augment the response to this exercise stimulus, with the focus on maximal exercise capacity. The influence of ACE-inhibition on strength, health-related quality of life and physical activity levels was also considered. In the final study, pilot work was undertaken to assess the role of nitrate supplementation on endurance exercise capacity and oxygen consumption during submaximal exercise.

The work contained in this thesis aims to address the following research questions:

1. Does the ACE genotype influence exercise characteristics in COPD patients as assessed by incremental cycle ergometry?
2. Does angiotensin-converting enzyme (ACE) inhibition alongside pulmonary rehabilitation augment the improvement in maximal exercise capacity in COPD patients?
3. Does nitrate supplementation in COPD patients improve exercise performance and reduce the oxygen cost of submaximal exercise?

Chapter 2: Description of Methods

2.1 Ethical approval

The studies in this thesis were approved by the London Bloomsbury Research Ethics Committee (REC reference 12/LO/0331) and the Research and Ethics Committee of Bromley (REC reference 13/LO/0372). Written informed consent was obtained from all participants and research was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

2.2 Subjects studied

Patients recruited to the studies in this thesis had a diagnosis of COPD made in line with current National Institute for Health and Care Excellence (NICE) guidance (O'Reilly, Jones et al. 2010). The severity of lung disease was categorised using the GOLD (Global Initiative in Obstructive Lung Disease) stage classification (table 2.1) (Rabe, Hurd et al. 2007).

Patients were recruited from outpatient clinics at the Royal Brompton and Harefield NHS Foundation Trust, referrals to the pulmonary rehabilitation service and through public outreach events including World COPD Day. Exclusion was made of any patients who possessed other comorbid factors that would limit their exercise capacity.

Stage	Lung function criteria
Stage I: mild	FEV ₁ /FVC <0.70 FEV ₁ ≥80% predicted
Stage II: moderate	FEV ₁ /FVC <0.70 50% ≤ FEV ₁ <80% predicted
Stage III: severe	FEV ₁ /FVC <0.70 30% ≤ FEV ₁ <50% predicted
Stage IV: very severe	FEV ₁ /FVC < 0.70 FEV ₁ <30% predicted or FEV ₁ <50% predicted plus chronic respiratory failure

Table 2.1: Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification of disease severity based on post-bronchodilator spirometry (Rabe, Hurd et al. 2007).

2.3 Anthropometric measurements

Height (cm) without shoes was measured using a wall mounted measure and weight (kg) measured using standardised scales. The body mass index (BMI) was calculated using the following equation:

$$\text{BMI} = \text{weight (kg)} / \text{height (m)}^2$$

2.4 Blood pressure measurements

Blood pressure was measured using an automated blood pressure monitor (Omron M6, Omron Healthcare Europe, Hoofddorp, Netherlands). Subjects rested for at least 10 minutes before any measurements were taken, with the subject in the seated position with their arm supported at the level of the heart. An appropriate cuff size was selected. A mean of three measurements was recorded and mean arterial pressure (MAP) calculated using the following equation:

$$\text{MAP} \approx \text{diastolic pressure} + \frac{1}{3} (\text{systolic pressure} - \text{diastolic pressure})$$

2.5 Body composition

2.5.1 Fat free mass measurement using bioelectrical impedance analysis

Fat free mass was determined using bioelectrical impedance analysis, a technique which works as electricity is conducted by dissolved ions and cell membranes act as incomplete capacitors. This technique uses the electrical impedance of body tissues to estimate total body water by measuring the electrical resistance between the wrist and ankle. A two-compartment model was implemented, assuming that adipose tissue contains no water and that fat free mass has a constant hydration of 73% water. A Bodystat 4000 device (Bodystat, Isle of Man, United Kingdom) was used and subjects were asked to lie supine for 10 minutes prior to measurement. Silver chloride electrodes were placed on the dominant hand and foot, at 2 cm proximal to the metacarpophalangeal joints and the ulnar level of the wrist on the dorsal aspect of the hand, and at 2 cm proximal to the metatarsophalangeal joints and level of the malleoli of the ankle, respectively.

2.5.2 Fat free mass calculation

Single-frequency, 50 Hz, bioelectrical impedance values were incorporated into a disease specific regression equation that accounts for height, weight and gender in order to allow calculation of fat free mass (Steiner, Barton et al. 2002). The use of such equations has been validated against other methods for assessing body composition, including dual energy X-ray absorptiometry and deuterium dilution (Schols, Wouters et al. 1991).

COPD males:

$$\text{FFM (kg)} = 8.383 + [(0.465 \times \text{Height (cm)}^2 / \text{Resistance (ohm)})] + [0.213 \times \text{Weight (kg)}]$$

COPD females:

$$\text{FFM (kg)} = 7.610 + [(0.474 \times \text{Height (cm)}^2 / \text{Resistance (ohm)})] + [0.184 \times \text{Weight (kg)}]$$

Fat free mass index (FFMI) was calculated by dividing FFM by height in metres squared (kg/m^2).

Individuals with a fat free mass index (FFMI) below 16 kg/m^2 in males and 15 kg/m^2 in females were considered to have evidence of fat free mass depletion (Steiner, Barton et al. 2002).

2.6 Quadriceps imaging

2.6.1 Mid-thigh cross-sectional computed tomography

A computed tomography (CT) scan of the upper legs midway between the pubic symphysis and inferior condyle of the femur was performed using a 64-slice CT scanner (Siemens SOMATOM Sensation 64, Erlangen, Germany) with the subject in the supine position. Each image was 10 mm thick and taken at 120 kV and 50 mA with a scanning time of 1 second, using modification of a previously designed protocol (Marquis, Debigare et al. 2002) to reduce the total radiation dose. The cross-sectional area of the musculature of the leg at the mid-thigh level and quadriceps was mapped using standard window settings for visualisation of soft tissues (centre 40HU, window width 380 HU) using Impax software (Impax 6.3, Agfa Healthcare, Greenville, United States of America). Measurements were taken from the ipsilateral side as that from which strength measurements were recorded (figure 2.1).

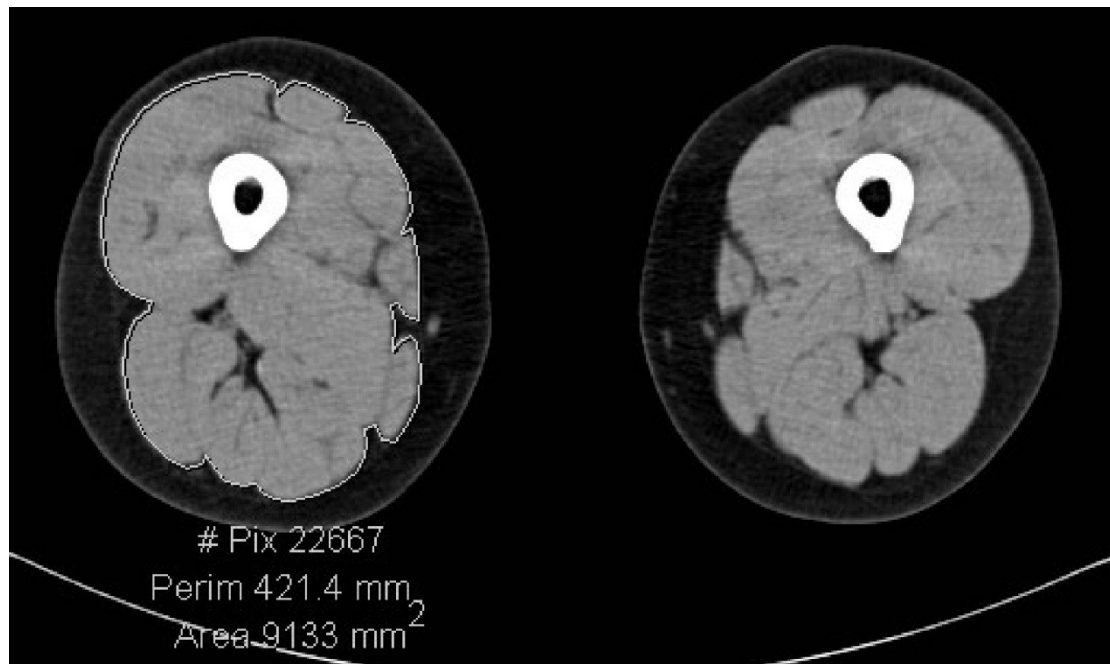


Figure 2.1: Mid-thigh computed tomography cross-sectional image. The mid-thigh cross-sectional area is outlined, from which the area of the femur is subtracted to give the mid-thigh muscle cross-sectional area.

2.7 Quadriceps muscle strength

The capacity of a muscle to generate force was assessed using both volitional (subject effort-dependent) and non-volitional (effort-independent) methods.

2.7.1 Volitional: isometric quadriceps maximal voluntary contraction

Maximum isometric quadriceps volitional contraction (QMVC) was measured using the technique of Edwards et al. (Edwards, Young et al. 1977) This is an isometric measurement, requiring the static contraction of the quadriceps muscle in the absence of a change in the muscle length or joint angle. Prior to each measurement calibration was performed using a standardised weight. Subjects were seated on a modified couch with the leg flexed at an angle of 90° relative to the edge. The right leg was tested unless it was not feasible to do so. An inextensible strap was placed around the subject's ankle and connected to a strain gauge (Straininstall, Isle of Wight, United Kingdom; figure 2.2). The maximum force generated and sustained for at least 5 seconds during at least five maximum voluntary contractions with vigorous encouragement was measured, ensuring that the response

magnitude was not continuing to increase between efforts. A minimum of 30 seconds was allowed between each effort to allow for recovery.

Output from the strain gauge was processed as a digital output by a Powerlab recording unit (AD Instruments, Oxford, UK) and immediately visible online. This was analysed using LabChart software (LabChart version 7.1, AD Instruments, Oxfordshire, United Kingdom) sampling at 10 kHz. The QMVC was taken as the highest tension recorded for 1 second and recorded in kilograms.

In the original description of QMVC measurement, strength was normalised to body weight alone (Edwards, Young et al. 1977). However, a more advanced regression equation to allow calculation of the percentage predicted QMVC has been developed, identifying the variables age, gender and fat free mass as predictors of QMVC (Seymour, Spruit et al. 2010) with an r^2 of 0.51. The following equation is thus now commonly implemented.

Predicted QMVC force in kg =

$$56.2 - [0.30 \times \text{Age (years)}] + [0.68 \times \text{FFM (kg)}] - [0.15 \times \text{Height (cm)}] - (3.42 \text{ if Female})$$



Figure 2.2: Quadriceps maximal isometric voluntary contraction measured using a strain gauge.
This follows the technique of Edwards et al. (Edwards, Young et al. 1977).

2.7.2 Non-volitional: supramaximal magnetic femoral nerve stimulation

Twitch quadriceps tension (TwQ) was measured using the technique of Polkey et al. (1996) (Polkey, Kyroussis et al. 1996). The subject was positioned, with an inextensible strap placed around the ankle, and rested for 20 minutes before any measurements were recorded to ensure relaxation of

the quadriceps muscle. The right leg was tested unless it was not feasible to do so. A 70 mm figure of eight coil head powered by a double Magstim 200 stimulator (Magstim Co, Whitland, United Kingdom) was positioned in the femoral triangle just lateral to the femoral artery in an anatomical position in proximity to the femoral nerve. The output was processed by a Powerlab recording unit (AD Instruments, Oxfordshire, United Kingdom) and analysed using LabChart software (Labchart version 7.1, AD Instruments, Oxfordshire, United Kingdom) sampling at 10 kHz.

At least a 30 second interval was allowed to elapse between each stimulation to avoid potentiation. A series of stimulations at pre-determined levels were performed, and a stimulus response curve constructed to confirm supramaximality (figure 2.3). The mean tension provided by 5 stimulations at 100% magnetic stimulation level was analysed to calculate the twitch quadriceps tension. The degree of twitch potentiation was also assessed by magnetic stimulation at 100% stimulation 5 seconds after a maximal quadriceps volitional contraction manoeuvre.

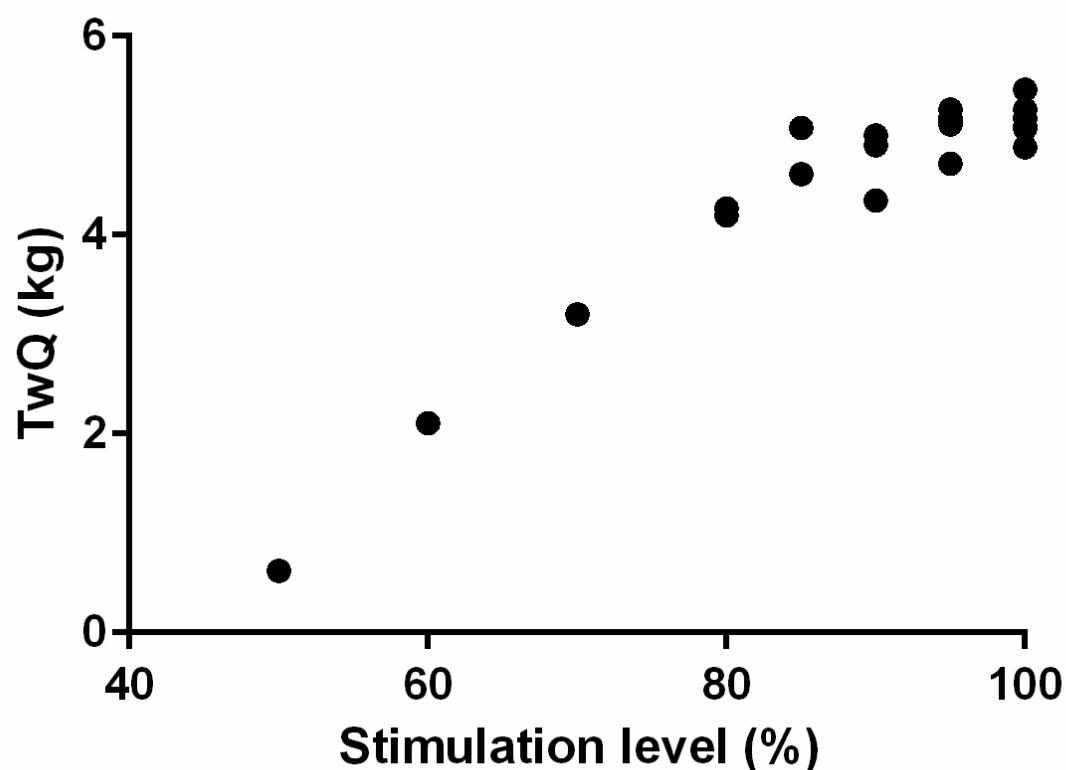


Figure 2.3: Example data of quadriceps twitch (TwQ) response. Note a plateau is reached indicating supramaximality has been achieved.

2.8 Pulmonary function testing

Measurements were made in the lung function department of the Royal Brompton Hospital according to international guidelines and with rigorous quality assurance in place with a Jaeger master lab system (CompactLab system, Jaeger, Wurzburg, Germany). The equipment used underwent daily calibration checks. Spirometry, carbon monoxide diffusing capacity, plethysmographic lung volumes (total lung capacity, residual volume, functional residual capacity) and arteriolised blood gases were measured in accordance with European Respiratory Society (ERS)/ American Thoracic Society (ATS) guidelines (Macintyre, Crapo et al. 2005, Miller, Hankinson et al. 2005, Wanger, Clausen et al. 2005).

Spirometry was performed by asking the subjects to take a deep inspiration to total lung capacity (TLC) and then perform a maximal forced exhalation through a bacterial filter attached to a disposable mouthpiece. This manoeuvre was repeated until 3 values within 5% of each other were obtained, and the highest value for forced expiratory volume in 1 second (FEV₁) and forced vital capacity (FVC) were recorded.

Lung volumes were measured using a body plethysmography technique. Gas transfer was measured by the inhalation of a known concentration of carbon monoxide (CO) and after a single breath hold measuring the end expiratory concentration of CO, with adjustment for alveolar volume.

Standardised lung function testing reference equations were based on the European Coal and Steel Community (ECSC) reference values (Gibson 1993). All lung function tests were performed with patients taking their regular inhaled medication.

2.9 Cycle ergometry testing

2.9.1 Incremental cycle ergometry

A symptom limited incremental exercise test was performed on a cycle ergometer (Ergoselect 100, Ergoline, Bitz, Germany) with metabolic measurements collected using a mouthpiece (Masterscreen

CPX metabolic cart, CareFusion, Basingstoke, UK) and analysed using JLAB software (JLAB Lab Manager version 5.32, Jaeger, CareFusion, Basingstoke, UK). Following a 2 minute rest period and 1 minute free cycling workload increased by 5 Watts every 30 seconds with subjects being asked to maintain a speed of 60-70 revolutions per minute (figure 2.4). Measurements were taken breath-by-breath of pulmonary oxygen uptake (VO_2), carbon dioxide production (VCO_2), minute ventilation (VE), respiratory rate (BF) and tidal volume (VT). These were averaged in the final 30 seconds of the rest period to provide the resting values, and the final 30 seconds of loaded cycling for the peak exercise values, with the exclusion of aberrant breaths causing by coughing and swallowing.

Arteriolised capillary blood gas samples were taken from the earlobe during the rest period and at peak exercise to give oxygen and carbon dioxide tensions. Peak workload was assessed as the highest workload sustained for a 30 second time period. This provides an effective evaluation of whole body exercise capacity, accounting for cardiorespiratory and skeletal muscle function, and has been used in large trials such as the NETT study (Fishman, Martinez et al. 2003). Previous work has shown leg fatigue is more likely to limit cycling-based exercise than walking tasks (Man, Soliman et al. 2003), and thus may be more discriminatory when assessing interventions that may influence skeletal muscle function.



Figure 2.4: Subject performing cycle ergometry testing.

2.9.2 Endurance cycle ergometry

Following a 2 minute rest period and 2 minutes free cycling, the workload increased to 70% of the subject's previous peak workload achieved on incremental cycle ergometry. Subjects were asked to maintain a speed of 60-70 revolutions per minute. Measurements were taken breath-by-breath of pulmonary oxygen uptake (VO_2), carbon dioxide production (VCO_2), minute ventilation (VE), respiratory rate (BF) and tidal volume (VT). The endurance time was calculated as the time from the beginning of loaded cycling to the point of test cessation due to symptom limitation. Rolling 8-breath averages were used in the isotime analysis, with the exclusion of aberrant breaths causing by coughing and swallowing.

2.9.3 Oxygen uptake efficiency slope (OUES)

Using breath-by-breath data measurement was made of the oxygen uptake efficiency slope (OUES). This describes the relationship between oxygen uptake (VO_2 , ml/min) and the logarithm of total ventilation (VE, L/min) during exercise [$\text{VO}_2 = a \log_{10} \text{VE} + b$], the slope of this linear relationship (a) representing the OUES (figure 2.5). The slope was calculated from the initiation of loaded cycling up the ventilatory threshold, if this could be identified, and the line visually inspected for each subject to ensure a linear fit was acceptable. The OUES provides a useful integrated index of cardiovascular, pulmonary and skeletal muscle functional reserve (Baba, Nagashima et al. 1996), a steeper slope representing more efficient oxygen uptake and a shallower slope indicating greater ventilation being required for any given oxygen uptake. The OUES has been shown to be more reproducible than other measures such as VO_2 peak, being reliable even if the subject has exercised with submaximal effort (Hollenberg and Tager 2000). The OUES correlates highly with VO_2 peak and is measurably lower in cardiorespiratory disease states (Baba, Nagashima et al. 1996, Hollenberg and Tager 2000).

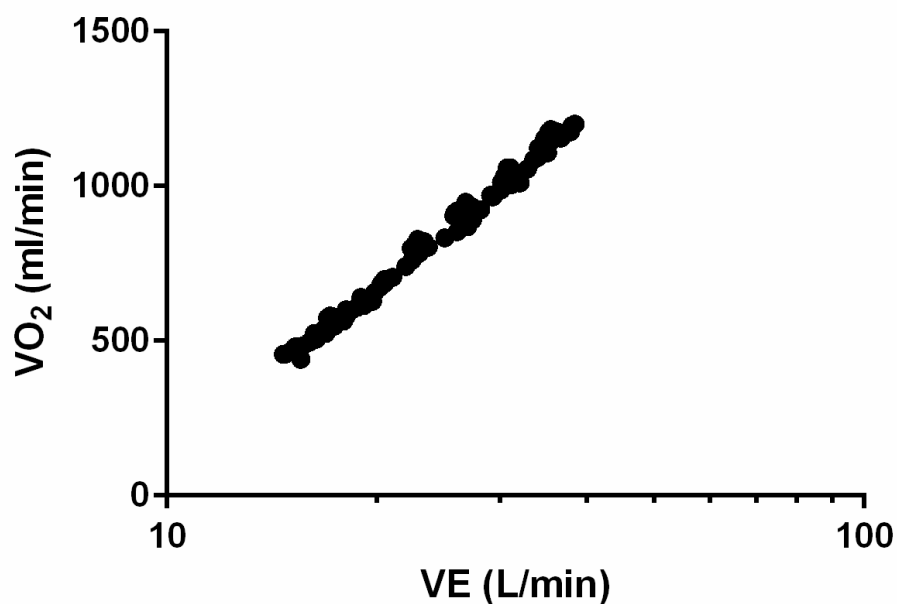


Figure 2.5: Example data of the oxygen uptake efficiency slope (OUES). The pulmonary oxygen uptake (VO_2) is plotted against the logarithm of the minute ventilation (VE), the slope of the relationship representing the OUES.

2.9.4 VE/VCO₂ slope

The slope of minute ventilation (VE) to pulmonary carbon dioxide (VCO₂) production provides a measure of the level of ventilation needed to clear carbon dioxide production during exercise (figure 2.6). This index of ventilatory efficiency is affected by the peripheral muscle ergoreceptor response, pulmonary dead space and pulmonary blood flow. Linear regression was performed of the relationship from the initiation of loaded cycling up to the ventilatory compensation point, or to peak exercise in those individuals that did not meet the ventilatory compensation point. The slope was visually analysed for each subject to ensure it was linear. The VE/VCO₂ slope is known to be elevated in COPD (Paoletti, De Filippis et al. 2011) through a combination of factors, including increased pulmonary dead space leading to ventilation-perfusion mismatching. Some patients with severe emphysema, however, may be limited in their ability to increase minute ventilation in response to the increasing metabolic demands during exercise due to dynamic hyperinflation and the respiratory mechanical constraints this induces. These individuals tend to have low VE/VCO₂ ratios (closer to those seen in health), and because of their inability to increase minute ventilation develop hypercapnia during exercise (Paoletti, De Filippis et al. 2011, Teopompi, Tzani et al. 2014).

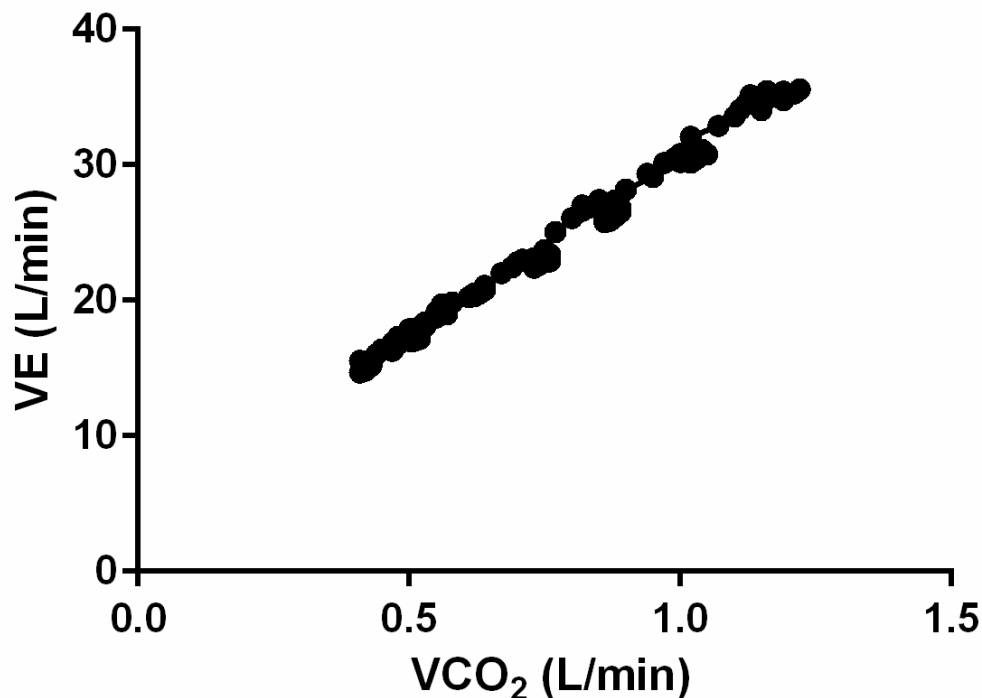


Figure 2.6: Example data of the VE/VCO₂ slope. The slope of minute ventilation (VE) to pulmonary carbon dioxide production (VCO₂) relationship provides a measure of ventilatory efficiency.

2.9.5 VD/VT relationship

The dead space ventilation may be measured using the VD/VT relationship calculated using the equation $VD/VT = (PaCO_2 - P_ECO_2) / PaCO_2$ where VD is the dead space, VT tidal volume, $PaCO_2$ arterial partial pressure of carbon dioxide and P_ECO_2 is the mixed expired partial pressure of carbon dioxide. This provides an index of lung gas exchange efficiency.

2.10 Venous blood sampling and processing

20 ml of blood was obtained by venesection into gel activator tubes for serum and EDTA tubes for plasma collection. Plasma samples were processed within 30 minutes of collection and centrifuged at 4°C and 520 g for 10 minutes. Serum samples were mixed and then left upright at room temperature for 30 minutes to ensure clot formation, and then centrifuged at 18°C and 304 g for 10 minutes. The resulting supernatant was pipetted into 2 ml polypropylene cryotubes, taking care to avoid any contamination from the pellet containing cellular debris, and frozen at -80°C prior to batch analysis.

Routine blood analysis for renal function, C-reactive protein (CRP) and fibrinogen was conducted by the Pathology Department, Royal Brompton and Harefield NHS Foundation Trust.

2.10.1 Serum ACE measurement

Serum ACE was measured using an enzyme assay and spectrophotometric method (Beckman-Coulter, California, USA) performed by the Pathology Department, Royal Brompton and Harefield NHS Foundation Trust. The synthetic tripeptide substrate N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG) is broken down to furylacryloylphenylalanine (FAP) and glycylglycine, accompanied by a decrease in absorbance at 340 nm using a spectrophotometric detector (Maguire and Price 1985). Calibration was provided by test solutions of known ACE concentration to provide an indicator of ACE activity.

2.10.2 Plasma nitrate and nitrite measurement

All plasma samples were obtained by venesection in lithium heparin tubes and immediately spun for 15 minutes at 1620 g and 4°C. The resulting supernatant was pipetted into 2 ml polypropylene cryotubes, taking care to avoid any contamination from the buffy coat containing the white cells/platelets atop the erythrocyte pellet, and immediately frozen at -80°C.

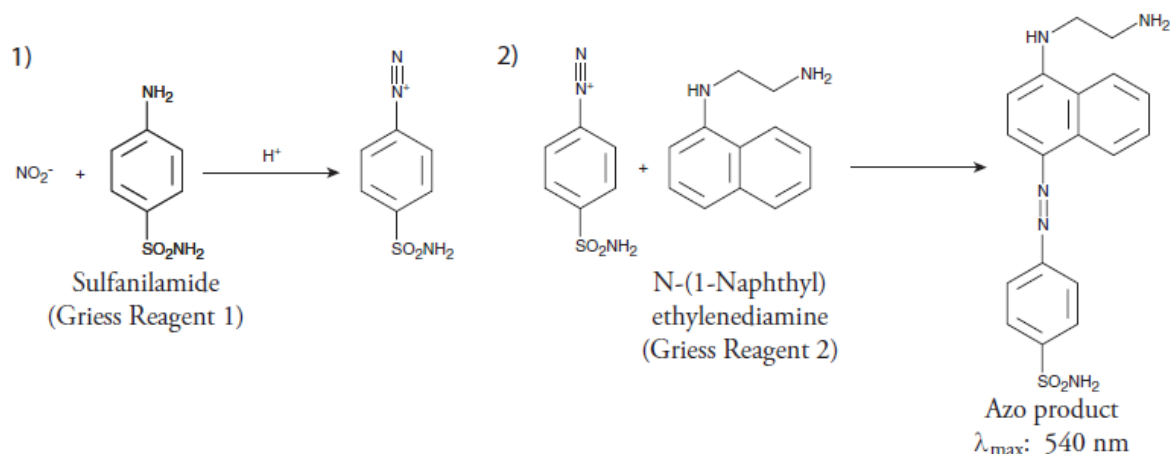


Figure 2.7: Griess reaction for measurement of plasma nitrate metabolites. Image provided by the University of Southampton with permission.

Nitrate and nitrite plasma levels were assessed using a post-column diazo coupling reaction (Griess reaction) in combination with high-performance liquid chromatography (HPLC) (Eicom NOx analyser, ENO-20, San Diego, USA) (Rassaf, Bryan et al. 2002) (figure 2.7). This analysis was performed in conjunction with Ms Magda Minnion at the University of Southampton. The sample was first injected into the pre-column, and then nitrate and nitrite separated on the analytical column where nitrate was retained for longer. Nitrite then passed through the reduction column without any reaction and then reached the Griess reagent containing sulphanilamide and N-1-naphthylethylenediamide dihydrochloride, generating diazo compounds, with absorbance measured at 540 nm using a spectrophotometric detector (Tsikas 2007) (figure 2.8).

Nitrate later passed through the reduction column and was reduced to nitrite, through reaction with cadmium and reduced copper, before undergoing the same diazo coupling reaction, thus appearing

as a second peak (figure 2.9). The peak areas of absorption of solutions of know standard concentrations were then compared to those produced by the test samples to provide measures of plasma nitrate and nitrite.

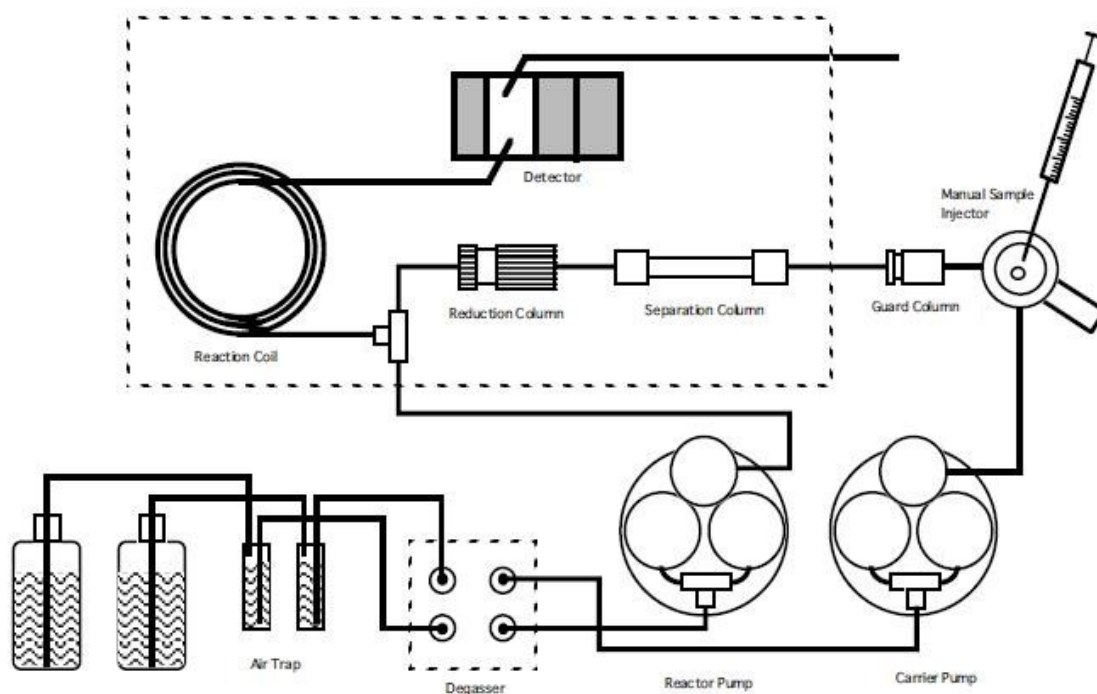


Figure 2.8: Schematic diagram of high-performance liquid chromatography and spectrophotometry method for plasma nitrate and nitrite measurement. Image provided by the University of Southampton with permission.

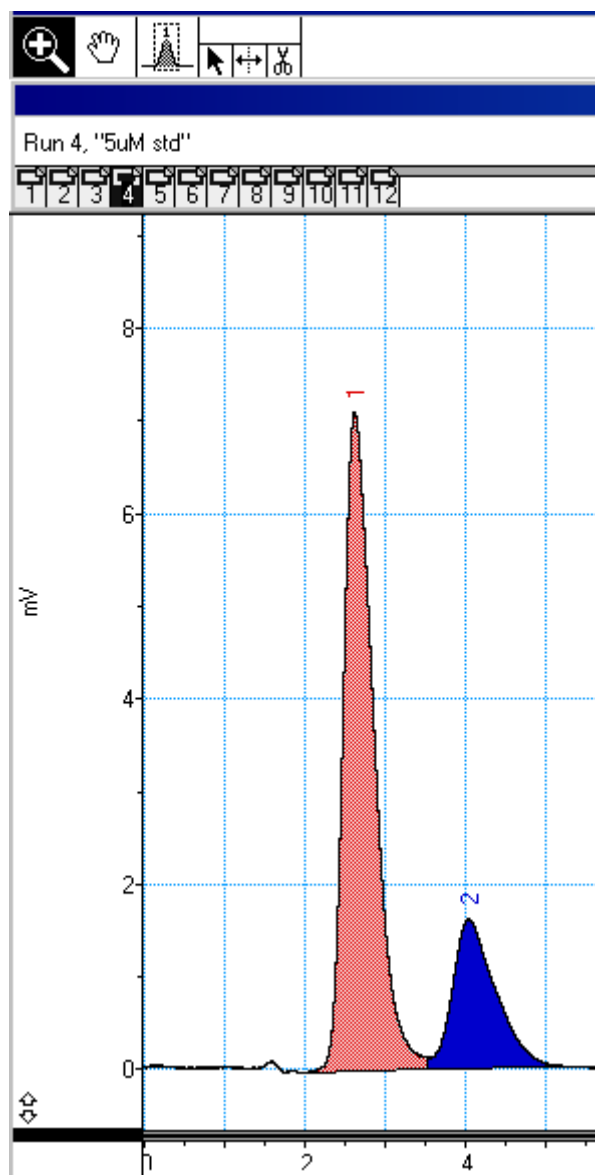


Figure 2.9: Example chromatogram. Peak 1 represents nitrite and 2 represents nitrate. Image provided by the University of Southampton with permission.

2.11 ACE genotyping

Saliva samples were collected using ORAgene DNA self-collection kits (DNA genotek, Ottawa, Canada) from which buccal cells were extracted to obtain DNA. ACE genotyping was conducted by the Department of Cardiovascular Genetics, Rayne Institute, University College London via a polymerase chain reaction (PCR) with amplification using a 3-primer method which included an I-specific oligonucleotide. The use of a primer which anneals to the I-specific oligonucleotide avoids preferential amplification of the shorter D allele and mistyping of the heterozygotes (ID) as homozygous (DD).

Primer ratios corresponded to 50 pmol of *ACE1* (D-specific oligonucleotide) and *ACE3* (common oligonucleotide) and 10 pmol *ACE2* (I-specific oligonucleotide) in a 20 µl reaction. This yielded amplification products of 84 base pairs for the D allele and 65 base pairs for the I allele, which were visualised by using ethidium bromide staining on 7.5% polyacrylamide gels, as has previously been described (O'Dell, Humphries et al. 1995) (figure 2.10). Two independent staff verified the genotype obtained, and replica PCRs were set up without the I-specific primer (*ACE2*) to confirm the presence of the D allele. Any discrepancies were settled by repeat genotyping.

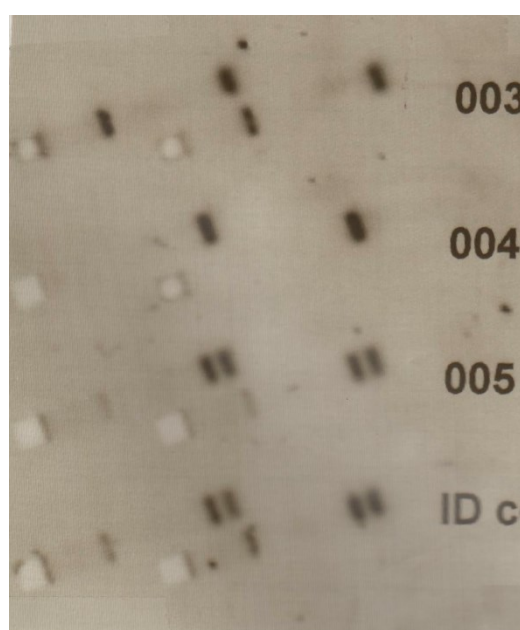


Figure 2.10: Example of PCR plate for ACE genotype. Subject 003 is homozygous for the I allele (II), subject 004 is homozygous for the D allele (DD) and subject 005 is a heterozygote (ID), with a control heterozygote as the lowest band. Image provided by University College London with permission.

2.12 Physical activity monitoring

2.12.1 SenseWear ProArmband

Patients were asked to wear a physical activity monitor to record daily step count and that includes a piezoelectric triaxial accelerometer measuring the body's acceleration in three axes (SenseWear Pro Armband, Bodymedia, Pittsburg, USA). The device also includes physiological measurements of galvanic skin response, heat flux and skin temperature. These are incorporated to provide an

estimate of energy expenditure, which has been validated against the gold standard of indirect calorimetry in a COPD population (Hill, Dolmage et al. 2010, Van Remoortel, Raste et al. 2012).

The armband was placed over the body of the triceps muscle of the right arm (figure 2.11), and the subject asked to wear it continuously for a week except when bathing and showering. Data was analysed over a period of 5 days including 2 weekend days, with a minimum of 92% duration on body taken as sufficient recording time to allow accurate analysis. Average daily step count and physical activity were analysed from the collected data using SenseWear investigational software (SenseWear Professional version 7.0, Bodymedia, Pittsburgh, USA).



Figure 2.11: SenseWear Pro Armband in situ.

Measurements of total energy expenditure (TEE) and sleep energy expenditure, as a marker of resting energy expenditure (REE), were used to calculate the physical activity level ($PAL = TEE/REE$). A $PAL \geq 1.70$ indicates a moderately to extremely active person, 1.40-1.69 indicates a sedentary person and $PAL < 1.40$ indicates an extremely inactive person (Manini, Everhart et al. 2006).

2.13 Health-related quality of life assessments

2.13.1 The Medical Research Council (MRC) dyspnoea scale

The MRC dyspnoea scale comprises 5 statements (figure 2.12) that encompass the range of respiratory disability from none (grade 1) to incapacitating dyspnoea (grade 5). The score correlates well with the results of both other breathlessness scales and with lung function measurements (Stenton 2008).

The MRC Breathlessness Scale

Grade	Degree of breathlessness related to activities
1	Not troubled by breathlessness except on strenuous exercise
2	Short of breath when hurrying on the level or walking up a slight hill
3	Walks slower than most people on the level, stops after a mile or so, or stops after 15 minutes walking at own pace
4	Stops for breath after walking about 100 yds or after a few minutes on level ground
5	Too breathless to leave the house, or breathless when undressing

Figure 2.12: Medical Research Council (MRC) dyspnoea scale.

2.13.2 St. George's Respiratory Questionnaire for COPD (SGRQ-C)

The SGRQ-C is a 40 item self-completed questionnaire that assesses 3 domains of health status in COPD patients; symptoms, activities and impacts on daily life (see Appendix). Each domain is scored out of 100, a higher score being indicative of poorer health status, and a total score is calculated from the individual component domains. The SGRQ-C is validated in assessing health-related quality of life in COPD and has been proven to be responsive to intervention (Meguro, Barley et al. 2007). A change of 4 points is considered clinically significant (Jones 2002, Schunemann, Griffith et al. 2003, Jones 2005).

2.13.3 COPD Assessment Test (CAT)

The COPD Assessment Test is an 8 item self-completed questionnaire where subjects rate their responses on a scale of 0-5, the maximum total score being 40 and a higher score being indicative of poorer health status (figure 2.13). This questionnaire is simple and easy to complete independently and thus provides a user-friendly method of assessing health-related quality of life in COPD. The CAT is validated in assessing health status in COPD (Jones, Harding et al. 2009), and a change of 2 points is considered likely to represent a clinically significant difference (Kon, Canavan et al. 2014).

Your name :

Today's date:



COPD Assessment Test

How is your COPD? Take the COPD Assessment Test™ (CAT)

This questionnaire will help you and your healthcare professional measure the impact COPD (Chronic Obstructive Pulmonary Disease) is having on your wellbeing and daily life. Your answers, and test score, can be used by you and your healthcare professional to help improve the management of your COPD and get the greatest benefit from treatment.

For each item below, place a mark (X) in the box that best describes you currently. Be sure to only select one response for each question.

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

	(0) (1) (2) (3) (4) (5)		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			

COPD Assessment Test and CAT logo is a trademark of the GlaxoSmithKline group of companies.
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Figure 2.13: COPD Assessment Test (CAT) questionnaire.

2.14 Pulmonary rehabilitation programme

Patients attended a standardised pulmonary rehabilitation programme delivered by a multi-disciplinary team and lead by a senior physiotherapist as per national and international guidelines (Nici, Donner et al. 2006, Bolton, Bevan-Smith et al. 2013). This programme was of eight weeks duration, delivering 3 sessions per week of exercise, 2 under direct supervision and 1 for the patient to undertake independently at home. The circuit-style programme was prescribed for the individual patient based on their baseline exercise capacity and was progressive in nature, including frequent re-evaluation and goal-setting. This was conducted alongside a regular educational component incorporating disease lifestyle and self-management advice. Each programme included regular aerobic training, particularly of the lower limbs, alongside strength training of both lower and upper limbs. Aerobic training included both treadmill and cycle exercise, with individuals prescribed exercise intensity at 60-80% predicted VO_2 peak, and record made of the time and resistance to inform goal setting. Strengthening exercises included both upper and lower resistive exercise with weights, including sit-to-stand, knee lifts/extension, bicep curls and push ups, with individuals recording the weight used and number of repetitions to allow progression. Education classes covered a variety of topics to encourage active patient participation and self-management, including diet, medication use, physical activity, coping methods, psychology, and recognising and management exacerbations.

Chapter 3: ACE genotype and Exercise Characteristics in COPD

3.1 Introduction

3.1.1 Background

The Angiotensin Converting Enzyme (ACE) gene, located on chromosome 17q23, demonstrates a functional polymorphism dependent on the presence (insertion, I allele) or absence (deletion, D allele) within an intron of a 287-base pair non-coding DNA domain. This results in three distinct genotypes, either homozygous (II or DD) or heterozygous (ID), with the genotypes having an approximate distribution of 25%, 25% and 50% respectively in a Caucasian population (Jones and Woods 2003). ACE activity is highest in those homozygous for the D allele (DD), intermediate in heterozygotes (ID) and lowest in those homozygous for the I allele (II). The I allele is thus associated with both reduced tissue (Costerousse, Allegrini et al. 1993) and serum (Brown, Blais et al. 1998) ACE activity, and in those possessing the I allele, angiotensin II levels are lower and bradykinin levels (whose breakdown is catalysed by the ACE enzyme) higher (Brown, Blais et al. 1998).

Research has suggested serum ACE levels may be elevated in COPD (Brice, Friedlander et al. 1995, Ucar, Yildirim et al. 1997, Ahsan, Ram et al. 2004), and thus some investigators have hypothesised a potential role for the ACE gene in the development of this disease (Busquets, MacFarlane et al. 2007, Simsek, Tekes et al. 2013). Given that angiotensin II is known to contribute to systemic inflammation it is feasible that intrinsically high levels of angiotensin II may invoke an exaggerated response to inhaled irritants, and an increased likelihood of developing COPD in those exposed to such agents. However, meta-analyses of several large studies suggest no real relationship exists between ACE genotype and COPD development (Lee, Nordestgaard et al. 2009, Pabst, Theis et al. 2009, Li, Wei et al. 2012, Li, Lan et al. 2013), with the exception of the possibility of an association between the DD genotype and the development of COPD in Asian populations (Li, Lan et al. 2013).

Despite these findings, it remains possible that ACE genotype status may in part determine the phenotype of those patients with already established disease, with evidence suggesting the I allele may be associated with a more stable disease course (Pabst, Theis et al. 2009). The German study by Pabst et al. studied 152 Caucasian patients with COPD compared to 158 healthy control subjects, with no indication of any association between the diagnosis of COPD and a distinct ACE gene polymorphism. In the COPD cohort, however, a significant association was found between the I

allele and a stable disease course, diagnosed as less than 3 hospitalisations over the preceding 3 years for pulmonary exacerbations. Regression analysis continued to demonstrate this relationship even when accounting for age, sex and smoking habits. The suggestion has thus been made that the I allele could be a disease-modifying gene, influencing an exacerbation phenotype.

The ACE gene is well recognised to contribute to exercise capacity in athletic individuals, with the presence of the I or D allele a contributor to determine endurance versus strength capacity. This has been an area of avid interest in groups exploring athletic potential, with the panacea of finding a gene target that may be the subject of future manipulation. Homozygosity for the I allele of the ACE gene (ACE-II genotype) is associated with improved endurance exercise performance (Montgomery, Marshall et al. 1998, Myerson, Hemingway et al. 1999, Zhang, Tanaka et al. 2003). This has been demonstrated in several independent cohorts and exercise modalities, including high altitude performance, long-distance running and repetitive elbow extension exercises. Conversely the D allele of the ACE gene has been linked to elite power-orientated athletic performance, such as sprinting and short-distance swimming (Myerson, Hemingway et al. 1999, Woods, Hickman et al. 2001).

3.1.2 Rationale and hypothesis

In COPD there is a general paucity of data relating to the potential role of the ACE genotype in determining the exercise characteristics of this population. In a group of 103 stable outpatients with COPD it has been shown that the presence of the deletion (D) allele is associated with preserved isometric quadriceps strength, with volitional testing showing a rising quadriceps strength associated with the number of D alleles (quadriceps maximal volitional contraction expressed as mean $\pm$ standard deviation, II genotype 31.4 $\pm$ 10.8 kg; ID genotype 34.1 $\pm$ 13.0 kg; DD genotype 38.3 $\pm$ 11.6 kg; $p=0.04$ linear trend), with the relationship strengthened further when account is made of fat free mass. A similar pattern was noted when non-volitional methods of testing were used (Hopkinson, Nickol et al. 2004, Hopkinson, Eleftheriou et al. 2006). In addition, further work has shown greater gain of strength following a 9 week isometric quadriceps strength training programme in healthy male D allele carriers (Folland, Leach et al. 2000). Of note, however, this gain was not seen in a manner associated with allele frequency which may call in question a mechanistic role for the D allele in this association (Folland, Leach et al. 2000). The present data does suggest that the D allele is important for strength in COPD, as is noted in healthy populations.

Limited work has been completed looking at the role of the ACE genotype in determining exercise characteristics in individuals with COPD. In a study of 61 COPD patients of Chinese ethnic origin comparison was made to 57 healthy control subjects, demonstrating maximal workload and aerobic work efficiency was greatest in those COPD patients possessing the II genotype, rather than ID or DD (Zhang, Wang et al. 2008). This indicates that the ACE pathway may be involved in the aerobic efficiency of skeletal muscle in COPD patients, in a manner that was not evident in control subjects. Thus far the influence of the ACE genotype on exercise characteristics has yet to be investigated in a non-Asian population and, given possible genotype influences on COPD development in such an ethnic group, this is an area that merits further work. In addition, given the possible multiple levels at which the renin-angiotensin system may influence exercise, exploring the influence of ACE genotype on integrated measures of exercise performance such as the oxygen uptake efficiency slope which integrates pulmonary, cardiovascular and skeletal muscle efficiency, is an area of interest. We hypothesised that measures of exercise capacity, assessed by incremental cycle ergometry, would be greater in COPD subjects possessing the I allele of the ACE gene.

3.2 Methods

3.2.1 Patient selection

All participants provided written informed consent prior to enrolment in the study which was conducted in line with the principles of the Declaration of Helsinki. This cross-sectional study recruited COPD patients identified through outpatient clinics at the Royal Brompton Hospital and through public outreach events including World COPD Day. A diagnosis of COPD was made in line with NICE and GOLD criteria (Rabe, Hurd et al. 2007, O'Reilly, Jones et al. 2010).

Study inclusion criteria included subjects diagnosed with COPD with evidence of at least moderate airflow obstruction ($FEV_1 < 80\%$ predicted, GOLD II-IV (Rabe, Hurd et al. 2007)). Exclusion criteria included patients within three months of pulmonary rehabilitation or within one month of a pulmonary exacerbation, those already taking medications influencing the renin-angiotensin system (ACE-inhibitors or angiotensin-receptor blockers), pregnancy and any other significant comorbidity that would limit subject mobility or ability to complete an exercise test. As this study used baseline data from an interventional study of ACE-inhibition (chapter 4) subjects were also excluded if they

possessed any pre-existing diseases that would either benefit from (ischaemic heart disease, cardiac failure and diabetes mellitus) or be adversely influenced by ACE-inhibition (renovascular disease), or had impaired renal function ($\text{eGFR} < 50 \text{ ml/min/1.73m}^2$).

3.2.2 Study design

This was an observational cross-sectional study. Baseline data was taken from the interventional study of ACE-inhibition as an adjunct to pulmonary rehabilitation (chapter 4) and thus the sample selected was a convenience sample.

3.2.3 Study measurements

All subjects underwent a clinical assessment including the recording of a clinical history and cardio-respiratory examination. Anthropometric measurements were made and bioimpedance used to assess the lean body compartment and fat free mass index (FFMI). ACE genotyping was undertaken for all subjects using a 3-primer polymerase chain reaction (PCR) amplification method. Health-related quality of life was assessed using the COPD Assessment Test (CAT) and St. George's Respiratory Questionnaire for COPD (SGRQ-C), and baseline dyspnoea burden was assessed using the Medical Research Council (MRC) dyspnoea scale.

All subjects underwent assessment of their daily physical activity levels using a multisensor armband, and calculations were made of daily step count and physical activity level (PAL) over a period of 5 consecutive days including 2 weekend days. Quadriceps strength was measured using both volitional and non-volitional methods, using the technique of supramaximal magnetic femoral nerve stimulation. Subjects completed full pulmonary function testing, including measures of lung volumes and gas transfer, and an incremental cycle ergometry test to exhaustion with continuous metabolic measurements.

Peak exercise values were those obtained during the final 30 seconds of the exercise test when the revolutions per minute (rpm) was still appropriately maintained. The slope of the minute ventilation and pulmonary carbon dioxide production (VE/VCO_2) was used to reflect the efficiency of the ventilatory response during exercise. Exercise efficiency was expressed as the peak oxygen

consumption as a function of the peak workload (VO_2 peak/WR peak), and calculation was made of the oxygen uptake efficiency slope (OUES) an index of cardiopulmonary and skeletal muscle reserve.

Further details of the conduct of the phenotypic assessments, conduct of the cardiopulmonary test examination and ACE genotyping using a polymerase chain reaction (PCR) method can be found in Chapter 2 (Methods).

3.2.4 Primary and secondary outcome measures

1. Effect of ACE genotype on percentage predicted peak pulmonary oxygen uptake achieved during incremental cycle ergometry – Primary outcome measure
2. Effect of ACE genotype on quadriceps strength measured using volitional and non-volitional methods
3. Effect of ACE genotype on serum ACE levels
4. Effect of ACE genotype on the oxygen uptake efficiency slope (OUES) and ventilatory efficiency (VE/VCO_2 slope)

3.2.5 Data analysis and statistics

Data are presented as mean $\pm$ standard deviation and analysed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, California, USA). Actual genotype frequencies were compared with expected frequencies predicted by the Hardy-Weinberg equilibrium equation, and Pearson's Chi-Squared test used to assess for any significant deviation from equilibrium and for the effect of genotype on categorical variables. Between groups comparisons were conducted using one-way analysis of variance (ANOVA), followed by post-hoc correction for more than 2 groups using Tukey's multiple comparisons test. When genotypes were grouped and then compared to allow assessment of importance of the I allele, comparison was performed using 2-tailed, unpaired t-tests. The relationships between percentage predicted peak pulmonary oxygen uptake, age, quadriceps strength, FFMI and pulmonary function parameters were analysed using both univariate and multivariate linear regression models. A p value < 0.05 was considered to be statistically significant.

3.3 Results

3.3.1. Recruitment process

Of the 80 subjects recruited to the interventional study (Chapter 4), 78 subjects completed both baseline phenotyping visits, with 2 subjects withdrawing consent prior to completion of these visits. All 78 subjects completed an incremental cycle ergometry test which was symptom-limited in duration. 59 subjects recorded a valid period of physical activity monitoring allowing analysis, with the remaining 19 subjects recording less than the pre-specified 92% on body time over 5 consecutive days including a weekend. No patients were taking long-term corticosteroids and all were of Caucasian origin, with the exception of one female of Afro-Caribbean origin.

3.3.2 ACE genotype frequencies

Among 78 subjects with COPD, 16 possessed the ACE II genotype polymorphism (21%), 37 the ID genotype (47%) and 25 the DD genotype (32%) (figure 3.1). In this cohort the ACE genotype distribution was similar to that previously reported in UK Caucasian populations (Myerson, Hemingway et al. 1999, Hopkinson, Nickol et al. 2004) and was consistent with Hardy-Weinberg equilibrium ($\chi^2=1.12$, $p=0.57$).

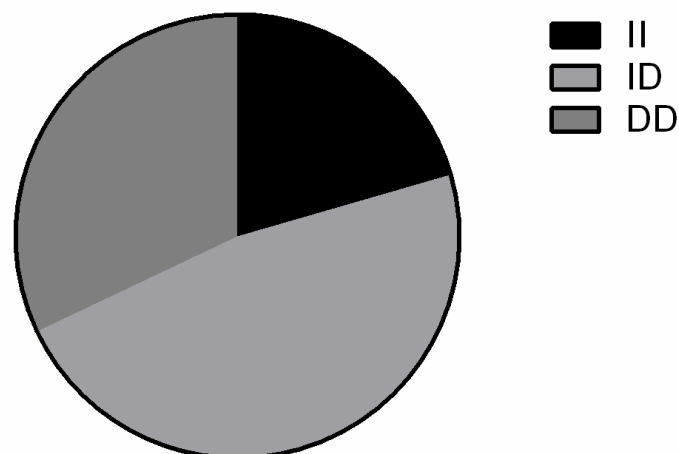


Figure 3.1: Observed ACE genotype frequency in the COPD cohort. I indicates the insertion allele and D the deletion allele. The frequencies observed were consistent with Hardy-Weinberg equilibrium.

3.3.3 ACE genotype and serum ACE level

The serum ACE levels measured in the different genotypes followed the expected pattern, with lower levels of ACE activity in those homozygous for the I allele, intermediate in the ID heterozygote group and the highest levels in those homozygous for the D allele. Thus the serum ACE activity level increased with the frequency of the D allele possessed (II 19 ± 8 IU/L < ID 31 ± 18 IU/L < DD 45 ± 22 IU/L; $p < 0.0001$; figure 3.2).

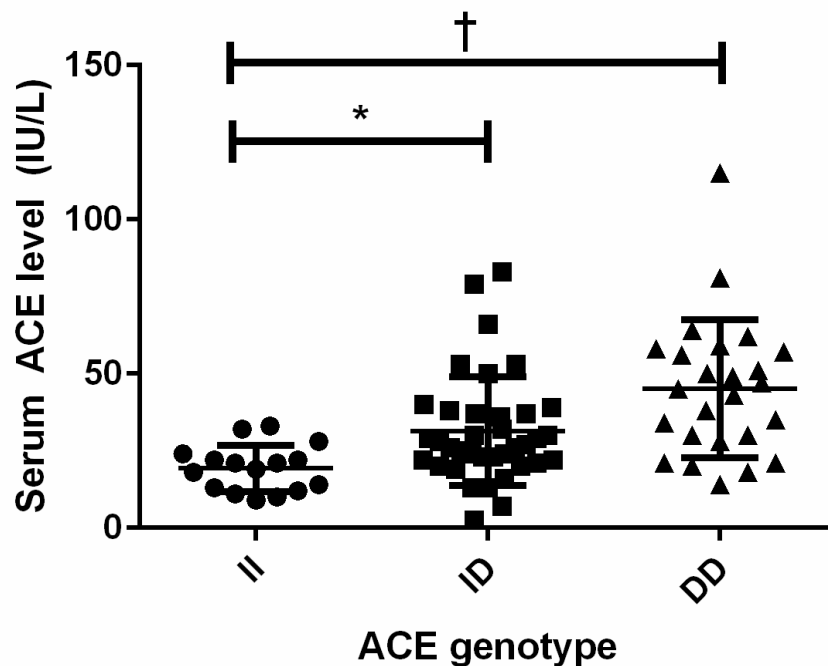


Figure 3.2: Serum ACE levels as related to genotype. The mean and standard deviation are represented by the line and cross-bar respectively; *significantly different from II genotype, $p < 0.05$; †significantly different from II genotype, $p < 0.0001$. There was a numerically higher ACE serum level in the DD genotype group versus the ID genotype group although this just failed to reach statistical significance ($p = 0.05$).

Abbreviations: II – II genotype; ID – ID genotype; DD – DD genotype.

3.3.4 Phenotypic characteristics of COPD patients by ACE genotype

There was no evidence of an association between ACE genotype and sex, age, body mass index, pulmonary exacerbation frequency or total prednisolone use in the preceding 12 months (table 3.1).

Baseline pulmonary function measures were not significantly different when comparing individual genotypes. However, when comparison was made of the DD genotype with the II and ID genotypes grouped there was a reduction in hyperinflation markers (residual volume (RV) and functional residual capacity (FRC) percentage predicted) in the DD group (table 3.1).

	II (n=16)	ID (n=37)	DD (n=25)	p value ANOVA	DD vs. II/ID
Age (years)	66 (6)	67 (8)	68 (10)	0.60	0.37
Sex (% female)	44	49	48	0.95	0.95
BMI (kg/m ²)	27.8 (6.2)	24.8 (5.3)	25.4 (6.4)	0.34	0.86
Smoking history (pack years)	58 (49)	49 (31)	39 (23)	0.20	0.12
Current smokers (%)	19	16	20	0.93	0.75
Exacerbation frequency*	2 (2)	3 (3)	3 (3)	0.40	0.25
Prednisolone use (mg)*	476 (439)	423 (530)	443 (714)	0.41	0.30
LAMA (%)	88	81	72	0.47	0.27
ICS-LABA combination (%)	81	84	64	0.18	0.06
MRC dyspnoea score	3 (1)	3 (1)	3 (1)	0.42	0.50
CAT score	18 (6)	19 (8)	17 (7)	0.47	0.25
SGRQ-C total score	48.15 (13.96)	50.18 (19.31)	44.86 (18.45)	0.53	0.28
SGRQ-C symptoms score	62.91 (18.12)	62.39 (19.98)	56.70 (21.73)	0.47	0.26
SGRQ-C activity score	69.86 (20.50)	70.61 (22.70)	63.99 (21.98)	0.49	0.23
SGRQ-C impacts score	30.19 (12.10)	33.91 (20.54)	29.18 (20.63)	0.60	0.44
FEV ₁ (L)	1.12 (0.43)	1.13 (0.58)	1.25 (0.49)	0.65	0.35
FEV ₁ (% predicted)	46.6 (22.4)	44.8 (20.2)	53.6 (19.2)	0.27	0.12
FVC (L)	3.32 (0.76)	3.04 (0.77)	3.02 (0.80)	0.43	0.57
TLCO _c (% predicted)	56.2 (26.1)	46.0 (19.4)	53.7 (23.0)	0.22	0.42
TLC (L)	7.60 (2.02)	7.39 (1.68)	6.57 (1.62)	0.11	0.037
TLC (% predicted)	129 (17)	127 (19)	117 (19)	0.07	0.021
RV (L)	4.18 (1.56)	4.29 (1.37)	3.55 (1.25)	0.10	0.06
RV (% predicted)	183 (53)	191 (57)	160 (53)	0.058	0.019
RV/TLC ratio (%)	54.0 (8.1)	57.5 (8.7)	53.4 (8.7)	0.14	0.15
FRC (% predicted)	161 (40)	169 (43)	145 (40)	0.08	0.035
PaO ₂ (kPa)	10.1 (1.3)	10.2 (1.7)	10.6 (1.5)	0.52	0.28
PaCO ₂ (kPa)	4.8 (0.6)	4.8 (0.7)	4.8 (0.4)	0.93	0.73
Daily step count [‡]	4844 (3889)	4863 (3503)	6633 (3508)	0.22	0.079
Daily PAL [‡]	1.39 (0.24)	1.45 (0.17)	1.45 (0.25)	0.63	0.63

Table 3.1: Baseline phenotypic characteristics of the subjects by ACE genotype.

Values are expressed as mean (standard deviation), except the categorical variables. The individual genotypes are compared by one-way ANOVA testing. Comparison is also made of the II/ID combined and DD genotypes using unpaired t-tests.

Abbreviations: II - II genotype; ID - ID genotype; DD - DD genotype; BMI - body mass index; LABA - long-acting beta agonist; LAMA - long-acting muscarinic antagonist; ICS - inhaled corticosteroid; MRC - Medical Research Council; CAT - COPD assessment test; SGRQ-C - St. George's respiratory questionnaire for COPD; FEV₁ - forced expiratory volume in 1 second; FVC - forced vital capacity; TLCO_c - carbon monoxide diffusing capacity; TLC - total lung capacity; RV - residual volume; FRC - functional residual capacity; PaO₂ - partial pressure of oxygen in arterialised blood gas; PaCO₂ - partial pressure of carbon dioxide in arterialised blood gas; PAL - physical activity level.

*Refers to number of pulmonary exacerbations requiring an increase in usual medication and dose of prednisolone received in the preceding 12 months

†59 patients had a minimum period of 92% on body sampling time over 5 days including 2 weekend days for the physical activity monitoring (14 II, 26 ID, 19 DD)

3.3.5 Muscle bulk and strength characteristics of COPD subjects by ACE genotype

Absolute fat free mass was not different between ACE genotypes (table 3.2). When considering the fat free mass index, ANOVA testing suggested a significant difference, however, post-hoc multiple comparisons showed no significant difference when comparing genotypes, although a trend towards significance was noted when comparing the II and ID groups (p=0.061; table 3.2).

	II	ID	DD	p value	DD vs.
	(n=16)	(n=37)	(n=25)	ANOVA	II/ID
FFM (kg)	47.4 (6.9)	44.1 (8.2)	45.7 (10.0)	0.37	0.86
FFMI (kg/m²)	17.2 (2.5)	15.7 (2.0)	16.8 (2.4)	0.038	0.23
MTMCSA (mm ²)	10683 (2171)	9508 (4827)	9762 (2626)	0.58	0.91
Quadriceps CSA (mm ²)	4649 (1049)	3867 (1194)	4406 (1174)	0.05	0.30
QMVC (kg)	32.3 (8.4)	27.1 (11.6)	32.2 (9.7)	0.10	0.17
QMVC (% predicted)	76.2 (16.8)	67.2 (22.7)	80.9 (20.7)	0.040	0.035
QMVC/FFM	0.68 (0.16)	0.60 (0.20)	0.71 (0.19)	0.08	0.073
QMVC/BMI	1.22 (0.43)	1.11 (0.43)	1.31 (0.45)	0.21	0.12
TwQ (kg)*	5.41 (4.5)	5.0 (2.8)	6.2 (3.2)	0.45	0.23

Table 3.2: Muscle mass and strength characteristics of the subjects by ACE genotype. Values are expressed as mean (standard deviation) and individual genotypes are compared by one-way ANOVA testing. Comparison is also made of the II/ID combined and DD genotypes using unpaired t-tests.

Abbreviations: II – II genotype; ID – ID genotype; DD – DD genotype; FFM – fat free mass; FFMI – fat free mass index; MTM – mid-thigh muscle; CSA - cross-sectional area; QMVC – quadriceps maximal volitional contraction; TwQ – quadriceps twitch response.

*64 patients had quadriceps twitch readings reaching supramaximality that were able to be assessed (14 II, 30 ID, 20 DD)

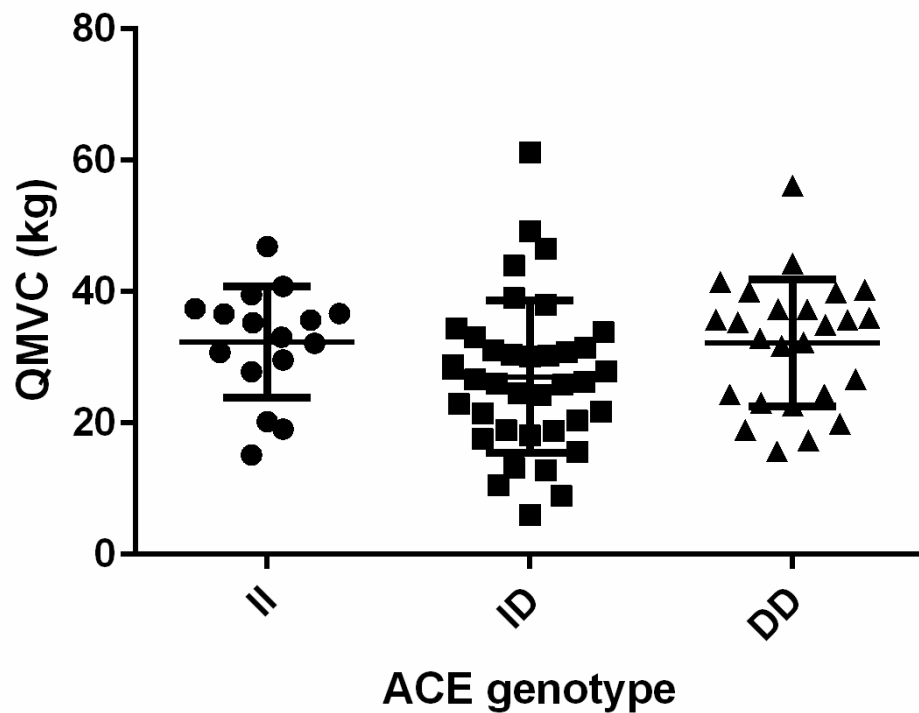


Figure 3.3: Quadriceps maximal volitional contraction versus ACE genotype in COPD subjects. The mean and standard deviation are represented by the line and cross-bar respectively. ANOVA – no significant difference between genotypes.

Abbreviations: II – II genotype; ID – ID genotype; DD – DD genotype; QMVC – quadriceps maximal volitional contraction.

Although no genotype dependent relationship with the absolute quadriceps maximal volitional contraction was identified (figure 3.3), using disease-specific prediction equations the percentage predicted QMVC was significantly higher in the DD genotype group compared to the ID group ($p=0.036$; table 3.2 and figure 3.4). There was also a numerical increase recorded in the QMVC/FFM ratio in the DD genotype group, although this failed to reach statistical significance (table 3.2 and figure 3.5).

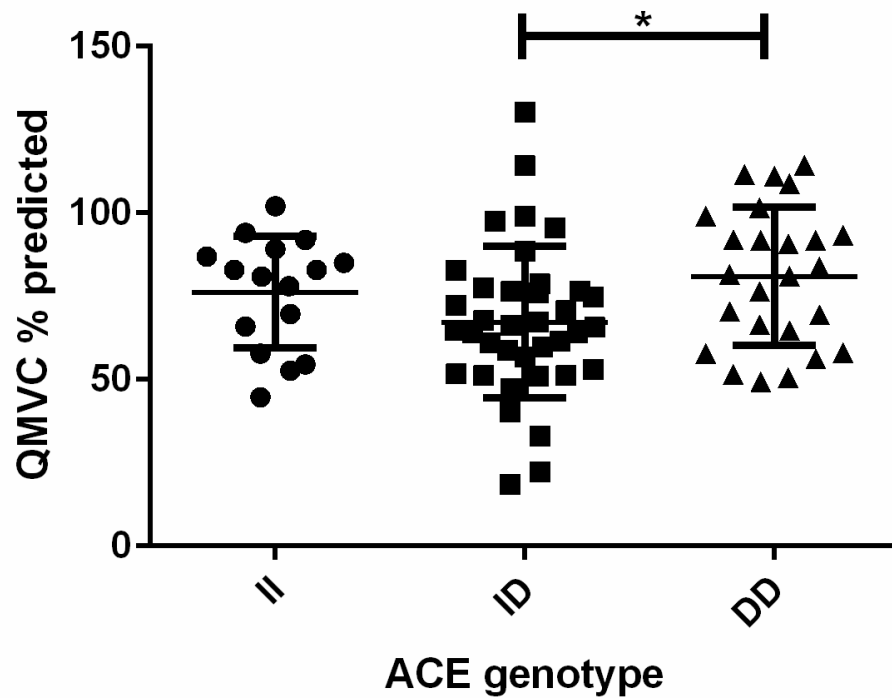


Figure 3.4: Quadriceps maximal volitional contraction percentage predicted versus ACE genotype in COPD subjects. The mean and standard deviation are represented by the line and cross-bar respectively. ANOVA – $p=0.040$, post-hoc Tukey’s multiple comparisons test showing a significant difference between the ID and DD genotypes ($p=0.036$).

Abbreviations: II – II genotype; ID – ID genotype; DD – DD genotype; QMVC – quadriceps maximal volitional contraction.

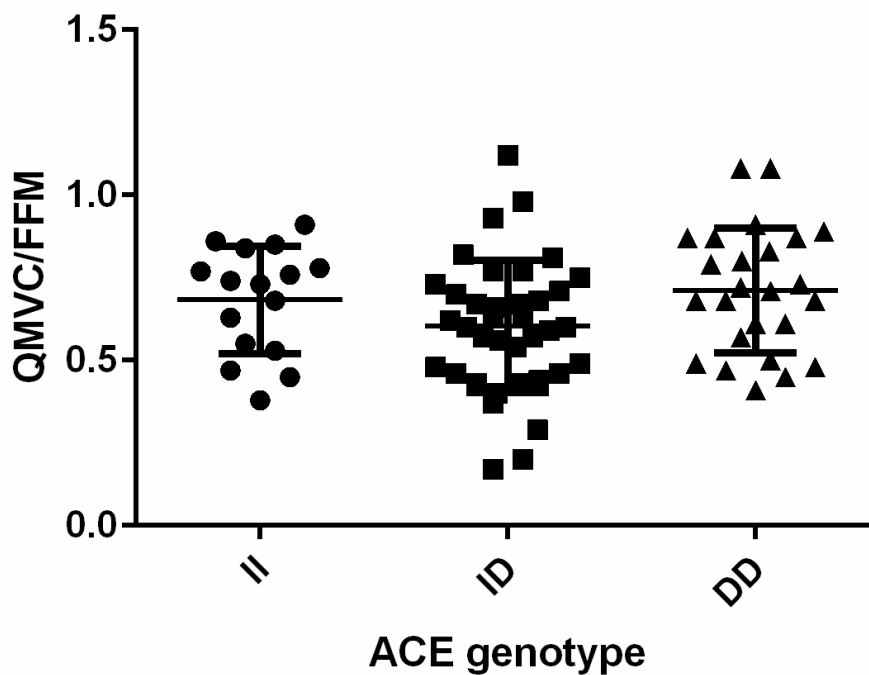


Figure 3.5: Quadriceps maximal volitional contraction corrected for fat free mass versus ACE genotype in COPD subjects. The mean and standard deviation are represented by the line and cross-bar respectively. ANOVA – no significant difference between genotypes.

Abbreviations: II – II genotype; ID – ID genotype; DD – DD genotype; QMVC – quadriceps maximal volitional contraction; FFM – fat free mass.

Muscle bulk assessed through computed tomography scanning did not show any differences in either mid-thigh muscle or quadriceps cross-sectional areas between genotypes (table 3.2 and figure 3.6).

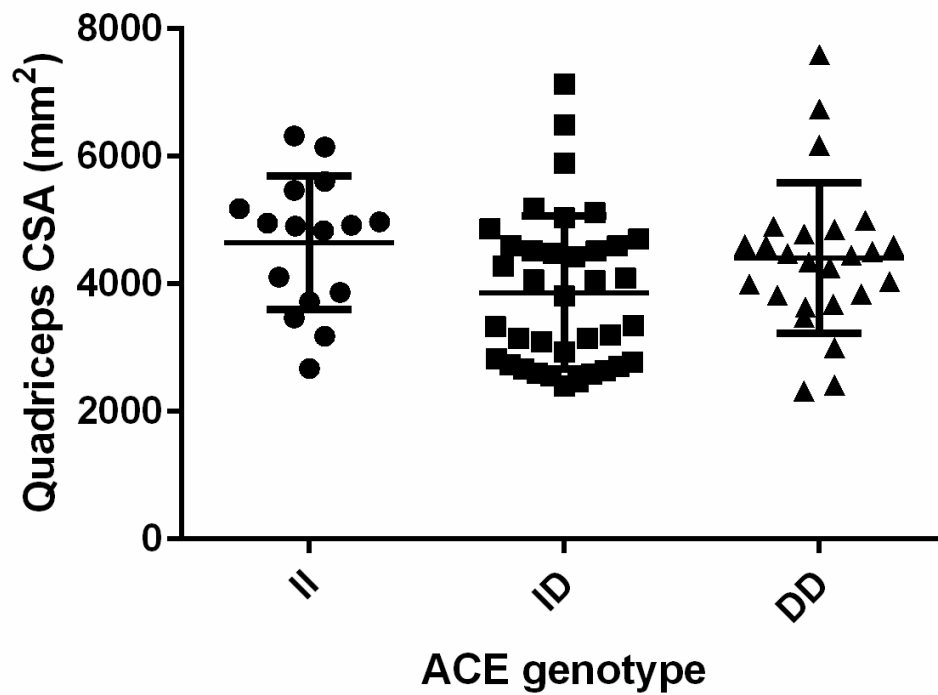


Figure 3.6: Quadriceps cross-sectional area versus ACE genotype in COPD subjects. The mean and standard deviation are represented by the line and cross-bar respectively. ANOVA – no significant difference between genotypes.

Abbreviations: II – II genotype; ID – ID genotype; DD – DD genotype; CSA – cross-sectional area.

In COPD subjects, quadriceps maximal volitional contraction showed a positive association with both fat free mass ($r=0.575$, $p<0.0001$; figure 3.7) and quadriceps cross-sectional area ($r=0.671$, $p<0.0001$; figure 3.7).

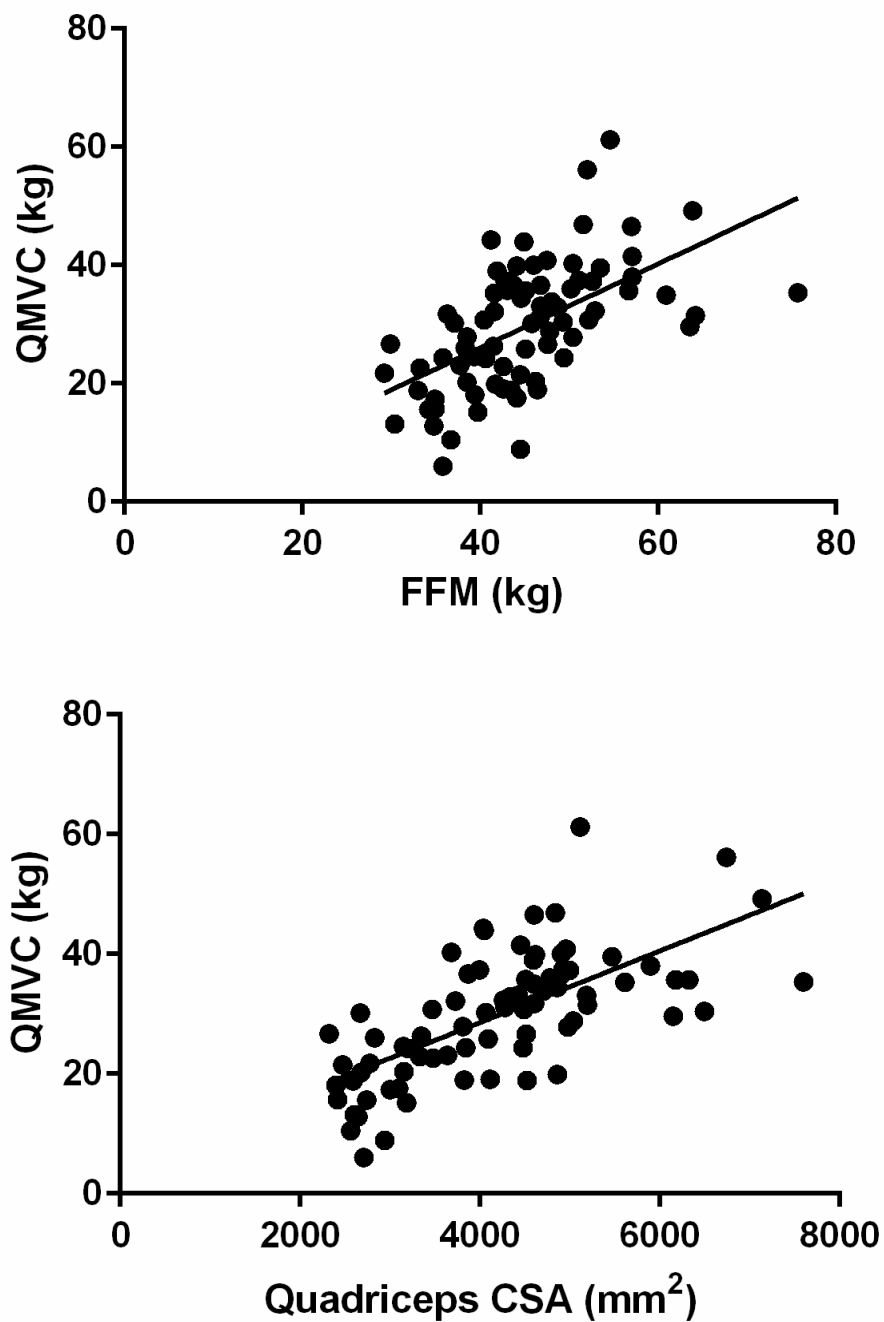


Figure 3.7: Quadriceps maximal volitional contraction versus fat free mass and quadriceps cross-sectional area in COPD subjects. QMVC vs. FFM upper panel, Pearson correlation $r=0.575$, $p<0.0001$; QMVC vs. quadriceps cross-sectional area lower panel, Pearson correlation $r=0.671$, $p<0.0001$. Fat free mass was assessed by bioimpedance and quadriceps cross-sectional area by computed tomography.

Abbreviations: QMVC – quadriceps maximal volitional contraction; FFM – fat free mass; CSA – cross-sectional area.

3.3.6 Exercise characteristics of COPD subjects by ACE genotype

Parameters relating to maximal exercise capacity were not significantly different between the genotype groups (table 3.3), including peak power and peak oxygen uptake, whether expressed as absolute values or percentage of the predicted value achieved. Assessment of ventilatory efficiency (VE/VCO₂ slope) and the oxygen uptake efficiency slope (an integrated measure reflecting combined pulmonary, cardiovascular and skeletal muscle efficiency) were again not significantly different between genotype groups.

	II (n=16)	ID (n=37)	DD (n=25)	p value ANOVA	DD vs. II/ID
Peak power (Watts)	49 (26)	49 (25)	52 (24)	0.87	0.44
Peak power (% predicted)	53 (31)	52 (21)	63 (38)	0.35	0.56
Peak VO ₂ (ml/min/kg)	13.9 (5.6)	14.5 (3.2)	15.5 (4.5)	0.26	0.21
Peak VO ₂ (% predicted)	67 (27)	66 (16)	73 (28)	0.49	0.23
VO ₂ peak/ WR peak (ml/min/kg/W)	27.9 (18.6)	23.6 (11.6)	22.0 (7.7)	0.57	0.39
WR peak/ VO ₂ peak (W/ml/min/kg)	3.41 (1.20)	3.33 (1.18)	3.32 (0.95)	0.96	0.88
Peak O ₂ pulse (ml/beat)	8.5 (2.8)	8.4 (2.0)	8.7 (2.4)	0.90	0.65
VE/VCO ₂ slope	27.63 (4.26)	31.53 (9.36)	31.06 (6.24)	0.23	0.35
OUES	1826 (339)	1664 (559)	1582 (492)	0.33	0.29

Table 3.3: Exercise characteristics of the subjects by ACE genotype. Values are expressed as mean (standard deviation) and individual genotypes are compared by one-way ANOVA testing. Comparison is also made of the II/ID combined and DD genotypes using unpaired t-tests.

Abbreviations: II – II genotype; ID – ID genotype; DD – DD genotype; VO₂ – pulmonary oxygen uptake; WR – work rate; VE – minute ventilation; VCO₂ – pulmonary production of carbon dioxide; OUES – oxygen uptake efficiency slope.

The peak pulmonary oxygen uptake (VO_2 peak) did not differ between genotype groups when expressed either as an absolute value or as a percentage of the predicted peak value (table 3.3 and figure 3.8).

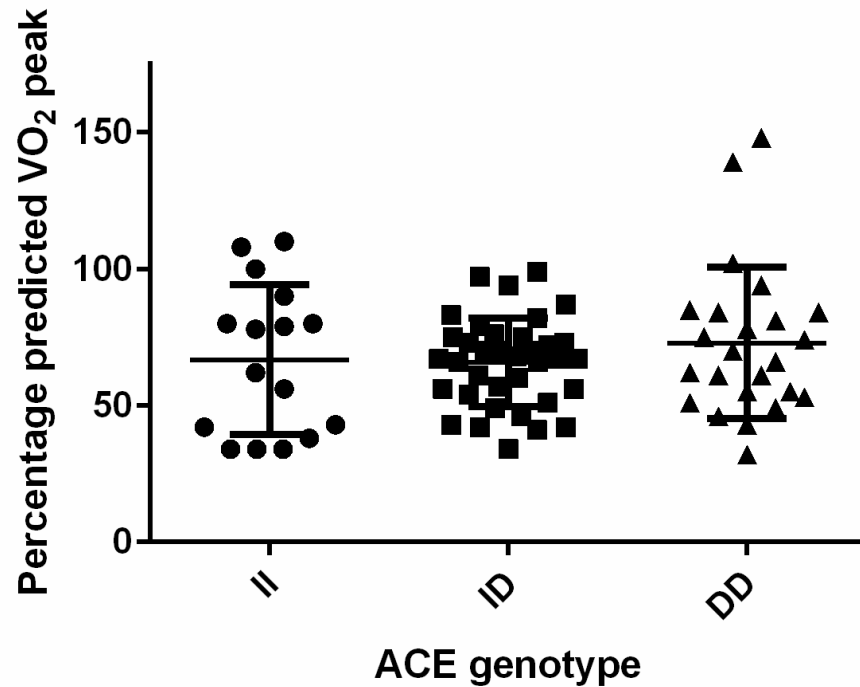


Figure 3.8: Percentage predicted VO_2 peak versus ACE genotype in COPD subjects. The mean and standard deviation are represented by the line and cross-bar respectively. ANOVA - no significant difference between genotypes.

Abbreviations: II – II genotype; ID – ID genotype; DD – DD genotype; VO_2 – pulmonary oxygen uptake.

Measures of ventilatory efficiency (as expressed by the VE/VCO_2 relationship) and overall body efficiency, as indicated by the oxygen uptake efficiency slope, were not different between genotype groups (figures 3.9 and 3.10).

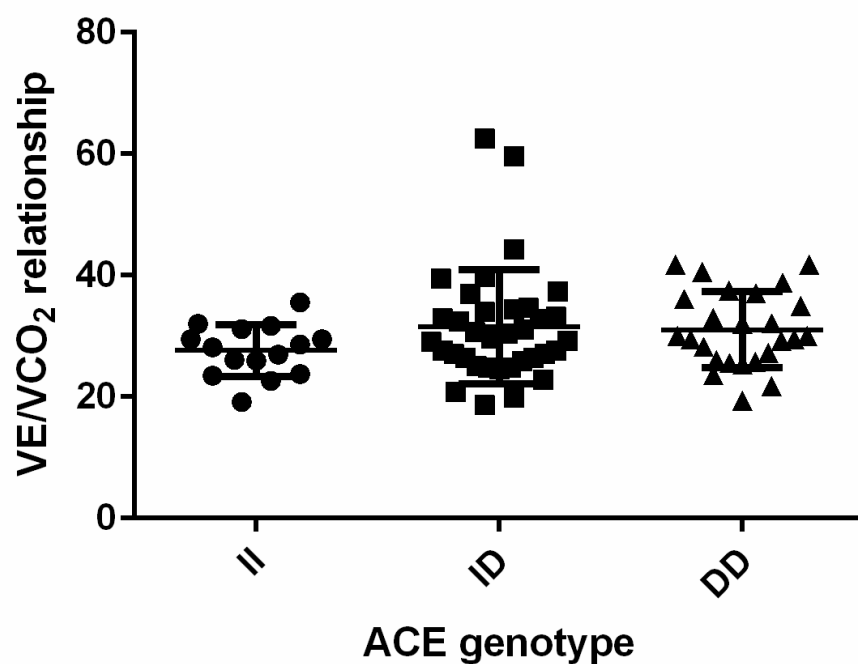


Figure 3.9: Ventilatory efficiency as expressed by the VE/VCO₂ relationship versus ACE genotype in COPD subjects. The mean and standard deviation are represented by the line and cross-bar respectively. ANOVA – no significant difference between genotypes.

Abbreviations: II – II genotype; ID – ID genotype; DD – DD genotype; VE - minute ventilation; VCO₂ – pulmonary production of carbon dioxide.

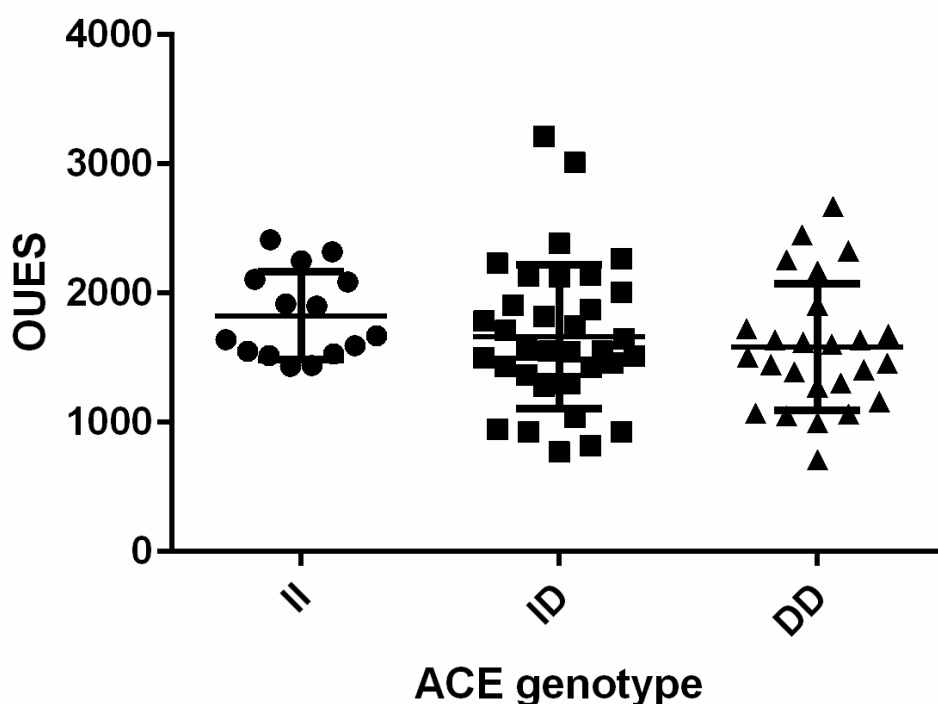


Figure 3.10: Oxygen uptake efficiency slope versus ACE genotype in COPD subjects. The mean and standard deviation are represented by the line and cross-bar respectively. ANOVA – no significant difference between genotypes.

Abbreviations: II – II genotype; ID – ID genotype; DD – DD genotype; OUES – oxygen uptake efficiency slope.

3.3.7 Predictors of peak pulmonary oxygen uptake

In this cohort of COPD patients, correlations were examined between baseline variables and percentage predicted VO_2 peak achieved during incremental cycle ergometry. The assumption of linearity was fulfilled for all continuous variables and percentage predicted of VO_2 peak achieved. Age, sex, FEV_1 percentage predicted, TLCO_c percentage predicted, residual volume to total lung capacity (RV/TLC) ratio, daily step count and physical activity level were all found to correlate with the percentage predicted VO_2 peak. FFMI, QMVC percentage predicted and ACE genotype did not show a significant correlation with this variable (table 3.4).

Factor	Pearson correlation value (r)	p value
Age (years)	0.262	0.022
Sex	0.446	<0.0001
FFMI (kg/m ²)	0.194	0.09
QMVC % predicted	0.110	0.34
FEV ₁ % predicted	0.700	<0.0001
TLCO _c % predicted	0.584	<0.0001
RV/TLC %	-0.462	<0.0001
Daily step count	0.563	<0.0001
PAL	0.323	0.013
ACE genotype	0.110	0.35

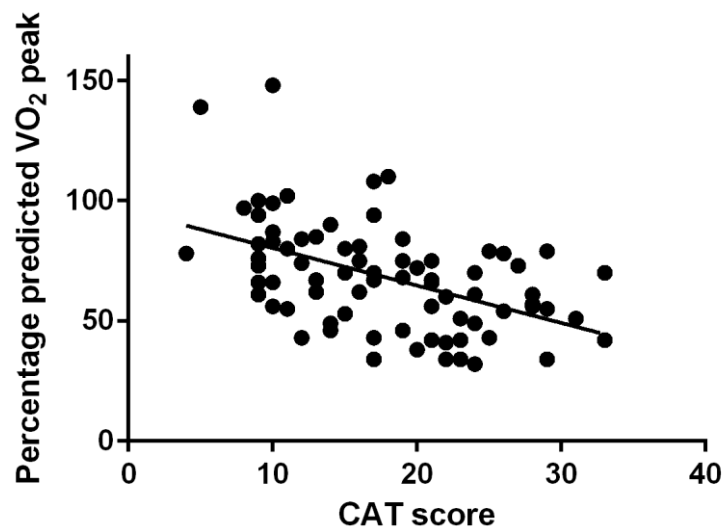
Table 3.4: Univariate correlates of percentage predicted VO₂ peak achieved in all COPD subjects.

Abbreviations: FFMI – fat free mass index; QMVC – quadriceps maximal volitional contraction; FEV₁ – forced expiratory volume in 1 second; TLCO_c – carbon monoxide diffusing capacity; RV – residual volume; TLC – total lung capacity; PAL – physical activity level; ACE – angiotensin converting enzyme (r and p values obtained from Pearson’s correlation coefficient).

A multiple regression model was used to predict percentage predicted VO₂ peak achieved during incremental cycle ergometry, incorporating the significant independent variables from univariate analysis (table 3.4). No influence was found of potential outliers on the regression analysis. Percentage predicted QMVC and FFMI were removed from the model as they were not identified as significant independent predictive factors. ACE genotype (whether grouped by possession of the I or D genotype) was not significantly associated with percentage predicted VO₂ peak. Age (r=0.24, p=0.004), sex (r=0.27, p=0.001), FEV₁ percentage predicted (r=0.38, p=0.002) and TLCO_c percentage predicted (r=0.32, p=0.005) were retained as independent predictors of percentage predicted VO₂ peak achieved, and produced the strongest model to predict this variable, giving an r² value of 0.634 (p<0.0001)

The health status of the COPD subjects was also assessed by both the COPD assessment test (CAT) score and St. George's respiratory questionnaire for COPD (SGRQ-C). A reduction in the percentage predicted VO_2 peak achieved during incremental cycle ergometry was associated with measures that indicated poorer health-related quality of life (figure 3.11).

a)



b)

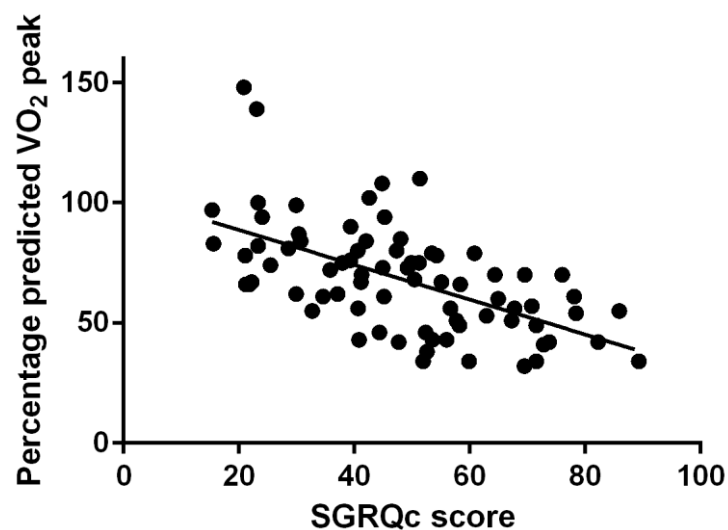


Figure 3.11: Percentage predicted VO_2 peak versus a) COPD assessment test (CAT) score and b) St. George's Respiratory Questionnaire for COPD (SGRQ-C) total score in COPD subjects. CAT score vs. percentage predicted VO_2 peak upper panel, Pearson correlation $r=-0.484$, $p<0.0001$; SGRQ-C total score vs. percentage predicted VO_2 peak lower panel, Pearson correlation $r=-0.579$, $p<0.0001$.

3.4 Discussion

3.4.1 Summary of results

In this observational study of a UK based COPD population, with at least moderate disease as assessed by the degree of airflow obstruction, there was no noted correlation between the ACE genotype and parameters of exercise capacity. This data, therefore, does not provide support to the previous study in a Chinese population suggesting that the I allele of the ACE genotype was associated with improved exercise measures.

3.4.2 Significance of the findings

In this population the distribution of genotypes was consistent with Hardy-Weinberg equilibrium and similar to that previously reported in UK Caucasian populations (Jones and Woods 2003, Hopkinson, Nickol et al. 2004). The pattern of serum ACE activity matched that expected for the genotype, with higher levels associated with increasing number of D alleles. The baseline phenotypic characteristics were very similar between genotype groups. Despite previous evidence suggesting that those possessing the I allele have a more stable disease course when examined retrospectively (Pabst, Theis et al. 2009), the exacerbation frequency and prednisolone use in the preceding 12 month period was equivalent between genotypes in this cohort. There was a trend towards reduced markers of hyperinflation in the DD genotype group, although this only reached statistical significance when comparison was made between the DD and II/ID genotypes grouped together.

Although previous research in COPD subjects has shown an association between the D allele and quadriceps strength (Hopkinson, Nickol et al. 2004), this current body of work did not show a genotype dependent relationship with absolute QMVC. However, the percentage predicted QMVC achieved was greater in the DD genotype group than the ID genotype group, and when comparison was made of the DD and II/ID groups. These current findings suggest other confounding factors may be at play.

Focussing on exercise characteristics, peak power and pulmonary oxygen uptake (either absolute values or percentage of the predicted achieved) were not different between genotype groups. In a

similar manner, integrated measures of ventilatory (the VE/VCO_2 slope) and whole body efficiency (measured by the oxygen uptake efficiency slope) were not statistically significantly different between ACE genotypes. Further, in both univariate and multivariate regression analysis ACE genotype did not contribute to predicting the percentage of predicted VO_2 peak achieved.

Previous studies of the ACE gene polymorphism have suggested a role for the I allele in elite athletic endurance performance (Montgomery, Marshall et al. 1998, Myerson, Hemingway et al. 1999). In the only study of a COPD population maximal workload was greater in subjects possessing the II allele, than ID or DD, suggesting ACE activity may influence the aerobic efficiency of skeletal muscle in COPD patients (Zhang, Wang et al. 2008).

Peripheral muscle strength is known to be an important contributor to exercise performance and in a patient population it may seem less intuitive to demarcate 'strength' and 'endurance' capacity as can be more easily understood in elite athletes whose performance may be at the extremes of ability. Patients with higher baseline quadriceps strength are known to perform better during endurance-based activities as demonstrated in the early work of Gosselink et al. In 41 consecutive COPD patients with moderate to severe disease referred for pulmonary rehabilitation walking distance, as assessed by the six minute walk, and VO_2 peak, assessed by incremental cycle ergometry, were influenced significantly by quadriceps strength in both univariate and stepwise multivariate regression analysis (Gosselink, Troosters et al. 1996). In addition, in ex vivo muscle models angiotensin II is known to be important in tetanic strength and skeletal muscle hypertrophy in response to mechanical loading (Gordon, Davis et al. 2001) and this may in part explain why individuals with high intrinsic angiotensin II levels (the DD ACE genotype) have higher quadriceps strength (Hopkinson, Nickol et al. 2004), in a similar manner to the well-recognised role of angiotensin II in cardiac myocyte hypertrophy. Thus, when considering exercise capacity, it may be that the role of angiotensin II levels in strength capacity outweighs any possible role of low angiotensin II levels in endurance when considering a COPD population.

Gosselink et al. demonstrated that quadriceps force correlated with both walking distance and peak pulmonary oxygen uptake assessed during incremental cycle ergometry in COPD subjects prior to starting a programme of pulmonary rehabilitation (Gosselink, Troosters et al. 1996). In addition, therapies aimed at improving quadriceps strength are known to improve exercise capacity. In a

randomised controlled trial of transcutaneous electrical muscle stimulation (TCEMS) in 18 moderate-severe COPD patients, those receiving TCEMS showed greater strength gains (39.0% TCEMS vs. 2.9% sham therapy) and this translated into improved walking capacity as assessed by the incremental shuttle walking test (36.1% TCEMS vs. 1.6% sham therapy) (Bourjeily-Habr, Rochester et al. 2002). This again reiterates that in this patient population strength is an important contributor to exercise capacity.

In furtherance of this, in subjects with COPD resistance training is a key component of pulmonary rehabilitation programmes alongside aerobic exercise (Bolton, Bevan-Smith et al. 2013, Spruit, Singh et al. 2013). Incorporating resistance training into rehabilitation programmes may translate to greater improvements in the performance of the activities of daily living and exercise capacity, particularly when performed at high training loads. However, although there is conflicting evidence that exercise capacity improves to a greater extent than with aerobic training alone despite notable improvements in muscle mass and strength (O'Shea, Taylor et al. 2009, Spruit, Singh et al. 2013).

Previous studies have suggested that strength adjusted for FFM is preserved in COPD (Franssen, Broekhuizen et al. 2005, Seymour, Spruit et al. 2010), suggesting that local effects may be involved, including the effect of physical detraining. This is interesting as in this current body of work the step count of those homozygous for the D allele was higher than either II or ID subjects, and at a level considered clinically significant, albeit not meeting statistical significance. Perhaps it could be construed that for some reason DD individuals maintain greater levels of physical activity and are less detrained than ID or II individuals, thus helping them to maintain their quadriceps strength as has been demonstrated in other cohorts (Hopkinson, Nickol et al. 2004) although differences in absolute strength were not clearly shown here. In part this may be due to milder disease as the DD group had lower markers of lung hyperinflation and a link between measures of lung hyperinflation and physical activity has been previously described (Shrikrishna, Patel et al. 2012). Early work by Serres et al. indicated that self-reported physical activity measures were related to both quadriceps endurance and peak pulmonary oxygen uptake (Serres, Gautier et al. 1998).

Of note the cohort studied by Hopkinson et al. (Hopkinson, Nickol et al. 2004) had significantly worse airflow obstruction as assessed by FEV₁ than this present cohort, which may explain some of the differences noted (FEV₁ expressed as mean $\pm$ standard deviation 34.4 $\pm$ 16.5% vs. 48.0 $\pm$ 20.5%). It is

possible that ACE activity levels influenced by genotype affect the response to detraining in more severe disease but not in milder disease. Thus this current group studied with less severe airflow obstruction may be more akin to healthy untrained individuals where genotype dependent influences on strength and exercise capacity are not always noted (Folland, Leach et al. 2000, Zhang, Wang et al. 2008). It may also be the case that differences in physical activity level contribute, and this has not previously been related to ACE genotype in COPD.

Although this study did not include a control group, previous work has failed to establish a role of the ACE genotype in healthy non-athletic subjects in either strength (Hopkinson, Nickol et al. 2004) or exercise capacity (Zhang, Wang et al. 2008). This suggests the ACE gene perhaps modifies other stimuli that become relevant in COPD, such as physical inactivity and detraining. In line with this previous work has shown that the D allele is associated with a greater gain in isometric quadriceps strength in response to training (Folland, Leach et al. 2000). Folland et al. studied 33 healthy recreationally active but untrained subjects exposed to a 9 week leg strength training programme. Although there was no genotype association with baseline quadriceps strength, following the training programme ID and DD subjects showing greater strength gains than the II genotype during isometric quadriceps strength training (strength gain expressed as mean $\pm$ standard error II $9.0 \pm 1.7\%$ vs. ID $17.6 \pm 2.2\%$ vs. DD $14.9 \pm 1.3\%$, ANOVA $p < 0.05$). Thus it remains possible that although no genotype dependent differences in exercise capacity were seen in this cohort, that there may ACE genotype related effects in the response to training.

3.4.3 Potential mechanisms by which ACE genotype may influence exercise characteristics

It is important to consider the potentially multiple levels at which the renin-angiotensin system (RAS) may influence exercise characteristics. Given there are several potential levels of action, some of which may have conflicting effects, it is possible that the overall effect of ACE genotype is dependent on the relative weighting of these effects in the cohort studied.

3.4.3.1 Skeletal muscle phenotype

Local renin-angiotensin pathways are thought to influence skeletal muscle phenotype. One model that has allowed us to explore this further is the ACE gene knockout mouse, where it is possible to manipulate the expression of the ACE genotype (denoted as “Ace” in mice). Wild type mice (Ace

+/-) possess significantly higher levels of ACE activity in the muscle than mutant heterozygote (Ace +/-) mice (Zhang, Shono et al. 2005), indicating genotype dependent changes in the skeletal muscle local renin-angiotensin system (RAS), and not just in the circulating serum. At a histological level in the skeletal muscle, ACE knockout heterozygous mice (Ace +/-) had significantly higher mean fibre capillary density, particularly around type IIa intermediate fibres than wild-type mice (Ace +/+), although the fibre type proportions were not significantly different between the groups (Zhang, Shono et al. 2005). These genotype dependent changes in capillarity may link to altered exercise characteristics by influencing blood flow to skeletal fibres with mixed aerobic and anaerobic capacity.

Limited work has been performed in this respect in human subjects, the only study being in a Japanese cohort. In this study 41 healthy human subjects were genotyped for the ACE gene and underwent concurrent *vastus lateralis* biopsies. Those individuals possessing the I allele were demonstrated to possess an increase in fatigue-resistant slow twitch type I fibres and a decrease in more fatigue-susceptible type IIb muscle fibres (Zhang, Tanaka et al. 2003). In this particular cohort of healthy subjects rather interestingly there were no genotype dependent differences in exercise capacity or strength despite ACE genotype associated skeletal muscle structural variations, which may call into question the importance of the histological changes noted.

3.4.3.2 Influences on pulmonary haemodynamics

Some COPD subjects may have pulmonary hypertension at rest, and even those with normal pulmonary pressures at rest may develop pulmonary hypertension during an exercise challenge, pulmonary hypertension being characterised by elevated pulmonary arterial pressure and pulmonary vascular resistance. Pulmonary hypertension in COPD involves pulmonary vascular remodelling, fibrosis, activation of cytokines, endothelial dysfunction and vasoconstriction, processes that may be contributed to by activity of the RAS (Bradford, Ely et al. 2010). Angiotensin II is a vasoconstrictor agent in the pulmonary circulation (Lipworth and Dagg 1994) and ACE activity has been implicated in the development of pulmonary hypertension in response to an acute hypoxic challenge in both a rat model (Nong, Stassen et al. 1996) and in healthy individuals (Cargill and Lipworth 1996), an effect that may be reduced by the administration of an ACE-I (Cargill and Lipworth 1996, Nong, Stassen et al. 1996).

Supportive evidence has been provided by a rat model of monocrotaline induced pulmonary hypertension, a plant-derived toxin, leading to elevated right ventricular systolic pressure, right ventricular hypertrophy, increased pulmonary vessel wall thickness and interstitial fibrosis, in association with increased mRNA levels of renin, ACE, angiotensinogen, angiotensin II type 1 (AT1) receptors and proinflammatory cytokines (Ferreira, Shenoy et al. 2009). Evidence also exists suggesting a potentially important role for angiotensin II in the pulmonary vascular responses to exercise, thus intrinsically low levels of angiotensin II (as with possession of the I allele of the ACE gene) may lessen the pulmonary vascular changes during exercise and thus improve exercise characteristics.

High ACE activity is associated with pulmonary hypertension and disturbed tissue oxygenation during an exercise challenge in a COPD population even when resting pulmonary pressures are normal (Kanazawa, Okamoto et al. 2000, Kanazawa, Otsuka et al. 2002, Kanazawa, Hirata et al. 2003). Furthermore, in a pilot randomised, double-blind, cross-over study of captopril versus placebo in patients with COPD undergoing right heart catheterisation during incremental exercise, lower ACE activity (either in relation to ACE genotype or induced by captopril) was associated with both improved pulmonary haemodynamics and oxygenation. Note was made of lower exertional pulmonary artery pressure, lower pulmonary vascular resistance, higher mixed venous oxygen saturations (reflecting oxygen delivery to peripheral tissues), and lower blood lactate levels (Kanazawa, Hirata et al. 2003). This may thus be one mechanism by which manipulation of the renin-angiotensin system is able to influence exercise performance in a COPD population.

As well as the important role of angiotensin II it should not be forgotten that ACE is also responsible for the breakdown of vasoactive kinins, including bradykinin, important in vasodilatory responses and also implicated in the pathophysiology of pulmonary hypertension seen in COPD. It has also been speculated that the ACE gene on chromosome 17 and the eNOS gene on chromosome 7 may interact in a manner to reduce the pathological consequences of activation of the RAS pathway (Ahsan, Ram et al. 2004). Bradykinin receptor polymorphisms have been shown to impact on the skeletal muscle phenotype in COPD (Hopkinson, Eleftheriou et al. 2006, Hopkinson, Li et al. 2008), with cross-sectional studies showing the gene polymorphism associated with reduced activity at the bradykinin receptor (+9/+9 BK₂R) to be associated with both reduced fat free mass and quadriceps strength in COPD patients (Hopkinson, Eleftheriou et al. 2006).

3.4.3.3 Endothelial function

The D-allele of the ACE gene has been found to be positively associated with endothelial dysfunction (Kuzubova, Chukhlovina et al. 2013), which may in part be due to an increased exacerbation frequency reported in those possessing the D allele (Pabst, Theis et al. 2009, Kuzubova, Chukhlovina et al. 2013). An association has also been made between the DD genotype and cardiovascular risk independent of other major risk factors (Cambien, Poirier et al. 1992, Nakai, Itoh et al. 1994). Thus high intrinsic levels of angiotensin II may be associated with both changes in endothelial function and cardiovascular disease that might influence exercise capacity. Both of these factors may suggest a role of ACE-inhibition in affecting exercise characteristics.

3.4.3.4 Complexity of the renin-angiotensin system

The renin-angiotensin system is a complex system. Whilst ACE1 is well recognised in its role catalysing the conversion of angiotensin I to angiotensin II, another isoform of ACE (ACE2) produces angiotensin-(1-7) [Ang-(1-7)] from angiotensin-II. This pathway helps to regulate the balance between the vasoconstrictive, proliferative (ACE1-angiotensin II-AT1 receptor) and the vasoprotective axis (ACE2-Ang-(1-7)-Mas) of the renin-angiotensin system. Activation of ACE2 has been shown to prevent the monocrotaline induced PH seen in a rat model (Ferreira, Shenoy et al. 2009), giving a glimpse into the potentially counter-regulatory influences of the renin-angiotensin pathway.

3.4.4 Critique of the method

This was an observational study of patients recruited both from hospital outpatients and community settings and this helped to establish a group representative of the COPD population with at least moderate disease as assessed by spirometry. However, study was not made of those with mild disease, and thus we cannot conclude that the ACE genotype may influence exercise characteristics in this group. However, use of cycle ergometry as a method to investigate exercise characteristics provided a well-established and reliable methodology and allowed the interrogation of integrated factors in response to exercise, including measures of ventilatory efficiency and the oxygen uptake efficiency slope.

No control group was included in this study and this is an obvious limitation, although previous work has suggested that in healthy but non-athletic individuals the ACE genotype does not contribute significantly to strength (Hopkinson, Nickol et al. 2004) or peak exercise characteristics (Zhang, Wang et al. 2008). Whilst we failed to establish a link between ACE genotype and exercise characteristics as assessed by incremental cycle ergometry at baseline this study does not rule out that the response to rehabilitative exercise may differ between genotype groups as has previously been suggested, and this remains an area that has not been thoroughly researched. Other parameters during the exercise test may also have been interesting to explore such as the oxygen uptake kinetics.

It is worthy of note that even within genotype groups there is a wide variation in the absolute level of ACE activity and this may help explain why it was difficult to find differences in exercise characteristics between distinct genotype groups, as individuals possessing different genotypes may actually have similar levels of angiotensin II activity. In addition, given the genotype distribution there are small numbers of individuals in the homozygous state, whether DD or II. It would thus be worth in future studies correlating absolute serum ACE activity with other clinical parameters. This study was also conducted using baseline data from a convenience sample of individuals entering an interventional trial and thus was not powered according to its primary endpoint, this is a major limitation and may explain some of the findings.

3.4.5 Conclusion

In summary, this study failed to establish any evidence of a link between ACE genotype and exercise characteristics in patients with at least moderate COPD, in contrast to previous work in an Asian COPD population and studies of athletic individuals. It is perhaps unsurprising that athletic individuals, particularly those participating in high level sport, may gain from the physiological advantage posed by possessing specific ACE genotypes as this may contribute to small improvements in performance abilities. In COPD individuals, although the detraining associated with the disease may be a perturbation of the same order of magnitude as athletic training, several other factors confound to influence exercise characteristics and thus a clear signal of the effect of the ACE genotype could not be seen in this cohort.

Chapter 4: Randomised Controlled Trial of ACE-inhibitor Therapy as an Adjunct to Pulmonary Rehabilitation in COPD

4.1 Introduction

4.1.1 Background

COPD is a multisystem disorder including skeletal muscle dysfunction, a well-recognised and common complication noted in both early and late disease (Seymour, Spruit et al. 2010). Quadriceps weakness is not simply an epiphenomenon, and has repeatedly been shown to be associated with clinically relevant outcomes such as impaired healthcare status (Shrikrishna and Hopkinson 2012), increased healthcare utilisation (Decramer, Gosselink et al. 1997) and mortality (Swallow, Reyes et al. 2007). The skeletal muscle thus provides an interesting target for intervention to improve disability in this patient population, particularly when the airflow obstruction is less amenable to change through therapy.

Pulmonary rehabilitation is a highly effective intervention that leads to improvements in quadriceps strength, exercise capacity and health-related quality of life in COPD (Nici, Donner et al. 2006, Bolton, Bevan-Smith et al. 2013). Patient with skeletal muscle dysfunction may, however, be responding suboptimally to such exercise based interventions, limiting the gain achieved, and even those who respond well tend to decline towards baseline by 12-18 months post intervention (Griffiths, Burr et al. 2000, Troosters, Gosselink et al. 2000). Thus, there is an unmet need for adjunctive agents, ensuring firstly that patients respond optimally and secondly to achieve maintenance of the response for as long as possible.

4.1.2 Rationale and hypothesis

Despite the RAS being most well-recognised for its role in water and salt homeostasis at a whole body level, local cellular (autocrine) and organ (paracrine) renin-angiotensin systems exist in many tissues. As a component of the circulating and tissue renin-angiotensin systems, the enzyme angiotensin-converting enzyme (ACE) is essential in both the production of angiotensin II and breakdown of vasoactive kinins, most notably bradykinin. There is evidence of upregulation of the renin-angiotensin pathway in COPD, especially when patients are hypoxic (Stewart, Waterhouse et al. 1994). The intramuscular renin-angiotensin system has been delineated as an important physiological pathway in skeletal muscle (Jones and Woods 2003), where angiotensin II type-1 (AT1) receptors are located.

Molecular data indicates that the renin-angiotensin system contributes to the skeletal muscle impairment seen in COPD through multiple mechanisms. Angiotensin II is noted to promote pro-inflammatory pathways, adversely affect insulin sensitivity and glucose handling, alter the balance of atrophy-hypertrophy pathways in favour of atrophy, and influence angiogenesis and vasodilatation (Shrikrishna, Astin et al. 2012). In addition, bradykinin has positive effects on insulin sensitivity (Henriksen and Jacob 2003), protects against oxidative damage (Yu, Li et al. 2008) and promotes angiogenesis (Shrikrishna, Astin et al. 2012), all components of effective skeletal muscle function.

Epidemiological studies of patient cohorts on ACE-inhibitors, thus with exogenously suppressed levels of angiotensin II and raised levels of vasoactive kinins, have demonstrated that such therapy is associated with preserved mass of the locomotor muscles (Di Bari, van de Poll-Franse et al. 2004), leg strength (Onder, Penninx et al. 2002) and walking speed (Onder, Penninx et al. 2002). These are, however, observational findings, the mechanisms behind which have yet to be fully investigated. In line with this, investigation of the ACE genotype polymorphism, in which some individuals have intrinsically low levels of serum and tissue angiotensin II, have demonstrated improved exercise characteristics in both healthy populations (Montgomery, Marshall et al. 1998, Myerson, Hemingway et al. 1999) and those with COPD (Zhang, Tanaka et al. 2003), with a greater peak workload achieved during incremental cardiopulmonary exercise testing. In health such individuals also demonstrate an improved mechanical efficiency in response to a training stimulus (Williams, Rayson et al. 2000). Further observational work has shown the bradykinin receptor polymorphism linked to reduced activity at the bradykinin receptor (+9/+9 BK₂R) is associated with reduced fat free mass and quadriceps strength in COPD (Hopkinson, Eleftheriou et al. 2006).

Early interventional studies have demonstrated that pharmacological manipulation of the renin-angiotensin system, to reduce angiotensin II activity, has been associated with clinical benefits on exercise capacity. In an elderly population with restricted mobility use of the ACE-inhibitor perindopril was associated with improved walking distance as assessed by the six minute field walking test (Sumukadas, Witham et al. 2007). In COPD patients improvements in both quadriceps strength (Andreas, Herrmann-Lingen et al. 2006) and maximal exercise capacity (Di Marco, Guazzi et al. 2010) have been associated with suppression of angiotensin II activity through the use of irbesartan and enalapril respectively. In fact in patients with moderate-severe COPD four weeks therapy with enalapril led to a 7% improvement in peak power achieved during incremental cycle

ergometry (Di Marco, Guazzi et al. 2010). These studies highlight the possibility of targeting the renin-angiotensin system to improve the response to exercise-based interventions such as pulmonary rehabilitation.

The efficacy of ACE-inhibition in altering exercise capacity in COPD has not always been confirmed however. In another study using COPD subjects selected for quadriceps weakness (defined by a quadriceps maximal voluntary contraction <120% body mass index), three months therapy with the ACE-inhibitor fosinopril was not able to induce an improvement in either quadriceps strength, quadriceps endurance or peak walking capacity (Shrikrishna, Tanner et al. 2014), raising the question of whether ACE-inhibition can be truly beneficial. The further use of animal models has suggested, however, that ACE-inhibition may act in synergy with exercise training in ensuring a skeletal muscle phenotype more favourable for exercise. Studies in rats have demonstrated improved capillarity and type I fibre proportions over that induced solely by an exercise-based intervention when angiotensin II activity is also suppressed (Minami, Li et al. 2007, Guo, Minami et al. 2010). This has led to the suggestion that a concurrent exercise stimulus may be required to ensure maximal benefit from manipulation of the renin-angiotensin system.

We thus investigated the hypothesis that the administration of an ACE-inhibitor with a concomitant course of pulmonary rehabilitation delivered to COPD subjects would augment the improvements in exercise capacity seen following the rehabilitative intervention. This work focussed on effects on exercise capacity, strength, health-related quality of life and physical activity. Patients recruited to the study were stratified by both ACE genotype polymorphism and baseline power output on incremental cycle ergometry in order to avoid any randomisation bias and additional confounding factors.

4.2 Methods

4.2.1 Patient selection

All participants provided written informed consent prior to enrolment in the study which was conducted in line with the principles of the Declaration of Helsinki. The trial was registered prospectively on a publicly accessible database (www.controlled-trials.com) reference

ISRCTN79038750 and approved by the London Bloomsbury Research Ethics Committee (REC reference 12/LO/0331).

Study inclusion criteria included patients diagnosed with COPD on the basis of NICE and GOLD criteria, of at least GOLD stage II severity, and those referred for pulmonary rehabilitation with an MRC dyspnoea score of at least 3, or 2 with evidence of functional limitation (Bolton, Bevan-Smith et al. 2013). Exclusion criteria included patients within three months of pulmonary rehabilitation or within one month of a pulmonary exacerbation, those already taking medications influencing the renin-angiotensin pathway (ACE-inhibitors or angiotensin-receptor blockers) or possessing pre-existing conditions that would benefit from ACE-inhibition (ischaemic heart disease, cardiac failure and diabetes mellitus), renovascular disease or impaired renal function (eGFR <50 ml/min/1.73m²), pregnancy and any other significant comorbidity that would limit subject mobility or ability to participate in pulmonary rehabilitation. Individuals with hypotension (defined as a systolic blood pressure less than 100mmHg) were also excluded from participation in the study.

4.2.2 Study design

The study was a single-centre, double-blind, randomised, placebo-controlled, parallel-group trial. Randomisation was stratified by the ACE genotype polymorphism of the subject (II, ID or DD; I representing the insertion allele, and D the deletion allele) and baseline peak power output on incremental cycle ergometry (using 50 Watts as a cut-off). The randomisation list was created by an independent statistician using 1:1 randomisation in blocks of 4 using consecutive numbers, and randomisation was performed by the Imperial College Clinical Trials Unit. Both the subjects and assessor were blinded to treatment allocation.

Subjects underwent baseline assessment and started treatment (enalapril/placebo) one week prior to the initiation of pulmonary rehabilitation. The multidisciplinary pulmonary rehabilitation programme was 8 weeks in duration offering a combination of exercise and educational sessions, incorporating both strength and aerobic training individualised to the patient as per both national and international guidelines (Nici, Donner et al. 2006) (Bolton, Bevan-Smith et al. 2013). The patients were offered 3 sessions per week, 2 under direct supervision and 1 to be undertaken independently, supervised sessions comprising one hour of exercise and one hour of education.

4.2.3 Intervention

Patients were randomly allocated to either ACE-inhibition (10mg enalapril once daily) or placebo (microcrystalline cellulose) for 10 weeks, starting one week prior to the commencement of the rehabilitation programme and continuing throughout its course until the end of pulmonary rehabilitation assessment.

4.2.4 Trial protocol

Visit 1 (screening): Informed consent was obtained and the following baseline assessments obtained.

- Review of inclusion/exclusion factors
- Blood drawn for renal function
- Saliva sampling for ACE genotype

Visit 2 (baseline assessments): The subjects underwent phenotypic assessment at baseline.

- Review of medical history
- Anthropometric measurements including BMI and bioimpedance for fat free mass index
- Spirometry
- Quality of life assessments including MRC dyspnoea scale, St. George's Respiratory Questionnaire for COPD (SGRQ-C), COPD Assessment Test (CAT)
- Measurement of vital signs
- Quadriceps maximal volitional contraction and quadriceps twitch force
- Computed tomography measurement of mid-thigh and quadriceps cross-sectional area
- Fasting bloods for renal function, inflammatory markers, serum ACE, BNP
- Physical activity monitoring for a period of one week using a triaxial accelerometer

Visit 3 (randomisation visit):

- Full pulmonary function testing including spirometry, gas transfer and plethysmographic lung volumes and capillary blood gases
- Incremental cycle ergometry

- Patient were randomised to receive either 10mg enalapril (ACE-inhibitor) or matched placebo which was started one week prior to commencing pulmonary rehabilitation

Visit 4 (safety visit):

Resting blood pressure and renal function were measured one week after initiation of therapy and the patients asked to report any potential side effects. If the increase of serum creatinine was >30% from baseline or patients were symptomatically hypotensive (systolic blood pressure less than 100 mmHg, or fall from baseline of greater than 10 mmHg with accompanying symptoms) they were withdrawn from the study and further investigations organised.

Visit 5 (end of pulmonary rehabilitation programme assessment):

Subjects reattended for assessment within one week of completion of the pulmonary rehabilitation programme, and continued therapy until completion of the study. All measurements taken at the baseline visits 2 and 3 were repeated. A pill count was made during this visit to assess drug compliance.

A summary of the trial schedule is indicated in table 4.1.

	Visit 1 Screening	Visit 2 Baseline phenotyping	Visit 3 Randomisation visit	Visit 4 Safety visit	Visit 5 Post PR assessment visit
Saliva sampling for ACE genotype	X				
Vital signs	X	X		X	X
Medical history & examination		X			X
Spirometry		X	X		X
Lung volumes & gas transfer			X		X
Renal function testing	X	X		X	X
Blood draw for inflammatory markers, ACE, BNP		X			X
Bioimpedance		X			X
Quality of life assessment		X			X
QMVC & quadriceps twitch		X			X
Mid-thigh CT		X			X
Physical activity monitoring		X			X
Incremental cycle ergometry			X		X

Table 4.1: Protocol for randomised controlled trial of ACE-inhibition as an adjunct to pulmonary rehabilitation

Abbreviations: ACE – angiotensin-converting enzyme; BNP – brain natriuretic peptide; QMVC – quadriceps maximal volitional contraction; CT – computed tomography; PR – pulmonary rehabilitation.

4.2.5 Primary and secondary outcome measures

1. Change in peak power achieved on incremental cycle ergometry from baseline following pulmonary rehabilitation - Primary outcome measure
2. Change in quadriceps maximal volitional contraction force following pulmonary rehabilitation
3. Change in health-related quality of life as assessed by the COPD Assessment Test (CAT) score and St. George's Respiratory Questionnaire for COPD following pulmonary rehabilitation
4. Change in the daily physical activity level following pulmonary rehabilitation

4.2.6 Data analysis and statistics

The change in peak workload achieved on incremental cycle ergometry from baseline was selected as the primary endpoint of the study, being well-validated in this population and frequently used in large studies such as the National Emphysema Treatment Trial (NETT study) (Fishman, Martinez et al. 2003) as it indicates the whole body (both cardiorespiratory and skeletal muscle) response to exercise. This endpoint is also more frequently limited by muscle fatigue than walking tests (Man, Soliman et al. 2003), thus cycle ergometry may be more discriminatory in assessing interventions designed to target the skeletal muscle. Maximal exercise capacity as assessed by incremental cycle ergometry has been shown to be associated with reduced angiotensin II activity in COPD through both genotype association (Zhang, Wang et al. 2008) and pharmacological studies (Di Marco, Guazzi et al. 2010).

Prior data indicated that following a course of pulmonary rehabilitation peak workload on incremental cycle ergometry increased from 55 ± 19 Watts to 63 ± 9 Watts (Mador, Bozkanat et al. 2004). Thus in order to have a confidence level of 0.05 with 80% statistical power, and to show an additional improvement of 10% with ACE-inhibitor therapy, 54 individuals were needed to complete the study. Allowing for a 10% drop out rate 60 patients needed to be enrolled. Account was also made for the fact that those subjects with endogenous low levels of ACE activity, possessing the ACE

II genotype with a predicted prevalence of 25%, may have no response to ACE-inhibitor therapy which thus increased the enrolment target to 80 subjects.

Statistical analysis was performed on a per protocol basis. Data are presented as mean $\pm$ standard deviation (SD) or 95% confidence interval, and are reported alongside the accompanying p value. Data were analysed and the figures prepared using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, California, USA). Categorical data are presented as percentages and comparisons performed using the Chi squared test. Data was compared using two-sided paired (for comparison pre and post rehabilitation) or two-sided unpaired (comparing treatment groups) t-tests, or the appropriate non-parametric test for data that was not normally distributed. A p-value <0.05 was considered to be statistically significant.

4.3 Results

4.3.1 Recruitment process

Subjects were enrolled from February 2013 through to March 2015 when enrolment was completed. 275 subjects with COPD were screened for eligibility for the study, 80 of which were enrolled and 78 underwent randomisation. There were 5 withdrawals in the placebo group and 8 in the treatment group. The CONSORT diagram (figure 4.1) provides further details of the recruitment and follow-up process.

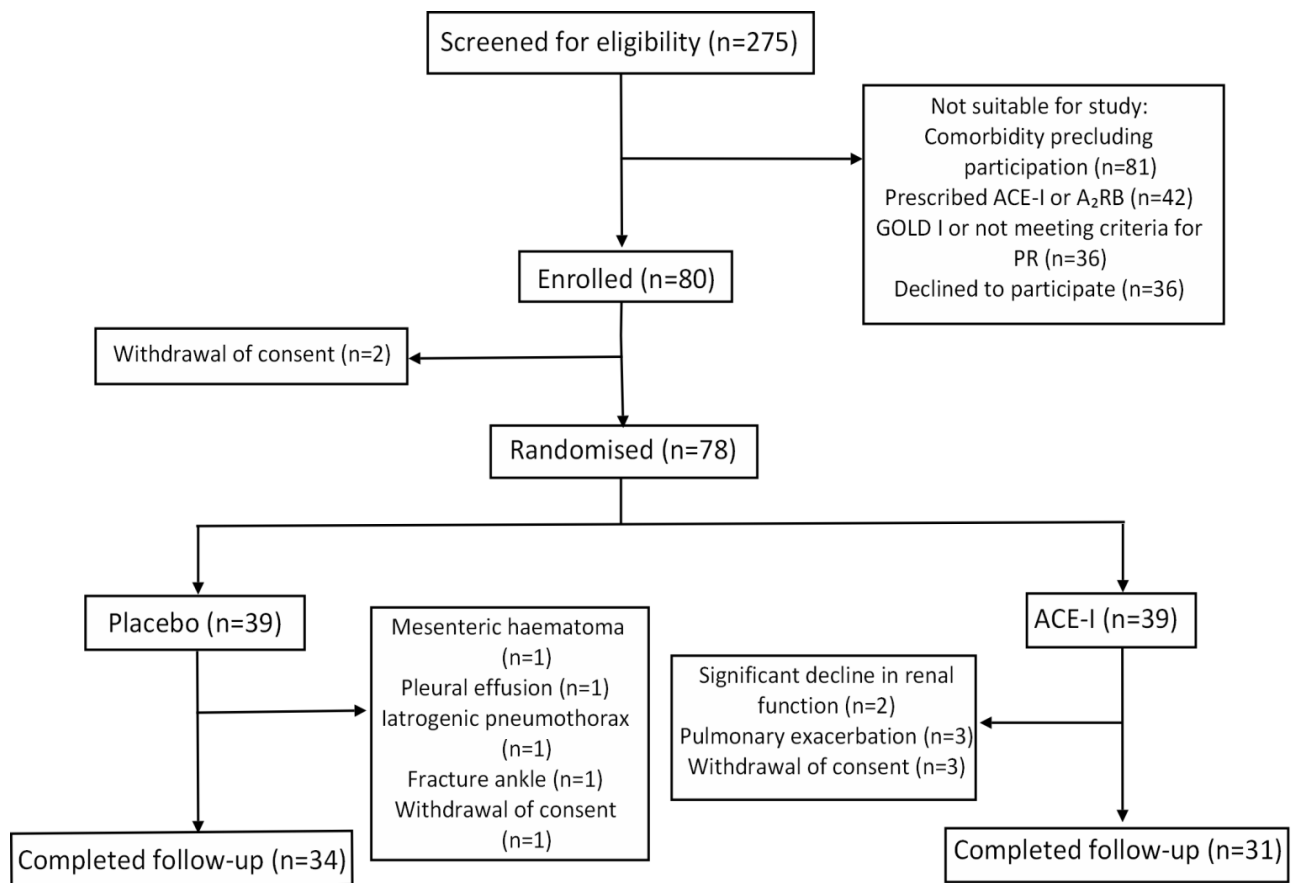


Figure 4.1: CONSORT diagram of enrolment and study completion.

Abbreviations: ACE-I - angiotensin-converting enzyme inhibitor; A₂RB – angiotensin II receptor blocker; GOLD – global initiative for chronic obstructive lung disease; PR – pulmonary rehabilitation.

4.3.2 Baseline characteristics of the treatment and placebo groups

The baseline characteristics of the group are presented in table 4.2. The participants were fairly typical of COPD subjects referred for pulmonary rehabilitation with a mean age 67 ± 8 years, FEV₁ $48 \pm 21\%$ predicted, systolic blood pressure 137 ± 18 mmHg, MRC dyspnoea score 3 ± 1 , quadriceps strength $73 \pm 22\%$ predicted and daily average step count of 5428 ± 3633 . 79% of the subjects displayed evidence of ventilatory limitation at baseline (as assessed by the ratio of peak ventilation to the estimated maximal ventilation of ≥ 0.9 (O'Donnell 2001)).

The placebo and treatment groups were well matched at baseline for age, gender, health-related quality of life, serum inflammatory markers, brain natriuretic peptide and physical activity levels. Although the BMI of the treatment group was statistically lower than those receiving placebo, this was not at a level that would be considered clinically significant.

The ACE genotypes were consistent with Hardy-Weinberg equilibrium in both placebo and treatment groups ($\chi^2=0.56$, $p=0.76$ placebo; $\chi^2=0.68$, $p=0.71$ ACE-inhibitor), and the distribution of genotypes was not different between the two arms. No patients in either arm were receiving long-term oral corticosteroid therapy, and the dose of corticosteroids required for the treatment of pulmonary exacerbations in the preceding 12 months was equivalent between the groups. All patients participating were of Caucasian background, with the exception of one female who was Afro-Caribbean.

	Placebo group (n=34)	ACE-I group (n=31)	p value
Sex (% female)	41	55	0.27
Age (years)	68 (7)	66 (10)	0.28
ACE genotype (II, ID, DD) %	21, 47, 32	23, 42, 35	0.92
BMI (kg/m²)	26.9 (5.9)	24.0 (4.6)	0.033
Systolic BP (mm Hg)	139 (17)	133 (15)	0.10
Diastolic BP (mm Hg)	79 (11)	78 (9)	0.73
Smoking history (years)	54 (33)	39 (25)	0.034
Current smokers (%)	18	16	0.87
MRC dyspnoea score	3 (1)	3 (1)	0.52
CAT score	18 (7)	17 (7)	0.65
SGRQ-C total	46.25 (18.59)	46.78 (17.68)	0.91
LAMA (%)	71	84	0.20
LABA-ICS (%)	79	71	0.43
Dose of prednisolone (mg)*	367 (443)	388 (613)	0.81
Calcium channel blocker (%)	15	10	0.45
Diuretic (%)	9	10	0.91
CRP (mg/L)	12 (33)	3 (3)	0.17
Fibrinogen (g/L)	3.3 (0.7)	3.3 (0.5)	0.72
Serum BNP (ng/L)	40 (28)	50 (114)	0.36
Average daily step count [†]	4883 (2668)	6685 (4234)	0.15
Average PAL [†]	1.39 (0.20)	1.49 (0.19)	0.10

Table 4.2: Demographic and baseline characteristics of the subjects (n=65).

Data shown are mean (standard deviation).

Abbreviations: ACE – angiotensin-converting enzyme; ACE-I – angiotensin-converting enzyme inhibitor; I – insertion allele; D – deletion allele; BMI – body mass index; BP – blood pressure; MRC – Medical Research Council; CAT – COPD assessment test; SGRQ-C – St. George’s respiratory questionnaire for COPD; LAMA – long-acting muscarinic antagonist; LABA-ICS – long-acting beta-agonist and inhaled corticosteroid; CRP – C-reactive protein; BNP – brain natriuretic peptide; PAL – physical activity level.

* Indicates dose of prednisolone for the treatment of pulmonary exacerbations in the preceding 12 months to the baseline assessment.

† Data is analysed from 53 subjects (29 placebo, 24 treatment arm) who recorded an adequate period for physical activity assessment.

4.3.2.1 Baseline pulmonary function and CPEX parameters in placebo and treatment groups

Despite a statistically significant difference in smoking pack year history (table 4.3) there was good matching of the placebo and treatment groups for lung function variables, including measures of airflow obstruction, gas transfer and hyperinflation at baseline. There was no statistically significant difference in baseline exercise capacity between the groups, or measures of ventilatory efficiency or the oxygen uptake efficiency slope.

	Placebo group (n=34)	ACE-I group (n=31)	p value
FEV ₁ (L)	1.31 (0.53)	1.10 (0.54)	0.12
FEV ₁ % predicted	51.6 (20.2)	48.2 (22.5)	0.37
FVC (L)	3.25 (0.67)	2.96 (0.88)	0.15
TLCO _c % predicted	54.2 (22.7)	51.1 (23.1)	0.59
RV (L)	3.85 (1.32)	4.08 (1.65)	0.53
RV % predicted	165 (51)	187 (62)	0.19
TLC (L)	7.16 (1.53)	7.10 (2.12)	0.89
TLC % predicted	120 (18)	128 (19)	0.11
RV/TLC ratio (%)	52.8 (8.5)	56.5 (9.0)	0.09
PaO ₂ (kPa)	10.4 (1.6)	10.4 (1.6)	0.87
PaCO ₂ (kPa)	4.7 (0.6)	4.9 (0.6)	0.22
Peak power on cycle (watts)	51 (22)	54 (29)	0.62
Peak power (% predicted)	53 (24)	64 (37)	0.34
Peak VO ₂ (ml/min/kg)	14.1 (3.1)	16.1 (5.4)	0.19
VE/VCO ₂ slope	31.26 (7.84)	30.16 (7.59)	0.38
OUES	1686 (485)	1658 (520)	0.73

Table 4.3: Baseline pulmonary function and cardiopulmonary exercise characteristics of the subjects (n=65).

Data shown are mean (standard deviation).

Abbreviations: ACE-I – angiotensin-converting enzyme inhibitor; FEV₁ – forced expiratory volume in 1 second; FVC – forced vital capacity; TLCO_c – carbon monoxide diffusing capacity; RV – residual volume; TLC – total lung capacity; PaO₂ – partial pressure of oxygen in arterialised blood gas; PaCO₂ – partial pressure of carbon dioxide in arterialised blood gas; VO₂ – pulmonary oxygen uptake.; VE – minute ventilation; VCO₂ – pulmonary carbon dioxide production; OUES – oxygen uptake efficiency slope.

4.3.2.2 Baseline muscle bulk and strength in placebo and treatment groups

There was a significant difference at baseline in body composition, with the placebo arm having a higher fat free mass. This did not, however, correlate with a difference in leg strength, whether measured using volitional or non-volitional techniques (table 4.4).

	Placebo group (n=34)	ACE-I group (n=31)	p value
FFM (kg)	48.2 (8.2)	42.1 (6.2)	0.0026
FFMI (kg/m²)	17.1 (2.3)	15.7 (1.8)	0.0089
QMVC (kg)	30.4 (11.0)	28.9 (10.1)	0.58
QMVC % predicted	72.9 (24.3)	73.6 (19.1)	0.90
TwQ (kg)*	5.3 (3.7)	5.6 (2.9)	0.71
MTMCSA (mm ²)	9969 (2012)	9120 (2417)	0.12
Quadriceps CSA (mm ²)	4348 (950)	4027 (1277)	0.27

Table 4.4: Baseline muscle bulk and strength characteristics of the subjects (n=65).

Data shown are mean (standard deviation).

Abbreviations: ACE-I – angiotensin-converting enzyme inhibitor; FFM – fat free mass; FFMI – fat free mass index; QMVC – quadriceps maximal volitional contraction; TwQ – quadriceps twitch response; MTMCSA – mid-thigh muscle cross-sectional area.

*Data is reported for 57 subjects (31 placebo, 26 treatment) who achieved a supramaximal twitch response.

4.3.3 Effect of ACE-inhibition on blood pressure parameters

In the placebo arm systolic blood pressure was unchanged from baseline (Δ -1 mmHg, 95% CI -5 to 4, $p=0.78$), whereas it was significantly reduced in the ACE-I arm (Δ -16 mmHg, 95% CI -22 to -11, $p<0.0001$) with a significant between group difference (-15 mmHg, 95% CI -21 to -9, $p<0.0001$) (figure 4.2). Similar changes were also noted with diastolic blood pressure (placebo Δ +1 mmHg, 95% CI -3 to 4, $p=0.71$ vs. ACE-I Δ -9 mmHg, 95% CI -11 to -6, $p<0.0001$; between group difference -10 mmHg, 95% CI -14 to -5, $p=0.0001$; figure 4.2).

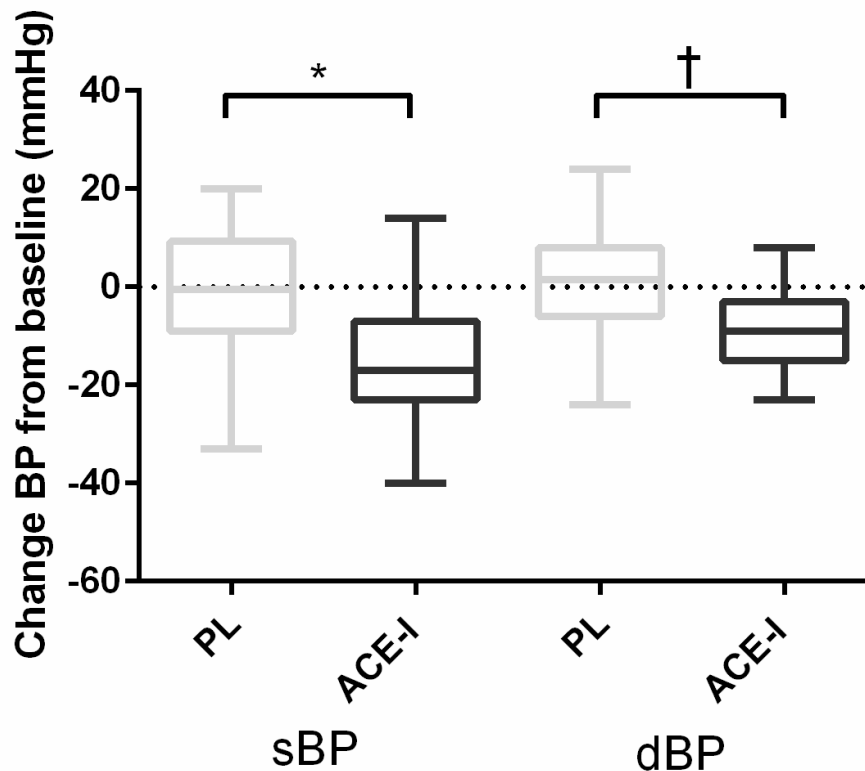


Figure 4.2: Change in blood pressure parameters from baseline to post pulmonary rehabilitation in the placebo (PL) and ACE-inhibitor (ACE-I) treatment arms. Data are presented as 25th-75th percentile with the solid line representing the median value, and whiskers the minimum to maximum values. Comparisons were made using unpaired t-tests; *p value <0.0001; †p value=0.0001.

Abbreviations: sBP – systolic blood pressure; dBP – diastolic blood pressure.

4.3.4 Effect of ACE-inhibition on serum ACE levels

There was a significant reduction in serum ACE levels in the ACE-I arm that was not seen in the placebo arm (placebo Δ +4 IU/L, 95% CI 0 to 8, $p=0.05$ vs. ACE-I Δ -18 IU/L, 95% CI -23 to -12, $p<0.0001$; between group difference -22 IU/L, 95% CI -29 to -15, $p<0.0001$; figure 4.3).

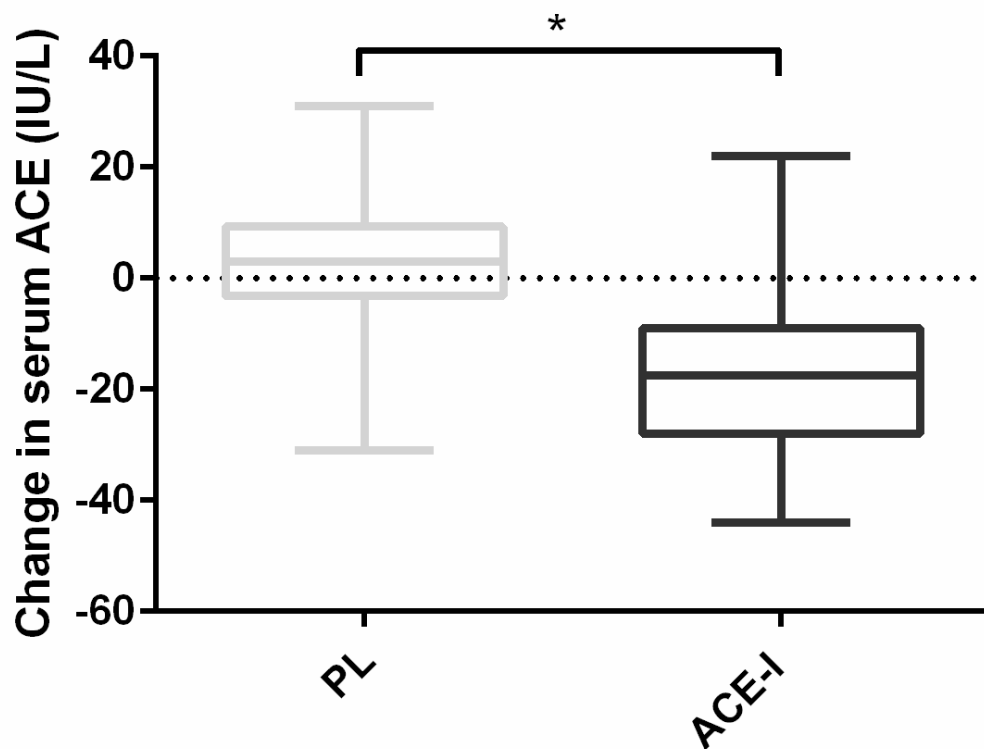


Figure 4.3: Change in serum angiotensin converting enzyme (ACE) levels from baseline to post pulmonary rehabilitation in the placebo (PL) and ACE-inhibitor (ACE-I) treatment arms. Data are presented as 25th-75th percentile with the solid line representing the median value, and whiskers the minimum to maximum values. Comparison was made using an unpaired t-test; *p value <0.0001.

4.3.5 Effect of ACE-inhibition on health-related quality of life, blood markers and pulmonary function

There was no between group difference in the change in symptom score assessed by the COPD Assessment Test (CAT) questionnaire (table 4.5). The health-related quality of life scores, as assessed by the SGRQ-C, improved in both treatment arms following pulmonary rehabilitation, including the total score (placebo Δ -3.14, 95% CI -5.26 to -1.01, $p=0.005$ vs. ACE-I Δ -4.66, 95% CI -7.85 to -1.46, $p=0.006$), with change noted particularly in the activity domain (placebo Δ -6.51, 95% CI -11.16 to -1.87, $p=0.007$ vs. ACE-I Δ -9.03, 95% CI -14.77 to -3.29, $p=0.003$). The magnitude of reduction was, however, equal in both treatment arms of the study, revealing no significant between group differences (total score between group difference -1.52, 95% CI -5.22 to 2.18, $p=0.42$; activity score between group difference -2.52, 95% CI -9.70 to 4.66, $p=0.49$).

Lung function variables including FEV₁, TLCO_c percentage predicted, measures of hyperinflation and arterialised blood gas parameters showed no significant between group differences in the change from baseline following pulmonary rehabilitation.

	Placebo group (n=34)	ACE-I group (n=31)	p value
ΔCAT score	-1 (3)	1 (4)	0.05
ΔSGRQ-C Symptoms	-0.55 (12.48)	-3.00 (11.43)	0.56
ΔSGRQ-C Activity	-6.51 (13.30)	-9.03 (15.65)	0.49
ΔSGRQ-C Impacts	-1.83 (7.82)	-2.62 (10.63)	0.52
ΔSGRQ-C Total	-3.14 (6.10)	-4.66 (8.71)	0.42
ΔFibrinogen (g/L)	-0.02 (0.62)	-0.02 (0.42)	0.98
ΔCRP (mg/L)	-6 (31)	2 (9)	0.20
ΔSerum BNP (ng/L)	-2.8 (23.0)	-3.6 (31.6)	0.91
ΔFEV ₁ (L)	-0.02 (0.10)	-0.01 (0.13)	0.91
ΔFEV ₁ % predicted	-0.02 (3.77)	-0.10 (6.68)	0.93
ΔTLCO _c % predicted	-1.45 (4.82)	-1.96 (5.61)	0.70
ΔRV % predicted	2.45 (10.07)	0.02 (16.51)	0.47
ΔTLC % predicted	1.36 (3.11)	1.14 (7.97)	0.29
ΔRV/TLC ratio (%)	0.39 (2.67)	0.09 (3.65)	0.70
ΔPaO ₂ (kPa)	-0.02 (1.16)	0.00 (1.12)	0.95
ΔPaCO ₂ (kPa)	0.08 (0.38)	0.02 (0.41)	0.60

Table 4.5: Change in health-related quality of life, blood markers and lung function measurements from baseline to post pulmonary rehabilitation.

Data are presented as mean (standard deviation). p values are for unpaired t-tests or the appropriate non-parametric test for data that was not normally distributed.

Abbreviations: ACE-I – angiotensin converting enzyme inhibitor; CAT – COPD assessment test; SGRQ-C – St. George’s respiratory questionnaire for COPD; CRP- C-reactive protein; BNP- brain natriuretic peptide; FEV₁ – forced expiratory volume in 1 second; TLCO_c – carbon monoxide diffusing capacity; RV – residual volume; TLC – total lung capacity; PaO₂ – arterial partial pressure of oxygen; PaCO₂ – arterial partial pressure of carbon dioxide.

4.3.6 Effect of ACE-inhibition on maximal exercise capacity

The peak power achieved on incremental cycle ergometry increased in both groups following pulmonary rehabilitation but the change was only significantly greater in the placebo group (placebo $\Delta+9$ Watts, 95% CI 5 to 13, $p<0.001$ vs. ACE-I $\Delta+1$ Watt, 95% CI -2 to 4, $p=0.62$; between group difference 8 Watts, 95% CI 3 to 13, $p=0.001$; figure 4.4). This was also reflected in the percentage change from baseline in peak power achieved, which was significantly greater in the placebo group (placebo $\Delta 23\%$, 95% CI 13 to 34 vs. ACE-I 8%, 95% CI -3 to 19; $p=0.009$).

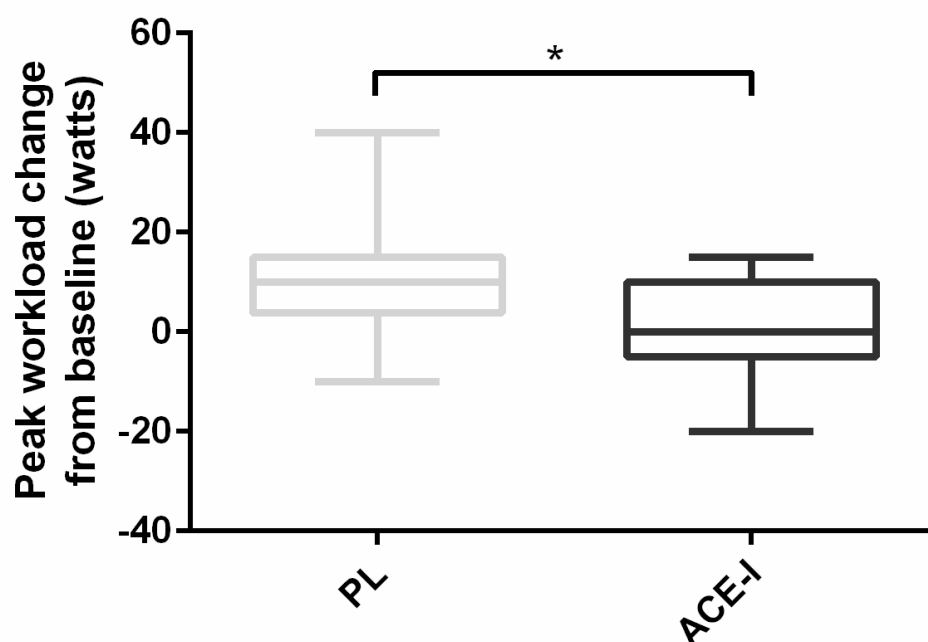


Figure 4.4: Change in peak workload during incremental cycle ergometry from baseline to post pulmonary rehabilitation in the placebo (PL) and ACE-inhibitor (ACE-I) treatment arms. Data are presented as 25th-75th percentile with the solid line representing the median value, and whiskers the minimum to maximum values. Comparison was made using an unpaired t-test; * $p=0.001$.

A similar pattern was seen in the change in peak pulmonary oxygen uptake (placebo $\Delta+1.37$ ml/min/kg, 95% CI 0.79 to 2.02, $p=0.0001$ vs. ACE-I $\Delta+0.33$ ml/min/kg, 95% CI -0.41 to 1.08, $p=0.45$; between group difference 1.04 ml/min/kg, 95% CI 0.08 to 2.01, $p=0.039$; figure 4.5).

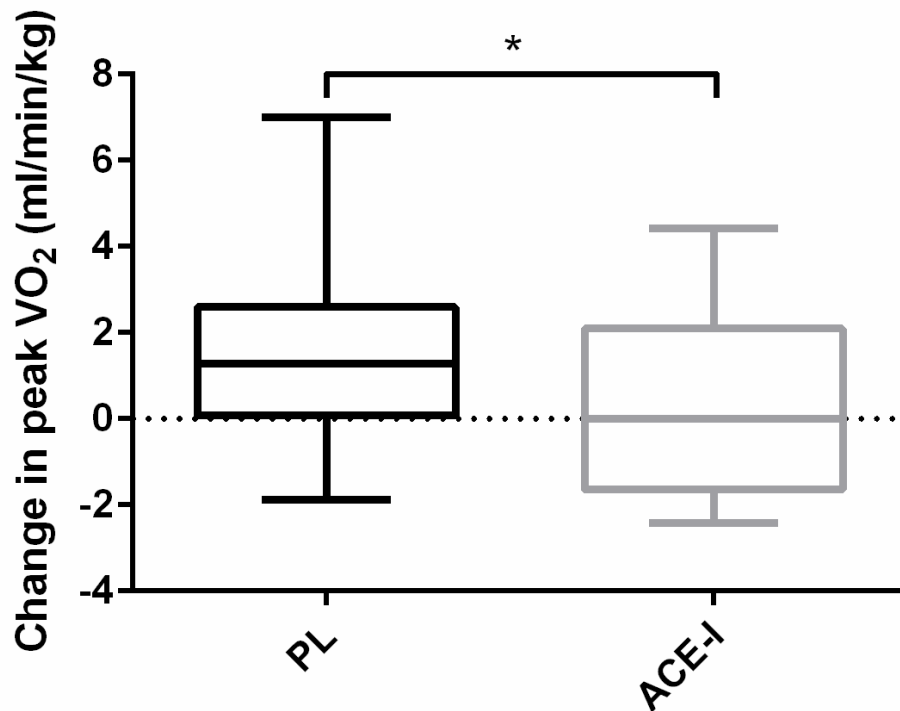


Figure 4.5: Change in peak pulmonary oxygen uptake (VO_2) during incremental cycle ergometry from baseline in the placebo (PL) and ACE-inhibitor (ACE-I) treatment arms. Data are presented as 25th-75th percentile with the solid line representing the median value, and whiskers the minimum to maximum values. Comparison was made using an unpaired t-test; * $p=0.039$.

The VE/VCO_2 slope, a measure of ventilatory efficiency, was not significantly changed in either treatment arm following rehabilitation (placebo $\Delta -1.25$, 95% CI -3.21 to 0.72 , $p=0.45$ vs. ACE-I $\Delta -0.87$, 95% CI -2.17 to 0.43 , $p=0.18$; between group difference 0.38 , 95% CI -2.02 to 2.78 , $p=0.57$). The oxygen uptake efficiency slope, however, was significantly increased following pulmonary rehabilitation only in the placebo arm, with a trend towards a significant between group difference (placebo $\Delta +151$, 95% CI 40 to 261 , $p=0.009$ vs. ACE-I $\Delta +29$, 95% CI -109 to 167 , $p=0.67$; between group difference 122 , 95% CI -49 to 292 , $p=0.08$).

	Placebo group (n=34)	ACE-I group (n=31)	p value
ΔPeak power on cycle (Watts)	9 (10)	1 (9)	0.001
ΔPeak power (% predicted)	9.9 (10.7)	2.0 (14.0)	0.013
ΔPeak VO₂ (ml/min/kg)	1.37 (1.87)	0.33 (1.99)	0.039
ΔVE/VCO ₂ slope	-1.25 (5.45)	-0.87 (3.28)	0.57
ΔOUES	151 (306)	29 (348)	0.08

Table 4.6: Change in exercise variables from baseline to post pulmonary rehabilitation.

Data are presented as mean (standard deviation).

Abbreviations: ACE-I – angiotensin-converting enzyme inhibitor; VO₂ - pulmonary oxygen uptake; VE - minute ventilation; VCO₂ - pulmonary carbon dioxide production; OUES - oxygen uptake efficiency slope.

4.3.7 Effect of ACE-inhibition on muscle bulk and strength

Following pulmonary rehabilitation there was no significant difference in the change in either mid-thigh muscle (placebo Δ+53 mm², 95% CI -120 to 227, p=0.67 vs. ACE-I Δ-52 mm², 95% CI -277 to 172, p=0.64; between group difference -105 mm², 95% CI -380 to 169, p=0.45) or quadriceps cross-sectional area (placebo Δ+81 mm², 95% CI -19 to 180, p=0.13 vs. ACE-I Δ+69 mm², 95% CI -14 to 152, p=0.10; between group difference 11 mm², 95% CI -140 to 117, p=0.86) as assessed by computed tomography scanning. There were no between group differences in the change in bioimpedance measures (table 4.7).

Following pulmonary rehabilitation both treatment groups increased their quadriceps maximal strength assessed volitionally, with the increase statistically significant in the placebo arm, although the difference between the groups failed to reach statistical significance (placebo Δ2.09 kg, 95% CI 0.45 to 3.73, p=0.014 vs. ACE-I Δ0.37 kg, 95% CI -1.57 to 2.31, p=0.70; between group difference 1.72 kg, 95% CI -0.76 to 4.20, p=0.17).

	Placebo group (n=34)	ACE-I group (n=31)	p value
Δ FFM (kg)	-0.72 (1.97)	-0.49 (1.38)	0.24
Δ FFMI (kg/m ²)	-0.31 (0.87)	-0.18 (0.54)	0.58
Δ QMVC (kg)	2.09 (4.70)	0.37 (5.29)	0.17
Δ QMVC % predicted	5.18 (13.21)	2.10 (13.62)	0.11
Δ TwQ (kg)*	0.09 (2.37)	0.34 (1.73)	0.89
Δ QMVC (kg)	2.09 (4.70)	0.37 (5.29)	0.17
Δ MTMCSA (mm ²)	53 (498)	-52 (601)	0.45
Δ Quadriceps CSA (mm ²)	81 (284)	69 (223)	0.86

Table 4.7: Change in muscle bulk and strength from baseline to post pulmonary rehabilitation.

Data are presented as mean (standard deviation).

Abbreviations: ACE-I – angiotensin-converting enzyme inhibitor; FFM - fat free mass; FFMI – fat free mass index; QMVC – quadriceps maximal volitional contraction; TwQ – quadriceps twitch response; MTMCSA - mid-thigh muscle cross-sectional area; CSA – cross-sectional area.

*54 patients had quadriceps twitch readings reaching supramaximality that were able to be assessed both pre and post pulmonary rehabilitation (29 placebo group, 25 ACE-inhibitor group)

4.3.8 Effect of ACE-inhibition on isotime CPEX parameters

Isotime was defined as the duration of the shortest of the two incremental cycle ergometry tests (pre and post pulmonary rehabilitation), and physiological variables were compared in the two tests at isotime to determine the change from baseline within each treatment group. The changes in isotime variables from baseline were then compared between the two treatment groups.

Modest differences were seen comparing isotime variables pre and post pulmonary rehabilitation (table 4.8). There was a trend towards a reduction in isotime minute ventilation in both groups

following pulmonary rehabilitation although no significant difference between the groups was found (placebo Δ -1.36 L/min, 95% CI -2.72 to 0.01, $p=0.05$ vs. ACE-I Δ -1.31 L/min, 95% CI -2.94 to 0.33, $p=0.11$; between group difference 0.05 L/min, 95% CI -2.02 to 2.13, $p=0.96$).

Whilst in the placebo treated group there was a trend for an improvement in the oxygen pulse at isotime (baseline 8.86 ml/beat, 95% CI 8.11 to 9.61 vs. post rehabilitation 9.28 ml/beat, 95% CI 8.27 to 10.28, $p=0.12$) this was not seen in the ACE-I treated group (baseline 8.38 ml/beat, 95% CI 7.46 to 9.32 vs. post rehabilitation 8.23 ml/beat, 95% CI 7.32 to 9.14, $p=0.40$), although the between group difference failed to reach statistical significance (table 4.8).

	Placebo group (n=34)	ACE-I group (n=31)	p value
HR (bpm)	-3 (12)	0 (12)	0.55
BF (breaths/min)	-2 (5)	0 (7)	0.29
VT (L)	0.007 (0.218)	-0.019 (0.253)	0.66
VE (L/min)	-1.36 (3.92)	-1.31 (4.47)	0.96
VO ₂ (ml/min/kg)	0.56 (1.80)	0.04 (1.86)	0.26
VCO ₂ (ml/min/kg)	0.42 (1.87)	0.00 (2.32)	0.43
Isotime power/VO ₂ ratio (W/ml/min/kg)	-0.15 (0.51)	-0.01 (0.40)	0.26
Oxygen pulse (ml/beat)	0.42 (1.47)	-0.15 (0.99)	0.08

Table 4.8: Change in isotime variables from baseline to post pulmonary rehabilitation.

Values are presented as mean (standard deviation).

Abbreviations: ACE-I – angiotensin converting enzyme inhibitor; HR - heart rate; BF - breathing frequency; VT - tidal volume; VE - minute ventilation; VO₂ - pulmonary oxygen uptake; VCO₂ – pulmonary carbon dioxide production.

4.3.9 Effect of ACE-inhibition on physical activity levels

Daily physical activity as calculated by the physical activity level (PAL) actually increased in the placebo arm but reduced in the treatment arm, producing a significant between group difference (table 4.9). This pattern was also noted in the daily step count, although this failed to reach statistical significance (table 4.9).

	Placebo group (n=34)	ACE-I group (n=31)	p value
ΔDaily step count	561 (2528)	-382 (2082)	0.30
ΔPAL	0.04 (0.15)	-0.06 (0.16)	0.030

Table 4.9: Change in physical activity levels from baseline to post pulmonary rehabilitation.

Data is presented as mean (standard deviation) and was analysed from 40 subjects (22 placebo, 18 treatment arm) who recorded an adequate period for physical activity assessment both at baseline and following rehabilitation.

Abbreviations: ACE-I – angiotensin converting enzyme inhibitor; PAL – physical activity level.

4.3.10 Effect of ACE-inhibition on exacerbation rate, pulmonary rehabilitation and drug compliance

There was no difference in either the pulmonary exacerbation rate during the period of pulmonary rehabilitation in either arm of the study (placebo 0.5 events, 95% CI 0.3 to 0.6 vs. ACE-I 0.5 events, 95% CI 0.1 to 0.8; p=0.31) or the rate of other adverse events (placebo 0.2 events, 95% CI 0.1 to 0.4 vs. ACE-I 0.3 events, 95% CI 0.1 to 0.5; p=0.61). Two patients in the ACE-I treated arm and one in the placebo treated arm reported dizziness, all episodes self-resolved and did not require cessation of therapy. Two patients in the ACE-inhibitor treated arm showed a significant decline in renal function beyond that selected to be an indication for study withdrawal (>30% increase in serum creatinine from baseline level). Withstanding those subjects who described a cough in the context of a pulmonary exacerbation, only one patient in the ACE-I treated arm described a persistent cough, but this did not lead to cessation of therapy.

When comparing the actual number of supervised pulmonary rehabilitation sessions attended by each treatment group (of a possible maximum of 16 sessions) the numbers, although statistically distinct, were fairly comparable (placebo 13, 95% CI 12 to 14 vs. ACE-I 11, 95% CI 10 to 12; $p=0.002$) and the difference is unlikely to have provided a more favourable training stimulus in the placebo group. Drug compliance was assessed through a pill return and count on the final assessment visit and was not significantly different between the groups (placebo 96% compliance, 95% CI 93 to 98 vs. ACE-I 96% compliance, 95% CI 94 to 99, $p=0.45$).

4.4 Discussion

4.4.1 Summary of results

The use of enalapril as an adjunct to a standardised programme of pulmonary rehabilitation rather than enhancing the peak work rate response to this rehabilitative intervention in fact reduced it, with a lower change in peak power measured during incremental cycle ergometry. This was associated with a smaller improvement in daily habitual physical activity in the enalapril treated group. Enalapril was clearly biologically active with reductions in both blood pressure parameters and serum ACE levels, suggesting that drug adherence was good. This data would thus not support the use of ACE-inhibitors to augment the response to pulmonary rehabilitation and may instead suggest caution should be applied in this context. Clinically it is important to note that this conclusion only applies to individuals without a clinical indication for ACE-inhibition.

4.4.2 Significance of the findings

Previous work has provided evidence suggesting a potential beneficial effect of ACE-inhibition on skeletal muscle function and thus the findings of this current study were not expected. This is, however, the first randomised controlled trial investigating ACE-inhibition as an adjunct to pulmonary rehabilitation in COPD. Many earlier human trials have been observational in nature looking at either epidemiological cohorts (Onder, Penninx et al. 2002, Di Bari, van de Poll-Franse et al. 2004) or those with intrinsic variability in angiotensin II levels due to genetic polymorphisms in the ACE gene (Montgomery, Marshall et al. 1998, Myerson, Hemingway et al. 1999, Zhang, Wang et al. 2008). The current findings highlight the importance of prospective randomised controlled trials, and it would be important to try to replicate these findings in another cohort, particularly as this was a single-centre study.

Early interventional studies suggested extrinsic reduction of angiotensin II activity would produce favourable effects on exercise capacity and strength in a COPD population. Work by Andreas et al. (Andreas, Herrmann-Lingen et al. 2006) in severe COPD subjects showed that administering the angiotensin II receptor blocker irbesartan for a period of 4 months lead to a numerical improvement in quadriceps strength, albeit not reaching statistical significance most likely due to a deficit of statistical power. Furthermore, in a small pilot study use of enalapril for 4 weeks in 21 patients with moderate to severe COPD lead to an improvement in both peak workload and oxygen pulse measured during incremental cycle ergometry (Di Marco, Guazzi et al. 2010).

Work by our own group in stable COPD patients selected for quadriceps weakness showed that 3 months therapy with the ACE-inhibitor fosinopril led to neither improved quadriceps strength or endurance, assessed with both volitional and non-volitional techniques, nor improved functional outcomes as assessed by the incremental shuttle walking test (Shrikrishna, Tanner et al. 2014). In fact in this study the change in quadriceps strength from baseline was measurably greater in the placebo than ACE-inhibitor treated group (placebo Δ +3.6 kg, 95% CI 2.1 to 5.0, $p<0.0001$ vs. ACE-inhibitor Δ +1.1 kg, 95% CI 0.03 to 2.2, $p=0.045$; between group difference +2.5 kg, 95% CI 0.7 to 4.3, $p=0.009$). This provides some support that, in line with this current study, ACE-inhibitor therapy may be in fact blunting changes in strength. One critique of this prior study was the lack of stratification by ACE genotype, with previous work showing that the ACE genotype can influence the response to training and emphasising the import of ensuring an even distribution of genotypes between treatment groups (Gosker, Pennings et al. 2004). In addition, the cohort of patients were selected on the basis of quadriceps weakness and rather unsurprisingly also displayed low levels of physical activity, with animal work suggesting that response to ACE-inhibition may require the provision of a concurrent training stimulus (Minami, Li et al. 2007, Guo, Minami et al. 2010). However, both of these issues were addressed in this current study which again showed no beneficial effect of ACE-inhibition, and in fact a detrimental effect on peak power gain.

Of note the cohort currently studied was particularly selected for the absence of any overt cardiovascular disease or other indication for ACE-inhibitor therapy, including ventricular failure and diabetes. Thus whilst ACE-inhibition alongside pulmonary rehabilitation does not appear to augment the physiological response, and in fact appears to attenuate it, this does not mean that ACE-inhibition per se is deleterious in all COPD patients referred for pulmonary rehabilitation. In fact it is well recognised that an increased risk of cardiovascular disease is a common systemic complication of COPD, and one in which the benefits of reduced angiotensin II activity are well established. A recently reported large meta-analysis demonstrated patients with COPD were far more likely to be diagnosed with cardiovascular disease than a non-COPD population (odds ratio 2.46, 95% CI 2.02-3.00, $p<0.0001$), including coronary arterial disease, arrhythmias, heart failure and arterial disease (Chen, Thomas et al. 2015), and there is a significant proportion of undiagnosed cardiovascular disease in this population (Barnes and Celli 2009). Thus, despite this current study failing to show a positive effect on augmenting the effects of pulmonary rehabilitation, it provides no support for cessation of ACE-inhibition therapy in those with a good indication for its use.

4.4.3 Potential mechanism of action of ACE-inhibitors

It is worth exploring the possible mechanisms by which the ACE-inhibitor enalapril may reduce the gains in exercise capacity in response to pulmonary rehabilitation, although the exact basis remains unclear and is a matter of speculation. Reductions in arterial blood pressure, both systolic and diastolic parameters, were noted in this study in line with recognised effects of ACE-inhibition in reducing total peripheral vascular resistance through vasodilatation. During exercise adequate blood flow is needed to ensure oxygen delivery to the exercising muscle and it may be that the reduction in perfusion pressure or vasodilatation to other vascular beds diverts blood flow away from actively exercising muscle. However, no direct evidence exists showing this and, at least in the resting state, ACE-inhibition in fact improves skeletal muscle blood flow through reductions in vascular resistance to the skeletal muscle bed (Ventura, Frohlich et al. 1984, Ventura, Frohlich et al. 1985).

Interestingly there is evidence that tissue capillarity is reduced in COPD (Saey, Michaud et al. 2005) and associated with muscle contractile fatigue, and that increased capillarity is one method through which rehabilitation is beneficial (Vogiatzis, Simoes et al. 2010). The renin-angiotensin system has also been implicated in maintaining normal vascular structure and reactivity in the microcirculation of skeletal muscle. In a normotensive rat model use of captopril has been associated with both a reduction in arteriolar diameter and arteriolar density of the skeletal muscle after 4 weeks of therapy, suggesting that ACE-inhibition may have a direct inhibitory effect on vascular growth (Wang and Prewitt 1991). As well as a reduction in maximum arteriolar diameter, the administration of captopril has been shown to impair the relaxation of skeletal muscle resistance arteries and arterioles in response to vasodilator stimuli, suggesting that ACE-inhibition affects both the structure and vascular tone, and thus the functional ability of arterioles supplying skeletal muscle to dilate (Frisbee, Weber et al. 1999). In further rat studies, the chronic administration of captopril has been associated with reduced exercise time on a constant speed treadmill exercise protocol accompanied by a greater rise in blood lactate at equivalent exercise, which has been postulated to relate to changes in microvasculature structure and function (Minami, Mori et al. 2004). Thus changes in vascularity of the skeletal muscle bed may be hypothesised to lie behind some of the adverse effects of ACE-inhibition on skeletal muscle function.

Although angiotensin II may be recognised to have adverse effects on skeletal muscle, as with cardiac myocytes it is noted to have an important influence in the hypertrophic response to mechanical loading and tetanic strength (Gordon, Davis et al. 2001). In COPD, individuals with high levels of angiotensin II, associated with homozygosity for the D allele of the ACE gene, have maintained quadriceps strength (Hopkinson, Nickol et al. 2004). It is well recognised that in COPD patients attending pulmonary rehabilitation, peripheral muscle strength is an important contributor to endurance exercise capacity (Gosselink, Troosters et al. 1996). It could perhaps be proposed that the role of high angiotensin II levels on strength and hypertrophic response to loading in COPD outweighs the effects of lower angiotensin II levels on endurance capacity, and inducing a reduction in angiotensin II levels pharmacologically attenuated strength capacity, thus leading to poorer peak exercise performance.

It is also worth considering the complexity of the renin-angiotensin system and how this may influence the response to ACE-inhibition. There are two recognised isoforms of ACE (ACE 1 and ACE 2), although the more well-recognised ACE 1 (usually referred to simply as ACE) is the only form present ubiquitously in the vasculature and expressed in human skeletal muscle, ACE2 being restricted to specific tissues only (heart, kidney and testis) (Donoghue, Hsieh et al. 2000). Other components of the RAS have been demonstrated to influence skeletal muscle function, including angiotensin (1-7), a cleavage product of angiotensin II, which has anti-atrophic effects and protects skeletal muscle from degradation, thus having the opposite effects to angiotensin II (Acuna, Pessina et al. 2014). It is certainly possible, therefore, that ACE-inhibition is acting on several regulatory pathways at a molecular level, some of which may counteract each other, highlighting the complexity of the renin-angiotensin system in the skeletal muscle (figure 4.6).

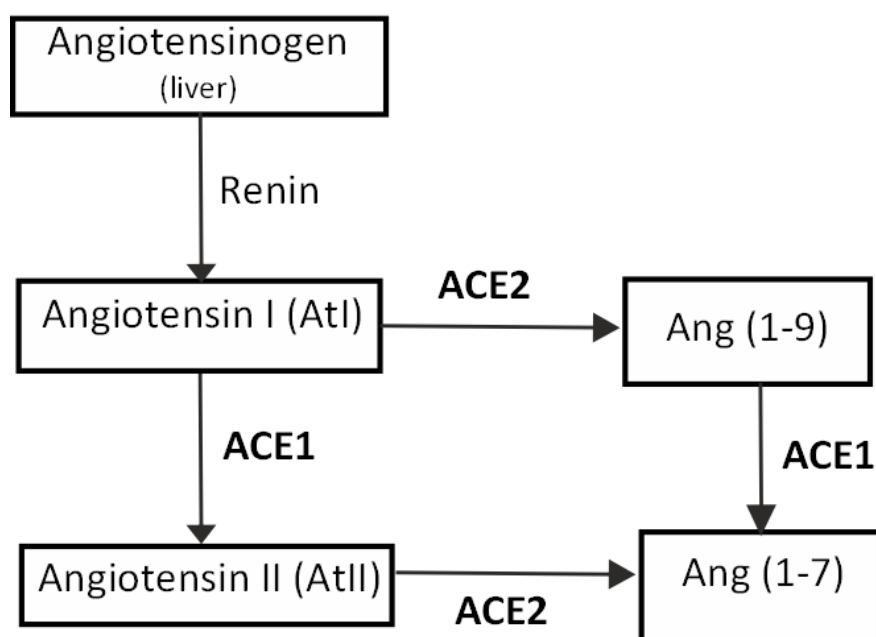


Figure 4.6: Renin-angiotensin pathway in skeletal muscle.

Abbreviations: ACE - angiotensin-converting enzyme; Ang - angiotensin.

Of interest in this work was the noted reduction in physical activity level of the subjects who received ACE-inhibition, in comparison to the rise seen in the placebo treated group. Some may speculate this was a consequence of hypotension, however, only two individuals in the ACE-inhibitor treated group reported dizziness, and in both cases this was transient, settled spontaneously and did not require cessation of therapy. Although the health-related quality of life questionnaires employed understandably focussed on respiratory disability, which may have missed other relevant symptom changes, patients in the ACE-inhibitor treatment group did not report feeling subjectively worse. The current data cannot fully explain this effect and we can only speculate on why this may have been seen.

4.4.4 Critique of the method

This trial was prospectively stratified and powered based on ACE genotype, precluding any discrepancy of the spread of the ACE genotype between treatment groups influencing the results. Some may speculate that perhaps it would be more effective to target those with intrinsically high angiotensin II levels (the DD genotype), who may benefit most from reduction of angiotensin II activity. It is also important to point out that previous work looking at rehabilitation responses in

COPD has demonstrated that those with intrinsically low ACE levels, through possession of the I allele of the ACE gene polymorphism, showed a greater response to exercise training (Gosker, Pennings et al. 2004), although in part this may have been explained by regression to the mean with this group being more physiologically detrained at baseline. However, no significant difference in peak workload response to pulmonary rehabilitation was found between genotypes whether either considering the placebo (II +4, ID +10, DD +11 Watt; $p=0.28$) or ACE-inhibitor (II 0, ID +1, DD +1 Watt; $p=0.96$) treated groups, although of note the study was not powered to allow effective subgroup analysis.

Account was made also in the power calculation of the fact that those individuals possessing the II genotype, thus with endogenous low levels of angiotensin II activity, may show a lower response to ACE-inhibition and thus we do not believe that the effects seen were due to underpowering. The treatment groups were well matched at baseline, lending confidence to the findings that the reduced effects of pulmonary rehabilitation in the ACE-inhibitor treated arm was not because of any underlying difference in the treatment groups.

Peak workload on incremental cycle ergometry, a strong physiological measure, was selected as the primary outcome measure. This has previously been shown to be influenced by both low angiotensin II levels through both the intrinsic ACE genotype (Zhang, Wang et al. 2008) and exogenous suppression of ACE activity through pharmacological means (Di Marco, Guazzi et al. 2010). In addition, taking continuous metabolic measurements allowed us to study measures of both ventilatory efficiency and integrated measures of exercise capacity including the oxygen uptake efficiency slope. Due to the power-time curve a relatively small change in maximal exercise capacity can translate to a large improvement in endurance time, so it was felt peak exercise capacity was a valid endpoint to select over endurance time. Although it cannot be excluded that ACE-inhibition might influence endurance exercise performed at a submaximal workload, the attenuation of improvement in peak exercise performance makes this unlikely.

It is important to note that other tests of exercise performance could have been selected beyond cycling ergometry, and may have allowed a more focussed approach to the study of skeletal muscle function, such as single leg measurements. Whilst cycle ergometry allows study of the combined

cardiovascular, respiratory and muscle components of exercise, single leg measurements may allow a more tailored approach to study specific outcomes, such as power or endurance capacity.

One area of discussion is over the choice of pharmacological agent selected. A decision was made to select an ACE-inhibitor over an angiotensin II receptor blocker to ensure additional effects on the prevention of kinin, particularly bradykinin, degradation. Bradykinin receptor polymorphisms have been shown to influence skeletal muscle phenotype in COPD (Hopkinson, Eleftheriou et al. 2006, Hopkinson, Li et al. 2008), and established experimental work has shown bradykinin to influence skeletal muscle metabolism through generation of nitric oxide, improved insulin sensitivity and reduced oxidative stress (Henriksen and Jacob 2003, Yu, Li et al. 2008).

Previous beneficial effects in COPD have been shown in controlled trials using perindopril (Sumukadas, Witham et al. 2007) and enalapril (Di Marco, Guazzi et al. 2010), although no effect was seen with fosinopril (Shrikrishna, Tanner et al. 2014). Given that enalapril has previously been noted in COPD to improve peak work rate, our primary outcome measure, this seemed an appropriate agent to choose. There was physiological evidence in the current study of adequate dosing and a treatment effect in terms of both reduced blood pressure parameters and serum ACE levels. This does not ensure, however, that there were effects on the renin-angiotensin system at the level of the skeletal muscle, and it would be challenging to explore this without sampling the skeletal muscle itself, which remains an area for future exploration. This work does not preclude the fact that different ACE-inhibitors may lead to differing findings, but is in line with previous work (Shrikrishna, Tanner et al. 2014), suggesting this may be a class effect. Relatively little evidence exists for how the renin-angiotensin system influences skeletal muscle fibre type and size, although limited work in healthy individuals suggests low levels of angiotensin II are associated with a higher type I skeletal muscle fibre proportion (Zhang, Tanaka et al. 2003). Thus the role of agents altering the renin-angiotensin pathways on muscular structure and function is deserving of further research.

It remains possible that the dose of enalapril administered may be of some significance. We selected the same dose (10mg once daily) as had been previously studied and noted to alter peak exercise capacity (Di Marco, Guazzi et al. 2010). We achieved physiological effects expected through ACE-inhibition, suggestive that a sufficient dose had been administered. However, it remains

possible that the dose required to alter parameters such as blood pressure is not that required to alter the milieu of the skeletal muscle. This remains an area of possible future research.

The trial design provided 10 weeks medication, started one week prior to any pulmonary rehabilitation sessions and for the duration of the full eight week programme up until the post rehabilitation assessments were undertaken. Such assessments were thus conducted in the week immediately following the conclusion of the programme, and as some patients experienced pulmonary exacerbations during the programme this may have blunted the response to the intervention, although given that the pulmonary exacerbation rate was equal in both treatment arms is unlikely to have influenced the between group response. It was unclear prior to the initiation of this study the period of time over which to administer an ACE-inhibitor to achieve effects on the skeletal muscle, although previous effects on exercise capacity have been achieved with shorter periods of administration (Di Marco, Guazzi et al. 2010) than used in the current study.

We noted that the ACE-inhibitor treatment group attended a slightly lower number of physiotherapist led rehabilitation sessions than those receiving placebo therapy. The actual difference in number is unlikely to explain the differences in outcomes, although we cannot be certain this did not contribute. The programme itself, whilst achieving an improvement in peak workload in the placebo treated group, in some aspects such as quality of life measures demonstrated only modest improvements following. Whilst it is known the prescribed exercise intensity of the programme, full data is not available on whether this was achieved during the sessions and compliance in those who attended, and this is a notable limitation of the study. Thus some of the findings noted may relate to the rehabilitation programme itself, and thus would need confirmation in a separate site and cohort of patients. It also remains conceivable that certain groups of COPD subjects may benefit from ACE-inhibition whilst others experience detrimental effects and use of mean data loses the fact that there will be significant variability in individual responses. The current study is not sufficiently powered to allow effective subgroup analysis beyond the chosen stratification variables to either confirm or refute this.

4.4.5 Conclusion

This randomised controlled trial demonstrated that use of the ACE-inhibitor enalapril alongside a programme of pulmonary rehabilitation in a COPD population, in the absence of a clinical indication for ACE-inhibition, reduced the response to exercise training compared to placebo. Thus the evidence presented does not support a role for ACE-inhibition in this context and instead suggests significant caution should be advised when considering ACE-inhibition to influence the skeletal muscle phenotype in COPD. The exact biological mechanisms underlying these findings remain an area of active research. Of note this study specifically excluded individuals with a pre-existing clinical indication for ACE-inhibition and thus it does not support the withdrawal of ACE-inhibitors from such patients prior to or during pulmonary rehabilitation.

Chapter 5: Randomised Controlled Trial of Acute Nitrate Supplementation on Exercise Performance in COPD

5.1 Introduction

5.1.1 Background

Skeletal muscle impairment is a common complication of COPD, affecting the ability of patients to participate in endurance activities (Schols, Mostert et al. 1991, Gosselink, Troosters et al. 1996). The use of agents to augment exercise capacity is a longstanding research goal in both healthy and disease states. Whilst pulmonary rehabilitation is a highly effective intervention for COPD patients, there is a need for adjunctive therapies that will help ensure both a maximal response to this rehabilitative intervention and maintenance of this beneficial effect for as long as possible.

5.1.2 Rationale and hypothesis

Exercise limitation is common in COPD, with patients frequently volunteering leg fatigue as a limiting factor during exercise (Man, Soliman et al. 2003). Alterations in the skeletal muscle phenotype of these patients (Jakobsson, Jorfeldt et al. 1995, Gosker, Hesselink et al. 2007) means they have reduced oxidative capacity and experience early anaerobic metabolism (Maltais, Simard et al. 1996, Natanek, Gosker et al. 2013). Thus any interventions that reduce the oxygen cost of exercise are likely to be beneficial, allowing patients to exercise for a longer duration and at a higher intensity, where the physiological gains are known to be greater (Casaburi, Patessio et al. 1991).

Nitric oxide (NO) is well recognised as an essential physiological mediator in the body, essential for nutrient and oxygen delivery as well as mitochondrial function. NO may be produced endogenously through the action of the nitric oxide synthase (NOS) family of enzymes, causing oxidation of L-arginine, converting it to NO and L-citrulline. NO may also be produced through the reduction of exogenous dietary-derived nitrate, found at high concentrations in green leafy vegetables and some root vegetables such as beetroot (Ysart, Miller et al. 1999). Dietary nitrate ingestion leads to a rise in plasma nitrate levels followed by a rise in plasma nitrite, an indicator of bioavailable NO, through the nitrate-nitrite-NO reduction pathway in a NOS-independent manner.

In health a link has been suggested between plasma nitrite levels and performed physical activity suggesting a possible role for the nitrate-nitrite-NO pathway in augmenting exercise performance

(Dreissigacker, Wendt et al. 2010). Much interest has thus been stimulated in the possible role of nitrate supplementation in facilitating exercise in healthy subjects. Various studies have investigated the role of nitrate supplementation in augmenting athletic performance with reported benefits including a reduction in the oxygen cost of submaximal workloads during a variety of exercise modalities in young, healthy individuals (Larsen, Weitzberg et al. 2007, Bailey, Winyard et al. 2009, Bailey, Fulford et al. 2010, Vanhatalo, Bailey et al. 2010, Lansley, Winyard et al. 2011), associated with improved measures of exercise performance (Bailey, Winyard et al. 2009, Bailey, Fulford et al. 2010, Vanhatalo, Bailey et al. 2010, Lansley, Winyard et al. 2011, Murphy, Eliot et al. 2012). The majority of studies have used beetroot juice as a reliable method of supplementing nitrate, the influence on oxygen consumption being dependent largely on nitrate itself since nitrate-depleted beetroot juice fails to elicit the same effect (Lansley, Winyard et al. 2011).

Although dietary nitrate supplementation has been shown to enhance exercise performance in health it is not clear if these effects can translate to COPD patients, some studies suggesting benefit (Berry, Justus et al. 2015, Kerley, Cahill et al. 2015) but with significant methodological issues including the placebo preparation selected. Other more robust studies have proved negative (Leong, Basham et al. 2015, Shepherd, Wilkerson et al. 2015). In the two latter studies, the dose of nitrate administered immediately prior to testing was below that which has been shown in health to induce improvements in exercise performance (Wylie, Kelly et al. 2013), and in one case (Leong, Basham et al. 2015) testing was performed after only one hour had elapsed from nitrate dosing when nitrite levels in the plasma may not yet have peaked. Both studies also failed to show an effect on blood pressure parameters suggesting the dosing regimen chosen may have been inadequate.

We thus investigated the hypothesis that acute nitrate dosing, in the form of beetroot juice concentrate, using a higher dosing regimen would improve exercise performance and reduce the oxygen cost of submaximal exercise in COPD compared to a robust placebo preparation.

5.2 Methods

5.2.1 Patient selection

All participants provided written informed consent prior to enrolment in the study which was conducted in line with the principles of the Declaration of Helsinki. Approval was gained from the Bromley Research Ethics Committee (REC reference 13/LO/0372) and the study was registered prospectively on a publicly accessible database (www.controlled-trials.com/ISRCTN66099139).

COPD subjects of GOLD stage II-IV were considered for inclusion in the study (Rabe, Hurd et al. 2007). Subjects were excluded from participation if they were receiving antibiotic therapy (either to treat current infection or on a prophylactic basis), as influencing oral bacterial populations has been shown to affect the response to an oral nitrate load (Govoni, Jansson et al. 2008). Subjects on long-term oxygen therapy and those receiving nitrate-based medication, or with a clinical indication for such, including ischaemic heart disease, peripheral vascular disease and heart failure, were excluded from the study. Other exclusion factors included significant impairment of renal function (as assessed by an estimated glomerular filtration rate (eGFR) <50 ml/min/1.73m²), hypotension (defined as a systolic blood pressure <100 mm Hg), treatment with phosphodiesterase type V inhibitors, subjects within one month of a pulmonary exacerbation or 3 months of pulmonary rehabilitation, or possessing any other significant comorbidity that would impact on mobility and ability to participate in the study.

5.2.2 Study design and randomisation

The study was a single-dose, double-blind, randomised, placebo-controlled, cross-over trial. Subjects were randomly allocated to the order in which they received either the active or placebo preparations using a computer-generated randomisation in blocks of 4 produced by an independent statistician, with consecutive numbers linked to active or placebo beverages. Following a minimum of a one week wash-out period subjects received the cross-over beverage. This ensured plasma nitrate and nitrite levels would have normalised before repeat testing (Jones 2014).

5.2.3 Intervention

140 ml of BEET-IT Sport Stamina Shot (James White Drinks, Ipswich, UK) containing 0.8 g nitrate and 140 ml of a matched placebo of beetroot juice, specifically depleted of nitrate by passage through an ion exchange resin prior to pasteurisation, were used in the treatment and placebo conditions respectively. The placebo preparation was identical in appearance and taste (Lansley, Winyard et al. 2011), and also produced beeturia, thus was indistinguishable to both the subjects and investigator. The dose chosen was intended to provide a bolus of nitrate (12.9 mmoles) in the treatment condition exceeding that shown necessary to reduce oxygen consumption during submaximal exercise in young healthy subjects (Wylie, Kelly et al. 2013). This was also a convenient dosing method taken as two 70 ml bottles which provided a readily acceptable method of achieving acute nitrate dosing, being easily ingested.

5.2.4 Study protocol

Visit 1 (screening):

- Review of inclusion/exclusion factors
- Spirometry
- Blood drawn for renal function

Visit 2 (baseline phenotyping):

- Medical history review
- Anthropometric measurements
- Vital signs
- Full pulmonary function testing including spirometry, gas transfer and plethysmographic lung volumes
- Capillary blood gases
- Quality of life assessments including MRC dyspnoea score, St. George's Respiratory Questionnaire for COPD, COPD Assessment Test score
- Quadriceps maximal volitional contraction
- Measurement of body composition by bioimpedance
- Fasting bloods for renal function, inflammatory markers

- Physical activity monitoring for a period of one week using a triaxial accelerometer
- Maximal symptom-limited incremental cycle ergometry

Visit 3 & 4 (dosing visits):

Prior to the dosing visits subjects were requested to avoid foodstuffs high in nitrate in the preceding 48 hours and any strenuous exertion in the preceding 24 hours. Request was made to match food and caffeine consumption on the two days of testing and subjects were asked to cease the use of any mouthwash or chewing gum, as this affects oral bacterial flora and thus the response to an oral nitrate load (Govoni, Jansson et al. 2008).

- Oral nitrate / matched placebo given at time 0 hours with preceding measurements of blood pressure and blood draw for nitrite and nitrate levels
- At 3 hours after dosing repeated blood pressure measurements, blood draw for nitrate and nitrite levels and endurance cycle ergometry performed at 70% peak workload achieved on baseline incremental cycle ergometry
- Blood draw at peak exercise for nitrate and nitrite levels

After a minimum period of 7 days the subjects crossed over and repeated the protocol with the alternative beverage. Exercise testing was conducted at the same time on each occasion.

Further details of the conduct of the phenotypic assessments, plasma nitrate metabolite measurement and conduct of the cardiopulmonary test examination can be found in Chapter 2 (Methods). A summary of the trial schedule is indicated in table 5.1.

	Visit 1 Screening	Visit 2 Baseline phenotyping	Visit 3 Dosing visit	Visit 4 Dosing visit
Medical history & examination		X		
Spirometry	X	X		
Lung volumes & gas transfer		X		
Renal function testing	X	X		
Blood draw for inflammatory markers		X		
Bioimpedance		X		
Quality of life assessment		X		
QMVC		X		
Physical activity monitoring		X		
Incremental cycle ergometry		X		
Venous nitrate metabolites			X	X
Endurance cycle ergometry at 70% peak power			X	X

Table 5.1: Protocol for randomised controlled trial of acute nitrate supplementation on exercise performance in COPD.

Abbreviations: QMVC - quadriceps maximal volitional contraction.

5.2.5 Primary and secondary outcome measures

1. Effect of nitrate supplementation on time to exhaustion during endurance cycle ergometry –
Primary outcome measure
2. Effect of nitrate supplementation on plasma nitrate and nitrite levels
3. Effect of nitrate supplementation on resting arterial blood pressure parameters

4. Effect of nitrate supplementation on area under the curve to VO_2 isotime during endurance cycle ergometry

5.2.6 Data analysis and statistics

A convenience sample size of 25 subjects was chosen to potentially identify a clinically meaningful signal. Data are presented as mean $\pm$ standard deviation and were analysed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, California, USA). Repeated measures analysis of variance (ANOVA) was utilised to compare plasma nitrate metabolites between treatment conditions and at each specific time-point. To allow the comparison of pulmonary oxygen consumption between the two conditions, individual CPEX periods were expressed as percentiles of isotime (defined as the duration of the shortest of the two endurance exercise tests). Individual responses were then grouped to allow analysis of VO_2 against percentage of isotime (plotted at the midpoint of each 10th percentile of isotime). The area under the curve was assessed for each individual subject and the two treatment conditions compared using a paired t-test. Resting, isotime and peak exercise parameters in the two treatment conditions were compared using paired t-tests, or the appropriate non-parametric test for data that was not normally distributed. A p-value <0.05 was considered to be statistically significant.

5.3 Results

5.3.1 Recruitment process

Subjects were enrolled from June 2013 through to April 2014 when enrolment was completed. 43 subjects were assessed for eligibility for the study, of whom 34 were suitable and approached with regards to study participation. 25 were then formally screened for involvement in the study all of whom passed screening and agreed to participate. 21 of these completed both dosing visits, with 4 subjects withdrawing prior to study completion. The CONSORT diagram below provides further details of the recruitment process.

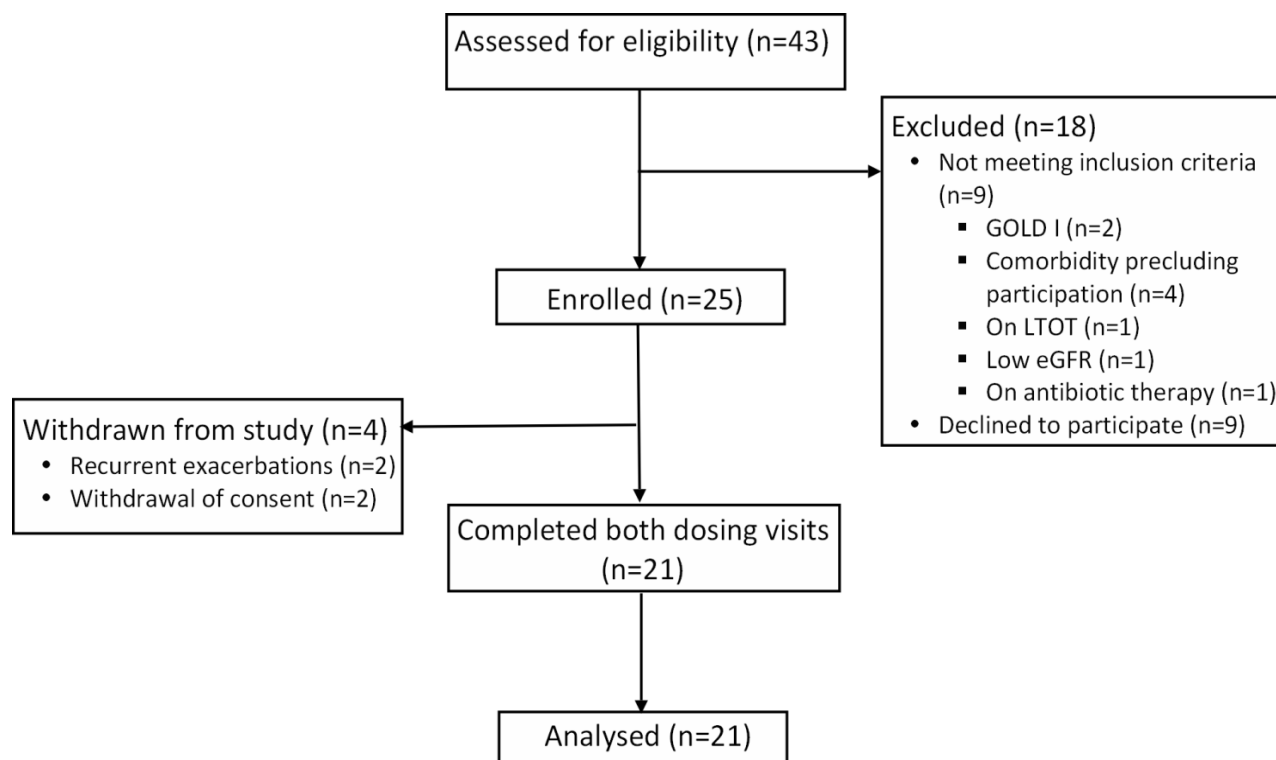


Figure 5.1: CONSORT recruitment diagram for enrolment and study completion.

Abbreviations: eGFR – estimated glomerular filtration rate; GOLD – global initiative for chronic obstructive lung disease; LTOT – long-term oxygen therapy.

5.3.2 Baseline characteristics

Sixteen men and five women completed both dosing visits of the study. The baseline characteristics of the group are noted in table 5.3. Patients were fully compliant with the dosing protocol and no adverse effects were noted during the dosing visits, with the exception of beeturia as expected (Bailey, Winyard et al. 2009, Wylie, Kelly et al. 2013).

Measurement	Mean (SD)
Sex (♂:♀)	16:5
Age (years)	68 (7)
Systolic BP (mm Hg)	137 (19)
Diastolic BP (mm Hg)	79 (7)
Smoking PYH	40 (29)
MRC dyspnoea score	2 (1)
CAT score	14 (7)
SGRQ-C total	35.37 (13.00)
BMI (kg/m ²)	25.2 (5.5)
CRP (mg/L)	6 (11)
Fibrinogen (g/L)	3.2 (0.7)
FFM (kg)	48.9 (8.4)
FFMI (kg/m ²)	16.9 (2.2)
QMVC (kg)	37.8 (10.7)
QMVC % predicted	87.8 (19.3)
Average daily step count	6669 (4436)
Average PAL	1.50 (0.20)
FEV ₁ (L)	1.33 (0.58)
FEV ₁ % predicted	50.1 (21.6)
FVC (L)	3.38 (0.91)
TLCO _c % predicted	54.1 (19.3)
RV (L)	4.01 (1.35)
RV % predicted	167 (53)
TLC (L)	7.56 (1.71)
TLC % predicted	119 (14)
RV/TLC ratio (%)	52.3 (8.7)
PaO ₂ (kPa)	10.7 (1.2)
PaCO ₂ (kPa)	4.7 (0.5)
Peak power on cycle (watts)	72 (31)
Peak VO ₂ (ml/min/kg)	18.0 (5.9)

Table 5.2: Demographic and baseline clinical characteristics of the subjects (n=21).

Data shown are mean (standard deviation).

Abbreviations: BP – blood pressure; PYH – pack year history; MRC – Medical Research Council; CAT – COPD assessment test; SGRQ-C – St. George’s respiratory questionnaire for COPD; BMI – body mass index; FFMI – fat free mass index; QMVC – quadriceps maximal volitional contraction; PAL - physical activity level; FEV₁ – forced expiratory volume in 1 second; FVC – forced vital capacity; TLCO_c – carbon monoxide diffusing capacity; RV – residual volume; TLC – total lung capacity; VO₂ – pulmonary oxygen uptake.

5.3.3 Effect of nitrate supplementation on venous nitrate and nitrite levels

Figure 5.2 demonstrates venous plasma nitrate measurements at all time points (baseline, post dosing and peak exercise). 3 hours following dosing with nitrate-rich beetroot juice plasma nitrate levels increased substantially ($37.0 \pm 16.4 \mu\text{M}$ baseline vs. $820.2 \pm 187.7 \mu\text{M}$ post dosing; $p < 0.0001$) and remained significantly elevated at peak exercise compared to baseline ($917.1 \pm 291.6 \mu\text{M}$ peak exercise; $p < 0.0001$). There were no significant changes in plasma nitrate concentrations following dosing with the placebo preparation ($45.7 \pm 15.8 \mu\text{M}$ baseline vs. $45.3 \pm 16.5 \mu\text{M}$ post dosing; $p = 0.997$) or at peak exercise ($42.8 \pm 20.0 \mu\text{M}$ peak exercise; $p = 0.86$; figure 5.2).

Plasma nitrite levels were below the quantifiable limit ($0.2 \mu\text{M}$) in all but three patients at baseline and remained so in the placebo condition but increased following nitrate supplementation to $1.57 \pm 0.98 \mu\text{M}$. In the nitrate treated condition the plasma nitrite levels remained elevated at peak exercise ($1.37 \pm 0.65 \mu\text{M}$). In the placebo treated condition there was no change in the plasma nitrite levels following supplementation.

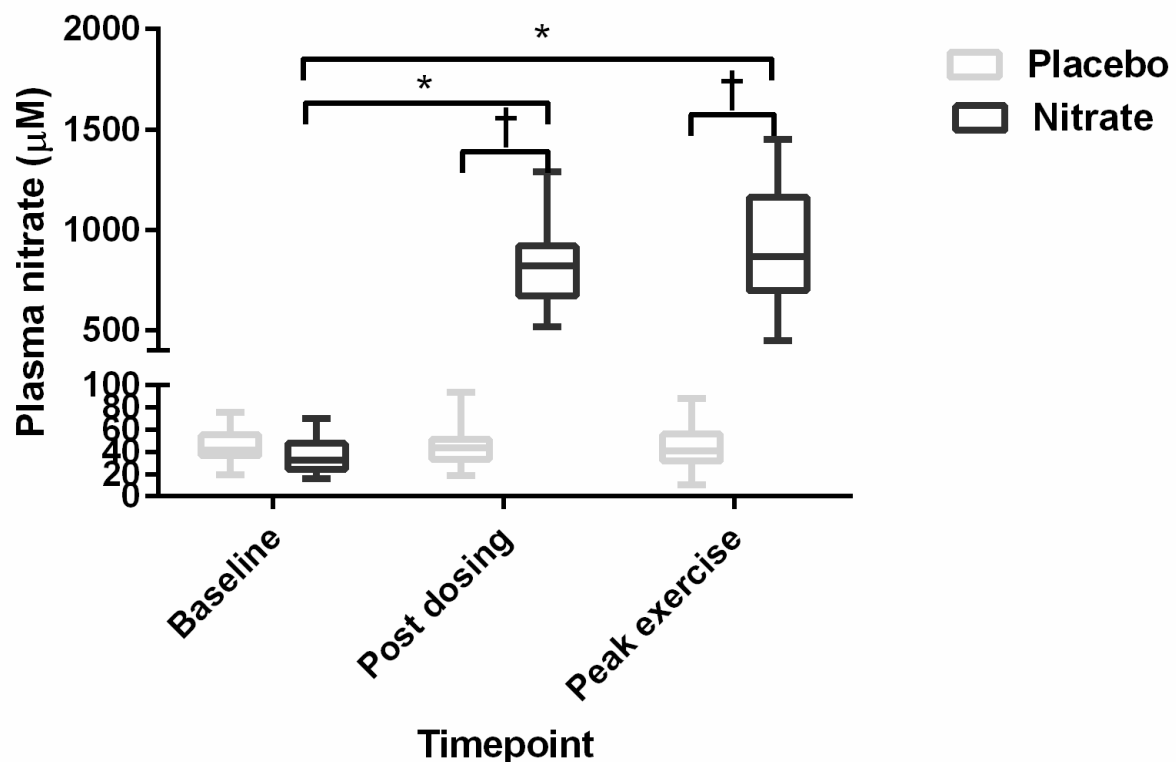


Figure 5.2: Alterations in plasma nitrate following dosing. Plasma nitrate concentrations prior to ('baseline') and following consumption of a nitrate-rich or placebo beverage. 'Post dosing' indicates a time point 3 hours following beverage consumption which was immediately prior to endurance cardiopulmonary exercise testing. 'Peak' was a time point at the point of exhaustion during endurance cycle ergometry testing. Data are presented as 25th-75th percentile with the solid line representing the median value, and whiskers the minimum to maximum values. Repeated measures analysis of variance (ANOVA) was utilised to compare plasma nitrate metabolites between treatment conditions and at each specific time-point; *significantly different from baseline, $p < 0.0001$; †significantly different from placebo group, $p < 0.0001$.

5.3.4 Effect of nitrate supplementation on blood pressure parameters

The changes in resting arterial blood pressure parameters following ingestion of nitrate-rich beetroot juice and placebo are shown in figure 5.3. There was no significant difference in the reduction in systolic blood pressure after dosing with nitrate or placebo (7 ± 10 mmHg nitrate vs. 8 ± 11 placebo mmHg; $p = 0.38$). The reduction in diastolic blood pressure was significantly greater in the nitrate supplemented group compared to placebo dosing (7 ± 8 mmHg nitrate vs. 1 ± 8 mmHg placebo; $p = 0.008$). There was a trend towards greater lowering of the mean arterial pressure (MAP)

following nitrate-rich beetroot juice versus placebo, although this failed to reach statistical significance (7 ± 8 mmHg nitrate vs. 3 ± 8 mmHg placebo; $p=0.07$). Reductions in blood pressure parameters were noted in both individuals who were normotensive and hypertensive at baseline.

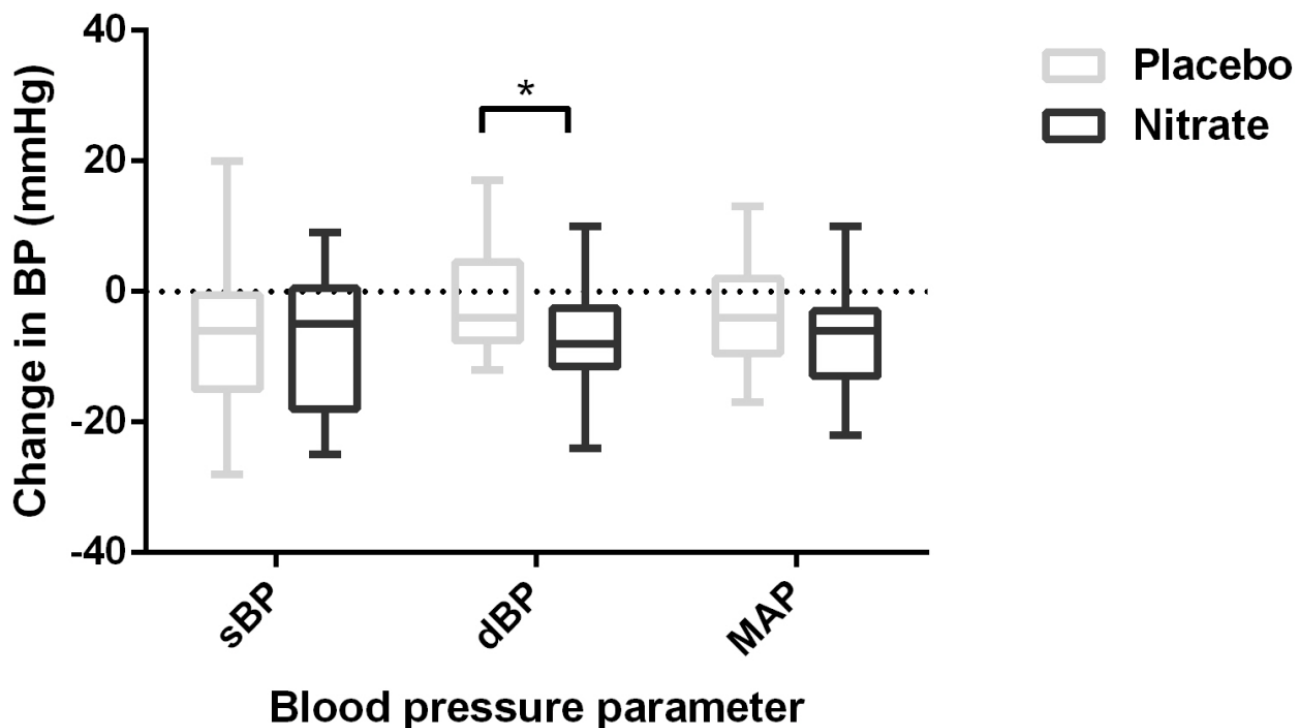


Figure 5.3: Alterations in resting blood pressure parameters following dosing. The change from pre-supplemented baseline in blood pressure parameters 3 hours following dosing with nitrate-rich beetroot juice or placebo preparation are shown. Data are presented as 25th-75th percentile with the solid line representing the median, and whiskers the minimum to maximum values. Paired t-tests were used to compare blood pressure parameters between treatment conditions; *significantly different from placebo, $p=0.008$.

Abbreviations: BP – blood pressure; sBP – systolic blood pressure; dBP – diastolic blood pressure; MAP – mean arterial pressure.

5.3.5 Effect of nitrate supplementation on endurance time

The individual endurance times during cycle ergometry performed at 70% maximum workload in both treatment conditions of the study are shown in figure 5.4. The median endurance time was not significantly different between the groups (5.65 (IQR 3.90-10.40) minutes nitrate vs. 6.40 (IQR 4.01-

9.67) minutes placebo condition; $p=0.50$). We also tested for the presence of an order effect, but this was not present (the change in endurance time if nitrate supplementation was administered first was -2.03 ± 4.64 minutes versus 1.92 ± 4.16 minutes if the placebo was given first; $p=0.13$).

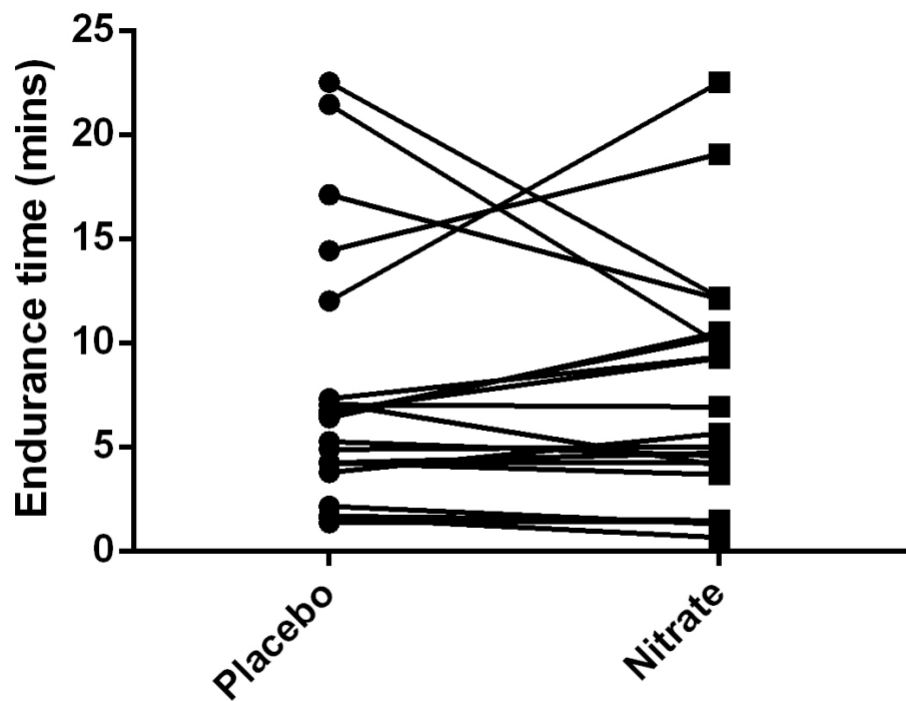


Figure 5.4: Endurance time during cycle ergometry at 70% maximal workload measured in the placebo and nitrate-rich beetroot juice dosing conditions. A Wilcoxon test was used to compare median values in the two treatment groups; no significant difference was found.

5.3.6 Effect of nitrate supplementation on resting, isotime and peak CPEX measurements

At rest there was no significant difference in minute ventilation or oxygen consumption (VO_2) between the two treatment conditions (table 5.3). At isotime (i.e. the longest duration of equivalent exercise completed under either exercise condition) both minute ventilation and pulmonary VO_2 were lower in the nitrate supplemented condition than the placebo treated (table 5.3). There was a numerical improvement in the power/ VO_2 relationship (P/O ratio) in the nitrate treated condition; however, this was not statistically significant. The relative oxygen pulse was reduced in the nitrate treated condition at isotime.

	Placebo	Nitrate-rich beetroot juice	p value
HR (bpm)			
Rest	85 (11)	87 (11)	0.06
Isotime	122 (17)	121 (20)	0.30
Peak	122 (19)	123 (18)	0.37
BF (breaths/min)			
Rest	16 (4)	17 (4)	0.10
Isotime	32 (8)	32 (5)	0.48
Peak	33 (6)	34 (6)	0.24
VT (L)			
Rest	0.93 (0.43)	0.87 (0.25)	0.33
Isotime	1.44 (0.39)	1.40 (0.47)	0.12
Peak	1.41 (0.40)	1.35 (0.41)	0.06
VE (L/min)			
Rest	13.27 (3.90)	13.37 (3.95)	0.34
Isotime	43.85 (13.94)	41.88 (14.85)	0.029
Peak	44.40 (14.11)	42.61 (14.67)	0.015
VO ₂ (ml/min/kg)			
Rest	4.5 (1.2)	4.4 (1.3)	0.32
Isotime	17.2 (6.0)	16.6 (6.0)	0.043
Peak	17.1 (5.5)	16.7 (5.7)	0.10
VCO ₂ (ml/min/kg)			
Rest	4.2 (1.2)	4.2 (1.3)	0.49
Isotime	17.1 (6.0)	16.7 (6.3)	0.14
Peak	17.1 (5.8)	16.7 (6.0)	0.14
Power/VO ₂ ratio (W/ml/min/kg)			
Isotime	2.92 (0.73)	3.03 (0.73)	0.05
Peak	2.91 (0.72)	3.02 (0.73)	0.07
SpO ₂ (%)			
Rest	95 (2)	96 (2)	0.26
Isotime	92 (4)	93 (4)	0.15
Peak	92 (4)	93 (4)	0.07
Oxygen pulse (ml/beat)			
Rest	3.8 (1.0)	3.6 (0.9)	0.12
Isotime	10.0 (2.6)	9.7 (2.3)	0.09
Peak	9.9 (2.6)	9.5 (2.2)	0.018
Relative oxygen pulse (ml/beat/kg)			
Rest	0.053 (0.013)	0.051 (0.014)	0.15
Isotime	0.143 (0.039)	0.138 (0.036)	0.043
Peak	0.140 (0.036)	0.134 (0.033)	0.012

Table 5.3: Rest, isotime and peak analysis of the cardiopulmonary exercise test (CPEX) parameters.

Data are shown as mean (standard deviation).

Abbreviations: HR - heart rate; BF – breathing frequency; VT – tidal volume; VE – minute ventilation; VO_2 – pulmonary oxygen uptake; VCO_2 - exhaled carbon dioxide production; SpO_2 – peripheral arterial oxygen saturation.

5.3.7 Effect of nitrate supplementation on VO_2 curves to isotime

There was a significant separation of the pulmonary VO_2 curves to isotime leading to a significant difference in the area under the curve between the two treatment conditions (figure 5.5).

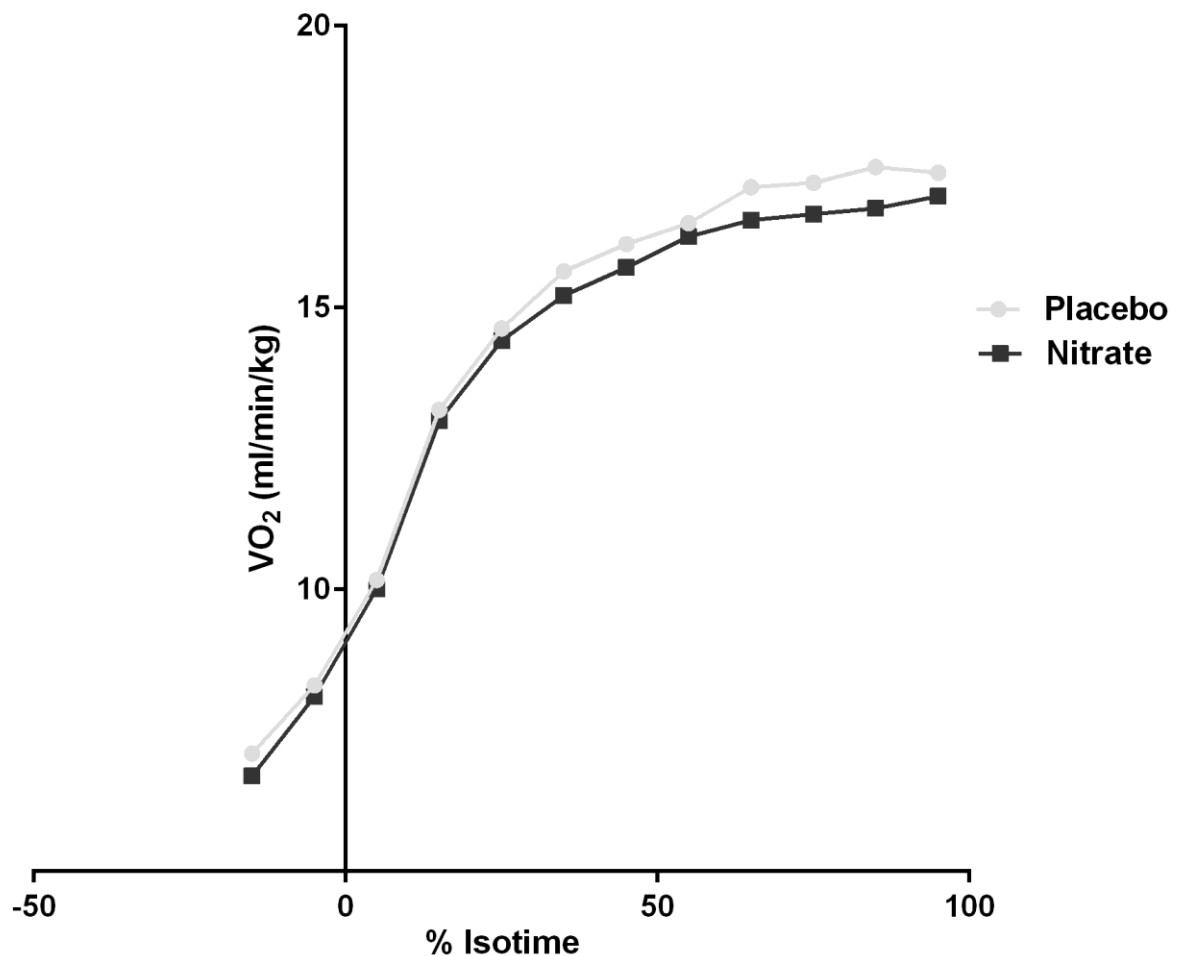


Figure 5.5: Isotime VO_2 analysis in the nitrate and placebo dosing conditions. The graph represents the VO_2 at each 10th percentile of isotime with 0% representing the initiation of loaded cycling at 70% maximal workload achieved on incremental cycle ergometry. The area under the curves to isotime were compared using a paired t-test, showing significant separation of the curves representing the two treatment conditions ($p=0.027$).

The area under the curve to VO_2 isotime was also calculated for each subject, enabling a comparison of the treatment conditions (figure 5.6).

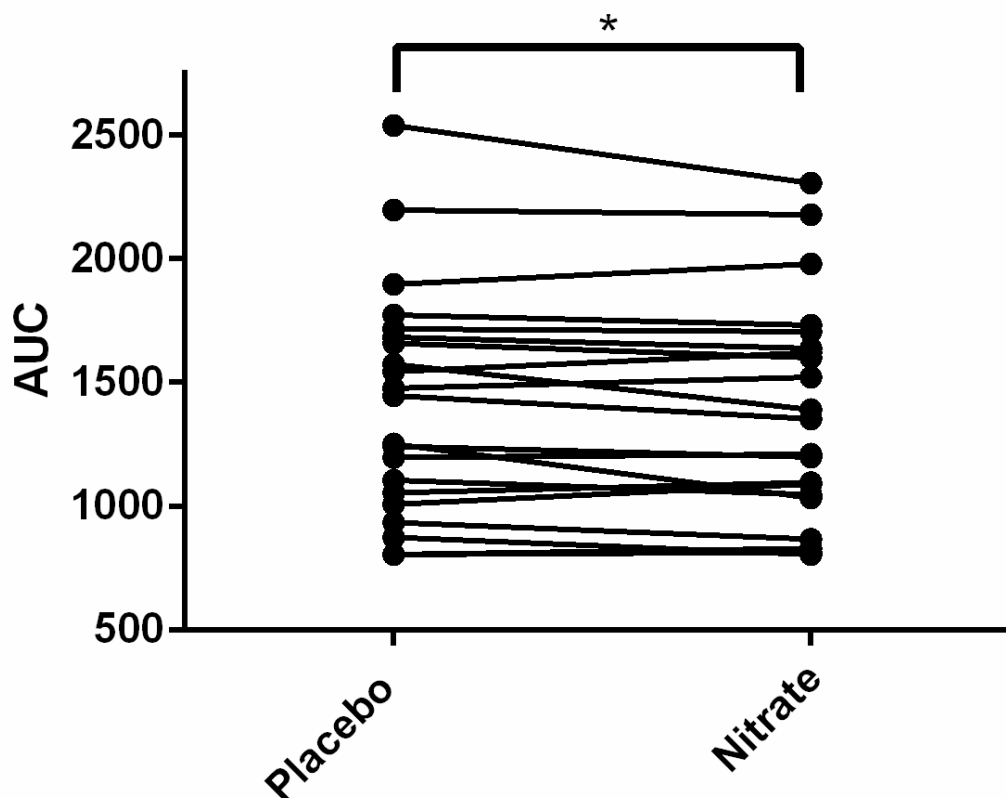


Figure 5.6: Individual responses for the area under the curve to VO_2 isotime. There was a significant difference between the treatment conditions, with a reduction in the mean area under the curve in the nitrate supplemented condition; * $p < 0.05$.

Abbreviations: AUC – area under the curve.

5.3.8 Effect of nitrate supplementation on ventilatory efficiency

For each individual subject the VE/VCO_2 slope, an index of ventilatory efficiency, was calculated. There was a significant reduction in the VE/VCO_2 slope in those receiving nitrate supplementation (29.2 ± 6.0 nitrate vs. 30.4 ± 7.1 placebo; $p = 0.038$).

5.3.9 Effect of nitrate supplementation on dead space ventilation

For each subject the dead space to tidal volume ratio (V_D/V_T) was calculated under both treatment conditions at both rest and peak exercise. There was no significant difference between the proportion of dead space ventilation in the nitrate treated condition than those receiving placebo in either the resting state (54% nitrate vs. 54% placebo; $p=0.42$) or at peak exercise (47% nitrate vs. 48% placebo; $p=0.30$).

5.3.10 Correlations of increase in plasma nitrate and change in resting diastolic blood pressure and isotime pulmonary VO_2

There was no noted correlation between the fold increase in plasma nitrate levels and either the percentage change in resting diastolic blood pressure ($r=-0.173$; $p=0.45$; figure 5.7) or percentage change in isotime pulmonary VO_2 ($r=-0.199$; $p=0.39$; figure 5.7).

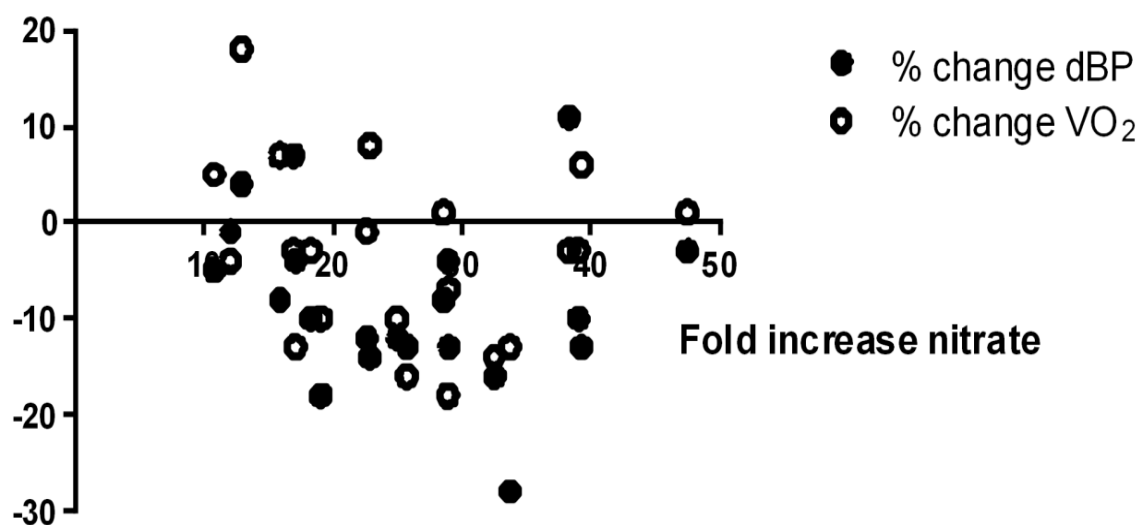


Figure 5.7: Correlation of fold increase in plasma nitrate and percentage change in resting diastolic blood pressure and isotime pulmonary VO_2 .

Abbreviations: dBP – diastolic blood pressure, VO_2 – pulmonary oxygen uptake.

5.3.11 Correlation of reduction in resting diastolic blood pressure and isotime VO_2

There was no noted significant correlation between either the absolute or percentage change in resting diastolic blood pressure and pulmonary VO_2 (absolute values $r=0.356$, $p=0.11$, figure 5.8a; percentage change $r=0.335$, $p=0.14$, figure 5.8b), although both appeared to be trending towards a positive correlation between these parameters.

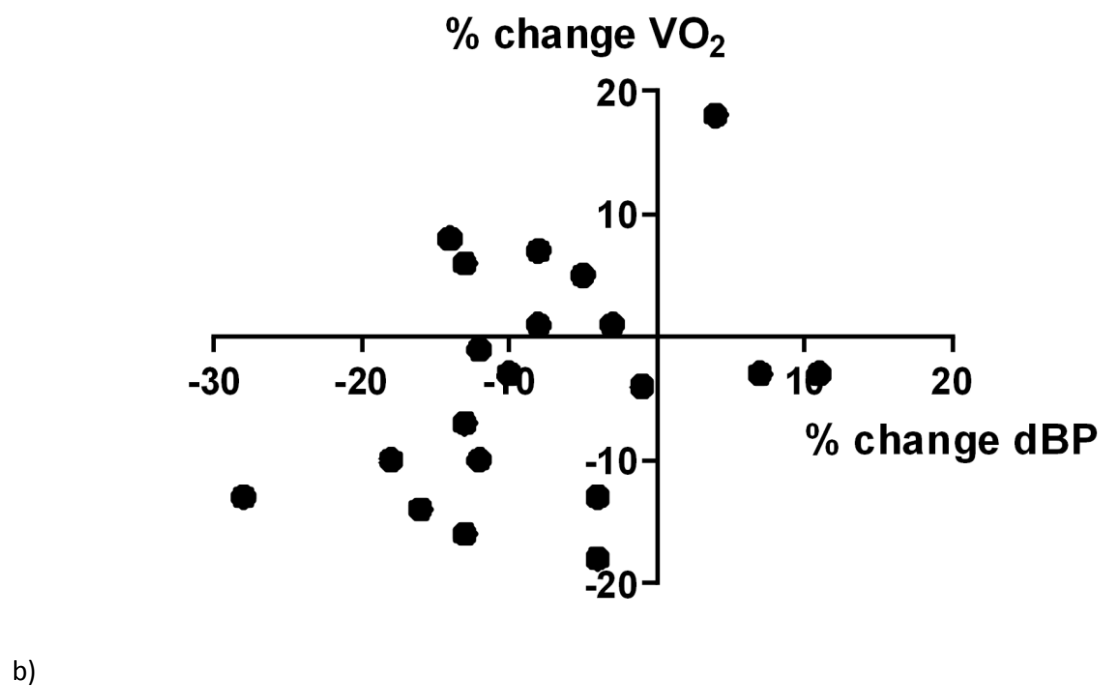
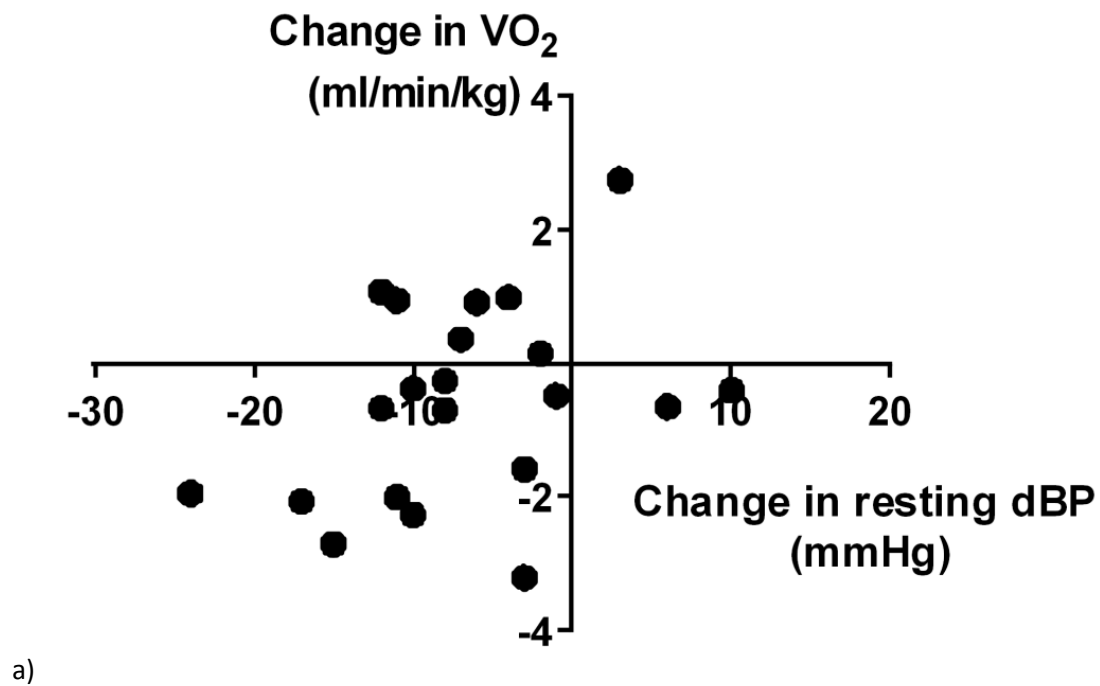


Figure 5.8: a) Correlation of absolute changes in resting diastolic blood pressure and isotime pulmonary VO_2 ; b) Correlation of percentage change in resting diastolic blood pressure and isotime pulmonary VO_2 .

Abbreviations: dBP – diastolic blood pressure, VO_2 – pulmonary oxygen uptake.

5.3.12 Effect of patient-related factors on percentage reduction in VO₂

The correlation between the percentage reduction in VO₂ at isotime and other patient characteristics was calculated to investigate if any particular phenotypic characteristics could predict the VO₂ response to nitrate supplementation (table 5.4). None were identified as predictive of correlating with the reduction of VO₂ at isotime.

Factor	r value	p value
Age (years)	0.112	0.63
FFMI (kg/m ²)	-0.043	0.85
QMVC % predicted	0.234	0.31
FEV ₁ % predicted	0.189	0.41
TLCO _c % predicted	0.329	0.15
Peak workload (Watts)	0.121	0.60
Peak workload % predicted	0.229	0.32
Peak VO ₂ (ml/kg/min)	0.209	0.36
Resting PaO ₂ (kPa)	0.180	0.44
Rest – peak PaO ₂ (kPa)	-0.022	0.93

Table 5.4: Correlation between phenotypic factors and the percentage reduction in pulmonary VO₂ at isotime.

Abbreviations: FFMI – fat free mass index; QMVC – quadriceps maximal volitional contraction; FEV₁ – forced expiratory volume in 1 second; TLCO_c – transfer factor for carbon monoxide; VO₂ – pulmonary oxygen uptake, PaO₂ – arterial partial pressure of oxygen.

5.4 Discussion

5.4.1 Summary of results

The major finding of this study was that 0.8 g of acute nitrate supplementation (providing 12.9 mmoles of nitrate), in the form of beetroot juice, was biologically active leading to a significant rise in plasma nitrate and nitrite levels, reduction in resting diastolic blood pressure and reduction in oxygen consumption at isotime during endurance cycle ergometry in a COPD population. However, this failed to translate to an improvement in exercise performance, as measured by the primary outcome of endurance time, although the biological signal seen suggests that this intervention warrants further investigation and consideration.

5.4.2 Significance of the findings

Our selected dosing regimen, 12.9 mmoles administered 3 hours prior to exercise testing, caused a 22-fold rise in plasma nitrate levels, which remained elevated at peak exercise. It was interesting to note that baseline plasma nitrate levels in this population were similar to those recorded in young recreationally active individuals (Jungersten, Ambring et al. 1997, Wylie, Kelly et al. 2013). This response to nitrate loading provides evidence that age-related changes in digestion, enterosalivary circulation and oral commensal bacterial flora do not ameliorate the response to an oral nitrate load. In fact, rather surprisingly, the plasma nitrate levels achieved exceeded those seen following the administration of a higher dose of nitrate in healthy young individuals (Wylie, Kelly et al. 2013). This may suggest possible altered nitrate metabolism and handling in COPD, although this has yet to be explored further.

We observed a significant reduction in resting diastolic blood pressure following acute nitrate supplementation as has previously been noted in healthy individuals (Larsen, Ekblom et al. 2006, Webb, Patel et al. 2008), seniors with peripheral vascular disease (Kerley, Cahill et al. 2015) and COPD patients (Berry, Justus et al. 2015, Kerley, Cahill et al. 2015). This contrasts with a previous study that failed to show a significant reduction in arterial blood pressure parameters in COPD following acute nitrate dosing (Shepherd, Wilkerson et al. 2015). That study, which also failed to show an effect on exercise performance or pulmonary VO_2 , used a lower acute dosing regimen than we selected, and achieved plasma nitrate levels that were four-fold lower than in this study ($215 \pm 84 \mu\text{M}$ vs. $820 \pm 288 \mu\text{M}$). Previous work has demonstrated that the blood pressure lowering effect and influence on pulmonary VO_2 are dose-dependent, and thus it is important to ensure adequate dosing

for this patient population. In this current work a higher dose of nitrate proved to be both safe and biologically active. As cardiovascular comorbidities are common in COPD (Chen, Thomas et al. 2015), an intervention which lowers blood pressure may lead to clinical benefit, outside of an effect on exercise performance.

In COPD subjects nitrate supplementation has been previously shown to improve endurance cycle ergometry time (Berry, Justus et al. 2015), although this study did not use a robust placebo (i.e. one which also caused beeturia) and, as noted by the authors, some individual responses were of a greater magnitude than could be explained by a plausible biological mechanism, causing skewing of the overall data. Despite this, and in line with this current work, a similar reduction in isotime pulmonary oxygen uptake was noted (of the order of 0.7 ml/min/kg), although this just failed to reach statistical significance ($p=0.099$) most likely due to underpowering (Berry, Justus et al. 2015). This lends support to the current findings regarding the influence of nitrate on the oxygen cost of submaximal exercise in this population.

In this study, ingestion of nitrate-rich beetroot juice caused a significant reduction in isotime VO_2 , indicating that the oxygen cost of achieving an equivalent exercise level was reduced. There was also a trend towards an improvement in the power/ VO_2 ratio, a measure of exercise efficiency, although this failed to reach statistical significance. The reduction of VO_2 at absolute isotime was of a similar magnitude (3-4%) to that recorded in healthy individuals following nitrate supplementation (Wylie, Kelly et al. 2013). Thus, selecting a higher dosing regimen led to biological effects and was achieved in both a safe and acceptable manner.

The level of ventilation needed to clear carbon dioxide production during exercise, as expressed by the minute ventilation/carbon dioxide output (VE/VCO_2) slope, is a measure of ventilatory efficiency and was also significantly lower in the nitrate-treated condition. In COPD patients the VE/VCO_2 slope is known to be elevated compared to healthy subjects (Teopompi, Tzani et al. 2014), in part because of increased physiological dead space and thus impaired ventilation-perfusion matching, with effective alveolar ventilation and oxygenation being maintained by an increase in minute ventilation (Elbehairy, Ciavaglia et al. 2015). The VE/VCO_2 slope has been associated with health status in COPD particularly with function as assessed by the Chronic COPD Questionnaire (CCQ) (Hornikx 2012), and thus interventions that reduce the VE/VCO_2 slope may help to improve

functioning in this patient group. Indeed subanalysis of data from the National Emphysema Treatment Trial (NETT) has shown LVRS to improve both ventilatory efficiency and functional outcomes, with those who most significantly improved their exercise capacity showing the greatest improvement in ventilatory efficiency (Armstrong, Dussault et al. 2015).

Studies in other population groups have demonstrated that exercise training leads to reductions in the VE/VCO₂ slope. A study of 10 student athletes completing a 90 day exercise programme as part of the Harvard University rowing programme (Murphy, Weiner et al. 2012), consisting mainly of endurance training, lead to a similar reduction in the magnitude of the VE/VCO₂ slope (from 21.3±0.3 pre-training to 20.0±0.5 post-training, p=0.02), and a reduction in the VE/VCO₂ slope was seen in the nitrate supplemented condition in this study (29.2±6.0 nitrate vs. 30.4±7.1 placebo; p=0.038). This change in the VE/VCO₂ slope in the student athletes was accompanied by a significant increase in the peak VO₂ (Murphy, Weiner et al. 2012), suggesting that relatively small changes in the VE/VCO₂ slope may be associated with clinically significant effects. Following acute myocardial infarction, a 12 week cardiac rehabilitation programme has been demonstrated to reduce the VE/VCO₂ slope in both those who sustained a 'large' myocardial infarct (from 30.2±4.8 pre-rehabilitation to 28.3±4.7 post, p<0.01), and in those with a 'small-medium' infarct (from 28.5±5.5 pre-rehabilitation to 27.7±3.9 post, p<0.01) (Sakuragi, Takagi et al. 2003). This was associated with significant improvements in both peak workload and peak pulmonary oxygen consumption (Sakuragi, Takagi et al. 2003).

Studies in cardiac disease have also demonstrated that the VE/VCO₂ slope is amenable to reduction through exercise training (Sakuragi, Takagi et al. 2003, Belardinelli, Georgiou et al. 2012). By reducing the oxygen cost, and thus ventilatory requirement, of submaximal exercise, nitrate supplementation may improve the efficiency of ventilation. Whilst it is not possible from the current data to elucidate the exact mechanism by which this is occurring, at isotime both minute ventilation and tidal volume were numerically reduced. As COPD patients frequently develop dynamic hyperinflation due to expiratory flow limitation during exercise, a reduction in minute ventilation at isotime may mean less dynamic hyperinflation develops, thus avoiding further worsening ventilation-perfusion matching and allowing a more efficient ventilatory response during exercise. It would thus be of interest to study more fully the effects of nitrate supplementation on ventilation-perfusion matching in COPD.

Despite a reduction in the oxygen cost of exercise and improvement in ventilatory efficiency, no improvement in exercise performance, as assessed by endurance cycle ergometry time, was noted. There are several possibilities that may explain this discrepancy. In part we saw wide variability in exercise performance during endurance cycle ergometry, and it is to be expected that day-to-day variability in patients' symptoms will influence performance (Kessler, Partridge et al. 2011), such that test-retest variability would be expected to exceed that seen in either healthy subjects or athletic individuals. Similar test variability has been seen in other studies of nitrate supplementation in COPD (Berry, Justus et al. 2015, Kerley, Cahill et al. 2015, Leong, Basham et al. 2015). Berry et al. introduced a familiarisation constant work exercise test into their protocol and adjusted the workload if this was outside a preset 4-10 minutes duration (Berry, Justus et al. 2015). In spite of this wide variability in endurance time was still evident (Berry, Justus et al. 2015).

In hindsight a trial run of the endurance cycle ergometry test may have helped to reduce this variability, but importantly we did not note the presence of an order effect. Other studies of COPD patients tend to have much larger samples sizes than our own (O'Donnell, Casaburi et al. 2011, Casaburi, Maltais et al. 2014) to account for this variability. Our findings reiterate the importance of true blinded studies in this field, where both treatment and placebo are indistinguishable to both the patients participating and investigators conducting the study.

Also to be considered is that despite nitrate supplementation leading to a reduction in the oxygen cost of exercise, this was a small change and may be insufficient to alter the rate-limiting factors impeding exercise performance in a population where patients experience ventilatory limitation. It may also be that a specific patient phenotype may benefit from this intervention which was not specifically targeted in this study. Of note we specifically excluded patients on long-term oxygen therapy, whereas one may not unrealistically speculate that this group may gain the greatest benefit from an intervention that reduces the oxygen requirement of exercise. Given the small sample size of our population it is not possible at the present time to draw further conclusions over which patients gained the greatest physiological benefit, and thus a more targeted approach merits further investigation in the future.

Other work in highly trained or elite athletes has not always revealed a reduction in the oxygen cost of exercise or improvement in exercise capacity following nitrate supplementation (Bescos, Rodriguez et al. 2011, Christensen, Nyberg et al. 2013, Wilkerson, Hayward et al. 2012), and there has been much discussion around 'responders' and 'non-responders' to nitrate administration. Part of this may be because of intrinsically high levels of plasma nitrate metabolites through pre-existing training induced upregulation of nitric oxide synthase (NOS) and thus greater endogenous activation of the nitrate-nitrite-NO pathway (McAllister and Laughlin 2006, McConell, Bradley et al. 2007). Thus well trained athletes having high endogenous levels of NO and an already efficient oxidative metabolism may not respond to supplementation of further nitrate via dietary means, or may require higher dosages to show a significant effect. Additionally such individuals are likely to choose a diet high in natural nitrate, again contributing to high natural levels of nitrate metabolites in the plasma. In the population studied here the baseline nitrate levels were similar to that seen in healthy, recreationally active individuals and a significant rise in plasma nitrate was achieved by dosing and thus the failure to increase exercise capacity would not seem to be explained by a blunted response to nitrate dosing. Effective subanalysis to identify subjects achieving greater physiological response to nitrate is not possible in this current work because of the small numbers involved, but should be the focus of future studies to help identify those who most benefit and can thus be the focus of therapy.

It is worth noting that the median endurance time in the nitrate treated group was numerically lower than in the placebo treated group and thus it may in fact be the case that nitrate supplementation does not induce improvements in exercise performance, and conversely may actually impede it despite changes in the oxygen cost. There are important parallels with the findings of this work and previous studies investigating the potential role of sildenafil in COPD patients with exercise-induced pulmonary hypertension. Sildenafil, a phosphodiesterase (PDE) type V inhibitor, prevents the degradation of guanosine monophosphate (cGMP), thus enhancing the effect of NO on the vasculature, including the pulmonary circulation. Despite the acute administration of sildenafil reducing the usual increase in pulmonary arterial pressure during submaximal exercise, this has not been associated with either improved cardiovascular measures (stroke volume and cardiac output) or improved maximal exercise performance (Holwerda, Rietema et al. 2008). Prolonged therapy with sildenafil for three months has also failed to improve either stroke volume or exercise capacity (Rietema, Holwerda et al. 2008), and sildenafil use as an adjunct to pulmonary rehabilitation did not enhance exercise outcomes (Blanco, Santos et al. 2013).

Conversely, some evidence may actually suggest potential detrimental effects of augmenting NO activity in COPD, and perhaps this negates any positive influence on reducing the oxygen cost of exercise. Blanco et al. demonstrated that sildenafil reduced hypoxic pulmonary vasoconstriction and thus created more ventilation-perfusion imbalance at rest, as assessed by the inert gas technique, with an associated reduction in resting oxygenation (Blanco, Gimeno et al. 2010). Similar effects have been seen in studies of inhaled NO (Barbera, Roger et al. 1996). In this current work there was no significant difference in arterial oxygenation (PaO_2) either at rest (10.7 ± 1.6 kPa nitrate vs. 10.7 ± 1.6 kPa placebo; $p=0.95$) or at peak exercise (10.2 ± 2.1 kPa nitrate vs. 10.2 ± 1.8 kPa placebo; $p=0.88$), and although arterial desaturation during exercise was noted it was of equal magnitude in both conditions. We did not measure ventilation-perfusion matching in this study and thus cannot comment on whether this occurred, although there was no alteration in either the resting or peak dead space ventilation (VD/VT) proportions following nitrate supplementation.

On the basis of the current work and pre-existing studies it remains unclear whether nitrate supplementation will augment exercise capacity in COPD, or effects on oxygen cost will be potentially off set by detrimental changes in ventilation-perfusion matching. Work thus far has indicated that nitrate supplementation either improved exercise capacity (Berry, Justus et al. 2015, Kerley, Cahill et al. 2015) or made no difference over placebo (Leong, Basham et al. 2015, Shepherd, Wilkerson et al. 2015), with no group thus far demonstrating significantly worsening of exercise capacity with nitrate supplementation.

5.4.3 Possible mechanisms of action

This was not a mechanistic study and the mode of action of nitrate in reducing isotime VO_2 is currently unclear. However, further mechanistic knowledge would allow the exploration of pharmacological therapies that may exploit the relevant pathways, and potentially allow greater manipulation than can be achieved with a natural source of nitrate.

There are several potential sites at which nitrate-related metabolites may be acting, including alterations to skeletal muscle blood flow (Bentley, Gray et al. 2014), efficiency of the mitochondrial electron transport chain (Larsen, Schiffer et al. 2011), energy requiring processes in skeletal muscle

including cross-bridge kinetics (Evangelista, Rao et al. 2010) and calcium-ATPase function (Ishii, Sunami et al. 1998), alterations to mitochondrial biogenesis (Jones 2014) and blood viscosity (Ashmore, Fernandez et al. 2015). The acute nature of the effect seen means changes in mitochondrial biogenesis or blood viscosity are less likely to be implicated. This does not, however, preclude that more prolonged periods of dosing could influence exercise energetics via these mechanisms. This is, however, beyond the scope of the current study.

Use of phosphorus-31 nuclear magnetic spectroscopy has suggested that nitrate supplementation acts, at least in part, via reduced ATP cost of muscle force generation (Bailey, Fulford et al. 2010). ATP generation via oxidative phosphorylation is dependent on the generation and maintenance of the electrochemical proton gradient. However, not all of this membrane potential is eventually coupled to ATP production due to proton leakage across the inner mitochondrial membrane and through uncoupling proteins and the adenine nucleotide translocase (ANT). An in-depth study of nitrate supplementation on skeletal muscle function was conducted by Larsen et al. (Larsen, Schiffer et al. 2011). Ex vivo it was shown that following three consecutive days of nitrate supplementation harvested human skeletal muscle mitochondria demonstrated increased oxidative phosphorylation efficiency over placebo supplementation. This correlated with a significant 3% reduction in whole-body oxygen consumption during submaximal exercise in the same healthy subjects from which the skeletal muscle biopsies had been obtained (Larsen, Schiffer et al. 2011). As it has previously been shown that ADP recovery times are prolonged in COPD patients selected for quadriceps dysfunction (Shields, Coissi et al. 2015), effects of nitrate on ATP generation are likely to be relevant in this group. In this current study given the acute effects of nitrate demonstrated changes to uncoupling proteins are unlikely to be the primary mechanistic action.

NO is known to reversibly inhibit cytochrome c oxidase, the terminal electron acceptor of the electron transport chain, leading to reduced proton leak through the inner mitochondrial membrane (Clerc, Rigoulet et al. 2007). Mitochondrial NOS is, in fact, located in close proximity to cytochrome c, and deletion of the domain that anchors NOS in the mitochondrial outer membrane leads to increased oxygen consumption (Gao, Chen et al. 2004). This provides further support that NO may be a physiological regulator of this enzyme, and may be the mechanism behind its mode of action in influencing the efficiency of oxidative phosphorylation.

5.4.4 Critique of the method

There are some methodological issues that merit further discussion. This study was intended as a pilot study, investigating the effects of a higher acute nitrate dosing regimen in a COPD population. Although negative it was not powered to its primary endpoint and it remains possible that studying a larger population may have led to a positive effect on endurance exercise capacity. However, previous data suggests that based on an endurance time of 528 ± 96 seconds from a comparable COPD population (Mador, Bozkanat et al. 2004), with a confidence level of 0.05 and 80% statistical power it would be necessary to study 22 individuals to identify a 60 second improvement in endurance time, which was close to the number that completed this study protocol. Given the wide variability in endurance time noted in our own sample, however, 271 individuals would have needed to be studied to see a similar improvement.

The use of a nitrate-depleted beetroot juice in this study provided a robust control preparation and significantly strengthens the validity of our findings. It should be noted that plasma nitrite levels fell below the limit of quantifiability in the majority of baseline samples. Part of the explanation for this, besides a small interfering peak apparently originating from the beetroot juice used in the current study, is that samples were not pre-treated with a thiol-blocking agent prior to centrifugation. Such treatment ensures there is no flux of nitrite across cell membranes, which may lower plasma concentrations following uptake by erythrocytes and white cells during processing. Despite this issue, high levels of plasma nitrite were still readily measurable following nitrate dosing, indicating an effective response to the oral nitrate load.

In retrospect it would have been useful to have obtained breathless scores throughout the endurance exercise test as this would have allowed some assessment as to whether the physiological changes in oxygen uptake correlated with any improvement in dyspnoea. This was not undertaken in the current work, but should be a consideration for future studies. This study tested the effect of nitrate supplementation during high intensity cycle exercise. It could be speculated that the effects may have differed if low intensity exercise or a different modality had been selected such as single leg exercise or arm crank exercise. This may allow a more focussed study of different aspects such as strength and endurance over whole body exercise tests.

5.4.5 Possible wider implications

In terms of more widespread use of nitrate supplementation in COPD there are several practical issues to consider. Patients were deliberately excluded if they were already receiving nitrate therapy or possessing comorbid factors meaning they would likely benefit from such. It is unclear if such individuals would benefit from dietary nitrate supplementation, and this has obvious implications given the frequent presence of cardiac comorbidities in patients with COPD (Chen, Thomas et al. 2015). The effects on resting diastolic blood pressure, however, indicate that nitrate supplementation may have potential beneficial effects beyond its influence on the oxygen cost of exercise.

Further research is required to understand how antibiotic therapy may affect response to an oral nitrate load and it is not uncommon that patients require antibiotic therapy during both acute exacerbations and, as is becoming more frequent practice, as a prophylactic measure in recurrent exacerbators. It is also important to consider a patient phenotype which may benefit most from nitrate supplementation. Certainly individuals in whom local changes in vascularity are a limiting factor in cellular oxygen uptake (Gagnon, Lemire et al. 2014) may be considered to be a possible target population. In such individuals the use of nitrate supplementation as an adjunct to rehabilitation might allow patients to achieve higher workloads during pulmonary rehabilitation and thus gain greater training benefits. In fact the use of nitrate supplementation as a possible adjunct to a standardised pulmonary rehabilitation programme is the subject of current investigation by our group (www.controlled-trials.com/ISRCTN27860457), powered to detect an improvement in incremental shuttle walk distance.

5.4.6 Additional issues to consider

In prospective observational population studies frequent consumption of cured meats containing added sodium salts of nitrate and nitrite, for their preservative and antimicrobial properties, has been associated with increased risk of COPD development, independent of cigarette smoking (Varraso, Jiang et al. 2007, Jiang, Camargo et al. 2008). This is speculated to be due to the damaging effect of reactive nitrogen species on the lung. This raises appropriate concern over the use of nitrate in this patient group. However, this association is more likely to be because of other unhealthy lifestyle choices associated with cured meat consumption (Chow 2008).

Additional concern has been raised over the ability of nitrate and nitrite to form dietary reactive nitrogen intermediates, which may combine with amines in the stomach forming *N*-nitrosamines which have been associated with carcinogenesis. Larsen et al. (Larsen, Schiffer et al. 2011) studied the tyrosine nitration of skeletal muscle proteins showing no actual increase under the condition of nitrate supplementation via a beetroot juice preparation over placebo. In addition, this nitrosation reaction may be inhibited by antioxidant substances, which are found in rich supply in vegetable sources such as beetroot (Jones, Bailey et al. 2011, Lundberg, Larsen et al. 2011). It is therefore a generally held view that nitrate in the form of natural vegetable products is highly unlikely to be harmful, although studies of long-term follow-up are lacking at present.

5.4.7 Conclusion

In conclusion, the acute administration of nitrate reduces oxygen consumption and improves ventilatory efficiency during submaximal exercise in COPD subjects, providing a possible new intervention to augment exercise capacity in this group. This did not translate into an improvement in exercise capacity as assessed by endurance cycle ergometry but given the safe and inexpensive nature of this intervention the results suggest further studies are warranted. A more targeted approach, specifying a particular phenotype may yield more functional benefit. Several studies have now shown the blood-pressure lowering effects of nitrate administered in the form of beetroot juice and, as cardiovascular comorbidities are common in COPD, this effect may also be of importance.

Chapter 6: General Discussion and Future Work

6.1 The ACE genotype, angiotensin enzyme inhibition and skeletal muscle dysfunction in COPD

6.1.1 Implications and future work

Pulmonary rehabilitation is a well-recognised and effective intervention for skeletal muscle dysfunction in COPD (Bolton, Bevan-Smith et al. 2013). However, there remain regional variations in its accessibility and availability (Green and Morgan 2002), limiting both its uptake and adherence. Hence agents that ensure the maximal physiological response to exercise training, and maintenance of this response for as long as possible, are in high demand. The search for pharmacological therapies to target skeletal muscle dysfunction in COPD thus remains an area of active research interest. In addition, there is a growing recognition of a subset of patients who are 'non-responders' to pulmonary rehabilitation (Troosters, Gosselink et al. 2001, Garrod, Marshall et al. 2006), and these individuals may benefit from targeted pharmacological treatment dependent on the individual factors which limit their exercise capacity. However, it is well recognised that baseline phenotypic variables are often poorly predictive of programme success, meaning it may be challenging to identify such individuals (Garrod, Marshall et al. 2006). Even individuals with COPD who have evidence of sarcopenia, which is known to be associated with poor health status and limit both exercise capacity and functional performance, are still capable of responding to pulmonary rehabilitation in a similar manner to those who are not sarcopenic (Jones, Maddocks et al. 2015).

The influence of genetics on exercise capacity has been of great interest in the field of sports medicine (Montgomery, Marshall et al. 1998). The observational work contained within this thesis failed to show a relationship between the angiotensin converting enzyme (ACE) genotype and baseline measures of exercise capacity in patients with at least moderate severity COPD, as assessed by the degree of airflow obstruction. This is not a particularly surprising finding as, in the presence of multisystem pathophysiology in COPD, there are likely to be several contributing factors, meaning small gains potentially related to genotype may fail to achieve significance in a small sample size.

Although there is a strong theoretical basis for manipulation of the renin-angiotensin system in COPD-related skeletal muscle dysfunction, the randomised controlled trial of enalapril as an adjunct to pulmonary rehabilitation reported in this thesis did not establish a beneficial effect, and in fact demonstrated attenuation of the improvement in exercise capacity as assessed by peak exercise

capacity during cycle ergometry, which was an unexpected finding. Whilst this is at odds with some previous attempts to manipulate the renin-angiotensin system (Andreas, Herrmann-Lingen et al. 2006, Di Marco, Guazzi et al. 2010), a randomised controlled trial of fosinopril (without adjunctive exercise training) in patients with COPD selected for skeletal muscle weakness also failed to show any beneficial effect (Shrikrishna, Tanner et al. 2014), and in fact showed a lower improvement in strength in the ACE-inhibitor treated over the placebo group. Whilst this previous study failed to stratify by ACE genotype, in this current body of work patients were stratified by both ACE genotype and baseline exercise capacity, strengthening the current findings, and lending support to the assumption that this is a class effect of ACE-inhibitors. This was the first study to investigate the role of ACE-inhibition with a concurrent programme of pulmonary rehabilitation and highlights the importance of well-designed prospective randomised controlled trials as my findings did not confirm assumptions from the body of prior observational data.

Research has suggested a beneficial effect of ACE-inhibition may only be seen when an adjunctive exercise stimulus is used. One mode of action of ACE-inhibition is through reduced bradykinin breakdown, and microdialysis experiments have shown bradykinin to be synthesised in skeletal muscle during contractile exercise, with increased bradykinin levels recorded in the gastrocnemius muscle during repetitive plantar flexion exercise (Langberg, Bjorn et al. 2002). Thus it is not unreasonable to assume that ACE-inhibition may potentiate the effects of bradykinin during exercise. Furthermore, in a Japanese study, administration of angiotensin II to mice reduced performed work to exhaustion, running distance and peak oxygen uptake, associated with increased skeletal muscle oxidative stress and inhibition of skeletal muscle mitochondrial respiration (Inoue, Kinugawa et al. 2012). Thus there are several potential modes of action by which ACE-inhibition alongside a concurrent exercise stimulus may be hypothesised to influence exercise performance.

Furthermore, Guo et al. (Guo, Minami et al. 2010) studied Wistar-Kyoto aged female rats tested under a variety of conditions, including following perindopril administration alone and with a combination of exercise training and perindopril administration. Chronic treatment with perindopril along with concurrent exercise training promoted adaptive changes in skeletal muscle including improved capillary density and type I fibre type percentage. However, of note this group showed no increase in running capacity over exercise training alone (Guo, Minami et al. 2010), indicating that changes at a histological level do not always translate to physiological improvements. Thus whilst no

improvement in exercise capacity was seen in this work, it is possible that histological changes were present in response to ACE-inhibition; it would perhaps have been preferable if I had taken muscle biopsies as well but the protocol was already significantly demanding for patients with significant disability due to their COPD.

Moreover it remains unclear the changes in skeletal muscle structure that underlie the effects promoted by ACE-inhibition. One study of young untrained but healthy subjects indicated an association between the ACE DD genotype and an increased proportion of anaerobic type IIb skeletal muscle fibres (Zhang, Tanaka et al. 2003). No studies have addressed the role of ACE genotype on skeletal muscle fibre type proportions in individuals with COPD where there are notable changes in the skeletal muscle fibre type distribution at baseline, with lower proportions of type I skeletal muscle fibres than seen in health (Jakobsson, Jorfeldt et al. 1990, Maltais, Sullivan et al. 1999). It is also unknown if 10 weeks therapy with an ACE-inhibitor would be sufficient to influence fibre type proportions and this is a possible area of future research, as this current body of work did not study potential histological changes at the level of the skeletal muscle.

It is thus evident that even when considering a localised renin-angiotensin system such as that seen in the skeletal muscle there are several conflicting potential beneficial and adverse effects of ACE-inhibition. These are summarised for clarity in table 6.1.

Beneficial effects of ACE-inhibition	Adverse effects of ACE-inhibition
Reduction of pro-inflammatory pathways	Reduced hypertrophic response to loading
Improved glucose handling	Reduced strength capacity
Promotion of anabolic pathways	Reduced perfusion pressure to skeletal muscle
Vasodilatation through bradykinin activity	Impaired arteriolar development
Effects on oxidative metabolism	Impaired vascular tone and vasoreactivity
Negation of TGF- β activity	

Table 6.1: Summary of the potential beneficial and adverse effects of ACE-inhibition on skeletal muscle function.

6.1.2 ACE-inhibition and cardiovascular disease in COPD

Despite a failure to show a beneficial effect of ACE-inhibition alongside a concurrent exercise stimulus on exercise parameters, it is important to note that patients with previously recognised cardiovascular disease were specifically excluded from the study. Whilst there are definite indications for ACE-inhibition in this context, which is a common comorbidity in COPD, there is also a growing recognition of the prevalence of undiagnosed cardiovascular disease in the COPD patient population. Observational data has supported the fact that COPD is associated with an increased risk of cardiovascular disease, even after adjustment is made for shared risk factors in both disease processes, such as smoking status (Mullerova, Agusti et al. 2013).

A recently reported large meta-analysis of observational studies attempted to quantify the relationship between COPD and cardiovascular disease, demonstrating that patients with COPD were statistically more likely to be diagnosed with cardiovascular disease than a non-COPD population (Chen, Thomas et al. 2015). Taking studies between 1980 and 2015, 29 data-sets were identified that were suitable for inclusion in the analysis. Compared with the non-COPD population, patients with COPD were more likely to be diagnosed with cardiovascular disease (odds ratio [OR] 2.46, 95% CI 3.02-3.00, $p < 0.0001$) and hypertension (OR 1.33, 95% CI 1.13-1.56, $p = 0.0007$) (Chen, Thomas et al. 2015). Understanding the link between COPD and cardiovascular disease is an important area of future study and will allow identification of individuals at highest risk of cardiovascular morbidity and mortality that may benefit from targeted therapy, including ACE-inhibition. The recognition and treatment of cardiovascular disease in COPD is thus an important focus of interest, and ongoing prospective work such as the ERICA study (Evaluating the Role of Inflammation in Chronic Airways disease) is investigating this in more detail (Mohan, Gale et al. 2014).

Consistent with these findings, research in patients hospitalised with an acute exacerbation of COPD, without a clinical diagnosis of an acute coronary syndrome or cardiac failure, has shown that biomarkers of cardiac dysfunction predict mortality independently of other indicators of severity and prognosis. This has been demonstrated for both troponin T (Chang, Robinson et al. 2011, Hoiseth, Neukamm et al. 2011) and N-terminal pro-B-type natriuretic peptide (NT-proBNP) (Chang, Robinson et al. 2011). Patients with elevated levels of both biomarkers have been shown to have a 15-fold

higher mortality at 30 days than individuals with normal levels (Chang, Robinson et al. 2011). Other work has shown that not only may cardiac biomarkers be raised in this group, but also serial electrocardiogram (ECG) changes may be present, with 1 in 12 hospitalised for an acute exacerbation of COPD meeting the criteria for myocardial infarction on the basis of troponin levels with accompanying clinical history and/or ECG changes (McAllister, Maclay et al. 2012). Although it is not yet possible to disentangle whether direct cardiac involvement is an important part of COPD exacerbations in some individuals, or whether elevation of such biomarkers is an indicator of the severity of the exacerbation, and the cardiac stress and ventricular dysfunction this promotes, it remains possible that some individuals may benefit from adjustment of their cardiovascular risk and targeting of the renin-angiotensin system may form part of this.

Other work has also shown that manipulation of the renin-angiotensin system in COPD is associated with a reduction in serum haematocrit (Andreas, Herrmann-Lingen et al. 2006) and may reduce the secondary erythrocytosis seen in hypoxaemic individuals with this condition. No change in serum haematocrit was noted following rehabilitation in either arm of this study, although it is not clear the time period over which this would be seen and the study was not powered to this endpoint. However, this effect may be of relevance as erythrocytosis is itself associated with cardiovascular disease, especially myocardial infarction (Sorlie, Garcia-Palmieri et al. 1981). Thus there are several levels by which manipulation of the renin-angiotensin system may adjust the cardiovascular risk seen in individuals with COPD.

Large epidemiological cohort studies also suggest beneficial effects for manipulation of the renin-angiotensin system in the long-term management of COPD patients. In a retrospective observational nested case control study of Quebec citizens of at least 65 years of age 946 COPD cases were identified. The combination of statin therapy and either ACE-inhibition or angiotensin-receptor blockade was associated with a reduction in COPD hospitalisation and mortality in both high (defined as COPD patients having previously undergone coronary revascularisation) and low cardiovascular risk groups (Mancini, Etminan et al. 2006).

Furthermore, a large retrospective national cohort study conducted in the USA involving greater than 11,000 patients 65 years or more of age hospitalised with an exacerbation of COPD demonstrated that pre-existing use of ACE-inhibitors or angiotensin receptor blockers was

associated with significantly reduced 90-day mortality (odds ratio 0.55, 95% confidence interval 0.46-0.66) (Mortensen, Copeland et al. 2009). This change was still present after adjusting for pre-existing demographic, comorbid and medication factors that may have confounded the findings. Data on the cause of mortality was not provided by this study, and thus it is difficult to draw definite conclusions on whether ACE-inhibitor use directly reduced COPD-related mortality, although an association was suggested. Thus this remains an observation that requires study in formal prospective randomised controlled trials. Although this current work would suggest that initiating ACE-inhibitor therapy for the purpose of enhancing the skeletal muscle response to a rehabilitative stimulus cannot currently be recommended, the beneficial effects of ACE-inhibition in terms of cardiovascular morbidity and mortality remains a possible future application and warrants further research.

6.1.3 Other potential beneficial effects of ACE-inhibition in COPD

Ventilation perfusion mismatching is a common feature of COPD, in part due to destruction of the pulmonary vasculature, vascular remodelling and pulmonary vasoconstriction, which may lead to pulmonary hypertension. Even subjects who do not display pulmonary hypertension in the resting state may during an exercise challenge. The RAS is thought to be implicated in this process, with evidence existing to suggest a potential important role for angiotensin II in the pulmonary vascular responses to exercise (Bradford, Ely et al. 2010). Supportive evidence has been provided by a rat model of monocrotaline induced pulmonary hypertension, where there are noted increased mRNA levels of renin, ACE, angiotensinogen, angiotensin receptors and proinflammatory cytokines (Ferreira, Shenoy et al. 2009).

Angiotensin II is a recognised vasoconstrictive agent in the pulmonary circulation (Lipworth and Dagg 1994) and ACE activity has been implicated in the development of pulmonary hypertension in response to an acute hypoxic challenge in both a rat model (Nong, Stassen et al. 1996) and in healthy individuals (Cargill and Lipworth 1996). These effects may be reduced by ACE-inhibition (Cargill and Lipworth 1996, Nong, Stassen et al. 1996). As well as the important role of angiotensin II it should not be forgotten that ACE is also responsible for the breakdown of vasoactive kinins, important in vasodilatory responses and may also be implicated in the pathophysiology of pulmonary hypertension seen in COPD.

Clinical studies have demonstrated that high ACE activity is associated with pulmonary hypertension and disturbed tissue oxygenation during an exercise challenge in a COPD population even when resting pulmonary pressures are within the normal range (Kanazawa, Okamoto et al. 2000, Kanazawa, Otsuka et al. 2002, Kanazawa, Hirata et al. 2003). Furthermore, research has demonstrated that low ACE activity is associated with improved pulmonary haemodynamics and oxygenation (Kanazawa, Hirata et al. 2003). Measurements of pulmonary pressure and vascular resistance were not recorded in this current body of work, but this remains one mechanism by which ACE-inhibitor therapy may influence exercise performance in COPD and is worthy of further study.

Other disease processes may allude to the scope of potential beneficial effects of ACE-inhibition. High levels of ACE activity, in association with the ACE genotype, has been shown to be increased in patients with acute respiratory distress syndrome (ARDS), and are associated with higher mortality in this condition (Marshall, Webb et al. 2002). This raises the possibility that altering levels of ACE activity may affect the response of the lung to acute injury. This has been hypothesised due to effects on pulmonary vascular permeability, vascular tone and fibroblast function, but remains an area yet to be fully researched.

Other research has concentrated on the study of active cigarette smokers and recognising those at particular risk of lung function decline and the development of COPD, potentially allowing the consideration of early intervention in those most at risk. The prospective longitudinal Lovelace Smokers Cohort study based in New Mexico studied 1170 ever-smokers with repeated spirometric testing over a minimum of 3 years. This allowed demarcation into rapid (≥ 30 ml/year), normal (0-29.9 ml/year) and no (< 0 ml/year) lung function decline as defined by post-bronchodilator FEV₁ (Petersen, Sood et al. 2014). Amongst those ever-smokers without baseline lung disease, rapid decline in lung function was a predictor for the development of COPD (Petersen, Sood et al. 2014). Interestingly an association was seen between use of ACE-inhibitors with the protection against such rapid lung function decline, particularly amongst those possessing cardiovascular disease, hypertension or diabetes (odds ratios of 0.48, 0.48 and 0.12 respectively; $p \leq 0.02$ for all analyses). Whilst this provides good evidence for the continuation of ACE-inhibitors in those with a pre-existing clinical indication, it also provides a basis for randomised controlled trials of the role of ACE-inhibitors in ever-smokers to investigate effects on lung function decline. This may provide one

mode to identify smokers at high risk of developing COPD and alter the natural history of the disease progression.

Whether such an observation is shown to be confirmed in prospective randomised controlled trials remains to be seen, and this thesis has emphasised the importance of testing hypotheses generated from observational work robustly. The mechanism behind this possible mode of ACE-inhibitors is also as yet unclear. It may be hypothesised that this is through mitigation of the pro-inflammatory effects of angiotensin II, including effects through TGF- β signalling (Cohn, van Erp et al. 2007). In support of this mice models show attenuation of the effects of angiotensin II reduced cigarette smoking associated lung injury (Podowski, Calvi et al. 2012), and this may be relevant to a human population.

Overall it is important to state that our particular application for ACE-inhibition, namely in augmenting the maximal exercise response to pulmonary rehabilitation, failed to show a positive effect. The value of ACE-inhibition as an adjunct to exercise training was not shown, and in fact evidence of a detrimental effect was noted, lending significant caution to manipulation of the renin-angiotensin pathway for this indication. However, given that patients were specifically excluded who received ACE-inhibition for other clinical indications, the conclusions cannot be extended to this group. In addition, there are several levels by which manipulation of the renin-angiotensin system may be of use in COPD, particularly with emerging evidence of the role of, often unrecognised, cardiovascular disease. It is only the design and implementation of prospective large randomised controlled trials that will provide future information of the role and potential benefits of these interventions.

6.2 Role of nitrate supplementation in augmenting exercise capacity in COPD

6.2.1 Implications and future work

The pilot work presented in this thesis demonstrated that nitrate administration to patients with at least moderate COPD was able to reduce oxygen consumption at isotime, however, this failed to translate to an improvement in endurance exercise capacity. The physiological changes may, however, be of relevance.

Research into nitrate supplementation has often shown conflicting results with some individuals showing benefit and others failing to do so. In part this may be influenced by baseline plasma nitrate and nitrite levels, and thus response to dosing. This may be a particular issue when examination is made of high level athletic subjects. Wilkerson et al. (2012) (Wilkerson, Hayward et al. 2012) enrolled 8 well trained club-level cyclists and following nitrate supplementation showed no significant change in power output during a laboratory based 50 mile time trial and no improved performance in a time trial setting.

The increase in plasma nitrite levels seen in the group was, however, lower following nitrate supplementation than seen in other studies, in part because of higher baseline nitrite and nitrate levels, as other groups have also demonstrated in athletic subjects (Jungersten, Ambring et al. 1997, Bescos, Rodriguez et al. 2011). The mean baseline plasma nitrite level of the subjects in the study by Wilkerson et al. (2012) (Wilkerson, Hayward et al. 2012) was recorded as 389 ± 107 nM as compared to the study by Lansley et al. (2011) (Lansley, Winyard et al. 2011) using healthy but non-trained persons who had a baseline plasma nitrite level of 197 ± 184 nM. The administration of 500 ml of nitrate-rich beetroot juice (containing 6.2 mmol nitrate) increased plasma nitrite levels by 105% in the untrained group (VO_2 peak 55 ± 7 ml/min/kg) (Lansley, Winyard et al. 2011) as opposed to only 21% in the well-trained athletes (VO_2 peak 63 ± 8 ml/min/kg) (Wilkerson, Hayward et al. 2012). Thus future studies need to consider not just the dose of nitrate administered but the baseline nitrate levels, as was performed during this current work, as this may influence the response noted.

The reasons why some individuals tend to respond to nitrate supplementation whilst others do not is an area which has yet to be fully understood. Whilst some of the reasoning may be due to baseline nitrate levels and absolute changes in plasma nitrate, other research has suggested the rationale is far from this straightforward. Christensen et al. (Christensen, Nyberg et al. 2013) failed to show a response in highly trained cyclists (VO_2 peak 72 ± 4 ml/min/kg), however in contrast to that seen in previous studies a subgroup comparison of 'responders' versus 'non-responders' showed no difference in the baseline nitrate levels (a surrogate measure of nitrite levels) indicating that probably several factors are at play and endogenous NO production is not the sole influence. It is also feasible that trained individuals have already established improved whole body efficiency,

meaning that there is less available room for further improvement in aerobic exercise performance and thus endurance capacity through nutritional interventions (Edwards, Holloway et al. 2011).

Areas of active research with regards to nitrate supplementation include the physiological mechanisms of action, which remains an area yet to be fully clarified. This current body of work did not allude to seek possible underlying mechanisms and more work is required in this field. Early work using ^{31}P -magnetic resonance spectroscopy suggested nitrate supplementation was active through reduced ATP turnover rate (Bailey, Fulford et al. 2010). This was supported by ex vivo work by Larsen et al. showed that three days supplementation with sodium nitrate (0.1 mmol/kg/day) was predominantly active through alterations to mitochondrial efficiency as measured in pooled isolated mitochondria (Larsen, Schiffer et al. 2011), with reduced measured adenine nucleotide translocase (ANT) and UCP3 uncoupling protein content.

This has been disputed in more recent work by Whitfield et al. who studied mitochondrial bioenergetics in a group of ten young active males following a seven day supplementation period with nitrate (13 mmol twice daily) in the form of beetroot juice (Whitfield, Ludzki et al. 2016). Despite a 3% reduction in whole-body oxygen consumption during submaximal exercise, no change was noted in mitochondrial leak respiration, uncoupling proteins or mitochondrial efficiency (Whitfield, Ludzki et al. 2016), as had been noted following sodium nitrate administration (Larsen, Schiffer et al. 2011). Whilst some have suggested that inorganic sodium nitrate may be acting in a different manner to nitrate derived from organic sources, it is unlikely that alterations to mitochondrial efficiency are the sole mode of action of nitrate supplementation, with reduced ATP cost of performed work also likely to play a role.

In line with this in a double-blind randomised study of nitrate-rich beetroot juice versus placebo over a period of 7 days in 19 healthy but untrained males, nitrate was demonstrated to improve excitation-contraction coupling of skeletal muscle in vivo, with improved involuntary force generation following low-frequency electrical stimulation (Haider and Folland 2014). This may explain some of the beneficial effects of nitrate on endurance exercise, where repeated submaximal activation of skeletal muscle fibres occurs. Similar responses have been seen in mouse models, associated with increases in myoplasmic free calcium concentration and greater expression of calcium handling proteins (Hernandez, Schiffer et al. 2012). This suggests some of this effect may be

due to improved calcium handling, and thus greater calcium transient into the cytoplasm leading to improved force production, along with altered crossbridge sensitivity to calcium. This may lend benefit to individuals with muscle weakness and exercise intolerance, but to understand the possible translational benefit of nitrate supplementation requires a fuller understanding of its mechanistic activity on human skeletal muscle.

Another area of consideration is whether a degree of tolerability develops during more prolonged periods of nitrate supplementation. Relatively little data is available, although there has been no indication of tolerability up to 15 days of supplementation (Vanhatalo, Bailey et al. 2010). In mice chronic low-dose administration of nitrate over 8-10 weeks did not lead to significantly increased plasma and tissue levels of nitrate and nitrite as were seen in the acute setting, suggesting the development of a tolerance (Carlstrom, Larsen et al. 2010). Certainly this area of interest warrants further investigation in human subjects.

It has also yet to be studied whether nitrate supplementation may be a possible adjunct to exercise based rehabilitation programmes such as pulmonary rehabilitation. A multicentre prospective randomised controlled trial in COPD patients undergoing pulmonary rehabilitation is currently recruiting (www.controlledtrials.com/ISRCTN27860457) and seeks to answer this research question, being powered to detect a 20% improvement in incremental shuttle walking distance over placebo administration.

6.2.2 Other possible applications of nitrate supplementation

Interest has been stimulated in the possible role of nitrate supplementation in individuals who may experience resting hypoxaemia or develop such during exertion. An interesting study by Engan et al. (Engan, Jones et al. 2012) looked at 12 well-trained apnoea divers. This demonstrated that nitrate supplementation increased the maximal resting apnoea duration by 11% (Engan, Jones et al. 2012). This suggests nitrate may have effects beyond active locomotive exercise, having possible effects on oxygen consumption even in resting conditions, although the study included in this thesis failed to show an effect on resting pulmonary oxygen uptake.

Research also suggests nitric oxide may be an important mediator in the adaptation to life in a hypoxic environment. Observational studies of high altitude dwelling humans have shown higher levels of exhaled NO (Beall, Laskowski et al. 2001) and plasma nitrite (Erzurum, Ghosh et al. 2007) than lowlanders. This cannot be accounted for by increased dietary consumption of nitrate as the average daily consumption studied would have been insufficient to increase plasma levels of nitrate and nitrite significantly (Erzurum, Ghosh et al. 2007). This suggests such persons adapted to life at altitude have upregulation of endogenous NO that may help them meet this physiological challenge. In line with studies from sports medicine, comparisons between long-term Tibetan residents and newly acclimatised residents at altitude show lower maximal VO_2 values in the Tibetan group and higher workload at maximal exercise effort illustrating possible improved exercise efficiency of the Tibetan population (Ge, Chen et al. 1994). This suggests a possible causal mechanism related to upregulation of endogenous NO, although this has yet to be formally examined.

It is thus possible that nitrate supplementation may prove beneficial to individuals with exercise induced hypoxaemia and associated exercise intolerance. In fact this is an area of active research in a prospective randomised controlled trial specifically focussing on those with respiratory disease, including COPD, idiopathic pulmonary fibrosis and pulmonary hypertension (www.controlledtrials.com/ISRCTN14888729). Current research suggests a potential beneficial effect, with dietary nitrate administration previously shown to improve both oxygen delivery and muscle metabolic recovery, as measured by T2-weighted magnetic resonance imaging in healthy subjects performing single-leg knee extension exercise in hypoxic conditions (Vanhatalo, Jones et al. 2014). Thus nitrate may both assist muscle metabolism during hypoxic exercise and aid the recovery period, alluding to a possible clinical benefit.

As has been previously discussed cardiovascular comorbidities are an important extrapulmonary manifestation of COPD and thus nitrate supplementation may exert effects through changes in endothelial and cardiovascular function. COPD is a condition of older age, age being a recognised factor in cardiovascular pathophysiology. Endothelial function is known to be impaired in aged individuals and contribute to atherosclerosis, and this may, in part, be through impaired endothelial production of NO via endothelial nitric oxide synthase (eNOS) in response to normal physiological stimuli (Lauer, Heiss et al. 2008). This may be self-perpetuating as the ability of the vasculature to regulate nitrite homeostasis and produce nitric oxide is itself a requirement for healthy vascular

function. Thus, the ability to raise plasma and tissue NO levels through nitrate administration may mitigate the reduced endogenous ability of the vasculature to produce nitric oxide, and thus ameliorate the endothelial dysfunction seen in both disease states and in response to normal aging.

Nitrates may, therefore, have a role in treating the cardiovascular comorbidity in COPD and other conditions. In fact this is one area in which the potential beneficial effects of ACE-inhibition and nitrate supplementation may interact, as both are responsible for improving NO levels which influences both substrate delivery through vasodilatation and oxygen utilisation through mitochondrial respiration (Zhao, Bernstein et al. 1999). It has also been speculated that the ACE gene on chromosome 17 and the eNOS (endothelial nitric oxide synthase) gene on chromosome 7 may interact in a manner to reduce the pathological consequences of activation of the RAS pathway (Ahsan, Ram et al. 2004). Thus nitrate physiology remains an area of interest in understanding the pathophysiology and potential treatment modes in COPD.

6.2.3 Concluding remarks

This work that makes up this thesis has demonstrated that ACE-inhibition as an adjunctive agent to a standardised programme of pulmonary rehabilitation in a group of COPD patients with at least moderate airflow obstruction led to a reduction in the peak power gained following the intervention. In this context there appears to be no real benefit for considering manipulation of the renin-angiotensin axis for this purpose, and this has helped to draw a line under a proposed pharmacological adjunct. As has been highlighted previously individuals with a clinical indication for ACE-inhibition were deliberately not studied, and thus in this group there is no rationale for avoidance or cessation of therapy. This body of work does not exclude other potential benefits of ACE-inhibition, and certainly future exploration of the mitigation of the well-recognised heightened cardiovascular risk remains a promising research area.

The pilot study of nitrate supplementation demonstrated some physiological effects, and the reduction of oxygen cost at isotime during endurance cycle ergometry provides an interesting signal to consider further work, perhaps considering a more tailored approach in a group where reduction of the oxygen cost of exercise may lead to the greatest benefit. In addition, the effects on arterial

blood pressure parameters also provide a potential treatment modality to consider addressing the cardiovascular risk of this population.

Whilst conducting this research other more broad issues around COPD and pulmonary rehabilitation have become obvious. There are deficiencies in both the uptake of rehabilitation programmes and adherence to these, as well as the consideration of maintenance programmes to attend to the loss of effect with the passage of time. Both national and international assemblies recognise the import of continuing to conduct high quality research to improve patient-centred outcomes (Celli, Decramer et al. 2015).

The studies that make up this thesis have highlighted some important general principles when studying such a patient population that is known to be rather heterogeneous in its make-up. Whilst in this work a rather 'one-size fits all' approach was taken to considering ACE-inhibition as an adjunct to pulmonary rehabilitation and nitrate supplementation to improve endurance time, this work emphasises the importance of a more tailored approach to improving exercise capacity. Studies may thus benefit from studying enriched populations of patients and identifying in each individual what limiting factors during exercise affect them. This would allow the consideration of a personalised approach to use both a training modality and adjunctive agents that act synergistically with such a modality remains the optimal approach. Achieving this remains a challenge but in the era of personalised medicine one which should be pursued.

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Appendix

**ST. GEORGE'S RESPIRATORY QUESTIONNAIRE
for COPD patients**

(SGRQ-C)

*This questionnaire is designed to help us learn much more about how your breathing is troubling you and how it affects your life.
We are using it to find out which aspects of your illness cause you most problems, rather than what the doctors and nurses think your problems are.*

Please read the instructions carefully and ask if you do not understand anything. Do not spend too long deciding about your answers.

ID: _____

Date: ____/____/____ (dd/mm/yy)

Before completing the rest of the questionnaire:

Please select one box to show how you describe your current health:

Very good

☐

Good

☐

Fair

☐

Poor

☐

Very poor

☐

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UK/ English version COPD

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St. George's Respiratory Questionnaire PART 1

Questions about how much chest trouble you have.

Please select **ONE** box for each question:

Question 1. I cough:

- most days a week..... ☐ a
- several days a week ☐ b
- only with chest infections ☐ c
- not at all ☐ d

Question 2. I bring up phlegm (sputum):

- most days a week..... ☐ a
- several days a week ☐ b
- only with chest infections ☐ c
- not at all ☐ d

Question 3. I have shortness of breath:

- most days a week..... ☐ a
- several days a week ☐ b
- not at all ☐ c

Question 4. I have attacks of wheezing:

- most days a week..... ☐ a
- several days a week ☐ b
- a few days a month ☐ c
- only with chest infections ☐ d
- not at all ☐ e

UK/ English version COPD

2/7
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SGRQ-C - United Kingdom/English
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Question 5. How many attacks of chest trouble did you have during the last year?

3 or more attacks ☐ a

1 or 2 attacks..... ☐ b

none ☐ c

Question 6. How often do you have good days (with little chest trouble)?

no good days..... ☐ a

a few good days ☐ b

most days are good ☐ c

every day is good..... ☐ d

Question 7. If you have a wheeze, is it worse in the morning?

no ☐

yes..... ☐

St. George's Respiratory Questionnaire PART 2

8. How would you describe your chest condition?

Please select *ONE*:

- Causes me a lot of problems or is the most important problem I have ☐ a
- Causes me a few problems ☐ b
- Causes no problem ☐ c

9. Questions about what activities usually make you feel breathless.

For each statement please select *the box* that applies to you **these days**:

- | | True | False | |
|------------------------------------|--------------------------|--------------------------|---|
| Getting washed or dressed..... | ☐ | ☐ | a |
| Walking around the home..... | ☐ | ☐ | b |
| Walking outside on the level..... | ☐ | ☐ | c |
| Walking up a flight of stairs..... | ☐ | ☐ | d |
| Walking up hills..... | ☐ | ☐ | e |

St. George's Respiratory Questionnaire PART 2

10. Some more questions about your cough and breathlessness.

For each statement please select *the box* that applies to you **these days**:

	True	False	
My cough hurts	☐	☐	a
My cough makes me tired	☐	☐	b
I am breathless when I talk.....	☐	☐	c
I am breathless when I bend over	☐	☐	d
My cough or breathing disturbs my sleep.....	☐	☐	e
I get exhausted easily	☐	☐	f

11. Questions about other effects that your chest trouble may have on you.

For each statement please select *the box* that applies to you **these days**:

	True	False	
My cough or breathing is embarrassing in public	☐	☐	a
My chest trouble is a nuisance to my family, friends or neighbours.....	☐	☐	b
I get afraid or panic when I cannot get my breath	☐	☐	c
I feel that I am not in control of my chest problem.....	☐	☐	d
I have become frail or an invalid because of my chest.....	☐	☐	e
Exercise is not safe for me	☐	☐	f
Everything seems too much of an effort.....	☐	☐	g

UK/ English version COPD

5/7
continued...

SGRQ-C - United Kingdom/English
SGRQ-C_AU1.0_eng-GBori.doc

St. George's Respiratory Questionnaire PART 2

12. These are questions about how your activities might be affected by your breathing.

For each statement please select *the box* that applies to you **because of your breathing**:

	True	False	
I take a long time to get washed or dressed.....	☐	☐	a
I cannot take a bath or shower, or I take a long time	☐	☐	b
I walk slower than other people, or I stop for rests.....	☐	☐	c
Jobs such as housework take a long time, or I have to stop for rests....	☐	☐	d
If I walk up one flight of stairs, I have to go slowly or stop	☐	☐	e
If I hurry or walk fast, I have to stop or slow down	☐	☐	f
My breathing makes it difficult to do things such as walk up hills, carrying things up stairs, light gardening such as weeding, dance, play bowls or play golf	☐	☐	g
My breathing makes it difficult to do things such as carry heavy loads, dig the garden or shovel snow, jog or walk at 5 miles per hour, play tennis or swim.....	☐	☐	h

13. We would like to know how your chest trouble usually affects your daily life.

For each statement please select *the box* that applies to you **because of your breathing**:

	True	False	
I cannot play sports or games	☐	☐	a
I cannot go out for entertainment or recreation	☐	☐	b
I cannot go out of the house to do the shopping	☐	☐	c
I cannot do housework	☐	☐	d
I cannot move far from my bed or chair.....	☐	☐	e

St. George's Respiratory Questionnaire

14. How does your chest trouble affect you?

Please select *ONE*:

It does not stop me doing anything I would like to do ☐ a

It stops me doing one or two things I would like to do..... ☐ b

It stops me doing most of the things I would like to do..... ☐ c

It stops me doing everything I would like to do..... ☐ d

Thank you for filling in this questionnaire.

Before you finish, would you please check to see that you have answered all the questions.

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