Lower Limb Muscle Dysfunction in COPD: The Effects of Resistance Training and Nutritional Supplementation

By

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Declaration

I hereby declare that this thesis has been composed by myself and that, to the best of my knowledge and belief, it contains no material that has been previously submitted or accepted for a higher degree or written by another person.

Except where assistance has been acknowledged, the work described in this thesis was performed by me during a period of research in the Department of Respiratory Medicine at Glenfield Hospital in Leicester. The muscle biopsy analysis for target gene and protein expression, and serum insulin assays were performed by Despina Constantin at the University of Nottingham. Linzy Houchen-Wolloff supervised the lower limb resistance training programme with assistance from Samantha Harrison and Carolyn Sandland.

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Abstract

Introduction: Weakness and wasting of the lower limb muscles is a frequent extra-pulmonary manifestation of Chronic Obstructive Pulmonary Disease (COPD). Although the underlying lung pathology is largely irreversible, the impact of the disease on the lower limbs is potentially treatable. Resistance training (RT) is an effective intervention for improving lower limb function in COPD, while nutritional supplementation as an adjunct to RT can provide additional benefits in older adults. The functional impact of this therapeutic combination and the accompanying muscle cellular and molecular changes has not been previously explored in COPD.

Methods: This thesis explored the functional, molecular, and cellular responses of the lower limb muscles in COPD patients in response to high-intensity quadriceps isokinetic RT. The primary hypothesis was that RT-induced increases in lower limb muscle mass and strength in COPD patients would be mediated through the expression of genes and proteins associated with muscle mass regulation in humans **(chapter 6)**. Secondary aims were to determine whether protein-carbohydrate supplementation at the time of training would augment the gains in functional performance in COPD **(chapter 5)**, to study the muscle inflammatory and satellite cell profile in response to RT **(chapter 7)**, and to measure the training-induced changes in lower limb muscle mass using portable ultrasound **(chapter 8)**.

Results: All participants demonstrated significant improvements in lower limb muscle mass and strength following RT, but protein-carbohydrate supplementation did not provide additional benefits. Absolute work done during RT was lower throughout in COPD when compared to healthy controls (HCs). Except for myogenic signalling, the increased expression of anabolic, catabolic, and transcription factor proteins was blunted in COPD patients, and nutritional supplementation did not alter this response. Inflammatory cells in the lower limb muscles increased in response to acute RT in COPD, but returned to baseline with chronic training. Portable ultrasound was shown to be a reproducible and sensitive technique for measuring changes in lower limb muscle mass following RT in COPD.

Conclusions: This thesis demonstrates that the potential for lower limb muscle rehabilitation in response to RT is preserved in COPD, while post-exercise protein-carbohydrate supplementation did not augment the functional or molecular responses. Except for myogenic proteins, the molecular responses to training were uncoupled from the functional gains, and more closely related to the absolute workloads performed during training.

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Abbreviations

ACSA	Anatomical cross-sectional area
ANOVA	Analysis of variance
ARTP	Association for Respiratory Technology and Physiology
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BIA	Bio-electrical impedance analysis
BMI	Body mass index
BTS	British Thoracic Society
COPD	Chronic Obstructive Pulmonary Disease
COPD(P)	COPD patients allocated placebo supplements
COPD(S)	COPD patients allocated protein-carbohydrate supplements
CSA	Cross-sectional area
Ct	Cycle threshold value
CT	Computed tomography
DEXA	Dual energy x-ray absorptiometry
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase enzyme
ECW	Extracellular water
EMG	Electromyography

ERS	European Respiratory Society
4E-BP1	Eukaryotic initiation factor 4E binding protein-1
FEV_1	Forced expiratory volume in one second
FFM	Fat free mass
FFMI	Fat free mass index
FM	Fat mass
FOXO	Forkhead box class O
FVC	Forced vital capacity
GMA	Glycol methacrylate
GSK-3α	Glycogen synthase kinase-3 alpha
GSK-3β	Glycogen synthase kinase-3 beta
HMBS	Hydroxymethylbilane synthase
ICW	Intracellular water
IGF-1	Insulin-like growth factor 1
IL-6	Interlukin-6
kDa	Kilo Dalton
LSD	Least significant difference
MAFbx/atrogin-1	Muscle Atrophy F-box protein
MCID	Minimal clinically important difference
MHC	Myosin heavy chain
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
MRC	Medical research council

MRF	Myogenic-regulatory factor
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MRS	Magnetic resonance spectroscopy
mTOR	Mammalian target of rapamycin
MuRF1	Muscle Ring-finger protein-1
MVC	Maximal voluntary contraction
NE	Neutrophil elastase
nwCOPD	Nonwasted COPD patients
1-RM	One-Repetition maximum
p70S6K	70 kD ribosomal S6 protein kinase
PaO_2	Partial pressure of oxygen in arterial blood
$PaCO_2$	Partial pressure of carbon dioxide in arterial blood
PAV	Pre-set angular velocity
PCR	Polymerase chain reaction
PCSA	Physiological cross-sectional area
PI3K	Phosphoinositide 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PR	Pulmonary rehabilitation
QMVC	Quadriceps maximum voluntary contraction
Q_t	Quadriceps muscle thickness measured by ultrasound
RF_{csa}	Rectus femoris cross-sectional area measured by ultrasound
ROI	Region of interest

RT	Resistance training
SD	Standard deviation
SEM	Standard error of the mean
SFA	Skin fold anthropometry
SGRQ	St George's Respiratory Questionnaire
TBW	Total body water
T_{dexa}	Thigh lean mass measured by DEXA
TGF-β	Transforming growth factor-beta
TNF-α	Tumour necrosis factor-alpha
TwQ	Isometric twitch tension
Ub	Ubiquitin
UPS	Ubiquitin Proteasome system
VO_{2peak}	Peak oxygen uptake
wCOPD	Wasted COPD patients

CHAPTER 1

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a chronic lung disease characterised by the presence of airflow limitation that is not fully reversible (Rabe et al. 2007). Patients typically present with symptoms of breathlessness, wheeze, cough and sputum production. Worldwide, cigarette smoking is the most important risk factor for developing the disease. Chronic exposure to noxious particles and gases emanating from cigarette smoke results in an abnormal inflammatory response in the airways, lung parenchyma and pulmonary vasculature (Hogg 2004). Less commonly, COPD can develop after prolonged exposure to occupational dusts and indoor air pollution, the latter being especially common among women in developing countries (Hogg 2004). The diagnosis is made by spirometry, and in a subject with an appropriate history, COPD is confirmed by the demonstration of a post-bronchodilator FEV_1/FVC ratio < 0.7. The lung pathology in COPD is usually irreversible, and treatment is typically aimed at alleviating symptoms and improving quality of life.

There are approximately 850,000 people currently diagnosed with COPD in the UK. However, a significant number of patients – estimated to be over 2 million, remain undiagnosed, with a reported disease prevalence of around 13% in England and Wales

(Department of Health 2011). It is a leading cause for mortality, with over 25,000 reported deaths in 2011 (Office for National Statistics 2012). The incidence of COPD is expected to increase in line with tobacco consumption, and is projected to become the third commonest cause for death and fifth commonest cause for physical disability worldwide by 2020 (Murray and Lopez 1997). In the UK, one in eight emergency hospital admissions are for COPD exacerbations, making it the second largest cause for emergency admissions, and occupying more than one million "bed days" each year. The direct annual costs to the NHS is estimated to be around £500 million, with 50% of this expenditure relating to hospital care (Commission for Healthcare Audit and Inspection 2006). Hence the burden of COPD is substantial.

Skeletal Muscle Dysfunction in COPD

COPD is a disease that is not only confined to the lungs, but is also associated with a number of extra-pulmonary or systemic manifestations. This includes systemic inflammation, weight loss, lower limb skeletal muscle dysfunction, cardiovascular disease, osteoporosis and depression (Agusti et al. 2003). Lower limb dysfunction has been the most widely studied extra-pulmonary feature, given that difficulty walking is such a prominent symptom for many patients. When compared to similar aged healthy subjects, patients with COPD present with reduced mass and strength of the lower limb muscles. This compromises physical performance and impacts adversely on mortality, morbidity and healthcare utilisation, independent of the degree of lung function impairment (Decramer et al. 1997; Gosselink et al. 1996; Marquis et al. 2002; Swallow et al. 2007). This is important because skeletal muscle dysfunction may be a remediable feature in a disease where the primary lung pathology is largely irreversible. The skeletal muscles are therefore a

potential target for therapies aimed at improving exercise capacity and health status in this population. Despite the appreciation of the clinical importance of skeletal muscle dysfunction in COPD, little is known about the cellular and molecular mechanisms underpinning this phenomenon, or indeed whether the potential for muscle hypertrophy is preserved to the same degree as in healthy older people.

Exercise Training in COPD

Pulmonary rehabilitation (PR) is an important nonpharmacological treatment option in COPD that has been shown to improve exercise capacity, dyspnoea and quality of life, and a reduction in healthcare usage (Troosters et al. 2005). Exercise training forms the cornerstone of PR, and underpins most of the positive effects seen with this intervention. While endurance training in the form of cycling and walking exercises has been the most commonly used training modality, the addition of a resistance training (RT) component enhances muscle mass and strength (Ries et al. 2007). Resistance-type exercise has the advantage of inducing a relatively low ventilatory burden in COPD (Probst et al. 2006), thus allowing patients to exercise individual muscle groups such as the quadriceps without exceeding their ventilatory capacity (Richardson et al. 1999). This type of training has been shown to produce positive clinical effects, including improvements in lower limb muscle mass and strength (Bernard et al. 1999; Casaburi et al. 2004; Kongsgaard et al. 2004), exercise capacity (Spruit et al. 2002) and health-related quality of life (Vonbank et al. 2012) . However there are few data on the adaptations occurring at the cellular and muscle fibre level in response to RT. While inflammatory mechanisms have been postulated to play a role in the aetiology of skeletal muscle dysfunction in COPD, it is not known whether acute exercise and regular training influence local muscle inflammation in these

subjects. Impaired muscle regeneration due to reduced satellite cell activity has been postulated as a mechanism for the observed loss of skeletal muscle mass in COPD (Hansen et al. 2006). The distribution and training response of these myogenic precursor cells in the lower limb muscles of COPD patients is unknown.

In order to quantify the effects of RT on lower limb muscles in COPD, reliable measurements of muscle mass are required. Imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI) and dual energy x-ray absorptiometry (DEXA) have all been employed for this purpose. However, these radiographic techniques are not readily accessible for routine clinical use. In this regard, there is potential for the application of portable ultrasound as a bedside method for determining lower limb muscle mass. The technique has been used previously for measuring various indices of quadriceps mass in COPD (Seymour et al. 2009; Shrikrishna et al. 2012), but the responsiveness of the measurements to an intervention such as RT has not been studied.

Exercise Training and Nutrition

Not all patients with COPD can access or participate in exercise training (e.g. due to musculoskeletal impairments). Therefore there is considerable interest in developing anabolic and nutritional therapies that can either augment the effects of RT, or provide functional benefits to patients who cannot perform exercise training. Studies in healthy adults suggest that administration of protein and carbohydrate during recovery from a bout of acute RT, enhances post exercise muscle protein synthesis (Rasmussen et al. 2000) and inhibits post exercise muscle protein breakdown (Roy et al. 1997), resulting in net muscle protein accretion. Hence dietary supplementation combined with RT may be an effective

strategy to enhance muscle function. At the molecular level, the combination of RT and post-exercise dietary protein supplementation has been shown to induce skeletal muscle hypertrophy via activation of muscle anabolic signalling pathways (Campbell and Leidy 2007; Hulmi et al. 2009a; Rennie et al. 2004). The effect of this therapeutic combination of RT and dietary supplementation on functional and molecular outcomes has not been previously investigated in COPD.

To address the above mentioned gaps in knowledge, a detailed investigation of the clinical and molecular effects of RT on the lower limb muscles of COPD patients and healthy controls was undertaken.

Structure of Thesis

The overarching aim of this thesis was to explore the functional, molecular and cellular responses of the lower limb muscles in COPD patients when compared to healthy controls following a programme of high-intensity lower limb RT, and to determine in patients the impact of providing a protein-carbohydrate dietary supplement at the time of training. The **primary hypothesis** tested was that RT-induced increases in lower limb muscle mass and strength in COPD patients would be mediated through the expression of genes and proteins associated with muscle mass regulation in humans. We estimated the sample size required to address this hypothesis using data from our own work and other published reports documenting improvement in quadriceps isometric muscle strength following RT.

The **specific aims** of this thesis were as follows:

1. To ascertain whether the functional response to RT (muscle mass and strength) will be blunted in COPD patients when compared to healthy controls.
2. To determine whether differences in the functional outcome of RT are mirrored by differences in the responses of genes and proteins known to regulate muscle mass in healthy humans.
3. To determine whether the ingestion of protein-carbohydrate supplementation at the time of RT will confer additional gains in functional performance in COPD patients.
4. To investigate the impact of RT combined with protein-carbohydrate supplementation in COPD on the expression of genes and proteins linked with muscle mass regulation.
5. To examine the inflammatory and satellite cell profile of the lower limb muscles in COPD patients and healthy controls in response to acute resistance exercise and regular RT.
6. To investigate whether portable ultrasound can be used to detect changes in lower limb muscle mass in response to RT in COPD patients and healthy controls.

An account of normal skeletal muscle structure and function, muscle fibre types and the physiology of muscle contraction is given in **chapter 2**. I will then review the current literature on skeletal muscle dysfunction in COPD, including its aetiology and

clinical consequences. In addition, the effect of ageing on skeletal muscle function is also reviewed. In **chapter 3**, I will discuss the various methods for assessing peripheral skeletal muscle function in COPD. Therapeutic strategies that are currently available, with particular reference to RT and nutrition, to enhance peripheral muscle function in this population are also discussed. Lastly, an overview of the muscle adaptations to RT and the molecular events proposed to play a role in the maintenance of muscle mass in health is reviewed. **Chapter 4** describes the methodology used in the experimental work described in this thesis.

In **chapter 5**, I discuss the effects of RT combined with dietary supplementation on functional outcomes including muscle mass, muscle strength and whole-body peak exercise performance. In **chapter 6**, the effects of the intervention on genes and proteins associated with muscle mass regulation are described. COPD patients and age-matched healthy controls underwent lower limb high-intensity isokinetic RT, thrice weekly for 8 weeks. Patients were randomly allocated to receive a protein-carbohydrate supplement (Vitargo Gainers Gold, Swecarb, Sweden) or placebo throughout training. Measures of lower limb muscle mass and strength, whole-body peak exercise performance and vastus lateralis muscle biopsies were obtained at various time points during the course of the study (The gene and protein expression analysis of the muscle biopsies were performed by Dr Despina Constantin at the University of Nottingham).

Chapter 7 describes a study exploring the inflammatory and satellite cell profile of the lower limb muscles in COPD patients and healthy controls, and the response to acute and chronic resistance exercise training. A new immunostaining technique to examine the structural and cellular characteristics of skeletal muscle was developed as part of this study.

A subset of participants from the randomised controlled trial underwent ultrasound measurements of lower limb muscle mass before and after the RT programme. The reliability and responsiveness of these ultrasound-derived measures of lower limb muscle mass was studied and is described in **chapter 8.**

Chapter 9 summarizes the key findings of this thesis, the clinical implications and ideas for future research that has risen from this work.

CHAPTER 2

Skeletal Muscle Dysfunction in COPD

There is a substantial body of evidence to show that skeletal muscle dysfunction exists in COPD (ATS/ERS 1999). This is important because skeletal muscle dysfunction may be remediable, and could be a target for new therapies in a disease where the primary pulmonary pathology is essentially irreversible. To place the importance of skeletal muscle dysfunction in its appropriate context, I will first of all give an account of normal skeletal muscle anatomy and physiology. I will then go on to discuss the evidence for skeletal muscle dysfunction in COPD, its aetiology, and clinical consequences. Lastly I will briefly review the effects of ageing on skeletal muscle function.

Healthy Muscle Anatomy and Physiology

Gross Anatomy and Ultra structure of Skeletal Muscle

The skeletal muscle is made up of a large number of muscle fibres, which are long thin cylindrical cells containing multiple nuclei **(Figure 2.1a)**. The length and diameter of muscle fibres varies according to the length and size of individual muscles. A thin layer of connective tissue called the *endomysium* wraps around each muscle fibre to separate it from the adjacent fibres. Groups of up to 150 muscle fibres – referred to as fascicles, are

bound together by another layer of connective tissue – the *perimysium*. An additional coat of fibrous connective tissue – the *epimysium*, surrounds the entire muscle and binds the individual fascicles together.

Individual skeletal muscle fibres are enveloped by a thin, elastic membrane known as the *sarcolemma* **(Figure 2.1b)**, which consists of a plasma membrane and a basement membrane. Between the plasma and basement membranes lie myogenic stem cells known as *satellite cells*, which are involved in the repair and growth of muscle fibres. The cytoplasm of the muscle fibre is made up of multiple filamentous bundles known as *myofibrils* **(Figure 2.1c)**, which are approximately 1μm in diameter and run along the entire length of the fibre. Myofibrils in turn are made up of smaller functional subunits called *myofilaments* **(Figure 2.1d)** that lie parallel to the long axis of the myofibril. The two main proteins that make up the myofilament are *actin* (thin filament) and *myosin* (thick filament), which are assembled in a specific manner to form the basic repeating functional unit of a myofibril – the *sarcomere*. The sarcomere consists of thick myosin filaments that are anchored to a protein sheet (the Z-line) and overlap with the thin actin filaments. This arrangement gives the skeletal muscle its characteristic striated appearance. The sarcomeres are arranged in sequence along the myofibrils, and it is the interaction between actin and myosin within each sarcomere that enables a muscle to contract. The functional property of a muscle is largely determined by the length of the sarcomere, which is on average about 2.5μm long in the resting state (William D McArdle et al. 2001).

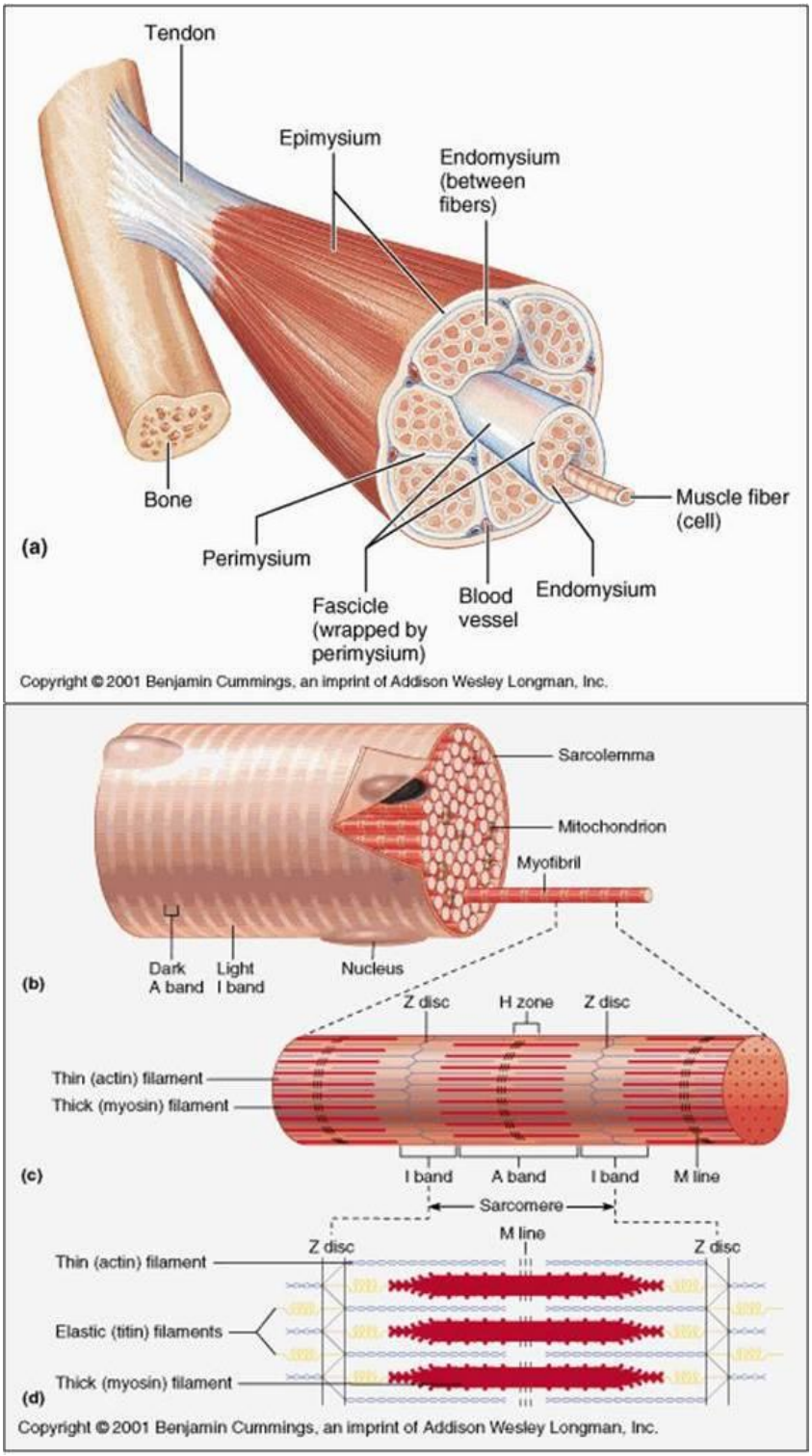


Figure 2.1 – Skeletal Muscle Structure
(a) Gross anatomy, (b) Muscle fascicle, (c) Myofibril, and (d) Myofilament

The Physiology of Skeletal Muscle Contraction

The myosin molecule is made up of 6 polypeptides – 2 heavy chains and 4 light chains. The myosin heavy chain contains a myosin head that binds to the actin molecule and forms the basis for muscle contraction. The myosin head region serves as the binding site for adenosine triphospate (ATP) and contains the enzyme adenosine triphosphatase (ATPase), which enables the hydrolysis of ATP into adenosine diphosphate (ADP) and inorganic phosphate (Pi), thus providing the energy for muscle contraction (Scott et al. 2001). The thin actin filament consists of 2 regulatory proteins – *troponin* and *tropomyosin*. Under resting conditions, tropomyosin binds to the active sites on actin, and prevents actin and myosin from binding together. The stimulus for muscle contraction is an action potential, which is conducted along the sarcolemma and leads to the release of calcium from the sarcoplasmic reticulum. The released calcium binds to troponin, which undergoes a conformational change and pulls tropomyosin away from the binding sites on actin. If ATP is also available, the myosin head attaches to the binding site on actin and pulls the thin filament along the thick filament leading to shortening of the sarcomere, a process termed as *cross-bridge cycling*. The end result is muscle contraction and force generation. Cross-bridge cycling continues as long as calcium and ATP are available, although the speed at which this occurs is mainly determined by the rate at which the myosin head ATPase can hydrolyse ATP.

Skeletal Muscle Fibre Types

Skeletal muscle contains a heterogeneous collection of muscle fibres with different morphological, physiological, biochemical and histochemical properties. Muscle fibres were originally classified according to their colour and contractile speed as “red”,

slow-twitch or "white", fast-twitch fibres. With advances in technology and staining techniques, it has become quite clear that a number of other differences also exist between fibre types, resulting in the development of several classification systems. However, the classification schemes do not always correlate with one another, so that fibres grouped together by one classification technique may be placed in a different group when a different classification technique is applied. Nevertheless for practical purposes, human skeletal muscle fibres are generally classified into 2 major groups – Type I (slow) and Type II (fast) fibres. Type II fibres are further divided into types IIa and IIx.

The most widely used scheme for classification of human skeletal muscle fibres is based on identifying the different forms of the myosin heavy chain (MHC) molecule **(Table 2.1)**. Three isoforms of the MHC molecule exist in human skeletal muscle – the slow isoform MHCI, and two fast isoforms MHCIIa and MHCIIx (previously labelled as MHCIIb). Various techniques are available for identifying these isoforms including histochemical staining for myosin ATPase activity, immunohistochemistry using antibodies raised against the various MHC isoforms, or gel electrophoresis of homogenized muscle tissue to separate the individual MHC isoforms. However, immunohistochemistry is considered to be the gold standard method since the MHC isoform correlates well with many of the metabolic properties of the muscle fibre (Richard L Lieber 2002). Another MHC isoform – MHCIIb, had been originally described in humans, and corresponded to the type IIb fibres identified by myosin ATPase staining. But studies have subsequently shown that this isoform is not expressed in human limb muscles, and what used to be described as MHCIIb was probably the MHCIIx isoform (Scott,Stevens,& Binder-Macleod 2001).

Fibre Type	I	IIa	IIx
Characteristic			
MHC Isoform	MHCI	MHCIIa	MHCIIx
Biochemical	Oxidative	Oxidative/Glycolytic	Glycolytic
Morphology			
Colour	Red	Red	White
Fibre CSA	Small	Medium	Large
Capillary density	High	Medium	Low
Mitochondrial density	High	High/medium	Low
Functional			
Contractile Speed	Slow-twitch	Fast-twitch	Fast-twitch
Endurance	High	Medium	Low
Tension generated	Low	Moderate	High
Fatigue resistance	High	High	Low

Abbreviations: MHC – Myosin Heavy Chain

Table 2.1 – Characteristics of human muscle fibre types Adapted from Kraus et al 1994, Maughan RJ et al 1997, Spangenburg et al 2003.

In addition to the pure types I, IIa and IIx isoforms, a proportion of muscle fibres in humans express both slow and fast (I/IIa) myosin isoforms, or a mixture of both fast (IIa/IIx) isoforms. These hybrid fibres constitute only about 5% of the total fibre type population in young subjects, but may be as high as 30% in the quadriceps muscle of elderly subjects (Andersen et al. 2000). Although there is wide variation in fibre type composition, on average the vastus lateralis muscle in healthy young adults consists of 50% type I, 40% type IIa and 10% type IIx fibres (Andersen and Aagaard 2000). The contractile speed of the IIx fibres is believed to be 10 times faster than the slow type I fibres, while the IIa fibres have intermediate contractile speed (Andersen, Schjerling, & Saltin 2000).

Nevertheless, the overall contractile property of a muscle will be determined by the relative proportion of each fibre type. Therefore, muscles that predominantly express the slow MHCI isoforms will have slow contraction speeds and vice versa.

Type I fibres are slow-twitch fibres that develop relatively little tension, depend mainly on aerobic metabolism and are fatigue resistant (Gosker et al. 2000). On the other hand, type IIx fibres are fast-twitch fibres that are capable of developing large tensions, and are unable to resist fatigue as they depend mainly on anaerobic metabolism. Type IIa fibres have intermediate properties and work under both aerobic and anaerobic conditions, but are relatively fatigue resistant.

Evidence for Skeletal Muscle Dysfunction in COPD

Impaired pulmonary mechanics alone does not explain the exercise limitation seen in COPD. Since the publication of the landmark study over two decades ago that first demonstrated the impact of lower limb dysfunction on exercise tolerance in COPD (Killian et al. 1992), there has been accumulating evidence to show that the skeletal muscles are structurally and functionally abnormal in these patients, and adversely affects exercise performance (Gosselink, Troosters, & Decramer 1996), quality of life (Mostert et al. 2000) and mortality (Marquis et al. 2002; Swallow et al. 2007). The quadriceps muscle has been the most commonly studied, since it is the primary muscle of locomotion, and is also readily accessible for biopsy studies.

At the cellular level, quadriceps muscle biopsies from COPD patients show a reduction in the proportion of type I muscle fibres (Gosker et al. 2002b; Hildebrand et al. 1991; Jakobsson et al. 1990; Whittom et al. 1998), with a proportional increase in type IIx fibre content (Whittom et al. 1998). In addition, the cross-sectional areas of all muscle fibre

types are reduced (Gosker et al. 2002a; Whittom et al. 1998). The activity of oxidative enzymes (Jakobsson et al. 1995; Maltais et al. 1996b) and the capillary density (Whittom et al. 1998) have also been observed to be lower in patients when compared to healthy controls. Overall this would suggest impaired aerobic function of the quadriceps muscle.

At a macroscopic level, reductions in muscle mass and strength, and increased muscle fatigability have been demonstrated. When compared to age-matched healthy controls, quadriceps strength is on average about 20-30% lower in patients with moderate to severe COPD (Bernard et al. 1998; Man et al. 2003; Man et al. 2005). There is also radiological evidence for reduced thigh muscle mass (Bernard et al. 1998; Marquis et al. 2002; Mathur et al. 2008; Vilaro et al. 2009), while several studies have demonstrated impaired quadriceps endurance (Allaire et al. 2004; Serres et al. 1998; Van't Hul et al. 2004), and increased susceptibility to muscle fatigue in response to exercise in this population (Mador et al. 2003).

There is evidence for abnormal muscle energy metabolism (Kutsuzawa et al. 1995) and early onset of lactic acidosis during exercise in COPD (Maltais et al. 1996b). This compromises the ability of the lower limbs to sustain repeated muscle contractions, leading to impaired exercise performance. Moreover, even when lung function is restored to normal by transplantation, exercise capacity remains reduced in these patients (Levy et al. 1993; Low et al. 1992). Similarly, improving lung function by drug therapy results in only modest improvements in exercise capacity (Grove et al. 1996), particularly when compared to PR.

Aetiology of Skeletal Muscle Dysfunction

The underlying mechanisms for skeletal muscle dysfunction in COPD remain unclear. However, there is general consensus that the aetiology is multifactorial, with a combination of systemic and local factors, contributing to varying degrees in individual patients. Factors that have been implicated include inactivity, nutritional depletion, hypoxia, corticosteroid use, oxidative stress, inflammation, abnormal muscle protein metabolism and impaired muscle regenerative capacity.

Inactivity and Deconditioning

This is increasingly regarded as a crucial factor contributing to skeletal muscle dysfunction in COPD. Patients generally tend to avoid activities that trigger breathlessness, and are therefore less active and sedentary when compared to their healthy counterparts. This leads to a vicious cycle where the avoidance of exercise and activity over a prolonged period results in progressive loss of fitness and deconditioning of the lower limb muscles. Studies using activity monitors have confirmed the presence of a reduction in total daily activity (Pitta et al. 2005; Singh and Morgan 2001) and localized lower limb activity (Walker et al. 2008) in COPD. The lower limb muscles are weaker when compared to the upper limb muscles (Bernard et al. 1998), while diaphragm strength seems to be preserved (Similowski et al. 1991). This supports the concept that inactivity results in deconditioning of the muscles that are habitually least activated (i.e. lower limbs). Many of the skeletal muscle abnormalities seen in COPD are comparable to that observed in detrained (Booth and Gollnick 1983), or immobilized (Appell 1990) healthy subjects, and in other chronic diseases where exercise limitation is a feature, such as chronic heart failure and chronic renal failure (Franssen et al. 2002). Lastly, the improvement in muscle function seen with

exercise training provides further evidence that disuse plays an important role in the development of skeletal muscle dysfunction in COPD.

Nutritional Depletion

Nutritional depletion and weight loss are quite common in COPD. This occurs due to an imbalance between dietary energy intake and expenditure. Increases in resting (Sergi et al. 2006) and total daily energy expenditure (Baarends et al. 1997b) have been reported in stable COPD patients when compared to healthy controls. In addition, acute exacerbations are accompanied by reduced caloric intake and increases in resting energy expenditure (Vermeeren et al. 1997). More recent data suggests that the decreased efficiency of ventilation (Kim et al. 2012) and higher ATP cost of lower limb muscle contraction (Layec et al. 2011) in COPD s may contribute to their elevated daily energy requirements. Energy expenditure is also influenced by protein turnover, and in COPD there is evidence for increased whole-body protein turnover that correlates with increased resting energy expenditure and reduced fat-free mass (FFM) (Kao et al. 2011; Rutten et al. 2006). The net result is a hypermetabolic state that may lead to weight loss if energy requirements are not maintained.

Depending on the population studied and disease severity, one-third (Schols et al. 1993) to one-half (Laaban et al. 1993) of all patients are estimated to be malnourished, and this impacts adversely on physical performance and health status (Engelen et al. 1994; Mostert et al. 2000). Nutritional depletion is also a marker of poor prognosis, independent of the degree of airflow limitation (Landbo et al. 1999). Importantly, even in patients with normal body weight, reductions in fat-free mass (FFM) have been observed (Schols et al. 1993). Reduced thigh muscle mass has been related to muscle weakness (Bernard et al.

1998), and when compared to body weight, is a better predictor of survival in COPD (Marquis et al. 2002). Moreover, some patients demonstrate an exaggerated systemic inflammatory response that may result in protein catabolism and loss of muscle mass (Eid et al. 2001; Schols et al. 1996).

Hypoxia

In COPD, hypoxia can be either intermittent or chronic, and may have adverse effects on skeletal muscle function. When healthy humans are chronically exposed to high-altitude hypoxia, their skeletal muscles demonstrate a number of morphological and biochemical alterations that are quite similar to that seen in COPD. Changes include a reduction in muscle strength (Caquelard et al. 2000), muscle endurance (Badier et al. 1994), fibre cross-sectional area (Hoppeler et al. 1990), and oxidative enzyme activity (Howald et al. 1990). Hypoxic COPD patients have a lower proportion of type I fibres (Gosker et al. 2002b), while there is evidence for improved muscle contractility in these subjects following the administration of supplemental oxygen (Zattara-Hartmann et al. 1995). It has been suggested that hypoxia triggers muscle dysfunction via a variety of mechanisms such as increases in pro-inflammatory cytokines (Takabatake et al. 2000) and oxidative stress (Heunks et al. 1999), or a reduction in anabolic hormone levels (Semple et al. 1980).

Corticosteroid Use

COPD patients are frequently treated with oral corticosteroids which have the potential to adversely affect skeletal muscle function. Severe peripheral muscle weakness and diffuse fibre atrophy – predominantly of the type IIx muscle fibres (Decramer et al. 1996), have been observed in patients with steroid-induced myopathy. There is also a close

relationship between skeletal muscle weakness and the average daily dose of corticosteroids received in the preceding six months (Decramer et al. 1994). Moreover, quadriceps strength and bulk are reduced in patients on long-term low dose corticosteroids (Bernard et al. 1998), although short burst therapy does not seem to adversely impact on muscle function (Hopkinson et al. 2004).

Oxidative Stress

Oxidative stress can affect muscle contractility (Reid 2001) and has therefore been suggested as a potential mechanism for muscle dysfunction in COPD (Couillard and Prefaut 2005). Increased oxidative stress has been observed in the stable state (Barreiro et al. 2008), during exacerbations (Rahman et al. 1997) and while performing local quadriceps exercise (Couillard et al. 2003). There is evidence for impaired skeletal muscle anti-oxidant capacity in COPD (Couillard et al. 2003), while treatment with anti-oxidants such as N-acetylcysteine may reduce exercise-induced oxidative stress and increase quadriceps endurance (Koechlin et al. 2004).

Inflammatory Mechanisms

While COPD is mainly an inflammatory disease of the airways, a number of studies have demonstrated the presence of low-grade systemic inflammation in the peripheral circulation (Di et al. 1994; Eid et al. 2001; Schols et al. 1996). In-vitro data suggests that pro-inflammatory cytokines such as TNF-alpha may induce muscle wasting (Reid and Li 2001), hence raising the possibility that inflammatory mechanisms might promote muscle dysfunction in this group of patients. Elevated IL-6 levels have been linked to reduced lower-limb (Debigare et al. 2003) and whole-body lean mass (Eid et al. 2001). In addition, COPD exacerbations are associated with increased IL-8 levels that

correlate negatively with quadriceps strength (Spruit et al. 2003). Patients with increased levels of circulating TNF-alpha fail to gain weight in response to nutritional supplementation during PR (Creutzberg et al. 2000). However, muscle dysfunction mainly affects the lower limbs in COPD, and the relative sparing of the diaphragm and upper limb muscles raises uncertainty about the role of systemic inflammation. Local muscle inflammation may be relevant, but to date there has been no data to support this (Barreiro et al. 2008; Crul et al. 2007).

Abnormal Skeletal Muscle Protein Metabolism

Skeletal muscle mass is determined by the relative rates of protein synthesis and protein degradation. Hence skeletal muscle atrophy may be a consequence of a reduction in muscle protein synthesis and/or an increase in protein degradation. While studies have demonstrated increased whole-body protein turnover in COPD (Engelen et al. 2000a) (Kao et al. 2011), the signalling pathways that regulate muscle atrophy and hypertrophy in this disease have not been fully investigated. At the whole-body level, decreased muscle protein synthesis (Morrison et al. 1988) and increased myofibrillar protein degradation (Rutten et al. 2006) have been reported in muscle wasted patients. There is currently no data on the synthesis and degradation rates of muscle-specific proteins in this population. However, biomarkers of muscle protein breakdown such as circulatory 3-methyhistidine (Ubhi et al. 2012) and urinary pseudo-uridine (Bolton et al. 2007) have been shown to be increased in underweight patients, and provide indirect evidence for increased muscle protein degradation.

Protein Synthesis Pathways

Research from cell line and animal models have identified that the PI3K (phosphoinositide 3-kinase)/Akt (protein kinase B)/mTOR (mammalian target of rapamycin) pathway plays a key role in the muscle atrophy-hypertrophy programme (Glass 2005). Anabolic stimuli such as exercise and insulin-like growth factor 1 (IGF-1) activate the PI3K/Akt/mTOR pathway and its downstream targets, resulting in protein synthesis (Glass 2005) **(Figure 2.2)**. The phosphorylated active form of Akt stimulates mTOR, which in turn activates p70S6K (70kDa ribosomal S6 protein kinase), and inhibits 4E-BP1 (eukaryotic initiation factor 4E binding protein-1), the net effect being increased protein synthesis. Phosphorylated Akt can also promote protein synthesis via a separate pathway by inhibiting the action of GSK-3β (glycogen synthase kinase-3β), which is a negative regulator of muscle growth.

Recent data in COPD has shown reduced muscle IGF-1 levels in cachectic patients when compared to non-cachectic patients (Vogiatzis et al. 2010). Decreased serum and muscle IGF-1 levels have also been observed during acute exacerbations (Crul, Spruit, Gayan-Ramirez, Quarck, Gosselink, Troosters, Pitta, & Decramer 2007;Kythreotis et al. 2009).

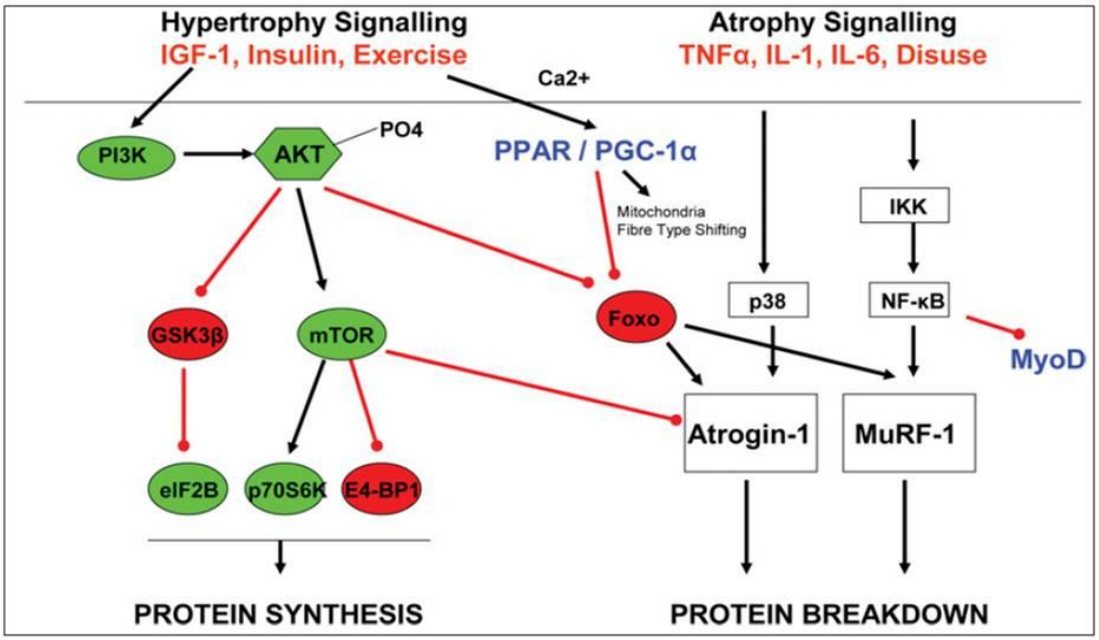


Figure 2.2 – Schematic diagram of muscle atrophy and hypertrophy pathways (Man et al. 2009)

Protein Degradation Pathways

Evidence suggests that the Ubiquitin Proteasome system (UPS) is the principal pathway responsible for cellular protein degradation and consequent muscle atrophy (Lecker et al. 1999). This is an ATP-dependent proteolytic system wherein proteins targeted for degradation are covalently attached to multiple molecules of ubiquitin (Ub) by a series of three enzymatic steps consisting of E1 (Ub-activating), E2 (Ub-conjugating) and E3 (Ub-ligase) enzymes (Murton et al. 2008) **(Figure 2.3)**. The target protein (ubiquitinated protein) undergoes degradation in the cylindrical 26S proteasome complex, with the Ub being released back into the cytoplasm for recycling. Target specificity is determined by the E3 Ub-ligase enzyme, several hundred types of which have been

identified in humans (Murton, Constantin, & Greenhaff 2008). Many components of the UPS – especially the muscle-specific E3-ligases, Muscle Ring-finger protein-1 (MuRF1) and Muscle Atrophy F-box (MAFbx/atrogin-1), may be up regulated by various factors that are especially relevant to muscle dysfunction in COPD such as muscle inactivity (Jones et al. 2004; Lecker et al. 2004), cytokines (Li et al. 2005) and glucocorticoids (Clarke et al. 2007). Also, the phosphorylated form of Akt, by inactivating the forkhead box class O (FOXO) transcription factors, down regulates the expression of MuRF1 and MAFbx/atrogin-1, hence blocking muscle protein breakdown.

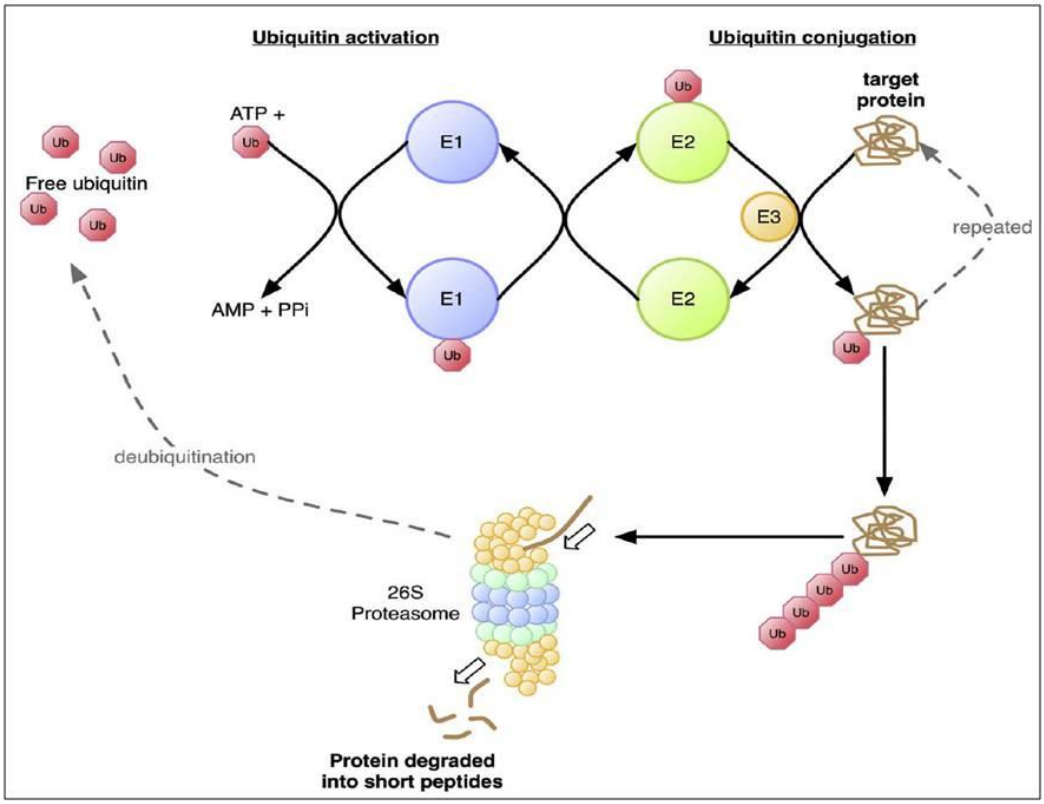


Figure 2.3 – Schematic diagram of the Ubiquitin Proteasome system (UPS) (Murton, Constantin, & Greenhaff 2008)

The activity of these protein degradation pathways have been examined in a number recent studies involving COPD patients. MAFbx/atrogin-1 mRNA and protein

levels were elevated in the quadriceps of muscle wasted COPD patients when compared to healthy controls (Lemire et al. 2012; Plant et al. 2010). In cachectic patients, the muscle levels of MAFbx/atrogin-1 mRNA and protein, MuRF1 mRNA, and total protein ubiquitination, were all increased in comparison to controls (Doucet et al. 2007; Fermoselle et al. 2012). In addition, elevated mRNA expression of FOXO-1 and FOXO-3 were noted in the lower limbs of muscle-wasted and cachectic COPD patients (Debigare et al. 2010; Doucet et al. 2007; Fermoselle et al. 2012). Interestingly in the study by Doucet et al., the activity of proteins involved in muscle hypertrophy signalling (p70S6K, 4E-BP1 and GSK-3β) was increased in a wasted sub-group, which the authors speculate might be a failed attempt at restoration of muscle mass. Another study has reported increased levels of MAFbx/atrogin-1 and myostatin, a negative regulator of muscle growth, in the quadriceps of COPD (Plant et al. 2010). In **chapter 5** I will describe a study examining the activity of various components of the UPS in the quadriceps of COPD and the response to RT.

Abnormal Myonuclear Turnover

Besides abnormal protein metabolism, muscle mass may also be influenced by changes in loss and accretion of myonuclei in muscle fibres (myonuclear turnover). Myonuclear loss is thought to occur by apoptosis, while myonuclear accretion and regeneration results from the fusion of stem cell-derived (satellite cells) myoblasts with adjacent myofibres. There is conflicting data on myonuclear apoptotic activity in COPD. While there are some studies showing increased muscle cell apoptosis in underweight patients when compared to normal weight patients and healthy controls (Agusti et al. 2002; Barreiro et al. 2011), this has not been observed by other groups (Gosker et al. 2003).

Impaired muscle regeneration has also been suggested as a potential mechanism for muscle dysfunction in COPD (Hansen et al. 2006). The maintenance, repair and regeneration of terminally differentiated adult skeletal muscle is facilitated by a small population of stem cells located along the periphery of the myofibre known as satellite cells (Kadi et al. 2004). These cells remain quiescent under normal physiological conditions, but are activated in response to stimuli such as exercise and muscle injury. This process is controlled by various growth and myogenic-regulatory factors (MRFs) such as MyoD, myogenin and myostatin. In COPD, the distribution of MRFs in the peripheral muscles of COPD have been reported by various groups, but except for increased myostatin mRNA expression (Man et al. 2010; Plant et al. 2010), none of the other MRFs have been noted to be significantly different between patients and age-matched controls at rest (Crul et al. 2007; Vogiatzis et al. 2007)

Clinical Consequences

A number of adverse clinical outcomes including reductions in exercise capacity, muscle strength, quality of live, and survival, have been linked to skeletal muscle dysfunction in COPD.

Impaired Exercise Performance

Studies have consistently shown that FFM is an independent determinant of exercise capacity in severe COPD (Schols et al. 1991a).Patients with decreased FFM have reduced functional (Mostert et al. 2000; Schols et al. 1993) and peak exercise capacity (Baarends et al. 1997a; Kobayashi et al. 2000).

Peripheral Muscle Weakness

Quadriceps strength has been shown to be directly proportional to thigh muscle cross-sectional area in COPD, and is lower when compared to similar aged healthy controls (Bernard et al. 1998). Besides, the quadriceps strength/thigh muscle CSA is preserved, which suggests that muscle weakness in COPD is mainly due to a loss of muscle mass. Several studies have shown that the strength of both the upper (Engelen et al. 1994; Mostert et al. 2000) and lower limbs (Engelen et al. 2000b; Gosselink, Troosters, & Decramer 1996) are impaired in patients with reduced FFM.

Impaired Quality of Life and Increased Healthcare usage

Patients with lower lean body mass report a significant reduction in health-related quality of life as measured by the St George's Respiratory Questionnaire (SGRQ) (Mostert et al. 2000; Shoup et al. 1997). Impaired health status is also related to peripheral muscle fibre-type composition, with worse SGRQ scores observed in patients with reduced type I fibre composition (Montes de et al. 2006). Moreover, quadriceps weakness in COPD is related to increased utilization of healthcare resources (Decramer et al. 1997).

Reduced Survival

While the association between low BMI and increased mortality has been a common observation in COPD (Landbo et al. 1999; Schols et al. 1998;Vestbo et al. 2006), in recent years it has become evident that it is the loss of muscle mass that is clinically relevant. Schols et.al. observed that median survival was nearly 50% lower in cachectic patients when compared to those with preserved muscle mass, even after adjusting for confounders such as age and disease severity (Schols et al. 2005). In addition, there is data

to show that reduced mid-thigh cross-sectional area, an index of muscle mass, is associated with reduced survival in COPD (Marquis et al. 2002). A similar negative impact on survival has been reported in patients with quadriceps weakness (Swallow et al. 2007).

The Effects of Ageing on Skeletal Muscle (Sarcopaenia)

The prevalence of COPD is more common with advancing age, and therefore age-related changes in skeletal muscle structure and function may be of relevance in this population. Ageing is associated with a reduction in muscle quality and quantity, and is termed *"Sarcopaenia"*, which in Greek translates to *"poverty of the flesh"*.

Definition of Sarcopaenia

The term sarcopaenia was originally coined to describe the decline in lean body mass that occurs with ageing (Rosenberg 1989). However, since impairment of muscle strength and functional status also accompanies this reduction in muscle mass, the definition of sarcopaenia has subsequently evolved to also include changes in muscle function. Currently sarcopaenia is defined as the loss of muscle protein mass, function and muscle quality that accompanies advancing age (Zinna and Yarasheski 2003). From an operational point of view, sarcopaenia is said to be present if the ratio of the appendicular skeletal muscle mass (in kilograms) to the square of the height (in square-metres), is less than 2 standard deviations below the mean of a young, healthy reference population (Baumgartner et al. 1998).

Epidemiology of Sarcopaenia

In humans, peak skeletal muscle mass is seen around the third decade of life. Subsequently, there is a progressive decline in muscle bulk, with significant losses

occurring by the end of the 5th decade (Janssen et al. 2000). It is estimated that beyond the age of 50, muscle mass is lost at the rate of 1-2% per year (Thomas 2007), leading to a sarcopaenia prevalence of about 25% (Iannuzzi-Sucich et al. 2002) in the sixth decade, rising to 50% in subjects over age of 80 (Baumgartner et al. 1998). The magnitude of muscle loss seems to be greater for the lower-limb muscle groups, with over 40% of the CSA of the vastus lateralis being lost between the ages of 20 and 80 (Lexell 1995). This may reflect reduced activity of these muscles as a consequence of leading a more sedentary lifestyle with advancing age. In addition, disability rates have been shown to be higher in sarcopaenic men and women, indicating the importance of muscle mass in maintaining functional ability (Baumgartner et al. 1998).

Muscle Morphology in Sarcopaenia

Data from biopsy studies, obtained mainly from the quadriceps muscle, have demonstrated a number of alterations in the ageing skeletal muscle. The observed decrease in whole-muscle cross-sectional area appears to be due to the predominant atrophy of type II fibres, although type I fibres are also affected to a lesser extent (Doherty et al. 1993; Lexell and Downham 1992). While cross-sectional data comparing young and elderly muscles seemed to indicate a shift in fibre composition, with a higher relative proportion of type I fibres in older individuals (Jakobsson et al. 1988; Larsson et al. 1978), there is some uncertainty as to whether this is true when subjects are followed-up long term. In a longitudinal study of 65 year old men who underwent quadriceps biopsies before and after a 12-year period, the proportion of type I fibres were shown to be reduced when compared with baseline (Frontera et al. 2000). The absolute number of muscle fibres are also reduced, such that the muscles of the very elderly have about 50% fewer type I and type II fibres

when compared to the muscles of a 20-year old (Lexell et al. 1988). The age-associated loss in α-motor neurons are believed to be responsible for some of these alterations in muscle mass and fibre number (Doherty, Vandervoort, & Brown 1993).

Other changes include "fibre-type grouping" (clusters of type I and type II fibres found together, as opposed to the "chessboard" type of even arrangement of different fibre types in the young), and the increased co expression of different MHC isoforms within the same fibre – so called "hybrid fibres" (Andersen et al. 1999). These alterations are thought to be secondary to a chronic neuropathic process resulting from progressive denervation and partial reinnervation of muscle fibres with ageing (Andersen, Terzis, & Kryger 1999; Oertel 1986). In addition, a recent study has demonstrated a reduction in the satellite cell content of type II fibres in elderly individuals (Verdijk et al. 2007).

Aetiology of Sarcopaenia

Multiple, interrelated factors are likely to be involved in the development of sarcopaenia. Muscle mass depends upon the relative rates of muscle protein synthesis and breakdown. Although basal rates of muscle protein synthesis and breakdown are not impaired, the ageing muscle seems to exhibit "anabolic resistance", whereby the protein synthetic response to anabolic stimuli like food intake or resistance-type exercise, is blunted (Koopman and van Loon 2009). Other possible mechanisms include reduced physical activity, altered endocrine function (testosterone, growth hormone, insulin-like growth factor-1), decline in food intake, and loss of α-motor neurons.

Summary

Skeletal muscle dysfunction is quite common in COPD, and its clinical importance is illustrated by the negative impact on exercise tolerance, quality of life and survival. In recent years, clinical and laboratory-based studies have thrown further light on the mechanisms responsible for muscle dysfunction in this disease. Although its aetiology is multifactorial, inactivity seems to be a key driver, as evidenced by the improvements in muscle function seen with exercise training. However, it is not clear whether skeletal muscle dysfunction in COPD differs from other chronic diseases or age-related sarcopaenia, where inactivity and deconditioning play an equally important role. In **chapter 3**, I will discuss the various strategies available for treating skeletal muscle dysfunction, and in particular assess the role of RT and nutrition in enhancing muscle function in this population.

CHAPTER 3

Assessment and Treatment of Skeletal Muscle Dysfunction in COPD

In **chapter 2** I discussed the proposed mechanisms and clinical consequences of skeletal muscle dysfunction in COPD. Although treatments aimed at improving airway function provide symptomatic benefit, a significant number of patients remain disabled as a consequence of the extra-pulmonary effects of the disease – namely skeletal muscle dysfunction. In this chapter I will discuss the various methods for assessing peripheral skeletal muscle function in COPD, focussing particularly on the lower limb muscles. I will also review the strategies that are currently available for treating muscle dysfunction, especially the role of RT and nutrition. The macroscopic and microscopic muscle adaptations occurring in healthy adults in response to RT will also be discussed.

Measuring Skeletal Muscle Function in COPD

Measurement of peripheral skeletal muscle function, especially of the lower limbs, has an important role in the clinical assessment of disability in COPD, and for monitoring

the response to interventions such as exercise training. The main objectives of measuring peripheral muscle function in COPD are as follows:

1) To assess physical impairment
2) To assess the outcome of interventions like rehabilitation or nutritional therapy
3) To allow prescription of RT

There is potential for muscle function assessment to be used as a diagnostic tool to identify the aetiology of muscle dysfunction in COPD, although at present this is speculative because the underlying pathophysiology of skeletal muscle dysfunction has not been fully understood. There is evidence to show that mortality in COPD can be predicted from measures of peripheral muscle mass and strength (Marquis et al. 2002; Swallow et al. 2007). Therefore measurement of peripheral muscle function can potentially be used as a guide to prognosis in this disease. Clinically relevant aspects of muscle function in COPD include measures of muscle strength, muscle mass, and muscle performance during cardiopulmonary exercise testing.

Muscle strength

Strength refers to the force generating capacity of a muscle and can be determined using volitional or nonvolitional methods. Volitional methods of strength testing require maximum effort and are therefore subject to patient and operator motivation. Volitional strength can be measured during static or dynamic muscle contractions using different types of equipment **(Table 3.1)**. Conversely, nonvolitional testing is effort-independent, but technically demanding and not routinely used in clinical practice. Both volitional and nonvolitional techniques of muscle strength testing have been used in COPD.

Types of Muscle Strength Assessments

Volitional Testing
e.g. Tensiometry, 1-RM testing, Dynamometry (Mechanical or Electric), Computer Assisted Dynamometry

Nonvolitional Testing
e.g. Magnetic Femoral Nerve Stimulation

Types of Muscle Contractions

Static (Isometric) Contraction – The length of muscle fibres remain constant during the muscle contraction, and there is no movement of associated joints

Dynamic Contraction – Joint movements occur during this type of muscle contraction, and consist of either isotonic or isokinetic contractions

- *Isotonic* – There is shortening (concentric action) and lengthening (eccentric action) of a muscle throughout its range of motion around a joint

- *Isokinetic* – The speed of muscle contraction is fixed within a range of motion, and the force generated by the muscle encounters an opposing force relative to that applied to the testing device

Table 3.1 – Types of Strength Measurements and Muscle Contractions

Volitional Methods

Tensiometry

Cable tensiometers can be used to measure isometric muscle strength. For measuring quadriceps muscle force, a cable is strapped to the lower leg and connected to a tensiometer. When the knee is forcibly extended, increased tension on the cable leads to depression of the riser over which the cable passes. This leads to deflection of the pointer on the instrument to indicate the subject's isometric strength. This technique can measure strength at different joint angles and the equipment is light and portable. It has been used to

measure quadriceps strength in COPD (Simpson et al. 1992), although at present it is not routinely employed in clinical practice.

One-Repetition Maximum (1-RM)

This is a form of isotonic strength measurement that measures the maximum amount of weight that can be lifted during one repetition of a standard weight lifting exercise. It can be tested using standard gym equipment. When properly conducted, 1-RM testing has been shown to be a reliable and safe testing tool in most subjects including COPD (Kaelin et al. 1999). It can also be used to prescribe muscle strength training programmes and to measure changes in muscle strength after training (Casaburi et al. 2004; Clark et al. 2000; Simpson et al. 1992) . When compared to other methods, 1-RM testing has some practical advantages and may be more aligned with habitual physical tasks. However, no normative data exist for these tests, and the values obtained depend on the equipment used. 1-RM testing may be contraindicated in frail elderly subjects and in those with pre-existing cardiac disease. In these patients sub maximal multiple repetition tests may be performed, and 1-RM can be estimated using validated equations (Braith et al. 1993).

Dynamometry

Dynamometers are used to measure isometric muscle force. Application of an external force leads to either compression of a steel spring (mechanical dynamometer) or movement of an electronic force transducer (electronic dynamometer), to give an estimate of isometric muscle strength.

Figure 3.1 – Handheld Dynamometer

Handheld dynamometers **(Figure 3.1)** can be used to test the strength of specific upper and lower limb muscles. As an example – for the assessment of the knee extensors, the subject is seated with hips and knees flexed at 90°, the shoulders are stabilised by an assistant, and the dynamometer is placed over the lower leg, just proximal to the malleoli (Andrews et al. 1996). The force applied by the subject will give an estimate of isometric quadriceps strength. Various other muscle groups can also be tested by positioning the subject accordingly. However in order to get reliable readings, the assessor's strength should be greater than the specific muscle group being tested (Stratford and Balsor 1994). Therefore there is potential for greater operator variability with this technique. Reference values are available (Phillips et al. 2000; Stratford & Balsor 1994; van der Ploeg et al. 1991), and this device has been used to test peripheral muscle strength in COPD (Gosselink et al. 2000).

Handgrip dynamometers **(Figure 3.2)** are available to measure grip strength (Mathiowetz et al. 1984), and they have been used in several studies involving COPD patients (Bernard et al. 1998; Kutsuzawa et al. 1992; Kutsuzawa et al. 1995; Wilson et al. 1986). Reference ranges are also available for various age groups (Mathiowetz et al. 1985).

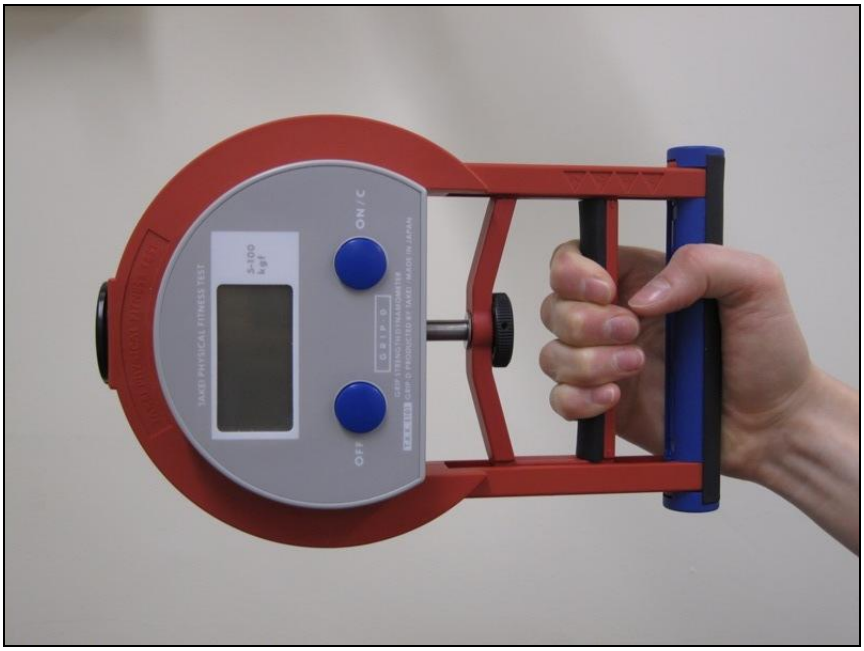


Figure 3.2 – Handgrip Dynamometer

Handheld devices are portable, easy to use, and cheaper than some of the more sophisticated instruments for measuring isometric strength. However, a number of errors can occur with these measurements. These can be avoided by ensuring that subjects are positioned in a standardised manner during testing. Sufficient training in the use of these devices is also needed to minimise variability of measurements.

Computer-Assisted Dynamometer (Isokinetic Dynamometer)

This is considered to be the gold standard method for muscle strength testing. Isokinetic testing uses the force-velocity characteristics of muscle contraction and allows the measurement of maximal muscle strength over a wide range of joint positions and velocities. An isokinetic dynamometer **(Figure 3.3)** contains a speed control mechanism that accelerates to a preset constant velocity with the application of force. Once a speed is attained, the device automatically adjusts to provide a force that opposes the force generated by the muscle through the range of motion. It can be used to measure isokinetic and isometric muscle strength of various muscle groups.

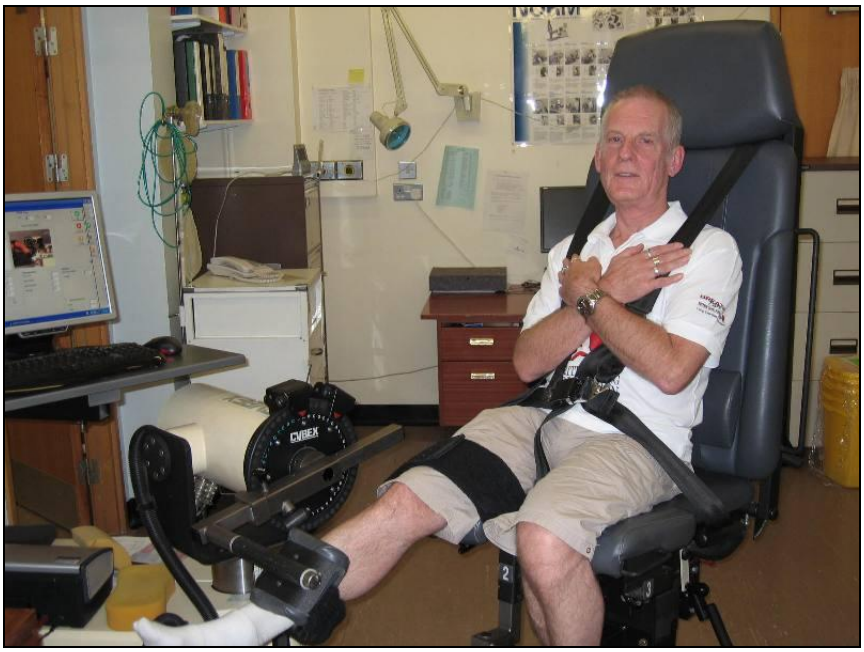


Figure 3.3 – Isokinetic Dynamometer

For example, in order to test the strength of the quadriceps using an isokinetic dynamometer (Cybex II Norm), the subject is seated in a chair with lumbar support and straps at the level of the shoulders, pelvis and thighs to minimise unnecessary movements. The padded lever arm of the dynamometer is attached with a strap to the shin of the leg to be tested, while the contra lateral leg is immobilised with padded support. The chair is positioned so that the axis of rotation of the knee joint is aligned with the axis of rotation of the dynamometer. The maximal range of movement at the knee joint is set with safety stops placed at the extremes of extension and flexion. Testing can be done using various protocols. In this thesis, quadriceps function was measured using the isokinetic dynamometer, with isometric quadriceps strength measured during a maximal static contraction with the knee at 70°, and isokinetic strength measured during a dynamic knee extension at 60°/second. Further details are given in **chapter 4**. In addition, it is also possible to determine both peak and total work performed during a set number of repetitions. For instance, if the number of repetitions is set at 30, peak isokinetic work will be calculated as the greatest amount of work done during any single knee extension over the course of the 30 contractions. Total isokinetic work is determined as the sum of the peak work done for each of the 30 contractions. In **chapter 5,** I describe the effects of high-intensity lower limb isokinetic RT on muscle mass, muscle strength and whole-body exercise capacity in COPD patients and healthy controls

In healthy subjects a good correlation exists between isometric and isokinetic measurements using this device (Borges 1989; Lord et al. 1992), and reference values are also available (Decramer, de, V, & Dom 1996; Neder et al. 1999). There is evidence to show that both isokinetic (Clark et al. 2000; Hamilton et al. 1995) and isometric muscle

strength (Decramer et al. 1994; Gosselink, Troosters, & Decramer 1996) are significantly lower in COPD when compared to healthy subjects.

Although isokinetic tests give accurate assessments of muscle strength, the equipment required is expensive, not widely available, and the measurements may not be related to functional ability of patients. Moreover, the clinical utility of the additional data that is provided by this method, including measures of torque and isokinetic work, remains uncertain.

Nonvolitional Methods

Motivation, which is an important issue in task performance, may influence muscle strength measured by maximal voluntary contraction (MVC) manoeuvres. Other issues like functional ability and patient cooperation may also affect these measurements. Therefore it is not uncommon to get sub maximal muscle activation during MVC testing (Allen et al. 1995). Nonvolitional methods of assessing muscle contractility overcome some of these limitations.

A technique using supramaximal magnetic stimulation of the femoral nerve has been developed as a nonvolitional method of assessing quadriceps strength (Polkey et al. 1996). This technique works on the principle that magnetic stimulation of the motor nerve results in a muscle twitch, and the ensuing tension or pressure generated has a constant relationship with maximal tetanic tension, or the true MVC, and therefore accurately reflects strength. A figure-of-eight coil is placed over the femoral nerve high in the femoral triangle, and a single supramaximal magnetic stimulus is applied. This results in femoral nerve depolarisation and yields isometric twitch tension (TwQ). The procedure is independent of patient effort, but may be uncomfortable, although most patients taking part

in studies will accept it. Quadriceps strength using this technique has been shown to be lower in COPD patients when compared with age-matched controls (Man et al. 2003). In addition, a drop in TwQ following exercise has been used as an indicator of muscle fatigue (Saey et al. 2003; Saey et al. 2006).

Nonvolitional testing is effort-independent and is particularly useful when detailed information about muscle physiology is required. Currently its application has mainly been in the research laboratory, and its role for measuring muscle function during rehabilitation, where voluntary exercise is prescribed requires further evaluation.

Muscle Mass

Other than cadaver analysis, there is no true gold standard method to directly measure human muscle mass. Measures of body composition, which divide the body into different compartments depending on the method used, can be used to indirectly estimate muscle mass. Of particular interest is the fat-free mass (FFM) compartment which contains functional muscle tissue. Depletion of FFM in COPD is associated with a number of adverse outcomes, and has been shown to occur even in patients with normal body weight (Schols et al. 1993). Measurement of FFM therefore gives better functional and prognostic information compared to simple measures of body weight. Moreover, increasing muscle mass is an important therapeutic goal for rehabilitation and nutritional support programmes, emphasising the importance of FFM measurement in COPD.

The choice of method for assessment of body composition will depend on the purpose for which the measurement is intended, in addition to the cost and availability of equipment. Furthermore, age-specific normal ranges for FFM have not been established making the identification of wasted patients difficult. FFM can be measured at the whole-

body or regional level, and the techniques that have found clinical applications in COPD are discussed.

Whole-body measurements

Skin Fold Anthropometry (SFA)

SFA is a cheap and simple bedside method that can be used to estimate body composition in routine clinical practice. By means of special callipers **(Figure 3.4)**, the thickness of the skin and underlying fat is measured at specific sites in the body – including the biceps, triceps, sub scapular and suprailiac areas. The sum of the skin fold measurements at the four sites is then placed in a regression equation that has been previously validated using hydro densitometry as the reference method, to give an estimate of body fat (Durnin and Womersley 1974). FFM is then obtained by subtracting fat mass from total body weight. This technique works on the assumption that the amount of subcutaneous fat in the sites chosen for measurement is proportional to total body fat. There are equations available to estimate regional quadriceps muscle cross-sectional area from measures of thigh circumference and skin fold thickness (Housh et al. 1995). But these have been developed in young men and may not accurately represent true muscle size in the elderly or in COPD patients (Mathur et al. 2008).

Figure 3.4 – Skin fold Callipers

Bioelectric Impedance (BIA)

Bioelectric impedance **(Figure 3.5)** is probably the most frequently used method for measuring body composition in clinical practice. It has the advantage of being relatively inexpensive, easy to use and is portable. It is based on the differential conductance of an electric current through the body compartments, with FFM – which contains all body fluids and electrolytes, being a better conductor of electricity than fat mass. With subjects in the supine position, ipsilateral self-adhesive electrodes are attached – two at the wrist and two at the ankle. In the single frequency method, a weak alternating current of 800 micro amps at 50 kHz is passed through the outer electrodes, and the voltage drop across the body is measured by the inner pair of electrodes which gives a measure of whole-body impedance. This information is then converted to a volume estimate based on the principle that the

impedance (or resistance) of a conducting system (the human body in this instance) is related to its length and cross-sectional area. This relationship is expressed as follows:

$V=L^2/R$

Where V is volume, L is length and R is resistance. In biological systems conduction of electricity occurs predominantly through water and so V represents total body water (TBW). Since TBW lies almost entirely within FFM rather than fat mass, FFM can be estimated from the following relationship:

$FFM \propto Height^2/R$

Figure 3.5 – Bioelectric Impedance

Regression equations for calculating FFM by this method have been derived for a number of different populations including patients with COPD, using a variety of reference methods for measuring body water (Kyle et al. 1998; Schols et al. 1991b).

Dual Energy X-ray Absorptiometry (DEXA)

DEXA **(Figure 3.6)** measures the differential attenuation of two low-energy x-ray beams by body tissues to provide a three compartment model of body composition – fat mass, bone-free lean mass and bone mineral mass. Bone mass is measured directly from its x-ray absorption, and soft tissue mass at each point is derived by subtracting bone mass from total mass. Lean and fat mass are distinguished by water content and their attenuation of x-ray beams is known from in vitro measurements. The subject is scanned longitudinally in the supine position using x-rays at two different energies. The differential attenuation of the x-ray beams as they pass through the body is measured and reconstructed by computer software, to provide quantitative information about the three body compartments. The entire scan takes less than 10 minutes to perform and analysis of regional limb and trunk FFM can also be done. **The technique is described in detail in chapter 4.**

Figure 3.6 – DEXA

DEXA has been suggested as a suitable reference method for the measurement of body composition, and has been validated against deuterium dilution in COPD (Engelen et al. 1998). It can be performed quickly, involves minimal radiation exposure and also gives information about bone mineral loss. It has the added advantage of being able to provide regional FFM measurements. Although this technique assumes constant intracellular hydration of bone-free lean mass (Jebb 1997), studies in which the hydration factor have been manipulated have shown that this method is less prone to errors (Kohrt 1995). DEXA was used to measure muscle mass in this thesis, and a study comparing this method with portable ultrasound to measure lower limb muscle mass and the responsiveness to RT is described in **chapter 8**.

In COPD, significant intermethod differences have been demonstrated for FFM measured using the various body composition techniques (Steiner et al. 2002). In a group of patients with moderate to severe COPD, FFM was estimated using SFA, BIA and DEXA prior to the commencement of pulmonary rehabilitation. Relative to DEXA, FFM was overestimated by SFA whereas it was underestimated by BIA. There was a systematic increase in bias with mean FFM for both DEXA versus BIA and DEXA versus SFA, although this was almost eliminated when FFM was corrected for height (Fat-Free Mass Index- FFMI). Moreover the sensitivity and specificity of BIA to identify nutritional depletion was superior to SFA, when they were both compared against DEXA as the reference method. These differences need to be borne in mind when choosing a method to assess FFM in clinical practice.

It is important to note that there are a number of limitations to whole-body FFM measurements. These techniques are based on the assumption that intracellular hydration

remains constant, which may not be the case in the elderly or in disease states. Therefore the true precision of these methods remain uncertain. SFA is subject to inter-observer variability, and sufficient experience is required by the operator to minimize this. Also, the inherent assumptions regarding the distribution of body fat may not hold true in COPD. Single frequency bioelectric impedance cannot reliably distinguish between extra cellular water (ECW) and intracellular water (ICW), and will therefore be affected by fluid shifts e.g. – oedema and diuretic therapy (Kushner et al. 1996). In addition, it may not be sensitive enough to detect changes in FFM in response to interventions like RT (Nelson et al. 1996; Sipila and Suominen 1995). In the case of DEXA, the equipment is expensive and not readily accessible, and despite its ability to determine regional limb FFM, it cannot reliably measure the size of individual muscles. It is also important to note that soft tissue mass measured using DEXA scanners from different manufacturers can give different results (Tothill et al. 1994).

Regional Muscle Mass Measurements

CT and MRI

These two radiographic techniques can be used to measure regional body composition and are considered to be the closest to a gold standard method for estimating human muscle mass. They can accurately measure the size and cross-sectional areas of individual muscles or muscle groups. In CT, collimated x-rays beams from an x-ray source are passed through the region of interest and the transmitted radiation is detected on the other side by an array of detectors. The difference in x-ray attenuation from different body

structures is related to the physical density of tissues, and a cross-sectional image of the scanned area is generated.

MRI on the other hand, does not involve ionizing radiation and provides better contrast between fat and muscle tissue. It is based on the principle that when the human body is placed in a strong magnetic field, hydrogen atoms in water can behave like magnets and attempt to align with the external magnetic field (Lukaski 1987). If a radiofrequency wave is then directed at the body tissues, the atoms tend to absorb energy and change their orientation in the magnetic field. When this radio wave is then turned off, the absorbed energy is emitted and the intensity of the signal is used to measure the number of hydrogen nuclei in the tissues, which is then used to create an image.

Reduced quadriceps cross-sectional area measured by CT has been shown to be a better predictor of mortality than body mass index in COPD (Marquis et al. 2002). Recent studies using MRI have shown that quadriceps mass, cross-sectional area and volume are lower in COPD patients when compared with age-matched controls (Mathur et al. 2008; Vilaro et al. 2009). CT and MRI have both been used to detect changes in quadriceps size in response to RT in COPD (Bernard et al. 1999; Kongsgaard et al. 2004). However, it is difficult to access these techniques in routine clinical practice due to the high cost of equipment and the need for technical expertise. Moreover CT involves exposure to ionizing radiation, which makes it unsuitable for repeat measurements.

Ultrasound

This non-invasive method can be used to measure thickness and cross-sectional areas of superficial muscles. It is based on the reflection of high-frequency sound waves

from tissue interfaces, which is then used to construct an image. The advantage of ultrasound is that it is portable, quick to perform and involves no exposure to ionizing radiation. Although ultrasound has been used extensively to measure muscle size in various populations (Bemben 2002), its application in COPD has so far been limited. Recent studies have demonstrated a good correlation between quadriceps strength and ultrasound measured cross-sectional area of the rectus femoris in COPD patients (Seymour et al. 2009; Shrikrishna et al. 2012). This technique can also potentially be used to quantify the muscle response to training. A study examining the responsiveness of ultrasound measurements of lower limb muscle mass in response to RT in COPD patients is described in **chapter 8**. However a number of measurement errors can occur with this method as it is more operator dependent than other imaging techniques. This can be minimised by avoiding excessive tissue compression during scanning, and ensuring that the probe is always placed perpendicular to the long axis of the limb being measured.

Muscle Performance during Cardiopulmonary Exercise Testing

In COPD, resting quadriceps muscle biopsies show impaired aerobic function as evidenced by reductions in oxidative enzymes and the proportion of type I fibres (Gosker et al. 2002a; Maltais et al. 2000; Whittom et al. 1998). But the primary role of the skeletal muscles is to maintain activity and body movements, and therefore from a functional point of view, important information regarding muscle performance can be obtained in the context of cardiopulmonary exercise testing **(Figure 3.7)**. In fact there is evidence to show that abnormal metabolic response to exercise may contribute to impaired whole-body exercise performance in COPD (Steiner et al. 2005). The measurements of relevance will include maximal oxygen uptake (VO_2 max), metabolic threshold, lactate and ammonia.

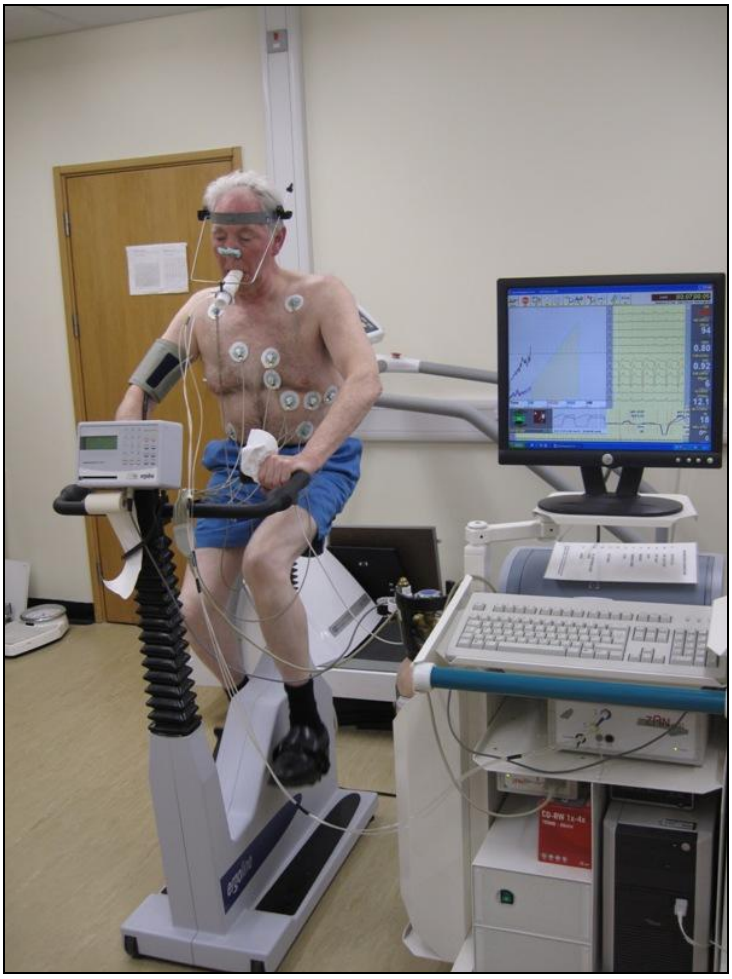


Figure 3.7 – Cardiopulmonary Exercise Testing

Maximal Oxygen Uptake

Maximal or peak oxygen uptake (VO_2 max) is the highest value for oxygen uptake that is attained during a standard incremental exercise testing protocol and is the best available index for the assessment of aerobic capacity (Christopher B.Cooper and Thomas W.Storer 2008). It usually requires the use of large muscle groups and is influenced by many factors including age, gender, body weight and the platform used for exercise testing. Since muscle metabolism is a major determinant of oxygen uptake during exercise,

abnormal muscle function is usually accompanied by a reduction in VO_2 max. However reduced VO_2 max on its own is not diagnostic for muscle dysfunction and examination of other physiological data including cardiovascular and ventilatory responses to exercise are required to make a proper assessment. In addition, COPD patients are more likely to show abnormal cardiopulmonary responses to exercise, thus making the interpretation of muscle dysfunction difficult. Nevertheless, VO_2 max provides a useful index of muscle aerobic function during exercise.

Metabolic Threshold, Lactate and Ammonia

During steady state exercise at low intensities, the energy for muscle contraction is provided predominantly from aerobic (oxygen dependent) sources. However, as intensity increases, anaerobic (oxygen independent) energy provision rises. In exercise testing terms, the metabolic or anaerobic threshold is defined as the point at which anaerobic metabolism becomes sufficient to cause lactate to appear in the blood. Exercise above the metabolic threshold cannot be sustained because the accumulation of lactate results in a metabolic acidosis and is an important cause of muscle fatigue. Although the transition from a predominantly aerobic to a predominantly anaerobic mode of exercise is not an abrupt process within the muscle, the metabolic threshold can usually be identified either directly from measurement of blood lactate or non-invasively from gas exchange measurements during incremental exercise testing.

The metabolic threshold is interpreted in relation to the predicted VO_2 max and normally occurs at around 50-70% of VO_2 max. Physical deconditioning and muscle abnormalities can result in low metabolic thresholds relative to VO_2 max, while it is increased with physical training. As with VO_2 max, metabolic threshold and lactate on their

own cannot be used to diagnose muscle disease, but has to be interpreted in conjunction with other variables obtained during cardiopulmonary exercise testing. In COPD an early rise in lactate and/or a premature metabolic threshold may be seen as a result of deconditioning although some patients may be unable to exercise sufficiently to reach the metabolic threshold if severe ventilatory limitation or lack of motivation is present.

A rise in ammonia levels during exercise is an indicator of the efficiency of energy provision during muscular contraction and has been associated with fatigue in healthy subjects. It has also been used to assess the skeletal muscle metabolic response to exercise in COPD but it's role in the wider assessment of skeletal muscle function in clinical practice remains uncertain (Calvert et al. 2008). 31P-Magnetic Resonance Spectroscopy (31P-MRS) is another technique that can provide useful information about skeletal muscle metabolic function during exercise. This technique can provide information about energy metabolism but remains mainly a research tool.

Treating Skeletal Muscle Dysfunction

Given that the aetiology of muscle dysfunction in COPD is multi-factorial, a single therapeutic approach is unlikely to rectify the problem in all patients.

Exercise Training

COPD patients frequently enter a downward spiral of breathlessness and inactivity, leading to deconditioning of the lower limb muscles and exercise limitation. Exercise training is an obvious way to improve muscle function, and is usually provided as part of a pulmonary rehabilitation (PR) programme. There is now overwhelming evidence to show that exercise training improves quadriceps function and quality of life in COPD

(Ries et al. 2007). Patients are able to sustain the required level of training intensity in order to induce favourable muscle adaptations (Maltais et al. 1996a; Maltais et al. 1997; Whittom et al. 1998). Moreover, metabolic changes such as increased oxidative capacity (Maltais et al. 1996a), reduced exercise-induced lactic acid production and improved muscle cellular bioenergetics (Sala et al. 1999), all provide confirmation that exercise training is a useful intervention in this population.

The general principles of exercise training in healthy subjects are equally applicable to the COPD population. Training should be of sufficient intensity, duration and frequency, in order to induce muscle adaptations and improve physical performance. In addition, the principle of training specificity implies that endurance training will induce muscle changes favouring endurance (e.g. increases in oxidative enzymes, fibre capillarity, mitochondrial density and proportion of type I fibres), while RT will yield muscle changes that improve strength (e.g. increases in fibre CSA and proportion of type II fibres). On the other hand, there is data from elderly subjects (Hepple et al. 1997b; Vincent et al. 2002) and COPD (Ortega et al. 2002; Simpson et al. 1992; Spruit et al. 2002) showing improvements in endurance performance after a programme of RT.

Endurance-type training in the form of walking and cycling exercises has been the most widely used in COPD. However, it has become increasingly clear that RT can provide additional advantages in terms of increasing peripheral muscle mass and strength, and guidelines now recommend the inclusion of RT in PR programmes (Ries et al. 2007). RT or strength training is a form of exercise whereby the muscle contracts against an external resistance. RT is believed to be the most potent stimulus for the growth of adult skeletal muscle (Spiering et al. 2008), and therefore represents a potential therapeutic strategy to

augment skeletal muscle mass and improve functional performance in wasting diseases such as COPD. The advantage of this form of exercise is that it induces less dyspnoea (Simpson et al. 1992), and relatively smaller groups of muscles can be trained without exceeding the ventilatory capacity of the patient (Richardson et al. 1999). A recent systematic review of RT in COPD has concluded that peripheral muscle RT is feasible, safe, and results in improved upper and lower limb strength that may carry over to the performance of some daily activities (O'Shea et al. 2009).

RT can be prescribed in a number of ways, and traditionally consists of lifting free weights, with training intensity set at 70% of the subjects' 1RM. This form of training was first used to successfully rehabilitate and improve quadriceps muscle strength in injured American soldiers returning home after the 2nd world war (Delorme 1945). Additional training variables such as the number of repetitions/sets, and level of resistance, can be varied to induce specific training adaptations. Training is generally progressed by increasing the resistance/weight, while keeping the number of sets and repetitions unchanged. Other modes of RT that are available include isokinetic dynamometry, elastic resistance bands or lifting one's own body weight (calisthenics). In this thesis, the isokinetic dynamometer (Cybex II Norm) was used to prescribe RT.

Nutritional Support

The adverse consequences of weight loss and muscle wasting have generated a lot of research interest into nutritional interventions to improve peripheral muscle function in COPD. Nutritional supplementation is generally provided with the aim of increasing body weight and/or to enhance exercise performance. Earlier trials in malnourished patients showed that caloric supplementation can lead to significant increases in body weight,

respiratory muscle strength and various measures of functional performance (Efthimiou et al. 1988; Rogers et al. 1992; Whittaker et al. 1990; Wilson et al. 1986). However these studies consisted of few participants, with variations in patient characteristics, types of supplementation and outcome measures. A systematic review conducted in 2005 concluded that nutritional support in stable COPD had no effect on anthropometric measures and exercise capacity (Ferreira et al. 2005). It should be noted that only 2 out of 11 studies included in this review (Schols et al. 1995; Steiner et al. 2003) had an exercise intervention (during PR) combined with supplementation. In contrast a more recent Cochrane review concluded that nutritional supplementation in malnourished COPD patients may increase body weight, respiratory muscle strength, fat-free mass (FFM), exercise capacity and quality of life (Ferreira et al. 2012). 5 out of 17 studies included in this review also underwent exercise training (Schols et al. 1995; Steiner et al. 2003; Sugawara et al. 2010; Sugawara et al. 2012; van Wetering et al. 2010), and suggests an additional anabolic effect when nutrition is combined with exercise training. Therefore it seems that nutrition in isolation may not be effective, and additional anabolic stimuli such as exercise training will be required to increase appetite and improve functional outcomes in COPD.

The importance of nutrition in enhancing sporting performance is well recognized, and the recommendation is that for individuals undertaking regular physical activity, carbohydrate-rich foods should provide 60-70% of their daily energy intake, protein 12-15%, with the remainder to be provided by fat (Williams 1995). Carbohydrates are a crucial source of fuel for sustained exercise, but their stores are limited, and if they are depleted during prolonged exercise, fatigue ensues. Carbohydrate supplementation during exercise can prolong endurance performance , and high-carbohydrate feeding several days

before exercise can increase muscle glycogen stores and enhance performance (Ivy 1999). Deconditioned individuals are particularly reliant on carbohydrate as a source of energy for sustaining exercise (Coggan A.R. and Williams B.D. 1995; Coyle et al. 1986), which may be especially relevant to COPD patients. Even though energy requirements decline with age, a similar amount of carbohydrate in the diet has been recommended for exercise in the elderly (Sacheck and Roubenoff 1999). Significant increases in muscle strength and thigh muscle CSA has been observed in elderly men given supplements consisting of predominantly carbohydrate and fat at the time of RT, when compared to no supplementation (Meredith et al. 1992). In COPD, various macronutrients and micronutrients have the potential to be used as ergogenic agents to augment the effects of exercise training, or to provide benefits to patients who cannot undergo training (e.g. due to musculoskeletal problems).

Previous studies combining exercise with nutrition in COPD used predominantly fat supplements, due to concerns that carbohydrates, which produce carbon dioxide when oxidized, might adversely affect ventilation in these patients (Efthimiou et al. 1992; Goldstein et al. 1989). Schols et al observed increases in weight (mainly fat mass) in patients receiving a predominantly fat-rich supplement during PR (Schols et al. 1995), although no additional improvements in physical performance were seen in supplemented patients compared to those receiving rehabilitation alone. However, subsequent evidence has suggested that carbohydrate-rich supplements, possibly due to more rapid gastric emptying, induce less postprandial dyspnoea than fat-rich supplements (Vermeeren et al. 2001). Besides, carbohydrates are an important source of fuel for prolonged exercise, and

as discussed earlier, may better meet the needs of the exercising patient. Steiner et al showed improvements in whole body exercise performance during aerobic training combined with carbohydrate supplementation compared with training alone (Steiner et al. 2003). These changes were linked to increases in body weight (again mainly fat mass) but occurred only in well-nourished patients. Baldi et al showed that in weight loosing patients, supplementation with essential amino acids during a 12 week PR programme led to significant gains in body weight and FFM, when compared to a control group that received no supplementation (Baldi et al. 2010).In a more recent study involving malnourished patients with chronic respiratory failure mainly due to COPD, a multimodal intervention (consisting of a predominant carbohydrate supplement, exercise and oral testosterone) resulted in significant improvements in exercise capacity (Pison et al. 2011).

Enhanced whole-body protein turnover (Engelen et al. 2000a; Kao et al. 2011) and loss of lean tissue mass have been consistently reported in COPD. Hence there is theoretical advantage in boosting muscle protein synthesis by providing protein supplements to these patients. Studies show that physical training combined with post-exercise dietary protein supplementation leads to skeletal muscle hypertrophy via activation of the mTOR signalling pathway (Campbell & Leidy 2007; Hulmi et al. 2009b; Rennie et al. 2004). There is evidence that muscle protein synthesis via this signalling pathway is blunted in the elderly (Cuthbertson et al. 2005; Kumar et al. 2009). In addition, the timing of protein supplementation in relation to RT may also be important. Data shows that following RT, protein synthesis is greatest in the first three hours post training when compared to 24 and 48 hours later. Since protein availability is essential for optimum protein synthesis, the provision of protein supplementation immediately after RT has

potential advantages. In fact a study in elderly subjects has shown that quadriceps muscle hypertrophy occurred only in those given protein immediately after RT, compared to 2 hours later (Esmarck et al. 2001). The regular ingestion of protein supplements with prolonged RT has been shown to significantly augment the gains in muscle mass and strength in both young and older subjects (Cermak et al. 2012). Furthermore, increasing insulin availability can acutely inhibit muscle protein breakdown (Gelfand and Barrett 1987; Greenhaff et al. 2008), and is a process that is known to be dysregulated in the elderly (Kumar et al. 2009; Wilkes et al. 2009). The impact of this therapeutic combination of RT with dietary protein-carbohydrate supplementation on functional and molecular outcomes was examined in this thesis and is described in **chapters 5 and 6** respectively.

Creatine supplementation has been tried in COPD, based on its positive effects on muscle mass in healthy subjects. Although one study demonstrated an increase in muscle mass and strength with creatine supplementation during PR (Fuld et al. 2005), the findings were not replicated in a subsequent larger trial (Deacon et al. 2008). A recent systematic review has concluded that creatine supplementation does not improve functional outcomes or health-related quality of life in patients undergoing PR (Al-Ghimlas and Todd 2010). Lastly, there is some data to show that polyunsaturated fatty acid (PUFA) supplementation during PR may improve exercise capacity in COPD (Broekhuizen et al. 2005; Sugawara et al. 2012).

Anabolic Hormones

The illicit use of anabolic hormones for enhancing physical performance is a significant problem amongst elite athletes, but in COPD this form of therapy may be advantageous, especially in underweight patients. Anabolic hormones either on their own

(Yeh et al. 2002),or when combined with endurance exercise training (Creutzberg et al. 2003; Ferreira et al. 1998; Schols et al. 1995) have been shown to increase lean body mass, but with no additional gains seen in muscle strength or exercise capacity. A similar increase in lean body mass has been reported when testosterone is combined with RT, with some modest improvements in maximal exercise capacity (Casaburi et al. 2004). Growth hormone combined with PR is reported to show increases in lean body mass in underweight patients, but had no effect on functional outcomes (Burdet et al. 1997). There is currently not enough evidence to recommend the routine use of anabolic or growth hormones in patients with COPD.

Other treatments

Short-term oxygen therapy in hypoxaemic COPD patients may improve exercise tolerance and muscle aerobic capacity, although the long-term effects on muscle function remain unknown (Levy et al. 1997). Non-invasive ventilation has been shown to increase body weight in cachectic patients, but it is unclear whether this is due to an increase in muscle mass, since body composition was not measured in this study (Budweiser et al. 2006). The use of appetite stimulants in under-weight patients can produce increases in body weight, mainly fat mass, but improvements functional outcomes have not been observed (Weisberg et al. 2002).

Adaptations to Resistance Training in Health

In this section I will discuss the skeletal muscle adaptations occurring at the molecular, cellular and whole-muscle level in healthy individuals in response to acute and chronic RT. While neural adaptations seem to be important during the early stages of

training, morphological adaptations occur with chronic exercise, resulting in muscle hypertrophy. The training response in elderly subjects will also be reviewed.

Neurological Adaptations

Neural adaptations refer to the enhanced ability of the nervous system (i.e. the motor unit) to activate and recruit muscle fibres during the performance of a specific strength task. They contribute to increases in muscle strength following RT, and result from enhanced learning and coordination. The rapid increase in muscle strength seen at the onset of training, the specificity of the training response and the effects of cross-training, are all taken as indirect evidence for neural adaptations in response to RT. When compared to muscle size, there is a disproportionately larger increase in muscle strength with RT, particularly during the first few weeks of training. This is thought to occur as a result of an increase in whole-muscle specific tension (Folland and Williams 2007). In addition, the training response is task-specific, such that dynamic RT (e.g. isotonic or isokinetic training) will lead to greater improvements in dynamic lifting strength (1-RM) rather than isometric strength (Dons et al. 1979; Rutherford and Jones 1986). Also, RT of one limb has been shown to increase the strength of the contra lateral untrained limb (Tracy et al. 1999), suggesting a cross-over training effect which is assumed to stem from central neurological adaptations. Direct evidence for increased muscle activation comes from data showing enhanced activity of surface electromyography (EMG) recordings after RT (Hakkinen and Komi 1983). However, there are issues with regard to the measurement, interpretation, and reproducibility of this technique.

Cellular and Morphological Adaptations

Muscle Fibre Hypertrophy and Satellite Cell Response

The multi-nucleated adult muscle fibre consists of post-mitotic nuclei that are considered to be incapable of dividing any further. Therefore at the cellular level, the predominant adaptation to RT is thought to be fibre hypertrophy (increase in size or cross-sectional area of individual muscle fibres) rather than fibre hyperplasia (increase in the total number of muscle fibres). This is reflected at the whole-muscle level as an increase in anatomical cross-sectional area (ACSA) or muscle volume. The magnitude of the hypertrophic response is greater for type II fibres, which also possess higher specific muscle tension, the net result being an increase in whole-muscle specific tension after RT (Folland & Williams 2007). It is assumed that each muscle nucleus can control the mRNA expression and protein synthesis for only a finite volume of cytoplasm, a concept known as the *myonuclear domain* (Hawke 2005).

Muscle fibre hypertrophy during the early stages of RT results from an increase in the size of each myonuclear domain (i.e. existing myonuclei increase their rate of protein synthesis and support a moderate increase in cytoplasmic area) (Kadi et al. 2005). However beyond a certain upper limit, referred to as the *"myonuclear domain ceiling"*, further increases in muscle size can only occur by increasing the number of myonuclei (and hence an increase in the number of myonuclear domains) (Petrella et al. 2008). This is achieved by the incorporation of satellite cells into existing muscle fibres. Satellite cells are undifferentiated myogenic stem cells that lie between the muscle fibre plasma membrane and basement membrane. They are usually quiescent, but are activated in response to signals like resistance exercise, and undergo cell division and differentiation to produce

new myonuclei. Increasing the number of myonuclei enhances the fibre's capacity for transcription, protein synthesis and growth.

Inflammatory Responses

RT leads to myofibril damage, sarcomere disruption and muscle soreness (Proske and Allen 2005). This muscle damage is characterised by a stereotypical inflammatory response that involves the invasion of neutrophils and macrophages into muscle tissue. The role of these cells is to remove debris and release proinflammatory cytokines like interlukin-6 (IL-6), transforming growth factor-beta (TGF-β) and tumour necrosis factor-alpha (TNF-α) (Peake et al. 2005). Cytokine release leads to satellite cell activation, proliferation and differentiation, ultimately resulting in skeletal muscle hypertrophy (Hawke 2005). A study exploring the inflammatory and satellite cell response to acute and chronic RT was performed as part of this thesis and is described in **chapter 7.**

Muscle Architecture

In theory, the maximum force a muscle can generate will depend on its physiological cross-sectional area (PCSA), which requires the measurement of muscle volume, angle of fibre pennation and estimation of fibre length. A muscle (e.g. quadriceps) is described as *"pennate"*, if its line of action does not match the line of action of its constituent fibres, and the angle formed between these 2 lines of action is referred to as the *Angle of Pennation* or the *Pennation Angle*. For a nonpennate muscle (e.g. biceps), the angle of pennation will be zero. It is difficult to accurately measure all these parameters in routine clinical practice, nevertheless there is evidence to suggest that the pennation angle increases with RT (Aagaard et al. 2001; Reeves et al. 2004). An increase in the pennation

angle will facilitate the packing of additional muscle fibres within the same ACSA, which effectively increases the PCSA, and hence muscle force. However, since the fibres are now pulling at a more oblique angle, the force applied to the tendon by the individual fibres may be reduced. The optimum angle of pennation has been estimated to be around 45° (Alexander R M and Vernon A 1975), although very few muscle groups are pennate to this extent. Therefore any increase in the pennation angle will be expected to contribute to increases in muscle strength even if the ACSA does not change.

Molecular Adaptations to Resistance Exercise and Training in Health

Resistance training is an effective strategy for enhancing skeletal muscle mass. However, the molecular adaptations occurring in response to acute resistance exercise and chronic RT have only become clearer in recent years. Resistance exercise results in a sequential cascade of events starting with muscle activation and recruitment, initiation of various intracellular signalling pathways, and increased myofibrillar protein turnover (Spiering et al. 2008). It is assumed that the cumulative effects of repeated resistance exercise will result in the summation of these acute responses leading to long-term adaptation and muscle protein accretion. I will give a brief account of the molecular adaptations that are thought to occur in response to acute and chronic RT in the healthy population.

Adaptations to Acute RT

An acute bout of resistance exercise leads to increased skeletal muscle protein turnover, with increases in both muscle protein synthesis (MPS) and muscle protein

breakdown (MPB), that can last for up to 48 hours post exercise (Phillips et al. 1997). At the molecular level, studies have consistently shown that the mRNA expression of various genes regulating MPB (ubiquitin proteasome pathway), MPS (phosphatidlyinositol 3 kinase/Akt/mammalian target of rapamycin pathway) and myogenesis (MyoD, myogenin) is increased in response to acute RT in healthy individuals, while the expression of myostatin – a negative regulator of muscle growth, is decreased (Deldicque et al. 2008; Hulmi et al. 2009b; Mascher et al. 2008). Dietary proteins are known to stimulate MPS, and when provided in the immediate post-exercise period, leads to a positive net protein balance, eventually resulting in muscle protein accretion (Phillips et al. 2005).

Adaptations to Chronic RT

While many studies have reported on the molecular events occurring in response to a bout of acute resistance exercise in healthy adults, there is very little data on the temporal changes in molecular signalling that occur following chronic RT. Therefore many researchers have extrapolated data generated from studies of acute RT to explain the adaptations seen with chronic RT. However there is currently no evidence to link the early exercise-induced changes in gene expression and activation of various signalling pathways with the muscle adaptations seen with chronic RT. In this thesis, the expression of candidate genes and proteins regulating MPS, MPB, myogenesis and transcription factors were determined from quadriceps muscle biopsies in COPD patients and age-matched healthy controls following RT, and are described in **chapter 6.**

Adaptations to Resistance Training in the Elderly

The ageing skeletal muscle retains its capacity for both neurological and morphological adaptations in response to RT. High-intensity RT performed over 6 to 12

weeks, has been shown to significantly increase muscle strength and muscle CSA in older adults (Fiatarone et al. 1990; Fiatarone et al. 1994; Frontera et al. 1988; Tracy et al. 1999).Training performed at a lower-intensity can also to lead to significant gains in quadriceps strength, but with no change in muscle size (Aniansson and Gustafsson E 1981). This suggests that neural adaptations may be an important mechanism for muscle strength gains in the elderly.

At a cellular level, hypertrophy of both type I and type II fibres have been demonstrated with progressive RT (Frontera et al. 1988; Pyka et al. 1994), although the increase in CSA has been shown to be greater for the type II fibres (Verdijk et al. 2009b). In addition, there is evidence for a shift in fibre composition; with an increase in type IIa and concomitant reduction in type IIx fibres (Hagerman et al. 2000; Hikida et al. 2000).

Although there have been suggestions that the ageing skeletal muscle may have a limited capacity for regeneration (Renault et al. 2002), the available evidence does not seem to support this hypothesis. A significant increase in quadriceps volume has been demonstrated in elderly subjects after 9 weeks RT, which is comparable to the response seen in young adults (Fiatarone et al. 1990; Tracy et al. 1999). This indicates that despite advancing age, the skeletal muscle retains its ability to undergo hypertrophy after training. Moreover, there is evidence for satellite cell activation and proliferation after RT in this population (Verdijk et al. 2009a), which leads to augmentation of the myonuclear pool and increased production of muscle proteins (Schulte and Yarasheski 2001; Tipton 2001).

Summary

Given the adverse impact of skeletal muscle dysfunction in COPD, measurement of peripheral muscle function (i.e. lower limbs) is becoming an important tool in the

clinical assessment of disability, and for monitoring the response to therapeutic interventions. A number of techniques are available for measuring various aspects of muscle function, although the choice of method will depend on the purpose for which the measurement is intended, in addition to the availability and cost of equipment. Lower limb exercise training is the key intervention that improves muscle function in COPD. While aerobic training improves muscle endurance, RT increases muscle mass and strength. Nutritional supplementation combined with RT can provide additional benefits, although the effect of a similar therapeutic combination has not been previously studied in COPD. Besides, the molecular adaptations occurring in response to acute and chronic RT have only begun to be elucidated in recent years. The hypothesis tested in this thesis is that lower limb RT combined with protein-carbohydrate supplementation will enhance anabolic and myogenic signalling, inhibit catabolic signalling, and result in improved muscle function in COPD.

CHAPTER 4

Materials and Methods

The main objective of this thesis was to explore the molecular, cellular, and functional responses to RT and nutritional supplementation in COPD patients and age-matched healthy controls. The thesis forms part of a Medical Research Council (MRC) funded randomised controlled trial (RCT) examining the effects of RT and supplementation on candidate genes and proteins associated with muscle mass regulation. This chapter describes the study design of the RCT, how subjects were recruited, and criteria used for inclusion and exclusion of participants. Several clinical and laboratory outcome assessments were performed in order to determine the impact of the RT programme, and these are also described. As part of this thesis, I developed an immunostaining technique to study the structure of skeletal muscles. A study exploring the inflammatory and satellite cell response in the quadriceps of COPD patients using this technique is reported in **chapter 7.** Portable ultrasound was used for the first time to assess the effect of RT on lower limb muscles in COPD, and the methodology is described in detail in **chapter 8**. The laboratory analysis for gene and protein expression was not performed by the author, but is described in **chapter 6**.

Subjects

Patients with COPD were recruited from outpatient clinics and from those referred for pulmonary rehabilitation at Glenfield Hospital in Leicester (UK). Stable outpatients who met clinical and spirometric criteria for moderate to severe COPD (FEV_1/FVC ratio <70%, FEV_1<50% predicted) with significant self-reported exercise limitation (MRC Grades 3, 4 or 5) were included in the study. Those on long term oxygen therapy and oral corticosteroids, or oral anticoagulants were excluded. Patients with co-morbid conditions, i.e. cardiovascular complications contributing to exercise limitation or preventing exercise training were also excluded. Healthy controls were recruited from the local population. Subjects were not taking part in any regular exercise training programs, and COPD patients who underwent pulmonary rehabilitation in the last 12 months were excluded. Approval was obtained from the Leicestershire and Rutland Research Ethics Committee (UK) and all participants provided informed written consent. The study was registered with the International Standard Randomised Controlled Trial Register (reference: ISRCTN22764439).

Study design

This was a double blind placebo-controlled trial of protein-carbohydrate supplementation given at the time of RT. All participants undertook fully supervised maximal intensity RT for 8 weeks as described below. Patients with COPD were randomly allocated in a double blind fashion to receive a dietary protein-carbohydrate supplement or a non-nutritive placebo at the time of training. Healthy control subjects all received placebo. Randomisation (random varying blocks of 2, 4 and 6 volunteers) was performed

by the Nottingham University Clinical Trials Unit using a web-based system. Randomisation was stratified for gender and muscle mass to ensure the treatment groups were matched at baseline. Outcome assessments were performed at baseline, 24 hrs after the first training session (muscle biopsy and plasma insulin only), 4 weeks, and at the end of the training intervention (8 weeks). The trial design and flow of patients is summarized in **Figure 4.1**.

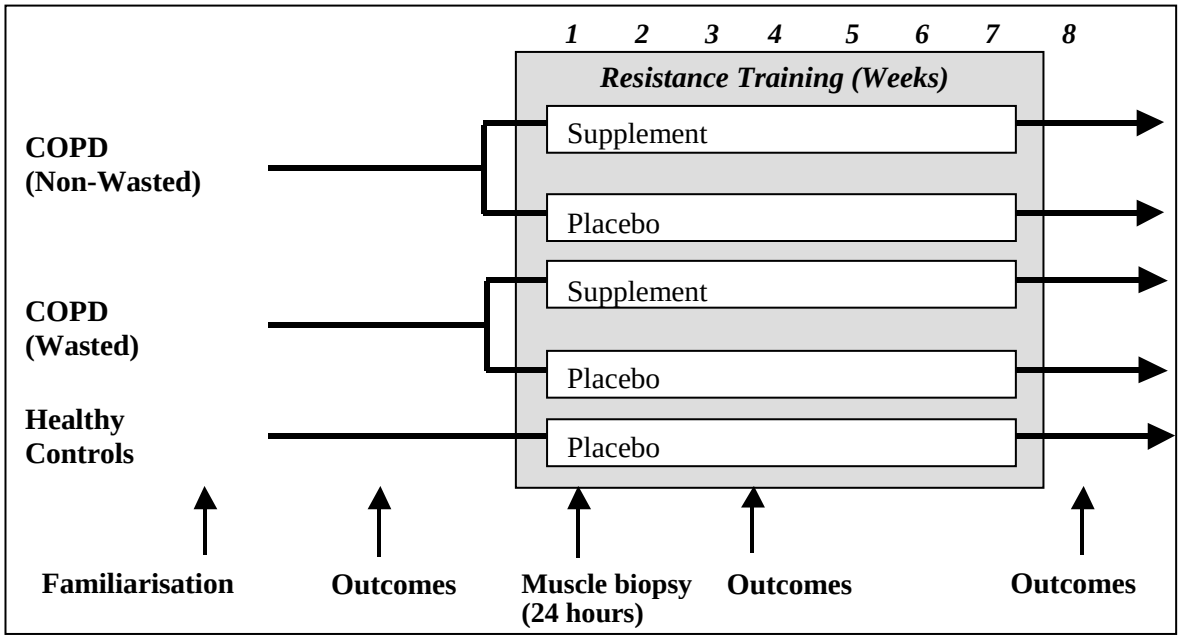


Figure 4.1 – Study Design

Interventions

Resistance training

All participants underwent 8 weeks of maximal intensity, bilateral, lower-limb RT on an isokinetic dynamometer (Cybex II Norm, Stoughton, MA, USA). Each exercise session was supervised by a trained physiotherapist, lasted around 30 minutes, and subjects attended thrice weekly for 8 weeks. Positioning and stabilisation was standardised according to the manufacturers' guidelines. Subjects were seated upright in a chair with lumbar support, and a seatbelt, thigh strap, and contra lateral limb stabiliser were used to ensure minimal movements of other body parts **(Figure 4.2)**. The padded lever arm of the dynamometer was attached with a strap to the shin of the leg to be trained, approximately 5cm above the lateral malleolus of the ankle. The chair monorail and back translation were positioned so that the axis of rotation of the knee joint is aligned with the axis of rotation of the dynamometer head. The maximal range of movement at the knee joint was set with safety stops placed at the extremes of extension and flexion. Subjects were instructed to push as hard as possible straightening the leg, from knee flexion to extension, and then to resist the lever arm bending the knee. 5 sets of 30 maximal knee extensions were performed at a pre-set angular velocity (PAV) of 180°/second. The contractions were isokinetic and concentric, with 1 minute rest between each set. Before and after each training session, subjects also performed a 1 minute continuous passive movement (flexion/extension) of the legs to act as a warm up/cool-down. Maximal knee extension effort was chosen in an attempt to ensure a high proportion of muscle fibre recruitment and volunteers were verbally encouraged at all times to elicit maximal effort.

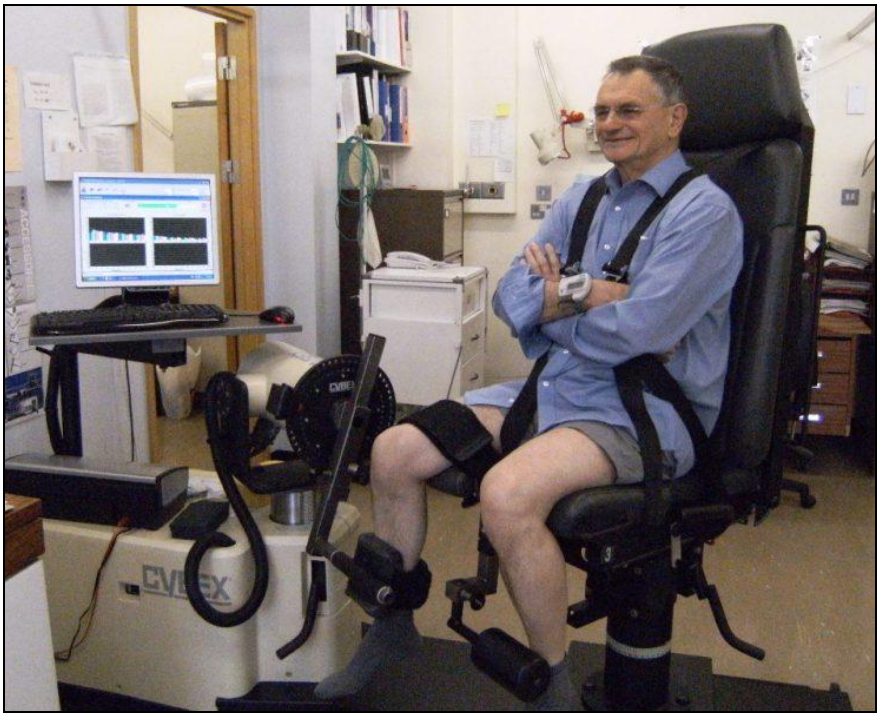


Figure 4.2 – Subject positioning on the Isokinetic dynamometer

The basic measurements recorded were the peak torque in Newton-metres (Nm) and total work done in Joules (J) for each of the five sets. The highest value for each leg was documented. During training, measures of oxygen saturation and heart rate were also recorded. After exercising one leg, the dynamometer chair was rotated around to train the contralateral leg using the same protocol. At the end of each exercise session, subjective measures of dyspnoea (Borg dyspnoea scale) and effort (Borg scale) were recorded. This training programme was chosen because it does not result in the rapid fatigue associated with slower velocities of contraction (thereby stimulating training adaptation), involves recruitment of fast and slow muscle fibres (Tesch et al. 1989), and has been demonstrated to completely restore lower limb muscle mass and increase isometric strength above

baseline following 2 weeks immobilisation-induced muscle wasting in young, healthy volunteers (Jones et al. 2004). This training protocol has also been shown to be tolerable in a pilot study involving frail COPD patients (William J.E.A. et al. 2007).

Nutritional Supplementation

COPD patients were randomly allocated to receive a protein-carbohydrate supplement or placebo throughout training. The rationale for supplementation was based on previous data in healthy individuals showing that the anabolic effect of RT could be further enhanced by providing dietary supplementation in the recovery phase after a bout of exercise (Esmarck et al. 2001; Rasmussen et al. 2000). In addition, a recent meta-analysis has concluded that protein supplementation during prolonged RT can significantly augment the gains in muscle mass and strength, when compared to RT alone in healthy younger and older subjects (Cermak et al. 2012). The supplement contained 19g protein (whey protein, milk protein isolate and glutamine peptide) and 49g glucose polymer carbohydrate (Vitargo® Gainers Gold, Swecarb, Sweden) made up to 500ml water **(Figure 4.3)**. This composition was chosen because data in healthy subjects suggested that 10 to 20g of protein would be sufficient to saturate post-exercise muscle protein synthesis (Bohe et al. 2003; Esmarck et al. 2001; Moore et al. 2009), while carbohydrate given at a dose of 1g/kg post exercise decreased myofibrillar protein breakdown, resulting in a net positive body protein balance (Roy et al. 1997). Besides the proportion of protein (25%) and carbohydrate (65%) in this dietary supplement is comparable to that given in a recent study in which significant improvements in functional outcomes were observed when supplementation was provided as part of a multimodal intervention (consisting of exercise, dietary supplements and oral testosterone) to malnourished patients with respiratory failure

predominantly due to COPD (Pison et al. 2011)Healthy participants were only given placebos. The impact of nutritional supplementation in healthy controls was not one of the research questions we were investigating since this question had been previously tested. At the time of designing the study, there was evidence for a beneficial effect of post-exercise dietary supplementation in healthy elderly subjects (Esmarck et al. 2001). Hence it was deemed not necessary to give the healthy controls any supplements during training. Besides the resources available from the MRC grant did not allow us to extend the study to include an additional group. The placebo was an identical volume non-nutritive non-caloric drink that contained flavourings in an attempt to match the taste of the supplement. The supplements and placebo looked identical both in powder form and after mixing with water. Participants were allocated a numbered treatment box at the start of the training programme which contained 24 sachets of either supplements or placebos. The numbers were generated after online randomisation and treatment boxes were labelled beforehand by an individual (Dr Louise Sewell) not related to the conduct of the study. Participants were responsible for identifying their treatment box and mixing the sachets in water, and the research team ensured that the supplements (or placebo) were ingested immediately after each exercise session. This is because greater increases in muscle mass have been reported in the elderly during training with immediate post-exercise amino acid intervention (Esmarck et al. 2001). Both participants and researchers were blinded to the nutritional intervention. **The full ingredients of both the supplement and placebo are given in appendix Ia and Ib.**

Figure 4.3 – Protein-Carbohydrate supplement

Outcome Assessments

Outcome assessments were performed at baseline, during and after the exercise training programme.

Lung Function

Spirometry was measured in the seated position to BTS/ARTP standards (Model R; Vitalograph, Buckingham, UK) (British Thoracic Society 1994). Values were expressed as a percentage of predicted values calculated from ERS regression equations (Quanjer et al. 1993). Subjects were asked to perform a forced expiratory manoeuvre, and the best of three attempts was recorded. Full lung function testing was performed in the respiratory physiology department at Glenfield Hospital by trained technicians using the helium dilution technique (Spiro Air, Medisoft, Belgium). Arterialised blood samples for PaO_2, $PaCO_2$, and pH were obtained at rest on room air, using the earlobe micro method (Bayer RapidLab 348, USA).

Anthropometry

Body weight was measured in light clothing using digital scales (SECA, Birmingham, UK) to the nearest 0.1kg. Height was measured to the nearest 0.1cm using a wall-mounted stadiometer (SECA). Body mass index (BMI) was calculated as weight/height^2.

Quadriceps Muscle biopsies

All participants underwent a quadriceps (vastus lateralis) muscle biopsy of the dominant leg at four time points – baseline, 24 hours after the first bout of exercise, at the mid-point of the training programme (4 weeks), and at the end of training (8 weeks). The biopsies were taken after a fast of at least 4 hrs and (apart from baseline samples) 24 hours after the previous training session.

Subjects were placed comfortably on an examination couch, either sitting up or in the supine position. By dorsiflexing the ankle, the rectus femoris muscle becomes more prominent, and the vastus lateralis is identified as lying along its lateral border. The depth of the muscle from the skin surface was measured by ultrasound, and the biopsy site was marked as the junction between the upper third and lower two-thirds of the thigh **(Figure 4.4)**. Muscle biopsies were obtained by a microbiopsy technique (Hayot et al. 2005) using the Magnum® core biopsy system (Magnum™ MG1522, Bard®). This consists of a spring-loaded reusable biopsy gun to which a 12-gauge disposable core biopsy needle (Magnum® Needle MN1210, Bard®) is attached. Using strict aseptic precautions, about 5 ml of 1% lidocaine is infiltrated, first into the skin and subcutaneous tissues, and then gradually deeper into the muscle fascia. A small 1cm longitudinal stab incision is then made and extended deeper down until the muscle fascia was pierced. The core biopsy

needle is attached to the biopsy gun and inserted through the incision. A muscle biopsy is obtained by pressing the trigger button on the biopsy gun, which unloads the spring and activates the needle to collect muscle tissue. The sample is immediately frozen in liquid nitrogen and stored at -80°C for further analysis. Using the same skin incision, 2 to 3 further biopsies were taken to ensure adequate quantities of muscle tissue were available for analysis. In a subset of participants, an additional biopsy was obtained for immunohistochemistry. This sample was transferred into ice cold acetone containing 2mM phenyl methyl sulphonyl fluoride and 20 mM iodoacetamide. At the end of the procedure, firm pressure is applied over the biopsy site for 5 minutes to prevent excessive bleeding. Steristrips are then placed over the incision site and covered by a water-proof dressing (Tegaderm). Finally a crepe bandage is firmly applied around the thigh in order minimise bleeding and bruising when the subject starts to mobilise. Written instructions were provided to remove the crepe bandage after 8 hours, and to keep the biopsy site dry for about 5 days.

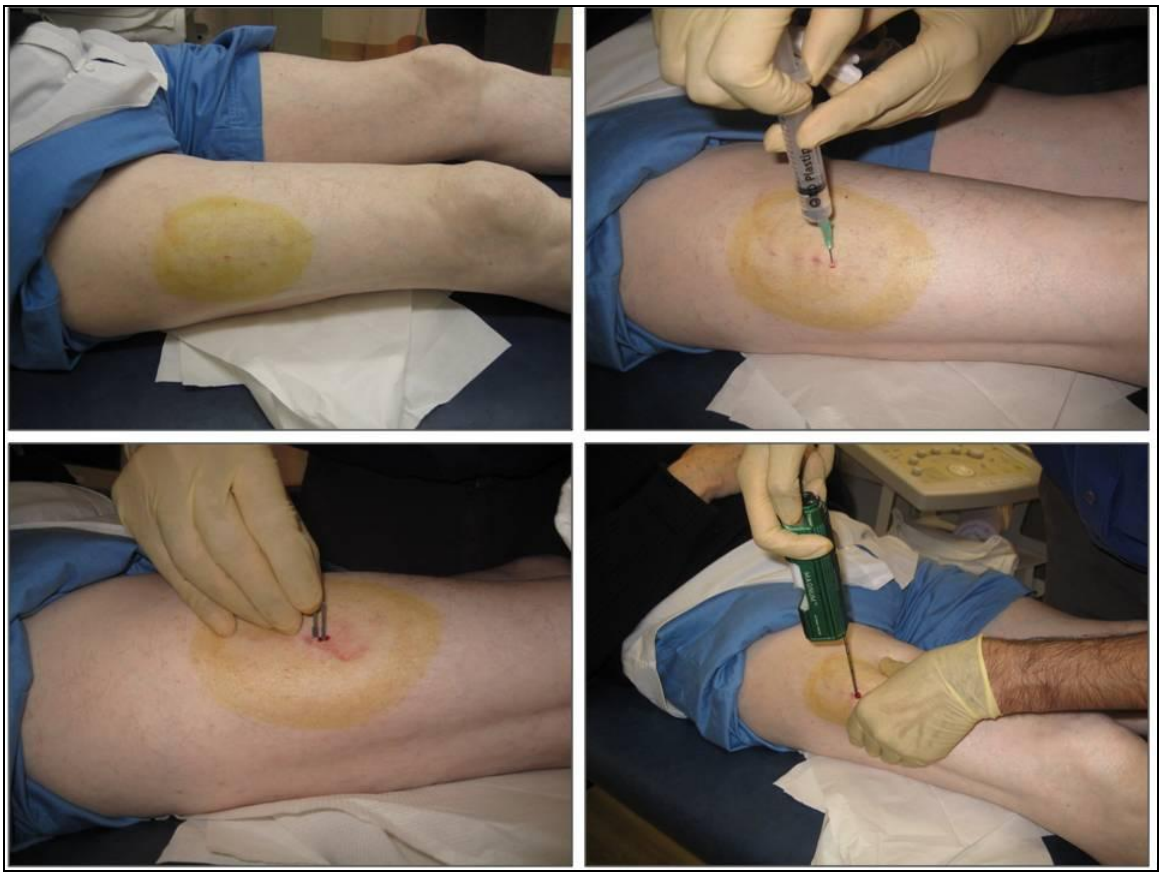


Figure 4.4 – Quadriceps muscle biopsy technique

Blood sampling

Venous blood was drawn in the fasted state at the same 4 time-points when muscle biopsy samples were obtained. Samples were immediately added to preservative, briefly left on ice and following centrifugation plasma samples were stored at -80°C until analysed for insulin concentration, using a Human Insulin specific RIA kit (Millipore, Billerica, MA, USA), according to the manufacturer' s protocol.

Muscle Strength Measurements

In this thesis, quadriceps muscle function was assessed using an isokinetic dynamometer (Cybex II Norm, CSMi, Stoughton, USA). Isokinetic testing is considered to be the gold standard method to determine muscle strength. However, the equipment is expensive, not widely available and measurements may not be related to functional ability of patients. This form of testing uses the force-velocity characteristics of muscle contraction and allows the measurement of maximal muscle strength over a wide range of joint positions and velocities. An isokinetic dynamometer contains a speed control mechanism that accelerates to a preset constant velocity with the application of force. Once a speed is attained, the device automatically adjusts to provide a force that opposes the force generated by the muscle through the range of motion. It can be used to measure isokinetic and isometric muscle strength of various muscle groups, including the quadriceps.

Isometric Quadriceps Strength

Before the first outcome assessments, subjects attended a familiarisation session for muscle strength determination. Positioning and stabilisation of the subject in the upright, seated position were standardised according to manufacturers' guidelines as previously described. Isometric strength of the quadriceps muscle group was determined during maximal voluntary contraction of the knee extensors, with the knee fixed at an angle of 70°. Subjects underwent three attempts at the manoeuvre, each separated by 30 seconds, on 2 consecutive occasions. The highest value obtained was recorded as the isometric strength.

Isokinetic Muscle Function

Isokinetic torque during knee extensor exercise was recorded at an angular velocity of 60°/s to maximise motor unit recruitment. Subjects performed 2 bouts of 5 repetitions of isokinetic knee extension, each bout separated by 1 minute rest. Isokinetic peak torque and work output were recorded during each contraction. The subjects' leg was held out so that the knee was straight (0 degrees) and range of movement was set between 10-80° flexion. The weight of the limb was measured to allow the computer system to correct for gravity in its calculations. The measurements recorded were peak torque (Newton-metres: Nm) and total isokinetic work (cumulative over a set) (Joules: J) for each of the sets.

Muscle Mass Measurements

Increasing muscle mass is an important therapeutic goal for rehabilitation and nutritional support programmes in COPD. No true gold standard method exists for the measurement of human muscle mass. Imaging techniques such as computed tomography (CT), magnetic resonance imaging (MRI) and dual energy x-ray absorptiometry (DEXA) can all be applied to measure lower limb muscle mass, although access to these machines may be an issue. Muscle mass can also be determined by indirect bedside methods such as skin-fold anthropometry, bioelectric impedance and ultrasonography. In this thesis both DEXA and portable ultrasound were used to measure lower limb muscle mass. A study comparing the responsiveness of DEXA and ultrasound to detect changes in lower limb muscle mass following RT is described in **chapter 8.**

Dual Energy X-ray Absorptiometry (DEXA)

Fat-free mass (FFM) – an index of muscle mass, was measured using DEXA **(also described in chapter 3)**. This technique measures the differential attenuation of two low-energy x-ray beams by body tissues, to provide a three-compartment model of body composition: fat mass, bone-free lean mass, and bone mineral mass. Bone mass is measured directly from its x-ray absorption, and soft tissue mass at each point, is derived by subtracting bone mass from total mass. Lean and fat mass are distinguished by water content, and their attenuation of x-ray beams is known from in-vitro measurements. The method assumes constant intracellular hydration of bone-free lean mass (Jebb 1997). DEXA is reproducible in COPD and has been validated against other methods in this population (Engelen et al. 1998; Steiner et al. 2002). The technique may also provide a better assessment of body composition in these patients (Miller et al. 2009).

Total body and thigh (hip to mid patella) FFM was measured using the Lunar Prodigy Advance (GE Healthcare, UK) bone densitometer. Subjects were scanned longitudinally in the supine position, with straps placed around the knees and ankles to hold them in position **(Figure 4.5)**. To determine FFM (lean mass) of the dominant thigh, a region of interest (ROI) was traced using custom analysis software. The upper limit of this ROI was the lowest point of the ischial tuberosity, while the lower limit was knee joint line. The pubic symphysis and the most lateral part of the thigh were used as the medial and lateral limits respectively (Visser et al. 1999) **(Figure 4.6)**. Total body FFM (g) was calculated as lean mass + bone mineral mass. The fat free mass index (FFMI) was calculated from height and total body FFM (FFMI=FFM/height^2). Subjects were deemed to have FFM depletion if their FFMI was below 16kg/m^2 for men and below 15kg/m^2 for

women. These criteria have been used previously to define muscle wasting in COPD and are predictive of poorer functional performance and quality of life (Baarends et al. 1997a; Mostert et al. 2000). All DEXA scans were performed at Glenfield hospital by the study investigators, who underwent prior training in the use of the equipment and interpretation of images.

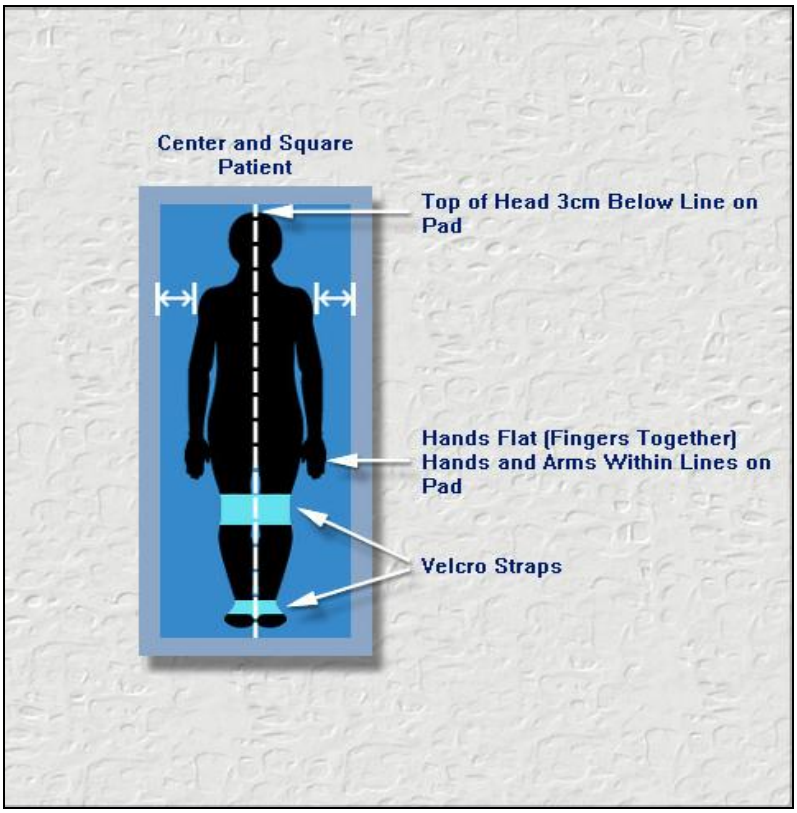


Figure 4.5 – DEXA subject positioning

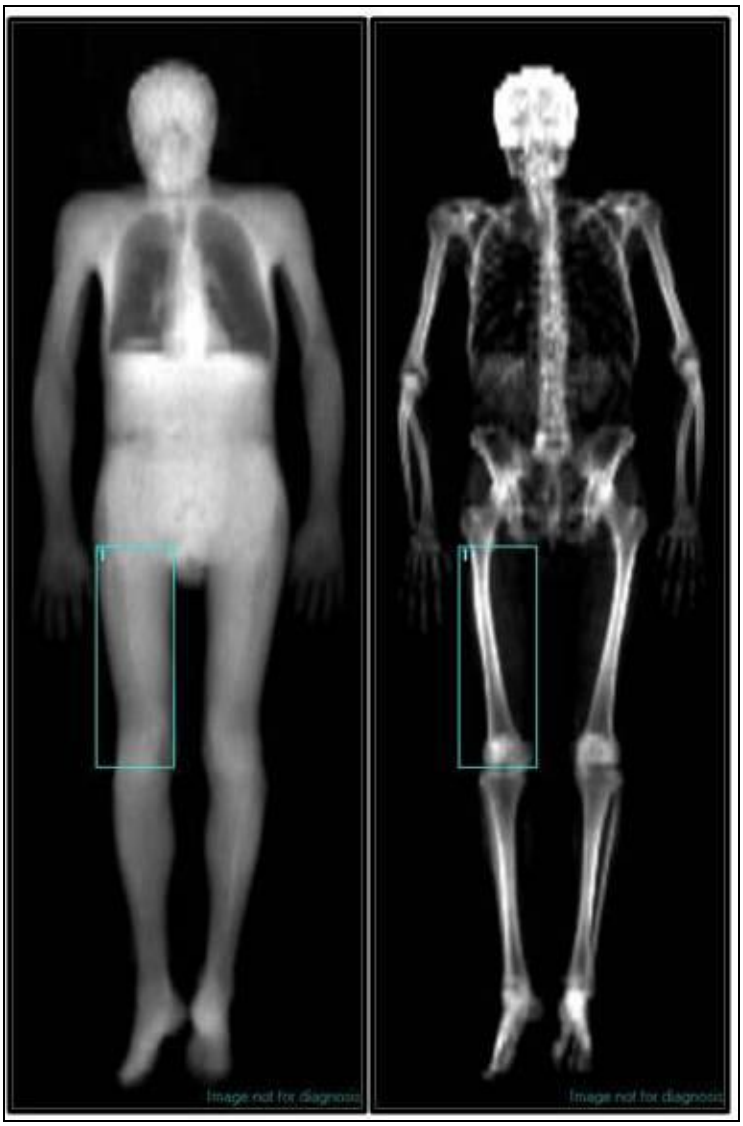


Figure 4.6 – Thigh FFM ROI

Cardiopulmonary Exercise Testing

After a familiarisation test at baseline, subjects performed a maximal (symptom-limited) incremental exercise test on an electrically braked cycle ergometer (Ergometric Er900; Ergoline GmbH, Bitz, Germany). After a 1 to 2 minute warm-up period, work rate was increased by either 10W (COPD) or 20W (healthy controls) every minute using a ramp protocol, to determine peak exercise capacity. Participants cycled at a cadence of 40-45 rpm and were encouraged to continue cycling at the required rate for as long as possible until symptom limitation. Peak work rate and breath-by-breath measurements of gas exchange and ventilation were recorded (Zan-600 ErgoTest, Meβgeräte GmbH, Oberthulba, Germany). Exercise testing was performed only at baseline and after completion of training.

An outline of the study visits is given in Table 4.1.

Visit	Consent	Practice exercise tests	Lung function tests	Muscle biopsy	Blood test	DEXA	Muscle strength	Cycle ergometry	Ultrasound	Resistance training and supplementation
1) Familiarisation	x	x	x						x	
2) Baseline						x	x	x	x	
3) Training session 1				x	x					x
4) 24 Hours				x	x					
Training week 1-4										x
5) Mid Point				x	x	x	x		x	x
Training week 5-8										x
6) Post study				x	x	x	x	x	x	

Table 4.1 – Study visit schedule

CHAPTER 5

Skeletal Muscle Functional Responses to Resistance Training and Protein-Carbohydrate Supplementation in COPD

In this chapter I will describe the effects of RT combined with protein-carbohydrate supplementation on important functional outcomes in COPD, including lower limb muscle mass, muscle strength and whole-body exercise performance. The impact of this training regimen on molecular signalling pathways is described in **chapter 6**.

Introduction

Patients with COPD present with impaired lower limb muscle mass and strength, which impacts adversely on exercise performance, quality of life and survival (Decramer et al. 1997; Gosselink et al. 1996; Swallow et al. 2007). Exercise training forms the cornerstone of pulmonary rehabilitation (PR), and is the key intervention that has been shown to improve peripheral muscle function in this population (Ries et al. 2007). While endurance training in the form of walking or cycling exercise is the most commonly applied training modality in PR, the addition of a Resistance Training (RT) component leads to significant gains in muscle mass and strength (Ries et al. 2007). The

beneficial effects of RT in COPD are observed either when it is performed in isolation (Casaburi et al. 2004; Kongsgaard et al. 2004; Lewis et al. 2007), or when combined with endurance training (Bernard et al. 1999; Mador et al. 2004; Ortega et al. 2002; Panton et al. 2004). However, not all patients can access or participate in exercise training (e.g. due to musculoskeletal impairments). Hence there is considerable interest in developing anabolic and nutritional therapies that can either augment the effects of RT, or provide functional benefits to patients who cannot perform exercise.

Resistance-type exercise is a powerful anabolic stimulus, wherein a single bout of RT not only accelerates muscle protein synthesis (MPS), but to a lesser extent also stimulates muscle protein breakdown (MPB) (Phillips et al. 1997). Studies in healthy adults suggest that administration of protein and carbohydrate during recovery from a bout of acute RT, enhances post exercise MPS (Rasmussen et al. 2000), and inhibits post exercise MPB (Roy et al. 1997), hence favouring net muscle protein accretion. Therefore dietary supplementation combined with RT may be an effective strategy to enhance muscle function. Indeed the regular ingestion of protein supplements with prolonged RT, has been shown to significantly augment the gains in muscle mass and strength in both young and older subjects (Cermak et al. 2012). The effect of this therapeutic combination of RT and dietary supplementation has not been previously investigated in COPD. The primary objective of exercise training is to improve functional performance. RT in COPD can also result in significant improvements in whole-body peak exercise performance (Spruit et al. 2002; Vonbank et al. 2012). Whether additional gains can be obtained by dietary supplementation is currently unknown.

The aim of this mechanistic study was to explore the effects of a controlled programme of high-intensity lower limb RT combined with protein-carbohydrate

supplementation on muscle mass, muscle strength and whole-body peak exercise performance in COPD patients. The study hypothesis was that protein-carbohydrate supplementation combined with RT would have greater effects on functional outcomes when compared to RT alone.

Materials and Methods

A detailed description of the methodology including study design and recruitment of participants is described in **chapter 4**. A brief summary of the methods relevant to this chapter are described below.

Interventions

Resistance training (RT)

All participants performed fully supervised, maximal voluntary lower limb RT on an isokinetic dynamometer (Cybex II Norm, Stoughton, MA, USA), thrice weekly for 8 weeks. Each session comprised 5 sets of 30 maximal knee extensions at an angular velocity of 180°/sec. The highest recorded peak torque (Nm) and total work done (J) from the 5 sets was recorded for each leg.

Nutritional supplementation

COPD patients were randomly assigned to receive either a dietary protein-carbohydrate supplement [COPD(S)] or an identical volume non-nutritive, non-caloric drink [COPD(P)] during RT. The supplement contained 19 g protein and 49 g glucose polymer carbohydrate (Vitargo Gainers Gold, Swecarb, Sweden). Healthy controls (HCs) received only the placebo intervention. Participants were instructed to ingest the supplement (or placebo) immediately after each training session.

Outcome Measurements

Outcome assessments were performed at baseline (after familiarisation), at mid-point (week 4), and at the end of training (week 8). Measures of whole-body exercise performance were determined only at baseline and week 8.

Quadriceps Muscle Function

Isometric quadriceps strength was determined during maximal voluntary knee extensor contraction (Cybex II Norm, Stoughton, USA). Isokinetic peak torque and isokinetic total work was recorded during 2 bouts of 5 repetitions (performed at an angular velocity of 60°/s).

Total Body and Thigh Lean Mass

Whole body and thigh lean mass (T_{dexa}) was measured using Dual Energy X-ray Absorptiometry (DEXA Lunar Prodigy Advance, GE Healthcare, UK). Fat free mass index (FFMI) was calculated from height and total body fat free mass. Patients were deemed to have muscle wasting if FFMI < 16kg/m^2 in men or < 15kg/m^2 in women (Schols et al. 2005).

Whole Body Exercise Performance

Subjects performed a symptom limited, exhaustive, incremental cycle ergometer test (at baseline and after completion of training only) (Zan-600 ErgoTest, Meβgeräte GmbH, Oberthulba, Germany).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 5.01 for Windows (GraphPad Software Inc, California, USA) and SPSS 18.0 for Windows (SPSS Inc, Chicago, USA). Parametric data were expressed as means (±SD) and non-parametric data were described as medians (interquartile range, ±IQR). One-way

ANOVA with Tukey's post-hoc test (or Kruskal-Wallis test for nonparametric data) was used to compare subject characteristics and outcome measures at baseline between the 3 groups [COPD(S), COPD(P), and HC]. The relationships between the various baseline outcome measures are described using Pearson's correlation coefficient. Training induced changes in functional outcomes were compared within-groups using paired t-tests, while between-group differences were analysed using one-way ANOVA. Data were analysed on an intention to treat basis. Statistical advice was taken from the Trent Institute for Healthcare Services Research at the time when the study protocol was written. The main functional outcome measure chosen was lower limb (quadriceps) muscle strength. This is an important functional outcome for patients undergoing RT, and for which there exists previous data. We estimated the sample size required to meet the study objectives using expected improvements in quadriceps isometric muscle strength following RT. Previous studies of RT in COPD patients have shown that a 20% difference between groups or a 20% improvement in quadriceps isometric strength after training would be significant (Bernard et al. 1999; Spruit et al. 2002). Using quadriceps strength data obtained at our institution, we estimated that 24 subjects would be required in each group to complete the study (90% power, significance level 0.05) to show a 10.0 Nm (SD 14.8 Nm) within-group improvement in isometric strength (Deacon et al. 2008). Allowing for a 25% dropout rate in the COPD group (the average for the outpatient rehabilitation programme at Glenfield Hospital), it was deemed that 30 patients with COPD (per group) and 24 healthy controls would need to be recruited. It was hypothesised that wasted patients might respond differently to the intervention, hence patients were stratified by FFMI during randomisation. A post-hoc subgroup analysis was performed comparing the functional outcomes in wasted and nonwasted

patients. All statistical tests were two-tailed and the threshold of statistical significance was a p value < 0.05.

Results

A total of 510 subjects were screened, of which 107 (81 COPD, 26 healthy controls) were recruited into the study. Common reasons for excluding subjects were – not meeting the inclusion criteria, unable to manage the frequency and intensity of training, or inability to have muscle biopsies (e.g. taking warfarin). Before randomisation and start of the first training session, 14 participants (10 COPD, 4 controls) either dropped out, or were withdrawn as exclusion criteria were met. Hence a total of 93 subjects (71 COPD, 22 controls) were randomised and started the RT programme. 38 COPD patients were randomised to receive the dietary supplements [COPD(S)], and 33 patients received placebos [COPD(P)]. There were 8 drop-outs in the COPD(S) group and 4 in the COPD(P) group. Among the healthy controls (HC), 1 participant dropped out (this was the spouse of a COPD patient who wanted to discontinue training as her partner had withdrawn from the study). Therefore at the end of 8 weeks, data was available for 30 and 29 patients in the COPD(S) and COPD(P) groups respectively, and for 21 HC. The consort flow diagram of study participants and the reasons for exclusion and drop-outs are given in **Figure 5.1**.

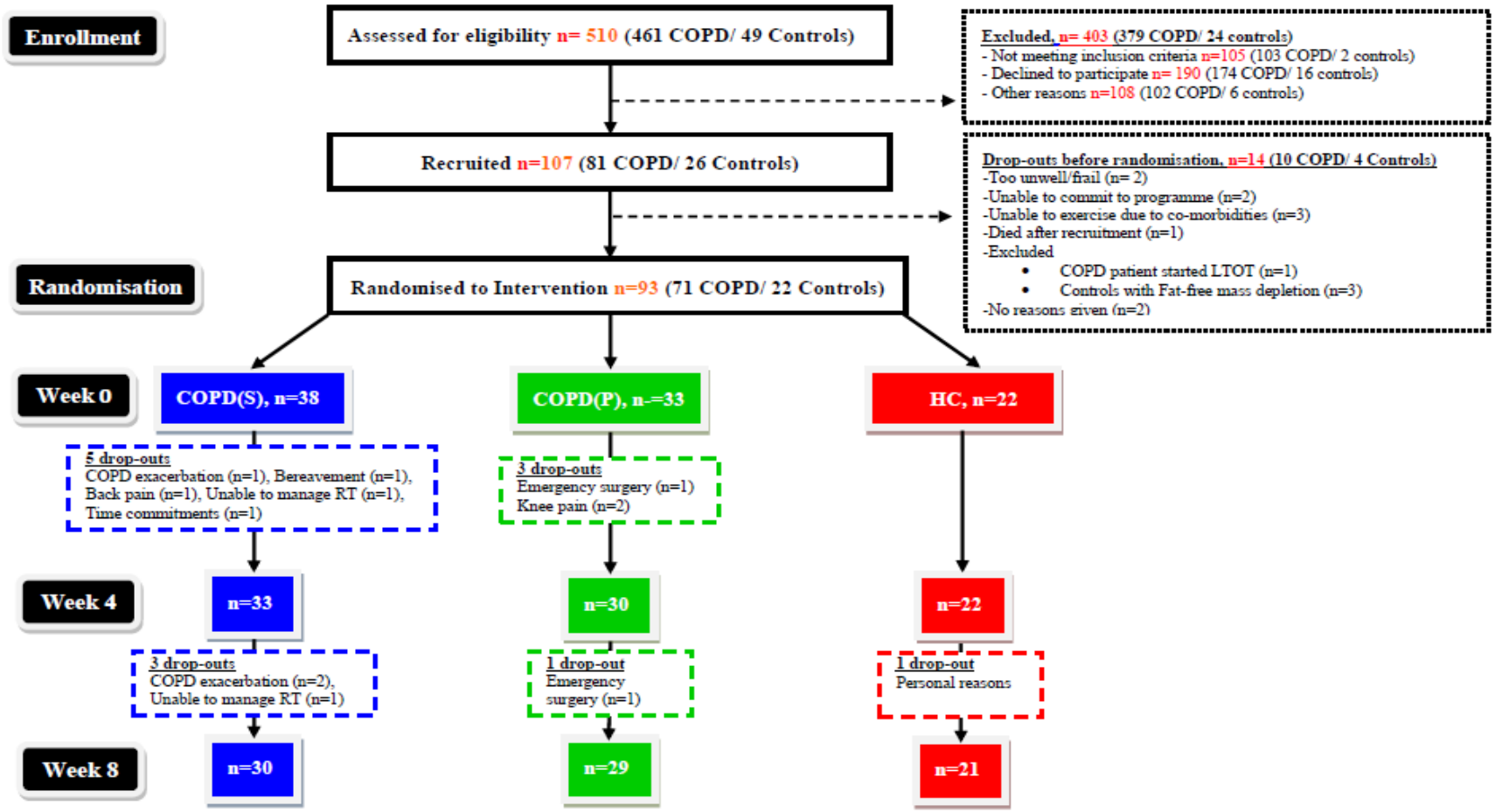


Figure 5.1 – Consort flow diagram of study participants

Baseline Characteristics

Table 5.1 shows the baseline characteristics of the three groups. Subjects were well matched for age. COPD patients had a significantly longer smoking history, worse lung function, and were subjectively more disabled when compared to HC, but there were no differences between the two COPD subgroups. Body weight, total body lean mass and T_{dexa} were comparable between the three groups. Quadriceps isometric strength, isokinetic torque and isokinetic total work was significantly lower in COPD(P) compared to HC, but not different from the COPD(S) group. Measures of cycle ergometry were significantly greater in HC when compared to COPD patients.

	COPD(S) (n=38)	COPD(P) (n=33)	HC (n=22)
Age (years)	68.6 (9.7)	67.6 (8.7)	66.5 (5.1)
Males (%)	24 (63.2)	18 (54.5)	11 (50.0)
Body weight (kg)	72.4 (15.7)	71.2 (17.0)	73.1 (10.2)
BMI (kg/m^2)	26.6 (4.8)	26.3 (5.6)	26.8 (2.7)
MRC Dyspnoea Scale (IQR)	3 (1)**	4 (1)**	1 (0)
Smoking pack years	48.4 (42.2)**	47.7 (30.9)*	14.9 (23.3)
FEV_1 (Litres)	1.1 (0.4)**	1.1 (0.4)**	2.6 (0.6)
FEV_1 % pred	46.9 (18.4)**	47.5 (16.8)**	105.0 (21.2)
FEV_1/FVC	39.8 (13.9)**	42.5 (11.7)**	72.1 (6.5)
FFMI (kg/m^2)	17.6 (2.6)	17.5 (2.7)	17.5 (1.7)
Muscle wasted subjects (%)	7 (18)	7 (21)	0 (0)
Total body lean mass (g)	45381.2 (9079.2)	45037.7 (9946.2)	45337.4 (8773.8)
T_{dexa} (g)	3950.7 (931.3)	3884.1 (1097.6)	4224.6 (862.2)
Quadriceps Isometric Strength (Nm)	111.0 (44.8)	109.6 (47.6)	137.7 (43.7)
Quadriceps Isokinetic Peak Torque (Nm)	81.5 (35.4)	76.3 (34.2)*	99.7 (36.3)
Quadriceps Isokinetic Total Work (J)	310.4 (145.5)	283.7 (141.9)*	378.5 (149.2)
Peak VO_2 (ml/kg/min)	14.9 (6.1)**	15.0 (5.0)**	22.7 (6.2)
Peak Work Rate (W)	48.3 (21.0)**	52.3 (24.1)**	119.6 (38.3)
Peak VE (l/min)	32.1 (10.7)**	32.2 (12.5)**	46.0 (17.1)

Table 5.1 – Baseline characteristics of study participants
All data presented as means (± SD). * p≤ 0.05; ** p≤ 0.01, significantly different compared to healthy controls.

In all three groups, a significant relationship was observed at baseline between thigh lean mass (T_{dexa}) and quadriceps isometric strength [COPD(S): r=0.67, p<0.0001; COPD(P): r=0.63, p<0.0001; HC: r=0.84, p<0.0001], isokinetic torque [COPD(S): r=0.66, p<0.0001; COPD(P): r=0.64, p<0.0001, HC: r=0.66, p<0.0001] and isokinetic total work [COPD(S): r=0.61, p<0.0001; COPD(P): r=0.55, p=0.0007; HC: r=0.80, p<0.0001] **(Figure 5.2)**. T_{dexa} was also related to cycle ergometry peak work in all participants

[COPD(S): r=0.43, p=0.02; COPD(P): r=0.73, p<0.0001; HC: r=0.73, p<0.0001] **(Figure 5.3)**. In addition, significant correlations were observed between cycle ergometry peak work and various measures of quadriceps function in COPD and HC **(Figure 5.4)**.

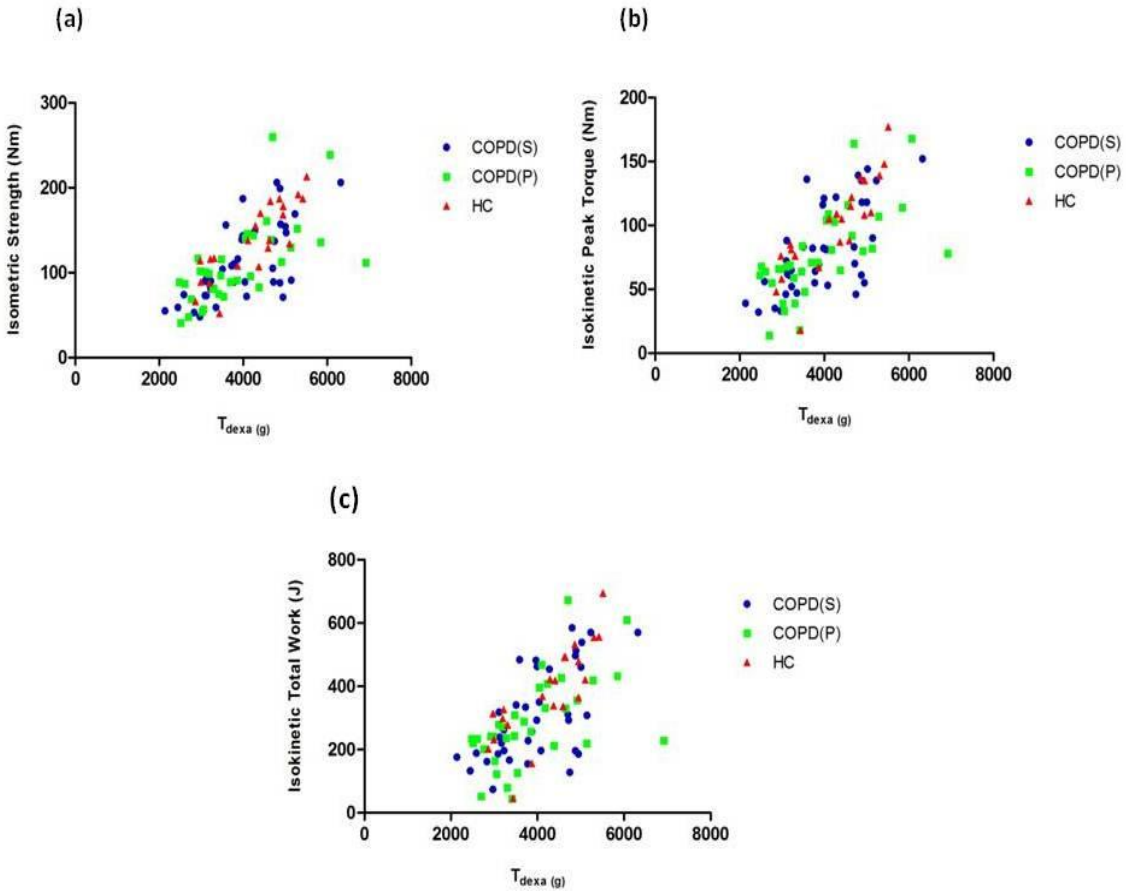


Figure 5.2 – Baseline relationships between thigh lean mass (T_{dexa}) and quadriceps function

(a) T_{dexa} vs. Quadriceps isometric strength
COPD(S): r=0.67, p<0.0001; COPD (P): r=0.63, p<0.0001; HC: r=0.84, p<0.0001.

(b) T_{dexa} vs. Quadriceps isokinetic peak torque
COPD(S): r=0.66, p<0.0001; COPD (P): r=0.64, p<0.0001; HC: r=0.66, p<0.0001.

(c) T_{dexa} vs. Quadriceps isokinetic total work
COPD(S): r=0.61, p<0.0001; COPD (P): r=0.55, p=0.0007; HC: r=0.80, p<0.0001.

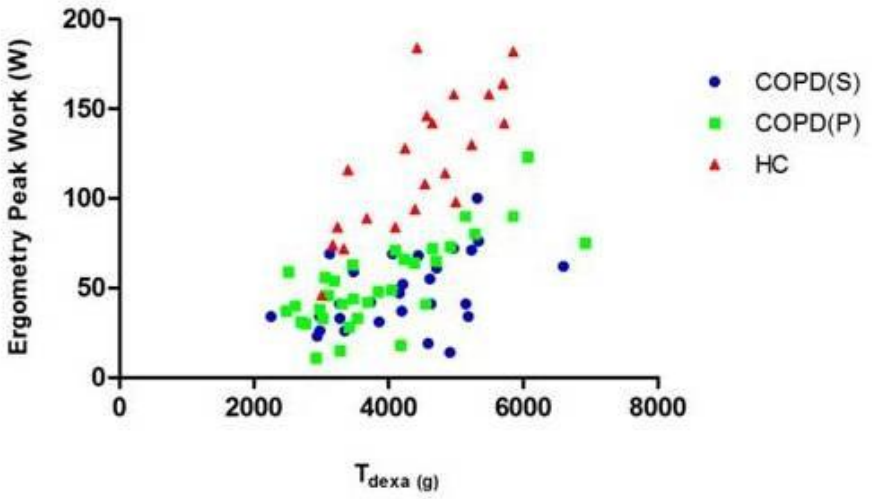


Figure 5.3 – Baseline relationship between T_{dexa} and cycle ergometry peak work

COPD(S): r=0.43, p=0.02; COPD (P): r=0.73, p<0.0001; HC: r=0.77, p<0.0001.

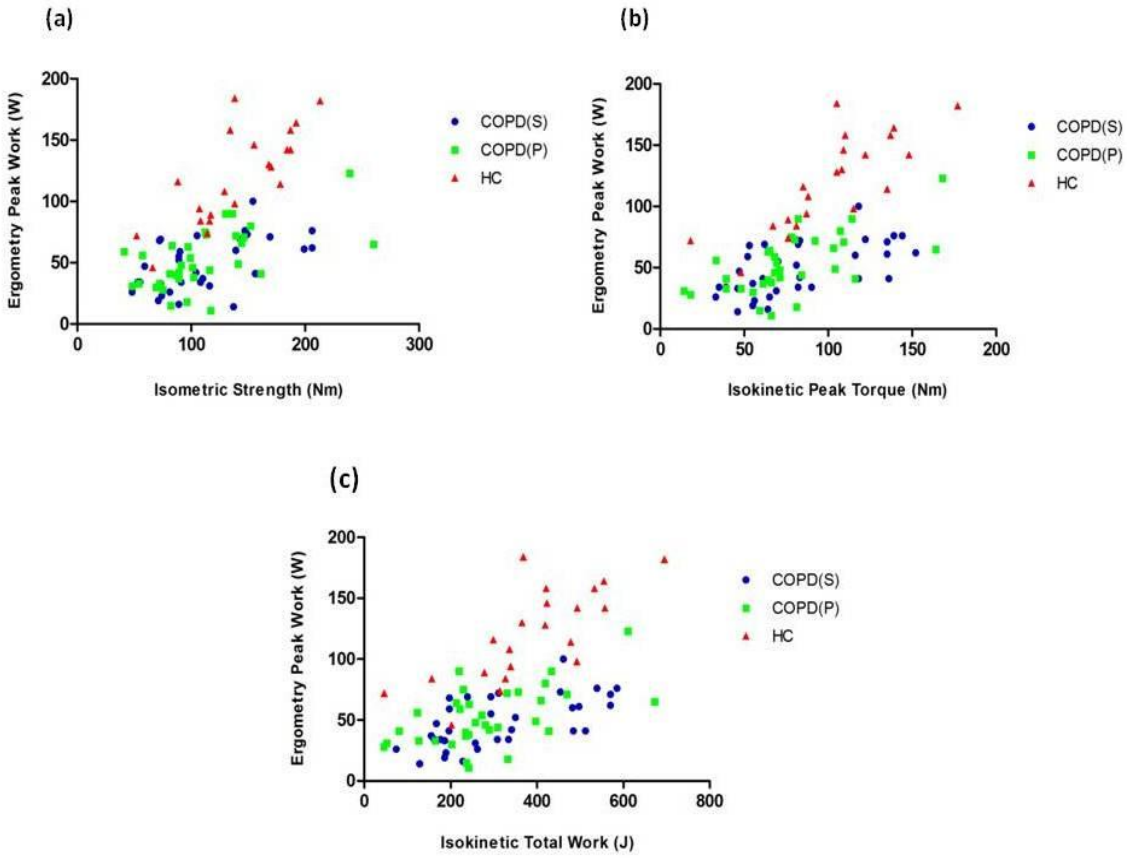


Figure 5.4 – Baseline relationships between cycle ergometry peak work and quadriceps function

(a) Cycle ergometry peak work vs. Quadriceps isometric strength
COPD(S): r=0.50, p=0.0025; COPD (P): r=0.58, p=0.0004; HC: r=0.77, p<0.0001.
(b) Cycle ergometry peak work vs. Quadriceps isokinetic peak torque
COPD(S): r=0.59, p=0.0002; COPD(S): r=0.63, p<0.0001; HC: r=0.79, p<0.0001.
(c) Cycle ergometry peak work vs. Quadriceps isokinetic total work
COPD(S): r=0.61, p=0.0001; COPD (P): r=0.55, p=0.0008; HC: r=0.74, p<0.0001.

Post Training

Table 5.2 shows the mean absolute change (SD) for the various outcome measures at the end of the RT programme **(relative changes illustrated in Figures 5.5, 5.6 and 5.7)**. All participants lost weight after training, but this was only significant in the COPD(S) [$p = 0.01$] and HC [$p \leq 0.001$] groups.

	Mean absolute change (SD)	95% CI	p value
Body weight (kg)			
COPD(S)	-0.8 (1.7)	-1.4, -0.1	0.01
COPD(P)	-0.3 (1.4) †	-0.9, 0.1	0.18
HC	-1.6 (1.7)	-2.4, -0.8	0.00
Total body lean mass (g)			
COPD(S)	76.1 (1031.0)	-308.9, 461.1	0.68
COPD(P)	205.2 (902.4)	-138.0, 548.5	0.18
HC	33.4 (937.5)	-393.2, 460.2	0.87
T_{dexa} (g)			
COPD(S)	180.1 (208.6)	102.1, 258.0	0.00
COPD(P)	230.3 (237.5)	140.0, 320.7	0.00
HC	232.6 (172.5)	154.0, 311.1	0.00
Quadriceps Isometric Strength (Nm)			
COPD(S)	19.6 (19.9)	12.1, 27.0	0.00
COPD(P)	16.5 (18.5)	9.5, 23.5	0.00
HC	16.8 (26.3)	4.8, 28.8	0.00
Quadriceps Isokinetic peak Torque (Nm)			
COPD(S)	17.7 (20.0)	10.2, 25.2	0.00
COPD(P)	19.7 (17.8)	12.9, 26.6	0.00
HC	12.7 (19.1)	4.0, 21.4	0.00
Quadriceps Isokinetic Total Work (J)			
COPD(S)	72.7 (98.7)	35.8, 109.5	0.00
COPD(P)	79.4 (71.5)	52.1, 106.6	0.00
HC	53.4 (88.8)	13.0, 93.9	0.01
Peak VO_2(ml/kg/min)			
COPD(S)	-0.4 (7.3) †	-3.4, 2.4	0.75
COPD(P)	2.3 (5.7)	0.1, 4.5	0.03
HC	4.8 (7.9)	1.1, 8.5	0.01
Peak Work Rate (W)			
COPD(S)	9.9 (14.1)	4.1, 15.6	0.00
COPD(P)	8.1 (12.2)	3.5, 12.8	0.00
HC	13.9 (14.6)	7.0, 20.7	0.00
Peak VE (l/min)			
COPD(S)	1.1 (11.7)	-3.5, 5.9	0.60
COPD(P)	5.5 (14.8)	-0.1, 11.2	0.05
HC	11.6 (15.5)	4.3, 18.9	0.00

Table 5.2 – Mean change (95% CI) and p values for within-group differences in functional outcomes at the end of 8 weeks

† Significantly different to control subjects (p≤0.05)

Significant within-group change in T_{dexa} was observed in all COPD patients [COPD(S): 4.6%; COPD(P): 6.2%] and HC (5.4%) **(Figure 5.5)**, although the differences between the groups were not significant. The increase in T_{dexa} was detectable at the halfway stage (week 4) of the RT programme.

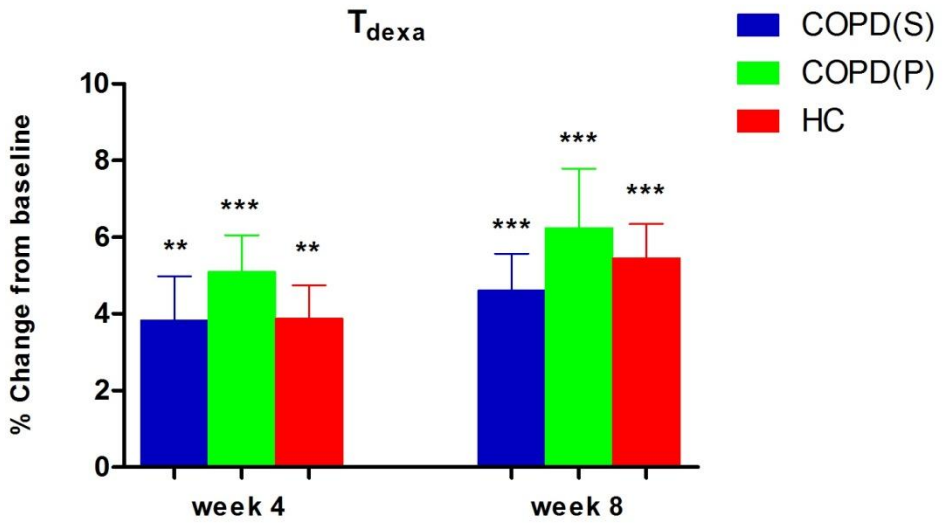


Figure 5.5 – Percentage changes from baseline in T_{dexa} at weeks 4 and 8 of training
Data presented as Means (SEM)
$^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$. Wilcoxon signed rank test.

Significant increases in quadriceps isometric strength [COPD(S): 20.4%; COPD(P): 16.9%; HC: 11.9%], isokinetic peak torque [COPD(S): 27.6%; COPD(P): 33.7%; HC: 13.5%] and isokinetic total work [COPD(S): 31.6%; COPD(P): 42.6%; HC: 17%] were observed in all groups after 8 weeks **(Figure 5.6)**. This was detected at the mid-point of the training programme (week 4), and measures of dynamic (isokinetic) quadriceps function showed larger increases than static (isometric) measures. However there were no statistically significant differences between the groups.

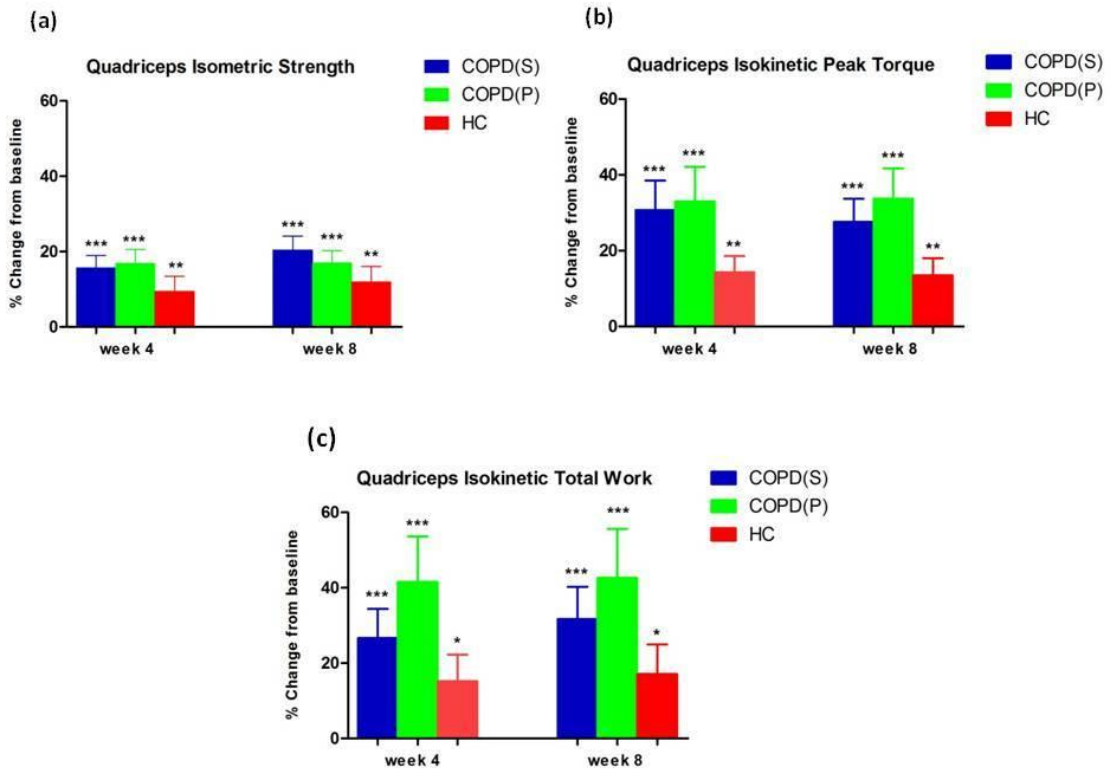


Figure 5.6 – Percentage change from baseline in measures of quadriceps function
(a) Quadriceps isometric strength, (b) Quadriceps isokinetic torque, and (c) Quadriceps isokinetic total work
Data presented as Means (SEM)
***p<0.001, **p<0.01, *p<0.05. Wilcoxon signed rank test.

Peak VO_2 increased after training in COPD(S) [12.7%], COPD(P) [20.1%] and HC [22.4%], but this was only significant in the latter two groups **(Figure 5.7a)**. When compared to HC, the change in peak VO_2 was significantly lower in the COPD(P) group ($p \leq 0.05$). Peak work rate also improved in all three groups after the intervention [COPD(S): 29.5%; COPD(P): 21.7%; HC: 12.3%] **(Figure 5.7b)**. There were no differences between the groups for any of the cycle ergometry outcomes.

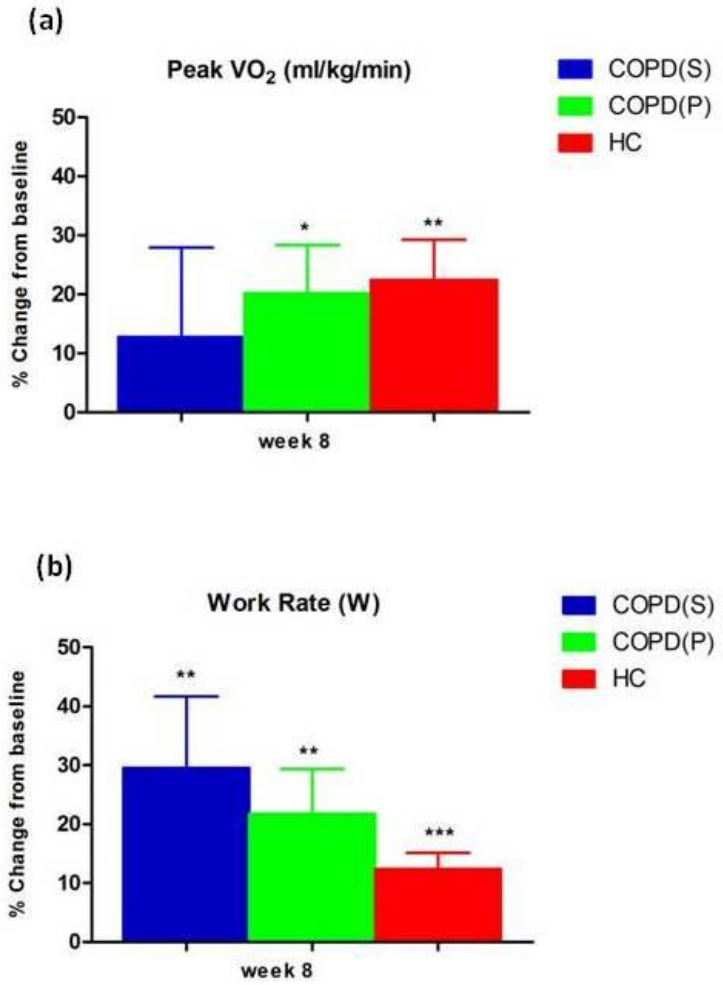


Figure 5.7 – Percentage change from baseline in measures of cycle ergometry performance

(a) Peak VO_2, and (b) Peak work rate.

Data presented as Means (SEM)

***p<0.001, **p<0.01, *p<0.05. Wilcoxon signed rank test.

Post-hoc sub-group analysis of wasted and non-wasted COPD patients

A post-hoc sub-group analysis was performed to compare the response to training and supplementation between wasted and non-wasted patients. 14 subjects had evidence of muscle wasting using FFMI criteria (Schols et al. 2005). Body weight, FEV_1, BMI, FFMI, total body lean mass, T_{dexa}, quadriceps muscle function and cycle ergometer peak workload were all significantly lower in the wasted COPD subjects at baseline **(Table 5.3)**.

	Nonwasted COPD (n=57)	**Wasted COPD (n=14)**	**p value**
Age (years)	68.5 (9.3)	66.8 (8.6)	0.54
Body weight (kg)	76.0 (15.0)	54.6 (6.9)	<0.0001
BMI (kg/m^2)	27.8 (4.7)	20.9 (2.7)	<0.0001
MRC Dyspnoea Scale (IQR)	3.0 (1)	4.0 (1)	0.28
Smoking pack years	49.2 (40.4)	43.8 (19.2)	0.63
FEV_1 (Litres)	1.2 (0.3)	0.9 (0.4)	0.02
FEV_1 % pred	48.8 (16.6)	40.5 (20.3)	0.11
FEV_1/FVC	42.6 (12.7)	34.7 (12.0)	0.04
FFMI (kg/m^2)	18.4 (2.3)	14.3 (1.1)	<0.0001
Total body lean mass (g)	47599.0 (8698.0)	35542.2 (5167.9)	<0.0001
T_{dexa} (g)	4164.2 (944.8)	2924.2 (515.7)	<0.0001
Quadriceps Isometric Strength (Nm)	117.6 (46.1)	80.8 (31.1)	0.006
Quadriceps Isokinetic Peak Torque (Nm)	83.7 (34.7)	60.1 (28.6)	0.02
Quadriceps Isokinetic Total Work (J)	316.8 (145.7)	221.3 (106.9)	0.02
Peak VO_2 (ml/kg/min)	14.9 (5.7)	15.2 (5.0)	0.88
Peak Work Rate (W)	53.2 (23.5)	37.2 (10.1)	0.02
Peak VE (l/min)	32.9 (11.9)	28.5 (9.7)	0.23

Table 5.3 – Baseline characteristics of wasted and nonwasted COPD patients

Training-induced improvements in quadriceps isometric strength tended to be lower in wasted COPD patients, but other functional measures were comparable between

wasted and nonwasted patients (**Figures 5.8 and 5.9**). The pattern of the training response was similar when protein-carbohydrate supplementation was combined with RT (**Figures 5.10 and 5.11**). The post-training changes in cycle ergometry performance tended to be lower in wasted subjects, although it was only significant for peak work rate ($p=0.04$), with no additional benefits seen with dietary supplementation **(Figure 5.12)**.

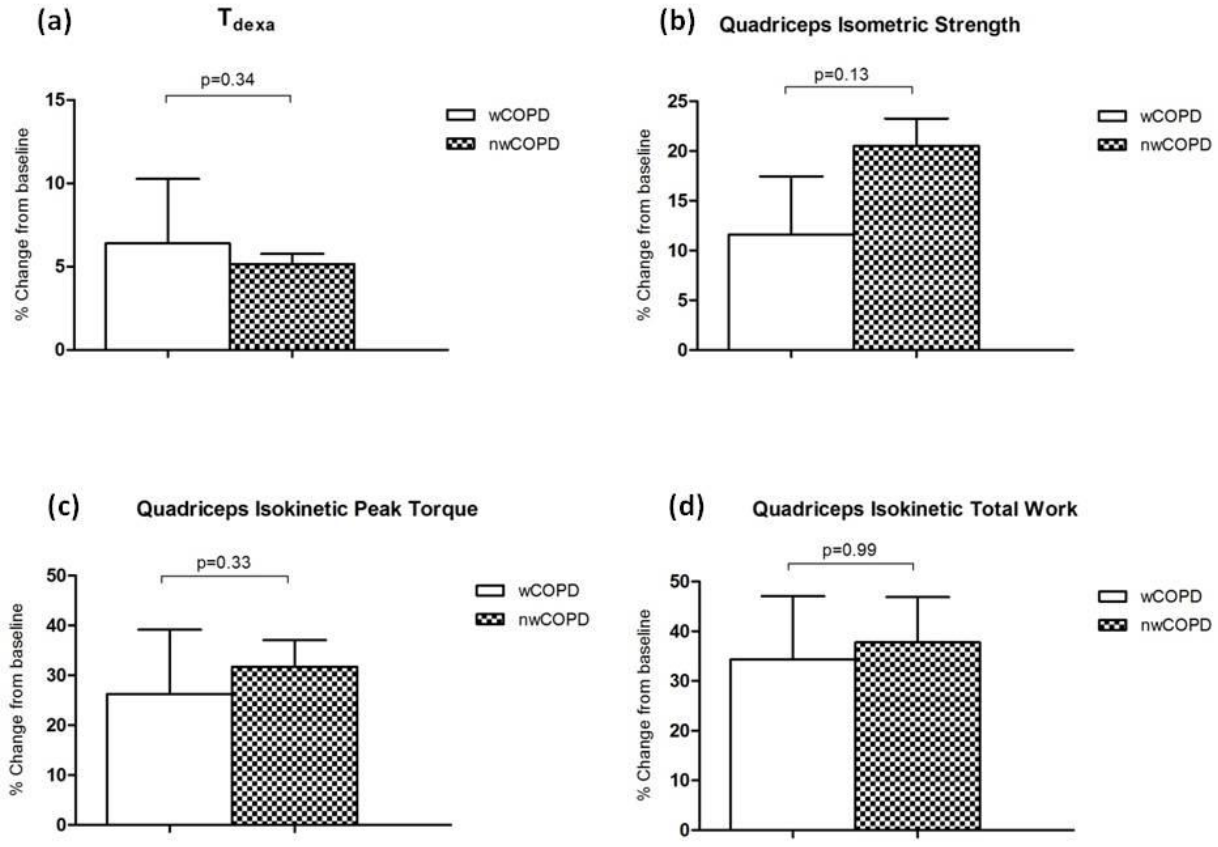


Figure 5.8 – Percentage changes from baseline in measures of quadriceps function in wasted (wCOPD) and nonwasted COPD (nwCOPD) subjects following RT

(a)T_{dexa}, (b) Quadriceps isometric strength, (c) Quadriceps isokinetic peak torque, and (d) Quadriceps isokinetic total work
Data presented as Means (SEM), p values calculated using Mann Whitney test

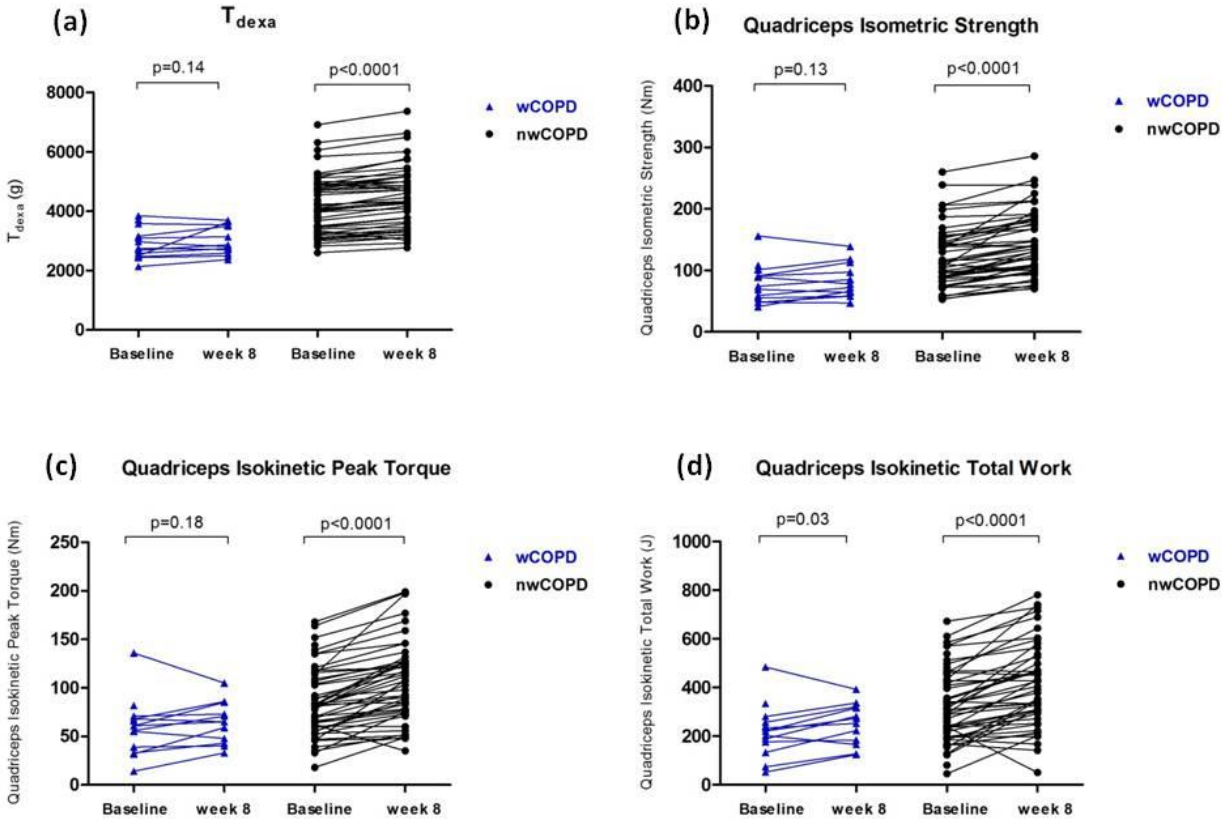


Figure 5.9 – Dot-plots depicting individual changes in measures of quadriceps function in wasted (wCOPD) and nonwasted (nwCOPD) subjects following RT

(a) T_{dexa}, (b) Quadriceps isometric strength, (c) Quadriceps isokinetic peak torque, and (d) Quadriceps isokinetic total work
p values calculated using paired t tests

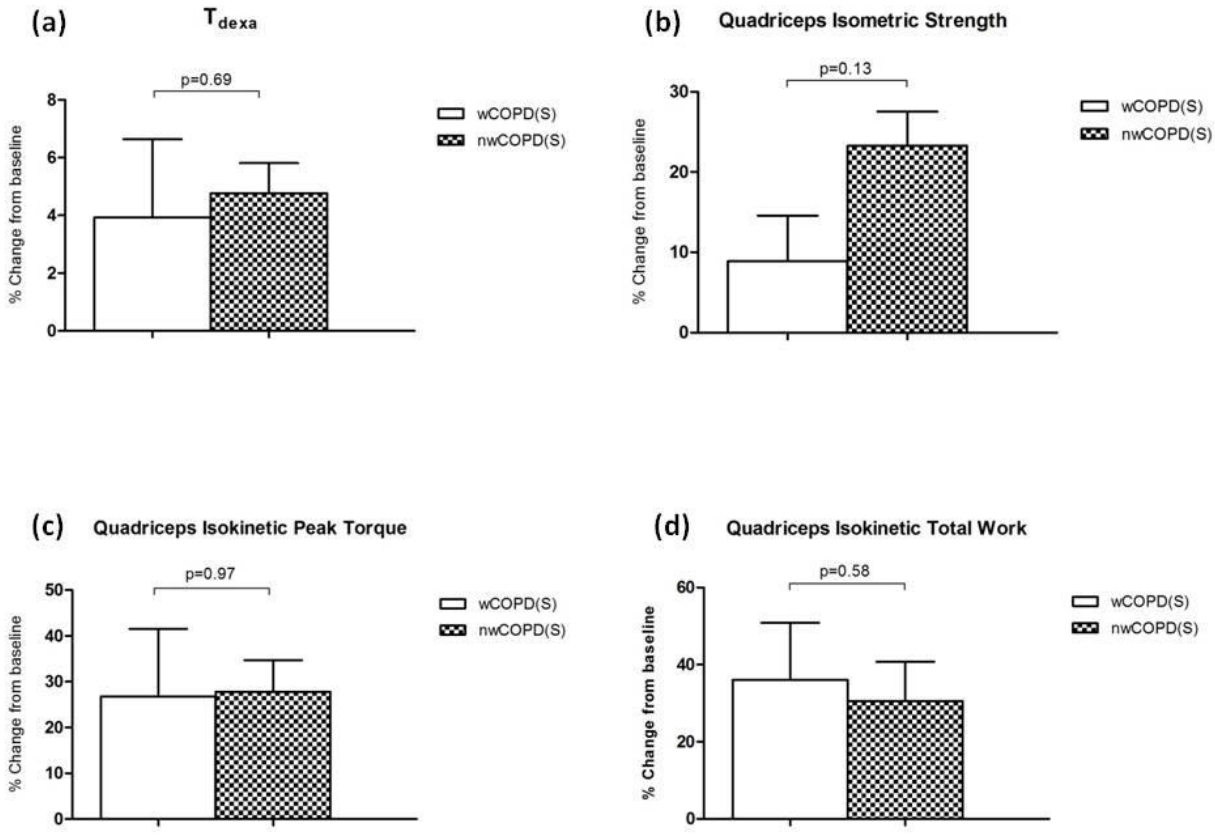


Figure 5.10 – Percentage changes from baseline in measures of quadriceps function in wasted [wCOPD(S)] and nonwasted COPD [nwCOPD(S)] subjects following RT combined with protein-carbohydrate supplementation

(a)T_{dexa}, (b) Quadriceps isometric strength, (c) Quadriceps isokinetic peak torque, and (d) Quadriceps isokinetic total work
Data presented as Means (SEM), p values calculated using Mann Whitney test

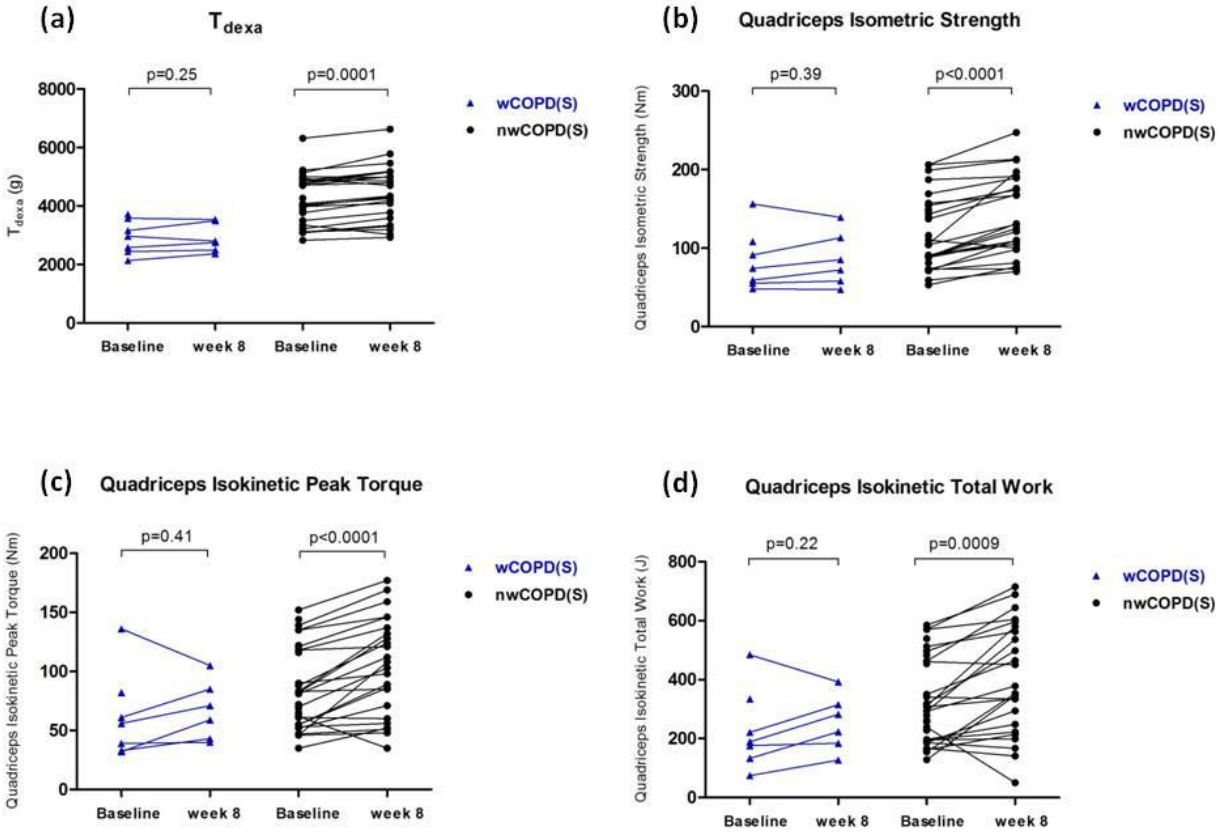


Figure 5.11 – Dot-plots depicting individual changes in measures of quadriceps function in wasted [wCOPD(S)] and nonwasted [nwCOPD(S)] subjects following RT combined with protein-carbohydrate supplementation

(a)T_{dexa}, (b) Quadriceps isometric strength, (c) Quadriceps isokinetic peak torque, and (d) Quadriceps isokinetic total work
P values calculated using paired t tests

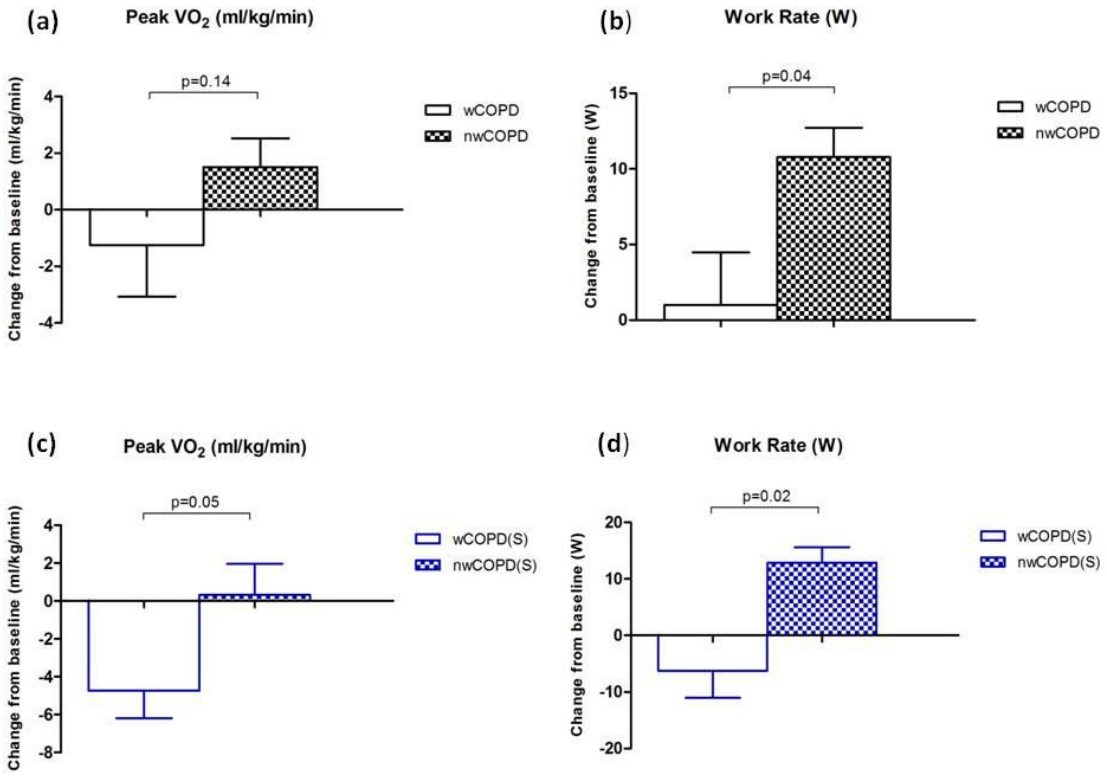


Figure 5.12 – Change from baseline in cycle ergometry performance following RT alone, and RT combined with protein-carbohydrate supplementation in wasted (wCOPD) and nonwasted COPD (nwCOPD) patients

(a) Peak VO_2 and (b) Peak work rate in wasted (wCOPD) and nonwasted COPD (nwCOPD) patients following RT

(c) Peak VO_2 and (d) Peak work rate between wasted [wCOPD(S)] and nonwasted COPD [nwCOPD(S)] patients following RT combined with protein-carbohydrate supplementation Data presented as Means (SEM). p values calculated using Mann Whitney test

Discussion

This study shows that high-intensity lower limb RT leads to significant increases in thigh muscle mass, quadriceps strength and whole-body peak exercise performance in COPD patients. However, protein-carbohydrate supplementation given at the time of training did not provide additional benefits in functional performance, over and above RT. HCs only ingested placebos during training, but demonstrated significant within-group improvements in functional outcomes that were comparable to patients with COPD. The mean absolute isokinetic work performed during RT was greater at all stages in HCs, but there was no difference in the rate of progression of work between the HC and COPD groups. This is discussed further in **chapter 6.**

This is the first study to examine the role of protein-carbohydrate supplementation as an adjunct to RT in COPD. This was a mechanistic exploration of the effects of supplementation combined with RT on functional outcomes, rather than the role of dietary protein-carbohydrate in isolation or alongside generic PR. The RT programme employed in this study has previously been shown to produce significant gains in lower limb muscle mass following immobilisation in healthy subjects (Jones et al. 2004). The composition of dietary supplements given during RT was based on previous data showing that protein-carbohydrate supplementation was effective in increasing protein synthesis in training studies involving young and elderly subjects (Esmarck et al. 2001; Rasmussen et al. 2000). Supplementation was provided immediately after each RT session, as the timing of protein intake appears to be crucial, with greater increases in muscle mass being reported in the elderly with immediate post-exercise amino acid intervention (Esmarck et al. 2001). The majority of participants tolerated the training regime, and although three participants

dropped out due to musculoskeletal problems (knee and back pain), this was not directly related to the RT programme.

Skeletal muscle mass is determined by the relative rates of protein synthesis and protein degradation. An increase in muscle mass (hypertrophy) occurs as a result of either an increase in MPS and/or a reduction in MPB. Resistance-type exercise is a powerful anabolic stimulus that promotes both MPS and MPB, albeit the latter to a lesser extent (Phillips et al. 1997). However net muscle protein balance remains negative in the absence of nutrient intake (Phillips et al. 1997). In healthy individuals, evidence suggests that provision of protein during recovery from acute RT leads to significant increases in MPS via activation of mammalian target of rapamycin (mTOR) signalling (Dreyer et al. 2008; Drummond et al. 2008; Miller et al. 2003; Rasmussen et al. 2000; Wilkinson et al. 2007) , while the co ingestion of carbohydrate seems to further inhibit the post exercise increase in MPB (Borsheim et al. 2004; Roy et al. 1997), hence improving net muscle protein balance. Therefore, it appears that post-exercise dietary supplementation may be an effective strategy to augment the anabolic response to training. However in this study, providing protein-carbohydrate supplementation at the time of RT did not provide additional benefits to patients with COPD. The reasons for this are unclear. It may be that the signal from RT overwhelms any additional effects of supplementation. Alternatively the quantity of supplementation provided may have been inadequate to elicit a response over and above RT. Previous data has shown that in elderly men undergoing prolonged RT, regular ingestion of protein (10g) immediately after each training session, lead to a significant increase in quadriceps mass at the end of 12 weeks (Esmarck et al. 2001). In the current study, patients received a higher dose of protein (19g); hence the amount of ingested

protein is unlikely to have been a limiting factor. Lastly, there are suggestions that in older people who habitually consume adequate amounts of dietary protein, additional improvements in functional performance are not obtained when RT is combined with protein supplementation (Campbell & Leidy 2007). Hence, another plausible explanation is that the supply of protein substrate is not a limiting factor for muscle growth in COPD.

T_{dexa} increased in all participants, indicating that RT increased net MPS within the quadriceps. Selective RT in COPD has been shown to significantly increase lower limb muscle mass and strength (Casaburi et al. 2004; Kongsgaard et al. 2004; Spruit et al. 2002). In the present study, the percentage increase in thigh muscle mass was around 5% in all groups, which is comparable to changes seen in healthy subjects undergoing a similar RT programme (Jones et al. 2004). Similarly, the magnitude of change in isometric (11 to 20%) and isokinetic (17 to 42%) quadriceps strength observed in the three groups is more or less comparable to previously published data (Casaburi et al. 2004; Kongsgaard et al. 2004). In addition, changes in dynamic (isokinetic) strength were greater than changes in static (isometric) strength in all subjects. This may reflect a specific adaptation to the RT programme which was also isokinetic in nature. The disconnect between the magnitude of training-induced changes in muscle strength and muscle mass suggests that factors other than increased muscle mass are responsible for producing muscle force. In the early stages of RT, neural adaptations may be involved in increasing muscle strength, but this was not directly measured in this study.

Although the training velocity in this study was set at 180°/sec, improvements in isokinetic function were seen at the testing velocity of 60°/sec. This would be in keeping with studies in healthy subjects demonstrating increased isokinetic peak torque at velocities

above and below the training velocity (Kraemer et al. 2000). Moreover, isokinetic training has been shown to improve measures of isometric strength (Kraemer et al. 2000), which was also observed in this study. In general, the improvements in thigh muscle mass and quadriceps strength tended to be greater in the COPD groups than HC, although this was not statistically significant. Since muscle mass and strength were lower in patients at baseline, the capacity to improve was perhaps far greater in this group when compared to HC.

Whole-body peak exercise performance (peak VO_2 and Peak Work) improved following RT in all subjects, and tended to be greater in HC when compared to the COPD groups. A similar outcome of RT on endurance performance has been reported previously (Spruit et al. 2002; Vonbank et al. 2012), while other studies have failed to demonstrate any significant changes in peak exercise performance following selective RT in this population (Casaburi et al. 2004; Clark et al. 2000; Ortega et al. 2002; Simpson et al. 1992). Given that lower limb weakness contributes to impaired whole-body exercise performance (Gosselink, Troosters, & Decramer 1996), one can speculate that enhanced quadriceps strength following RT might have allowed patients to improve their cycling performance. Dyspnoea and cardiopulmonary stress induced by lower limb RT has been observed to be lower when compared to whole-body endurance training in COPD (Probst et al. 2006). However, the angular velocity (180°/sec) of the isokinetic RT protocol used in this study sits in the middle of the spectrum of speeds available on isokinetic machines, and, as such, represents the centre of the muscular strength to endurance continuum (Kraemer et al. 2000). Hence, another plausible explanation is that the RT programme employed in this study led to some aerobic adaptation at the muscle level, resulting in

improved exercise performance. Indeed analysis performed by Linzy Houchen-Wolloff in a subset of study participants, confirms that this RT protocol sufficiently activated the cardio respiratory system, as evidenced by subjects working at a higher proportion of their maximum VO_2, VE and heart rate (Houchen-Wolloff 2012). Although whole-body exercise performance is not usually expected to improve following RT (Rutherford et al. 1986), there is evidence in elderly subjects (Frontera et al. 1990; Hepple et al. 1997a) and COPD (Spruit et al. 2002; Vonbank et al. 2012) showing enhanced peak exercise performance following this type of training. The change in Peak Work (9.9W to 13.9 W) observed in this study is comparable to previously published data (Spruit et al. 2002; Vonbank et al. 2012).

Post-hoc analysis after unblinding of treatment allocations showed that the improvements in quadriceps isometric strength tended to be lower in the wasted group, but other functional outcomes were comparable between wasted and nonwasted patients. A similar training response was observed when dietary supplements were provided with training, which suggests that additional supplementation was equally ineffective in both wasted and nonwasted patients. The provision of caloric nutritional supplements during PR to muscle-wasted patients with increased systemic inflammation, has previously failed to show any additional benefits in terms of body composition and energy expenditure (Creutzberg et al. 2000). On the other hand, a recent systematic review has suggested that nutritional support combined with exercise training can enhance body weight in malnourished COPD patients (Ferreira et al. 2012). Levels of systemic inflammatory markers were not been measured in the present study, and perhaps some of the wasted patients may have belonged to this “non-responder” category, hence reducing the effect of

supplementation on the whole group. However, the aim of supplementation in this study was to enhance functional muscle performance rather than body weight.

The study does have a number of limitations. Habitual diet of participants was not measured. This might be important, because as previously discussed, in subjects who habitually consume adequate amounts of dietary protein, RT-induced improvements in muscle performance are not further enhanced by the ingestion of additional protein during training (Campbell & Leidy 2007). Although this study was adequately powered to detect RT-induced within-group changes in functional outcomes, it may not have had insufficient power to detect between-group differences in response to supplementation, raising the possibility of a type II error. It's not known what a significant effect of supplementation over and above RT might be, but when comparing the supplemented and placebo COPD groups, the effects tended to be greater in the latter, making such an error unlikely. Muscle wasting was defined by applying FFMI cut-off values that have been shown to be predictive of mortality and morbidity in the COPD population (Schols et al. 2005). However by using this criteria, only few patients with muscle wasting (n=14) were recruited into this study. Therefore, the results of the post-hoc sub-group analysis comparing wasted and nonwasted groups should be interpreted with caution, as the analysis may have been underpowered to detect changes in the wasted patients. The use of arbitrary FFMI cut-off values may not reflect a condition of progressive wasting or cachexia, and it may be more useful to look at muscle mass as a continuous variable to identify clinically relevant wasted subgroups.

In conclusion, this study shows that high-intensity isokinetic RT in COPD leads to significant increases in thigh muscle mass, quadriceps strength and whole-body peak

exercise performance. However, protein-carbohydrate supplementation at the time of training does not provide additional benefits. When comparing the training response between wasted and nonwasted COPD patients, the provision of additional protein-carbohydrate supplementation was equally ineffective in both groups. .

CHAPTER 6

Skeletal Muscle Molecular Responses to Resistance Training and Protein-Carbohydrate Supplementation in COPD

The purpose of this thesis was to explore the skeletal muscle clinical and molecular response to RT and supplementation in COPD patients in comparison to healthy controls. In this chapter I will describe the effects of RT and protein-carbohydrate supplementation on molecular targets and pathways that have previously been shown to play a role in the maintenance of muscle mass in healthy individuals. The gene and protein expression analysis described in this chapter was performed at the University of Nottingham by Dr Despina Constantin, and I was involved in analysing and interpreting the data. The impact of training on clinical outcomes is described in **chapter 5**.

Introduction

Impaired skeletal muscle function is an important clinical feature of COPD (Agusti 2007; ATS/ERS 1999; Fabbri 2007). Patients with COPD present with reduced muscle mass and strength, which compromises physical performance and predicts morbidity and mortality independently from lung function impairment (Marquis et al. 2002; Schols et al. 1989; Swallow et al. 2007; Vestbo et al. 2006). This is important because skeletal muscle dysfunction may be a remediable feature in a disease where the primary lung pathology is largely irreversible (Steiner et al. 2012). Proof that this approach is beneficial is demonstrated by the efficacy of physical training in improving muscle function in COPD (Bernard et al. 1999; Kongsgaard et al. 2004).

Despite the appreciation of the clinical importance of skeletal muscle dysfunction in COPD, little is known about cellular and molecular mechanisms underpinning this phenomenon, or indeed whether the potential for muscle hypertrophy is preserved to the same degree as in healthy older people. Based on cross-sectional comparisons of COPD with aged-matched healthy subjects, it has been proposed that increased muscle protein breakdown is a major driver of muscle wasting in COPD (Debigare et al. 2008; Doucet et al. 2007; Plant et al. 2010). However, in the absence of data depicting temporal changes in muscle protein turnover or the expression of genes and proteins thought to regulate muscle mass in response to intervention, firm conclusions on the mechanistic and therefore clinical relevance of protein breakdown in muscle mass loss in COPD cannot be drawn. Importantly, training induced molecular adaptations will precede muscle mass gains and therefore important changes in gene and protein expression likely to control functional

adaptation may be missed if analysis is restricted to biopsy samples obtained when training is completed.

Physical training combined with post-exercise dietary protein supplementation is proposed to induce skeletal muscle hypertrophy via activation of mTOR signalling (Campbell & Leidy 2007; Hulmi et al. 2009a; Rennie et al. 2004). There is evidence that muscle protein synthesis via this signalling pathway is blunted in elderly volunteers (Cuthbertson et al. 2005; Kumar et al. 2009), and that the timing of supplementation in relation to chronic resistance exercise training (RT) may be an important factor in the magnitude of anabolic response in older people (Esmarck et al. 2001). The role of protein supplementation in combination with RT in patients with COPD has yet to be elucidated. Similarly, the impact of increasing insulin availability, which acutely inhibits muscle protein breakdown (Greenhaff et al. 2008), and is a process known to be dysregulated in the elderly (Kumar et al. 2009; Wilkes et al. 2009), has not been investigated in COPD.

These questions were addressed by conducting a detailed investigation of genes and proteins thought to play a central role in muscle mass regulation, in response to a controlled programme of maximal RT in patients with stable COPD and age matched control volunteers. Furthermore, responses were measured over the course of the RT intervention in a matched cohort of stable COPD patients who ingested post-exercise protein-carbohydrate dietary supplements. A substantially wider range of candidate genes and proteins were measured than in previous studies, and these responses were related to RT-induced changes in muscle mass and strength.

The study addressed the following specific hypotheses:

1) RT-induced increases in muscle mass and function would be blunted in stable COPD patients compared to age matched control subjects
2) This blunting in COPD patients would be mirrored by increased expression of genes and proteins associated with muscle protein breakdown and inhibition of muscle anabolic signalling and myogenesis
3) Post-exercise dietary protein-carbohydrate supplementation would inhibit muscle catabolic events and augment anabolic signalling and myogenesis thereby enhancing the functional effects of RT

Materials and Methods

A detailed description of the methods is provided in **chapter 4**.

Subjects

Patients with severe COPD (GOLD stage 3 or 4) and significant self reported exercise limitation (MRC grades 3, 4 or 5) were recruited. Age matched healthy controls were recruited from the local population. Approval was obtained from the Leicestershire Research Ethics Committee and all participants provided informed written consent. The study was registered with the UK National Research Register [(NRR) reference: N0123192026].

Study design

All participants undertook maximal intensity voluntary RT for 8 weeks. COPD subjects were randomly allocated in a double blind fashion to receive a dietary protein-carbohydrate supplement [COPD(S)] or a non-nutritive placebo [COPD(P)] at the time of

training. Healthy control subjects (HC) all received placebo. Outcome assessments and muscle sampling were performed at baseline, 24 hrs after the first training session (muscle biopsy and blood insulin only), 4 weeks, and at the end of training (8 weeks). **The trial design and flow of patients is summarised in chapter 5 (Figure 5.1).**

Interventions

Resistance training (RT)

Fully supervised maximal voluntary lower-limb RT was performed on an isokinetic dynamometer (Cybex II Norm, Stoughton, MA, USA) thrice weekly for 8 weeks. Each session comprised 5 sets of 30 maximal knee extensions at an angular velocity of 180°/sec. During training, peak torque (Nm) and total work performed (J) was recorded for each knee extension.

Nutritional supplementation

The supplement contained 19 g protein and 49 g glucose polymer carbohydrate (Vitargo Gainers Gold, Swecarb, Sweden) and was ingested immediately after each training session. The placebo was an identical volume non-nutritive and non-caloric drink. Healthy volunteers received only the placebo intervention.

Outcome Measurements

Muscle Biopsies

Vastus lateralis muscle biopsies were obtained after a fast of at least 4 hrs and (apart from baseline samples) 24 hrs after the previous training session. Samples were immediately snap-frozen, stored in liquid nitrogen and later analysed as described below.

Blood sampling

Venous blood was drawn in the fasted state and analysed for serum insulin concentration, using a Human Insulin specific RIA kit (Millipore, Billerica, MA, USA).

Quadriceps Muscle Function

Isometric quadriceps strength was determined during maximal voluntary knee extensor contraction (Cybex II Norm, Stoughton, USA). Subsequently, peak torque and total isokinetic work was recorded during 2 bouts of 5 repetitions (performed at an angular velocity of 60°/s).

Lean Mass

Whole body and thigh lean mass (T_{dexa}) was measured using Dual Energy X-ray Absorptiometry (DEXA Lunar Prodigy Advance, GE Healthcare, UK). Fat free mass index (FFMI) was calculated from height and total body fat free mass. Patients were deemed to have muscle wasting if FFMI < 16kg/m^2 in men or < 15kg/m^2 in women (Schols et al. 2005).

Whole Body Exercise Performance

Subjects performed a symptom limited, exhaustive, incremental cycle ergometer test (at baseline and after completion of training only) (Zan-600 ErgoTest, Meβgeräte GmbH, Oberthulba, Germany).

Muscle Biopsy Analysis

A wide range of genes (Quantitative reverse transcriptase PCR) and proteins (Western blotting with infrared detection) were measured at each sampling time-point. Candidate gene and proteins were selected on the basis of their known association with the regulation of muscle protein breakdown (20S proteasome, MAFbx, MuRF1 and ZNF216,

along with calpain-3), muscle protein synthesis (Akt, p70s6 kinase, GSK3α, GSK3β, 4EBP1 and Redd1), myogenesis (MyoD, myogenin and myostatin), transcription (members of the family of Forkhead transcription factors, FOXO1 and FOXO3 and RUNX1) and inflammation [Tumour necrosis factor-alpha (TNF-α) and interlukin-6 (IL-6) mRNA expression). This is the first time that such a comprehensive battery of molecular measurements have been made over the course of an intervention aimed at increasing muscle mass in COPD patients.

Quantitative RT-PCR

RNA was extracted from frozen muscle biopsies using TRI Reagent (Ambion, Huntingdon, UK), according to the manufacturer's protocol. First strand cDNA was then synthesised from 1 μg RNA using random primers (Promega) and Superscript III (InVitrogen). Additional reactions were performed, in which the reverse transcriptase was omitted to allow for assessment of genomic DNA contamination. All reactions were performed in the ABI 7900HT Fast Sequence Detection System (Applied Biosystems, Foster City, CA). Each well contained 2 μl of cDNA, 18 μM of each primer, 5 μM probe, and Universal Taqman 2X PCR Mastermix for fast reaction (Applied Biosystems) in a 25 μl final volume. Each sample was run in duplicate. Primers and MGB TaqMan probes (Applied Biosystems, Foster City, CA, USA) were designed such that probes spanned over exon-exon boundaries to avoid genomic amplification. Hydroxymethylbilane synthase (HMBS) was used as internal control, and all genes of interest were labelled with the fluorescent reporter FAM. The thermal cycling conditions used were: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Standard curves obtained by serial dilution of

cDNA were run in an initial stage in duplicate in two separate reactions: one with the primer gene set of interest, and one with the HMBS endogenous gene.

Ct values of the target gene were normalized to Ct values of the house-keeping gene in COPD patients and healthy control volunteers, and the final results were calculated according to the $2^{-\Delta\Delta Ct}$ method. The baseline for each subject was used as the calibrator and was set at 1. **Examples of amplification curves for some of the target genes are given in the appendix.** Ct values are indicated on the x-axis. The no template control (NTC) samples with only water, and which gives no amplification, is illustrated by the 2 traces under the green line. This suggests that there is no contaminant in the reaction mixture, and provides proof of quality of the data.

Western Blotting

Cytosolic and nuclear lysates were prepared from each muscle biopsy sample, and target protein expression was determined using Western blotting as described by Constantin et al (Constantin et al. 2011). Total protein concentration was measured using the Bradford assay (Bradford 1976). Protein samples were run on a 4-12% Bis-Tris acrylamide gel (Invitrogen, UK) for 2 hrs at constant 200 V and transferred on a polyvinylidene difluoride membrane (PVDF) overnight at constant 100 mA, in ice-cold buffers (4°C). The protein transfer was checked with Ponceau S red staining, before blocking the membrane in BSA, TBS, Tween for 1 hr at room temperature. Membranes were probed with the primary antibody overnight at 4°C. Antibodies to determine phosphorylated Akt1 (serine^{473}; PAkt1) and total Akt1 (60k Da), phosphorylated eukaryotic translation factor 4E-binding protein 1 (4E-BP1) (threonine$^{37/46}$; P4E-BP1) and total 4E-BP1 (15-20 kDa), phosphorylated p70

ribosomal S6 kinase (threonine^{389} Pp70S6K) (Pp70S6K) and total p70S6K (70 kDa), phosphorylated GSK3α (serine^{21}; PGSK3α) and total GSK3α (51 kDa), phosphorylated GSK3β (serine^{9}; PGSK3β) and total GSK3β (46 kDa), phosphorylated FOXO1 (serine^{256}; PFOXO1; 82 kDa) and total FOXO1 (78-82 kDa) and phosphorylated FOXO3 (serine^{253}; PFOXO3; 97 kDa) and total FOXO3 (82-97 kDa) and Redd1 (28 kDa) were obtained from Cell Signalling Technology (Danvers, MA, USA). MuRF1 (42 kDa) and MAFbx (42 kDa) antibodies, produced in-house by Pfizer Inc (USA), were provided to the department as gifts. Myostatin antibody (MW 45 kDa) was obtained from Novus Biologicals (Littleton, CO, USA). PDK4 (46 kDa), calpain-3 (94 kDa), Myogenin (34 kDa) and MyoD (35 kDa) antibodies were obtained from Insight Biotechnology (Insight Biotechnology Ltd, Middlesex, UK). 20S proteasome antibody (29 kDa) was purchased from Biomol. All proteins were visualized by developing with either an IRDye 800 labelled secondary anti-rabbit antibody or an IRDye 680 labelled secondary anti-mouse antibody (used in multiplex detection) and were further quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). The infrared signals have a greater dynamic linear range compared to chemiluminescence.

Statistical Analysis

The principle objective of this study was the investigation of skeletal muscle molecular responses to RT, and training combined with feeding in relation to gains in muscle function that would be clinically significant in patients with COPD. Meaningful changes in mRNA and protein expression across the range of planned targets are difficult to quantify but most human studies have enrolled in the region of 10 subjects. Based on

previous data from the PR programme at Glenfield Hospital, it was estimated that a 20% increase in muscle strength following training would be clinically and physiologically significant. To detect this strength difference (90% power, $\alpha = 0.05$), 25 patients would be required to complete training in each group. To account for predicted dropouts in the COPD group a larger number of subjects were recruited. The *a priori* aim was to recruit sufficient numbers of patients with low muscle mass (according to accepted criteria) to allow a subgroup analysis comparing the responses between patients with low and preserved muscle mass. However, the demanding nature of the study with the requirement for intensive physical training and repeated muscle sampling meant that sufficient patients with low muscle mass could not be recruited, and hence data for the whole cohort is presented.

Because observation of mRNA and protein changes over time was the primary objective of the study, only subjects who provided at least a baseline and 24 hour time-point muscle biopsy were included **(Figure 6.1)**. An independent Student t-test was used to compare baseline clinical and functional data between controls and COPD patients. Two-way repeated measures ANOVA, and when appropriate a Least Significant Difference (LSD) post-hoc test, was used to compare within group changes over time and differences between treatment groups [Healthy controls vs. COPD(P) vs. COPD(S)]. Training intensity progression was analysed using repeated measures ANOVA with Bonferroni corrections for multiple comparisons. Because of the non-ordinate nature of the molecular data, comparison of two independent groups was performed using Mann-Whitney's non-parametric analysis, and comparison of more than two time related groups was performed

using Friedman's non-parametric analysis. Data in tables and figures are expressed as mean ± SEM. Significance was set at the $p<0.05$.

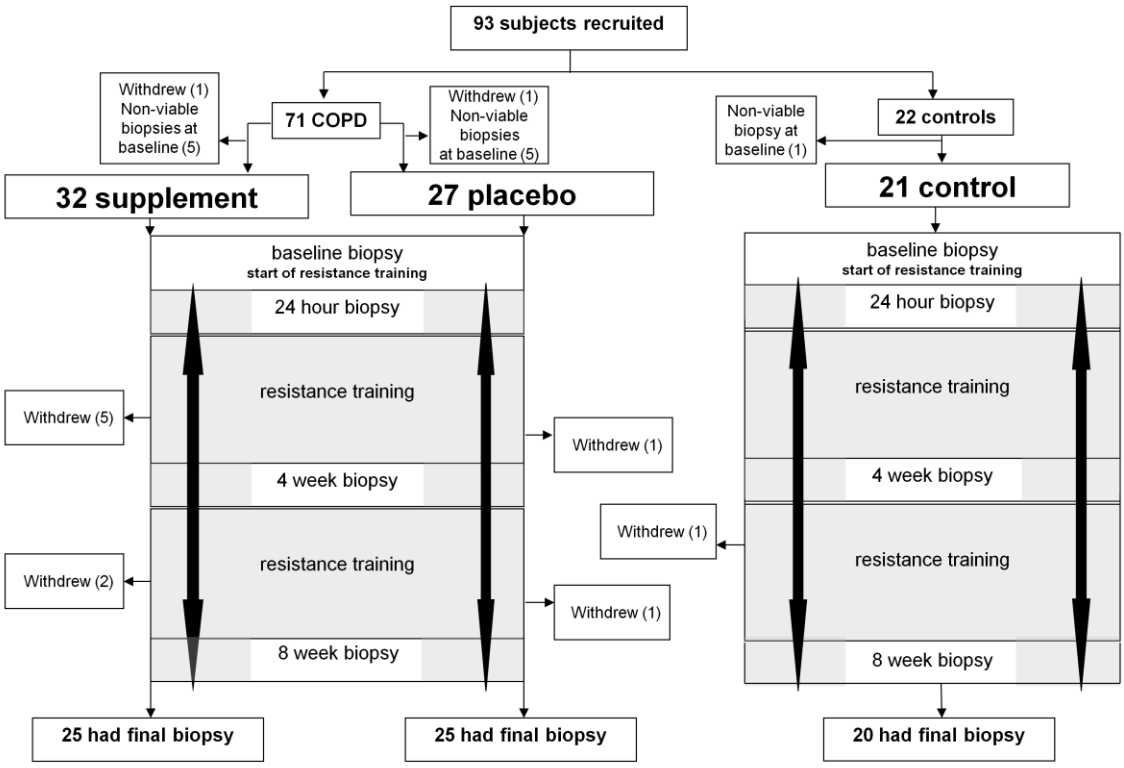


Figure 6.1 – Study Flow Diagram

93 subjects were recruited to the study (71 COPD and 22 HC) but only those with at least a viable baseline and 24 hour biopsy are included in the analysis (21 HC and 59 COPD). Non-viable biopsies at baseline (10): damaged samples/ defrosted.

Reasons for patient withdrawal at each stage, in all groups, are as follows:
COPD (S) = 1 unable to tolerate biopsy (after baseline/ before 24 hr biopsy), 2 exacerbation/ 1 back pain/ 1 family bereavement/ 1 too busy (wks 1-4), 1 exacerbation/ 1 advised to stop by GP (wks 4-8).
COPD (P) = 1 unable to tolerate biopsy (after baseline/ before 24 hr biopsy), 1 knee pain (wks 1-4), 1 unable to re-start training post-operatively (wks 4-8).
Healthy Control = 1 spouse of a COPD patient wanting to withdraw at the same time.

Results

59 patients with COPD and 21 healthy controls were included in the analysis. **Figure 6.1** shows the flow through the study and the number of viable muscle samples available for analysis at each time point.

Baseline (pre-training)

Physical characteristics

Baseline characteristics are shown in **Table 6.1**. There were no significant differences at baseline, between the COPD(P) and COPD(S) groups. Quadriceps muscle function and whole body exercise performance were lower in COPD patients than HC. Thigh lean mass (T_{dexa}) was lower in patients compared with HC, but this was not statistically significant. 7 COPD(P) and 6 COPD(S) subjects were deemed to be muscle wasted according to previously described criteria.

	Healthy Controls (n=21)	COPD(P) (n=27)	COPD(S) (n=32)
Age (years)	66.1 (1.0)	66.9 (1.7)	68.9 (1.7)
Gender (M:F)	10:11	14:13	19:13
BMI (kg/m^2)	26.8 (0.6)	25.5 (1.0)	26.6 (0.8)
FFMI (kg/m^2)	17.5 (0.3)	16.9 (0.4)	17.6 (0.4)
Muscle wasted subjects (n)	0	7	6
Smoking pack years	14.4 (5.1) * #	48.1 (5.6)	46.9 (7.9)
FEV_1 (Litres)	2.5 (0.1) * #	1.1 (0.0)	1.12 (0.0)
FEV_1 (% predicted)	105.0 (4.7) * #	45.8 (3.2)	47.7 (3.3)
FEV_1/FVC (%)	71.8 (1.4) * #	41.2 (2.1)	39.4 2.4)
T_{dexa} (g)	4190 (189)	3700(191)	3829(153)
Isometric Peak Torque (Nm)	135.8 (9.5) * #	109.3 (9.8)	105.9 (7.4)
Isokinetic Peak Torque (Nm)	98.0 (7.9) #	75.8 (6.9)	79.9 (6.0)
Isokinetic Peak Work (J)	373.8 (33.0) #	284.5 (28.2)	307.5 (25.2)
Cycle Peak VO_2 (ml/kg/min)	22.8 (1.4) * #	14.7(0.9)	14.4 (0.6)
Cycle Peak Workload (W)	119.9 (8.7) * #	51.1 (4.7)	48.9 (3.8)

Table 6.1 – Baseline Physical Characteristics.
Subjects who provided a minimum of baseline and 24 hr muscle biopsies were included in the analysis. Figures refer to mean (SEM).
*$p \leq 0.05$; HC vs. COPD(S), #$p \leq 0.05$; HC vs. COPD(P)

Molecular data

Baseline protein and mRNA expression levels in COPD patients as a whole and in HC are shown in **Tables 6.2 and 6.3** respectively. At baseline, MAFbx and MuRF1 protein expression, phosphorylation of p70s6kinase, Redd1 protein expression, Myogenin and MyoD protein expression and nuclear FOXO1 protein expression were significantly greater in COPD patients than HC.

Myostatin mRNA expression was greater in COPD patients at baseline but there were no other significant differences in gene expression between the groups.

	Healthy Controls (n = 21)	COPD (n = 59)
Muscle Protein breakdown		
20S proteasome	0.61 (0.11)	0.74 (0.07)
MAFbx	0.36 (0.05)	0.83 (0.12) *
MuRF1	0.30 (0.04)	0.69 (0.07) **
Calpain3	0.50 (0.08)	0.52 (0.04)
Muscle Protein synthesis		
Akt1	1.27 (0.37)	1.36 (0.11)
GSK3α	0.84 (0.26)	0.97 (0.11)
GSK3β	1.71 (0.38)	1.28 (0.19)
P70s6kinase	0.82 (0.10)	1.18 (0.10) *
4EBP1	1.03 (0.24)	1.11 (0.10)
Redd1	0.50(0.06)	0.85(0.06) **
Myogenesis		
Myostatin	2.70 (0.55)	3.18 (0.51)
MyoD	0.36 (0.05)	0.80 (0.09) **
Myogenin	0.45 (0.06)	0.86 (0.11) *
Transcription factors		
PFOXO1/FOXO1	1.83 (0.39)	2.22 (0.37)
PFOXO3/FOXO3	1.42 (0.22)	2.16 (0.31)
FOXO1	0.51 (0.04)	0.89 (0.07) **
FOXO3	0.58 (0.10)	0.78 (0.06)

Table 6.2 – Protein expression measured in muscle biopsy samples obtained at baseline
Figures refer to mean (SE) of relative optical density (normalized to either actin or lamin). The COPD group comprised of combined placebo and protein- carbohydrate supplemented volunteers at baseline (prior to intervention)
*p<0.05, **p <0.01 significantly different from control

	Healthy Controls (n = 21)	COPD (n = 59)
Muscle Protein breakdown		
20S proteasome	3.18 (1.12)	3.92 (1.09)
MAFbx	1.45 (0.26)	1.46 (0.13)
MuRF1	1.96 (0.54)	1.93 (0.21)
ZNF216	2.28 (0.92)	1.64 (0.38)
Calpain3	3.12 (1.22)	4.89 (0.7)
Muscle Protein synthesis		
Akt1	1.44 (0.28)	2.06 (0.3)
GSK3α	4.56 (1.67)	4.04 (0.9)
GSK3β	1.67 (0.39)	1.73 (0.19)
P70s6kinase	1.91 (0.69)	1.20 (0.18)
4EBP1	1.34 (0.22)	1.3 (0.17)
Redd1	1.42 (0.32)	1.81 (0.23)
Myogenesis		
Myostatin	1.41 (0.22)	2.19 (0.22) *
MyoD	1.57 (0.30)	1.62 (0.26)
Myogenin	2.54 (0.79)	1.99 (0.36)
Transcription factors		
FOXO1	1.97 (0.95)	1.86 (0.24)
FOXO3	1.7 (0.35)	1.52 (0.27)
RUNX1	4.49 (1.09)	5.60 (0.73)
Inflammatory cytokines		
TNFα	1.93 (0.49)	1.29 (0.42)
IL6	2.61 (0.92)	2.08 (0.32)

Table 6.3 – Muscle mRNA expression in muscle biopsy samples obtained at baseline
Figures refer to mean (SEM) mRNA expression relative to endogenous HMBS used as calibrator. HC = healthy controls.
* $p < 0.05$ significantly different from control

Training induced changes

Functional data

Thigh lean mass (T_{dexa}) increased significantly relative to baseline after 4 and 8 weeks of RT in HC [4.1(0.8)% and 5.4(0.9)%], COPD(P) [4.6(0.9)% and 6.2(1.7)%] and COPD(S) [3.9(1.3)% and 4.0(1.1)%] groups respectively **(Figure 6.2A)**. Isometric quadriceps strength also increased relative to baseline in all groups after 4 and 8 weeks RT [HC: 10.0(4.1)% and 12.4(4.2)%, COPD(P) 16.9(4.3) and 17.7(3.7)% and COPD(S) 14.6(2.8)% and 18.0(3.4)%]; **(Figure 6.2B)**.

There were no significant differences in the training induced gains in muscle mass or strength between the HC group and the COPD(P) and COPD(S) groups. Similarly, the absolute training induced increase in T_{dexa} was not different between the groups [HC: 232(40) g, COPD(P): 215(49) g and COPD(S): 148(43) g].

The weekly progression of total isokinetic work performed during training (weekly average) by each group is shown in **Figure 6.2C**. Mean absolute work performed by controls was significantly greater at all stages of training than in both COPD groups, but there was no significant difference in the rate of progression of work during training between the HC and COPD groups and between the COPD(P) and COPD(S) groups.

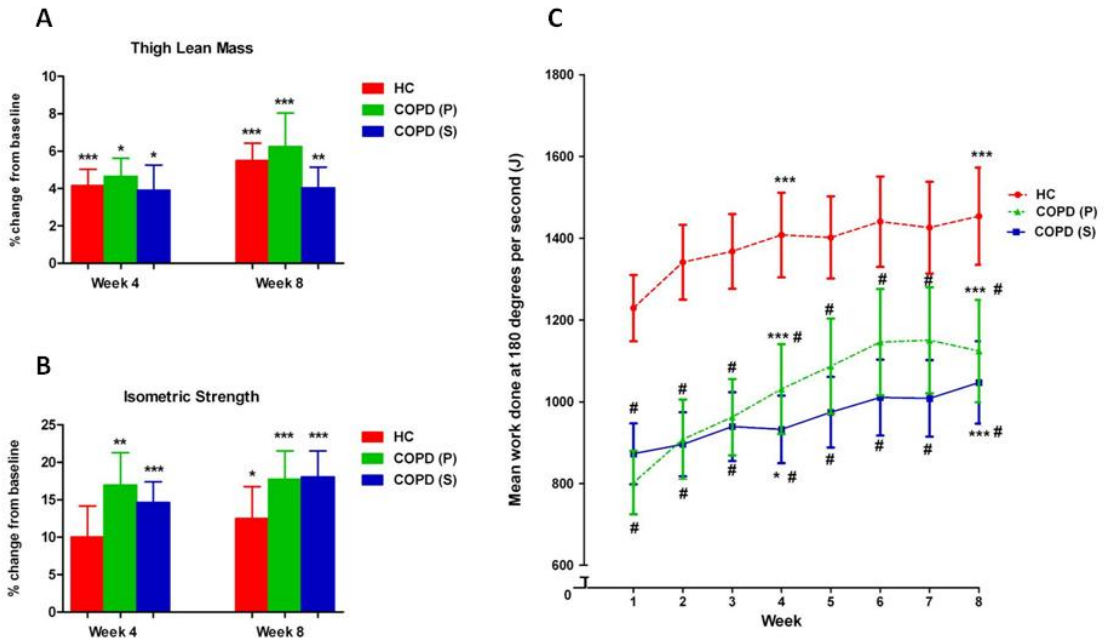


Figure 6.2 – Training-induced changes in muscle mass and strength and progression of work done

Changes (% ± SEM) from baseline in A: thigh muscle mass (T_{dexa}); B: Isometric peak torque in the healthy control group (HC; red bars), COPD placebo supplemented group [COPD(P); green bars] and COPD protein-carbohydrate supplemented group [COPD(S); blue bars].
* $p < 0.05$,** $p < 0.01$, *** $p < 0.001$; Significantly different from baseline
Panel C shows mean (± SEM) weekly total isokinetic work (180 degrees per second) performed during the 8 week training programme in the 3 experimental groups.
Significant difference compared to control group ($p<0.05$)

Protein expression

Muscle protein breakdown

In the HC group, expression of proteins involved in muscle protein breakdown was significantly increased 24hrs after the first bout of training and sustained at 4 and 8 weeks **(Figure 6.3A)**. In COPD the pattern of change in protein expression with training was broadly similar, but of smaller magnitude with fewer changes being statistically significant **(Figures 6.3B, 6.3C)**. In particular, the expression of MURF1 and MAFbx was unchanged in both COPD groups during training. There was no difference in the response to training between COPD(P) and COPD(S) groups.

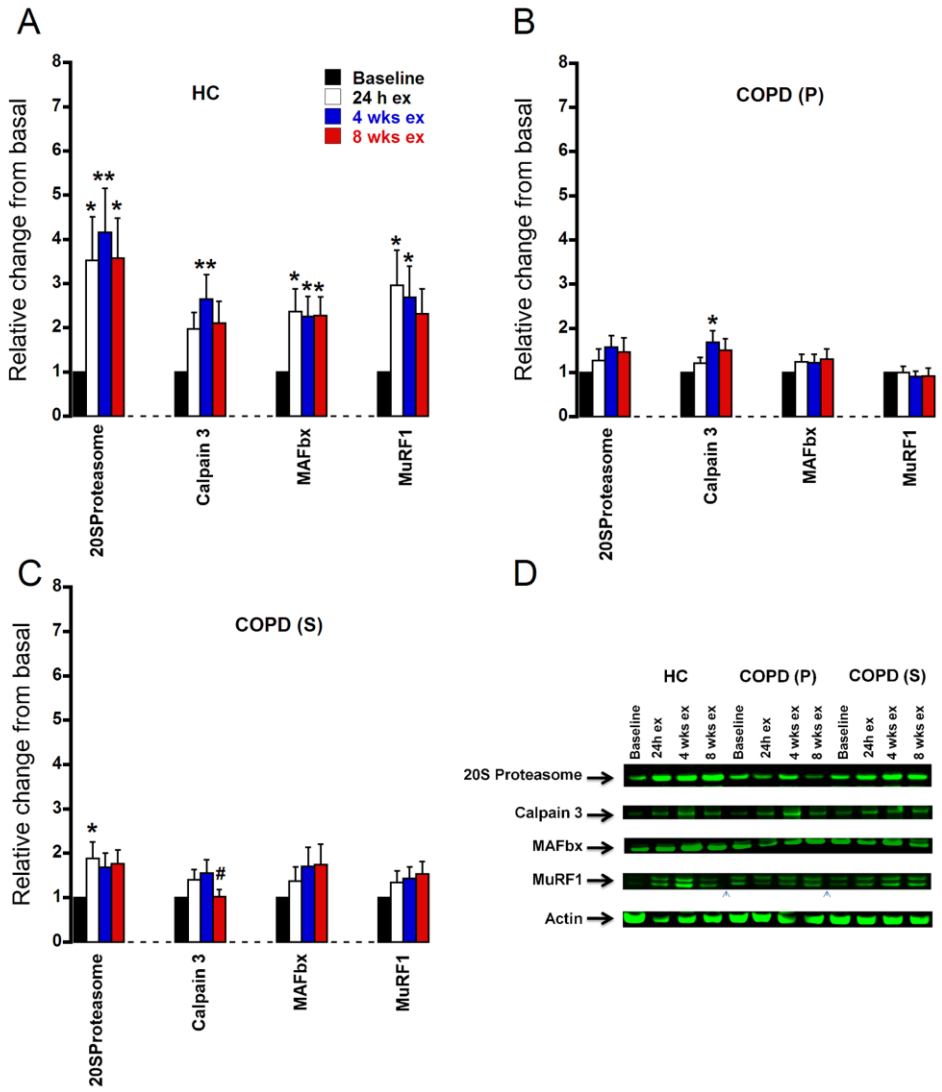


Figure 6.3 – Expression of target proteins regulating muscle protein breakdown in response to training and dietary supplementation

Protein expression (mean ± SEM) is represented as relative change from basal and was quantified by western blotting.

A: HC= Healthy Control subjects; B: COPD(P) = COPD patients receiving placebo supplements; C: COPD(S) = COPD patients receiving protein-carbohydrate supplements; D: Typical Western blot using IRdye 800 (green) secondary antibodies to quantify the catabolic proteins studied.

* $p < 0.05$, ** $p < 0.01$ Significantly different from baseline ($p < 0.01$);
$^{\#}$ $p < 0.05$ Significantly different from 24 hrs
ex: exercise

Muscle protein synthesis

In HC, there was an increase with training in the ratio of phosphorylated protein to total protein expression for all anabolic signalling proteins with the exception of PGSK3β/GSK3β ratio **(Figure 6.4A)**. The pattern of change in expression in COPD patients was similar, but training induced changes were of substantially lower magnitude than in HC **(Figures 6.4B, 6.4C)**. There was no difference in the magnitude of response when comparing COPD(P) and COPD(S) groups.

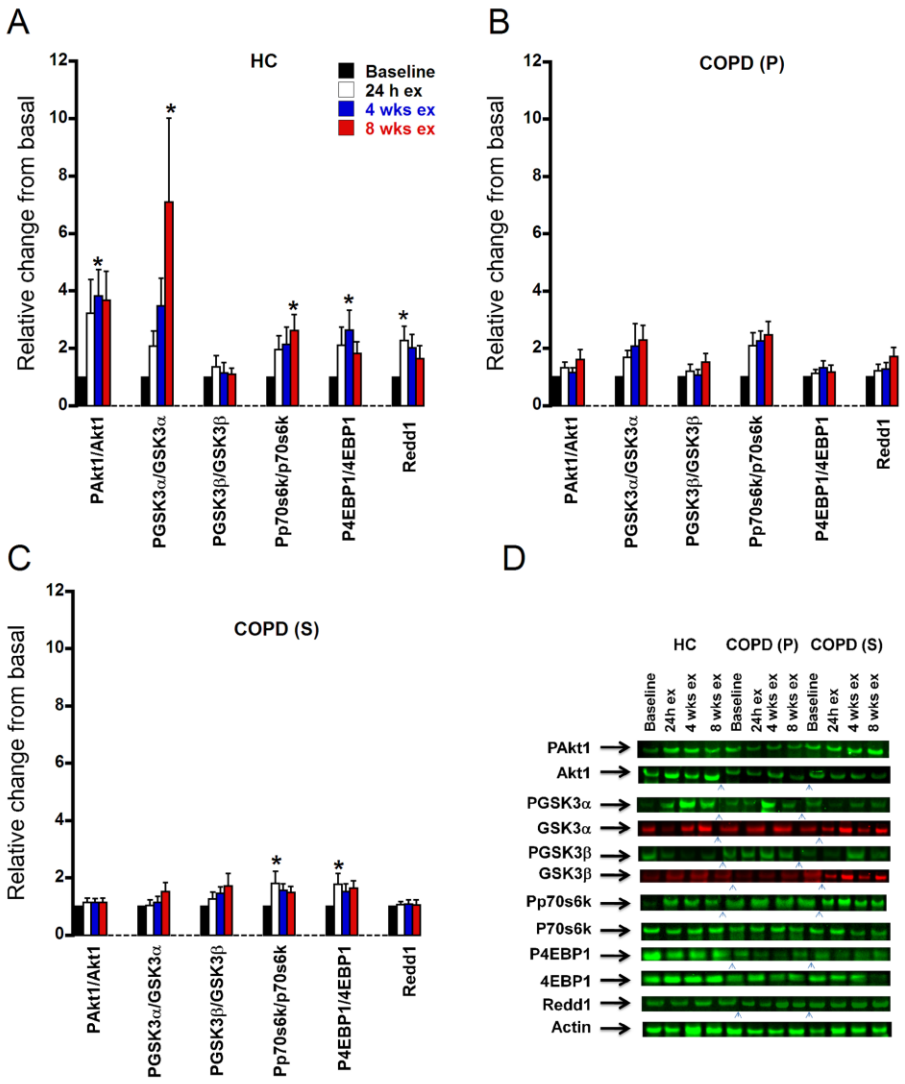


Figure 6.4 – Expression of target proteins regulating muscle protein synthesis in response to training and dietary supplementation

Protein expression (mean ± SEM) is represented as relative change from basal and was quantified by Western blotting.

A: HC = Healthy Control subjects; B: COPD(P) = COPD patients receiving placebo supplements; C: COPD(S) = COPD patients receiving protein-carbohydrate supplements; D: Typical Western blot using IRDye 800 (green) and IRDye 680 (red) secondary antibodies to quantify the anabolic proteins studied.

* Significantly different from baseline ($p < 0.05$)

ex: exercise

Myogenesis

Myostatin protein expression did not change significantly from baseline with training in either HC or COPD groups. There was a statistically significant increase in MyoD expression after 8 weeks training in all 3 groups, which was of the same magnitude across groups. There was a tendency for myogenin protein expression to increase with training in all groups, but this did not reach statistical significance **(Figures 6.5A, 6.5B, 6.5C, 6.5D)**. There was no difference in the pattern of response to training when comparing COPD(P) and COPD(S) groups.

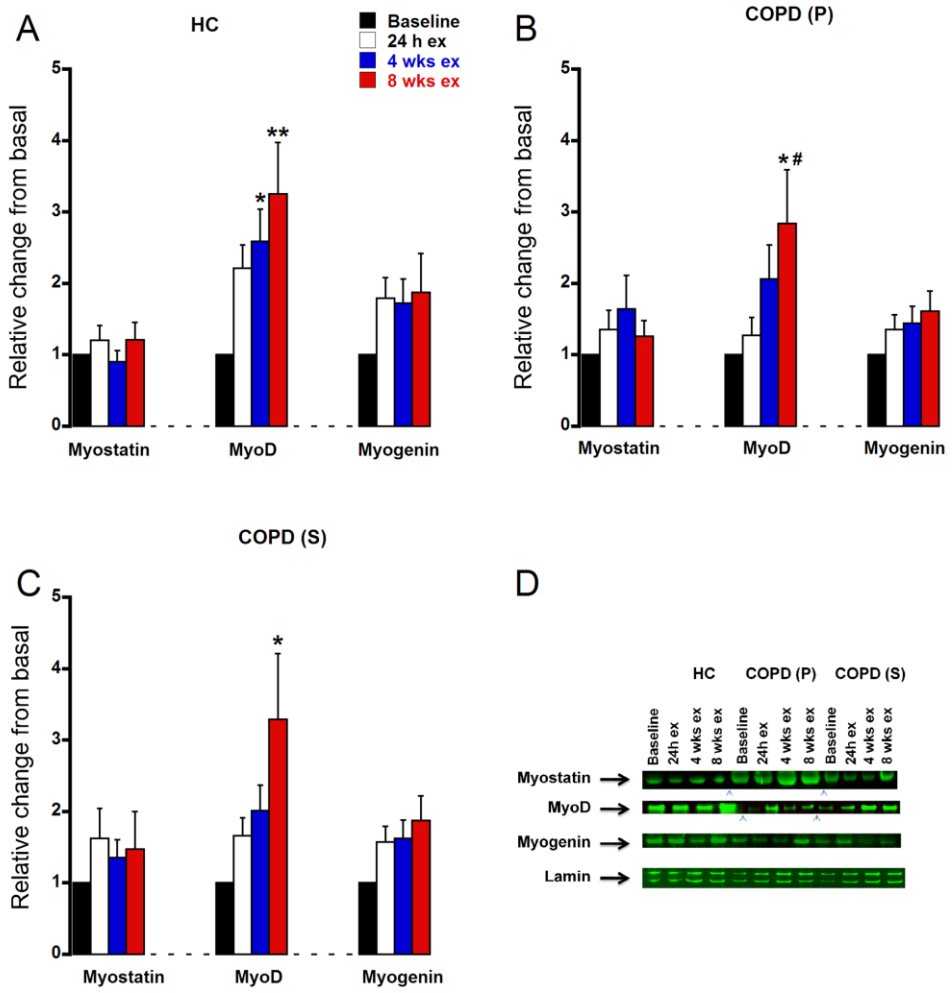


Figure 6.5 – Expression of target proteins regulating myogenesis in response to training and dietary supplementation

Protein expression (mean ± SEM) is represented as relative change from basal and was quantified by Western blotting.

A: HC = Healthy Control subjects; B: COPD(P) = COPD patients receiving placebo supplements; C: COPD(S) = COPD patients receiving protein-carbohydrate supplements; D: A typical Western blot using IRDye 800 (green) secondary antibodies to quantify the myogenic proteins studied.

* $p < 0.05$, ** $p < 0.01$ Significantly different from baseline ; $^{\#}$ $p < 0.05$ Significantly different from 24 hrs

ex: exercise

Transcription factors

Protein expression of total and of phosphorylated FOXO1 and FOXO3 transcription factors increased in all groups during training **(Figure 6.6)**. The magnitude of change was lower in both COPD groups compared to the HC group.

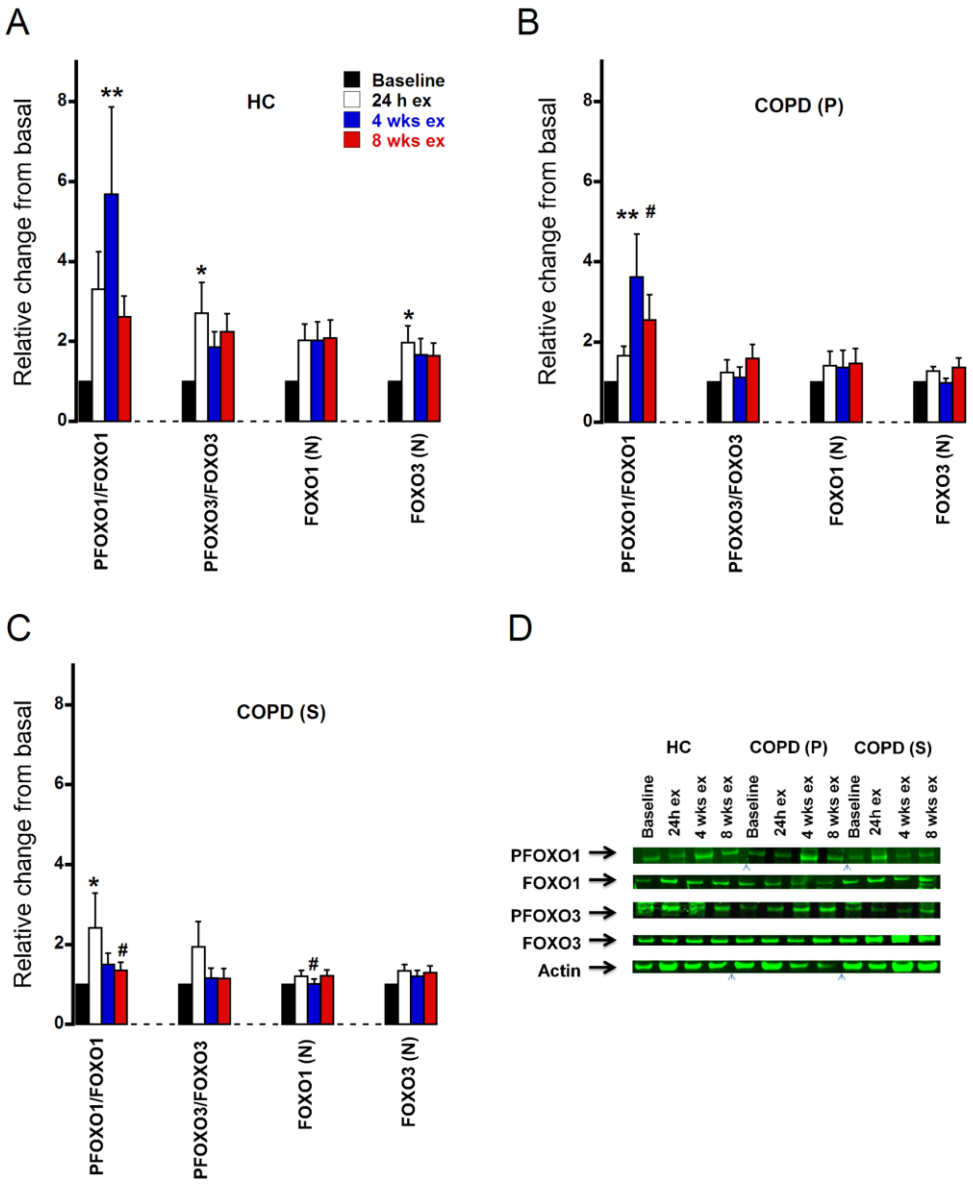


Figure 6.6 – Target transcription factor protein expression in response to training and dietary supplementation

Protein expression (mean ± SEM) is represented as relative change from basal and was quantified by Western blotting.

A: HC = Healthy Control subjects; B: COPD(P) = COPD patients receiving placebo supplements; C: COPD(S) = COPD patients receiving protein-carbohydrate supplements; D: A typical Western blot using IRDye 800 (green) secondary antibodies to quantify the transcription factors studied.

* $p < 0.05$, ** $p < 0.01$, Significantly different from baseline; $^{\#}$ $p < 0.05$ Significantly different from 24 hrs

ex: exercise

Training induced changes in mRNA expression

Expression of genes (mRNA) involved in muscle protein breakdown

In HC, expression of the majority of catabolic genes increased at 4 and 8 weeks of exercise, although (with the exception of calpain 3) this was not statistically significant **(Figure 6.7A)**. The COPD patients showed increased mRNA expression at 24 hrs, 4 and 8 weeks for MuRF1, 20S proteasome and ZNF216 although statistical significance was variable **(Figures 6.7B, 6.7C)**. There was no significant difference in the pattern of response to training between the COPD(P) and COPD(S) groups for the genes of interest.

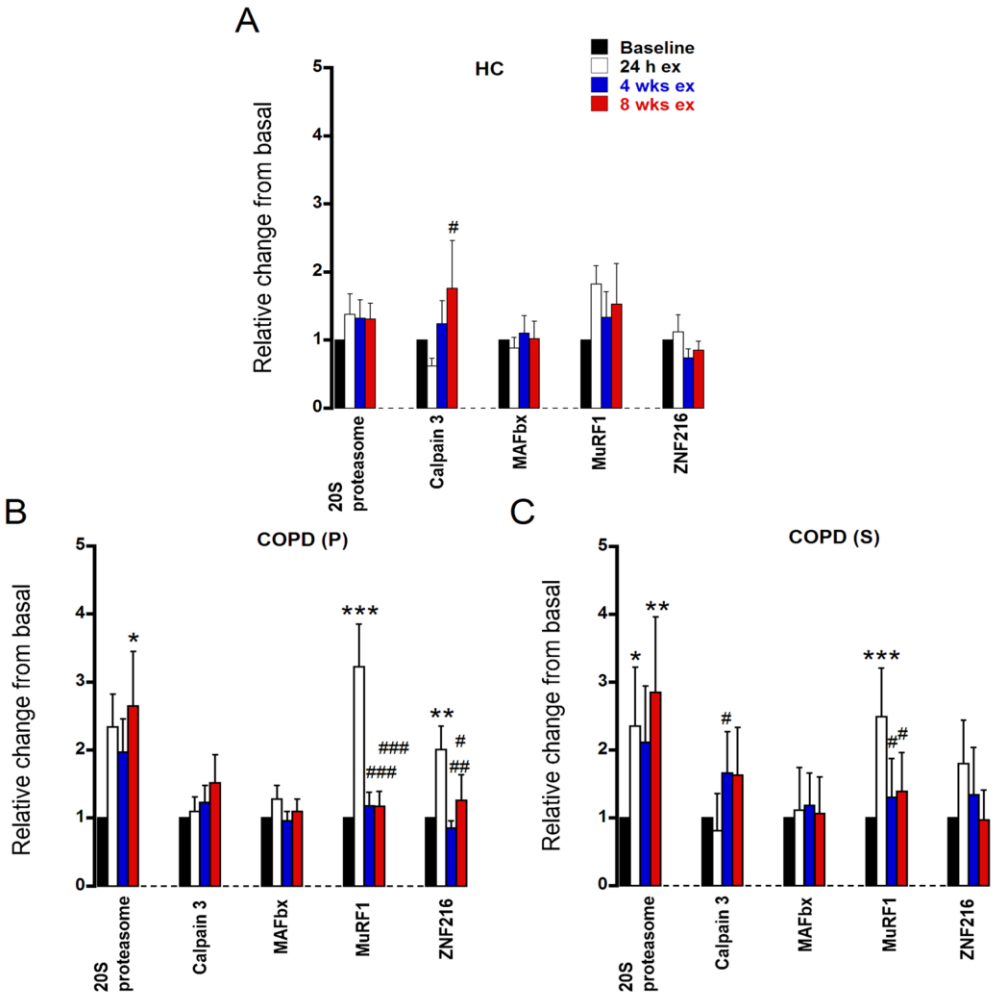


Figure 6.7 – Expression of target genes regulating muscle protein breakdown in response to training and dietary supplementation

Gene expression is represented as relative changes from basal. Values are expressed as $2^{-\Delta\Delta Ct}$ normalized to endogenous HMBS. Data represent mean ± SEM.

A: HC = Healthy Control subjects; B: COPD(P) = COPD patients receiving placebo supplements; C: COPD(S) = COPD patients receiving protein-carbohydrate supplements

* $p < 0.05$,** $p < 0.01$, Significantly different from baseline

$^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, Significantly different from 24 hrs

Expression of genes (mRNA) involved in muscle protein synthesis

In HC, there were increases in mRNA abundance although most were not statistically significant **(Figure 6.8A)**. The pattern of change in expression with training was similar for the COPD groups (although statistical significance was variable) with the exception of Akt1, which was up-regulated to a greater degree in the COPD groups **(Figures 6.8B, 6.8C)**.

There was a significant difference between the 2 COPD groups at 4 weeks ($p<0.01$) in the case of p70s6kinase mRNA expression.

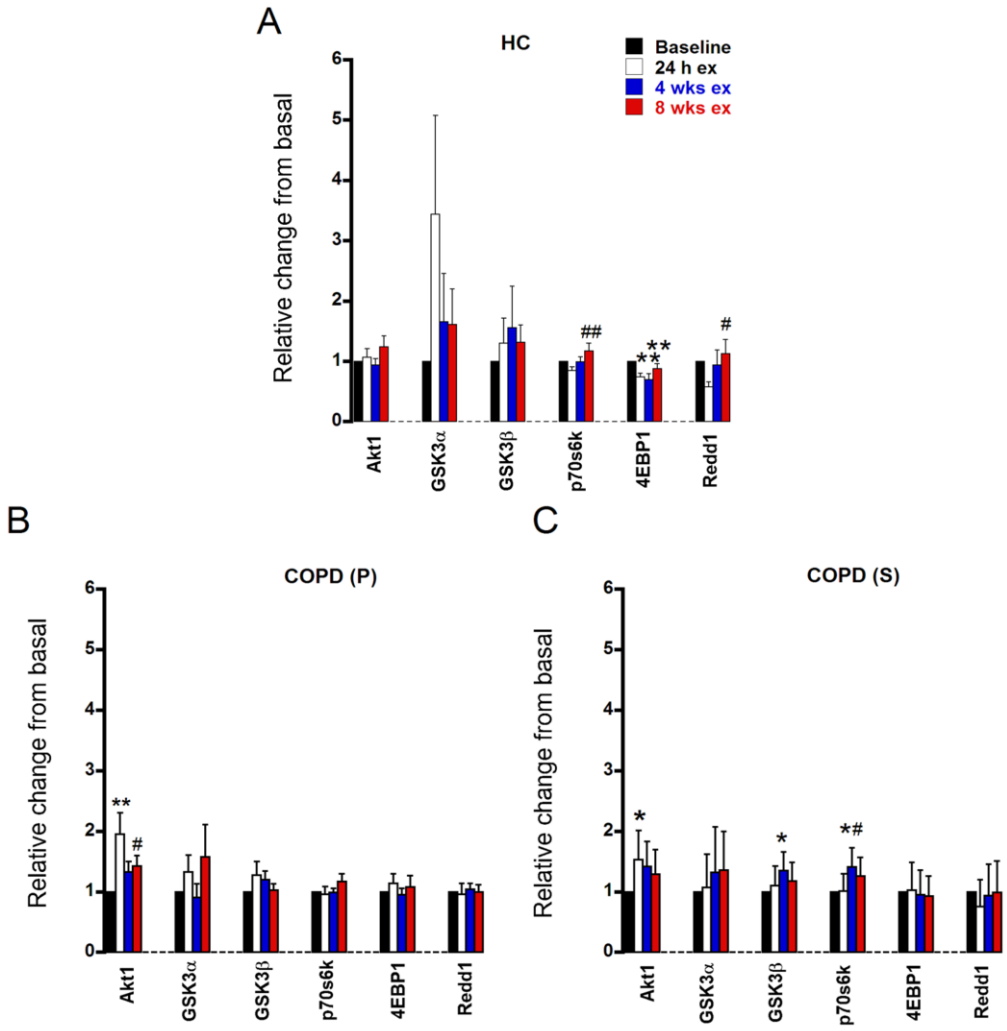


Figure 6.8 – Expression of target genes regulating muscle protein synthesis in response to training and dietary supplementation

Gene expression is represented as relative changes from basal. Values are expressed as $2^{-\Delta\Delta Ct}$ normalized to endogenous HMBS. Data represent mean ± SEM.

A: HC = Healthy Control subjects; B: COPD(P) = COPD patients receiving placebo supplements; C: COPD(S) = COPD patients receiving protein-carbohydrate supplements.

* $p < 0.05$, **$p < 0.01$, Significantly different from baseline;

$^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, Significantly different from 24 hrs

Expression of genes (mRNA) involved in myogenesis

In all 3 groups myostatin mRNA expression was significantly reduced at 24 hrs but had returned to baseline expression level at 4 and 8 weeks **(Figures 6.9A, 6.9B, 6.9C)**. MyoD and myogenin mRNA expression increased during training in all groups although statistical significance was variable.

There was also a significant difference between COPD(P) and COPD(S) groups in the expression of MyoD mRNA after 4 weeks of exercise training ($p<0.05$).

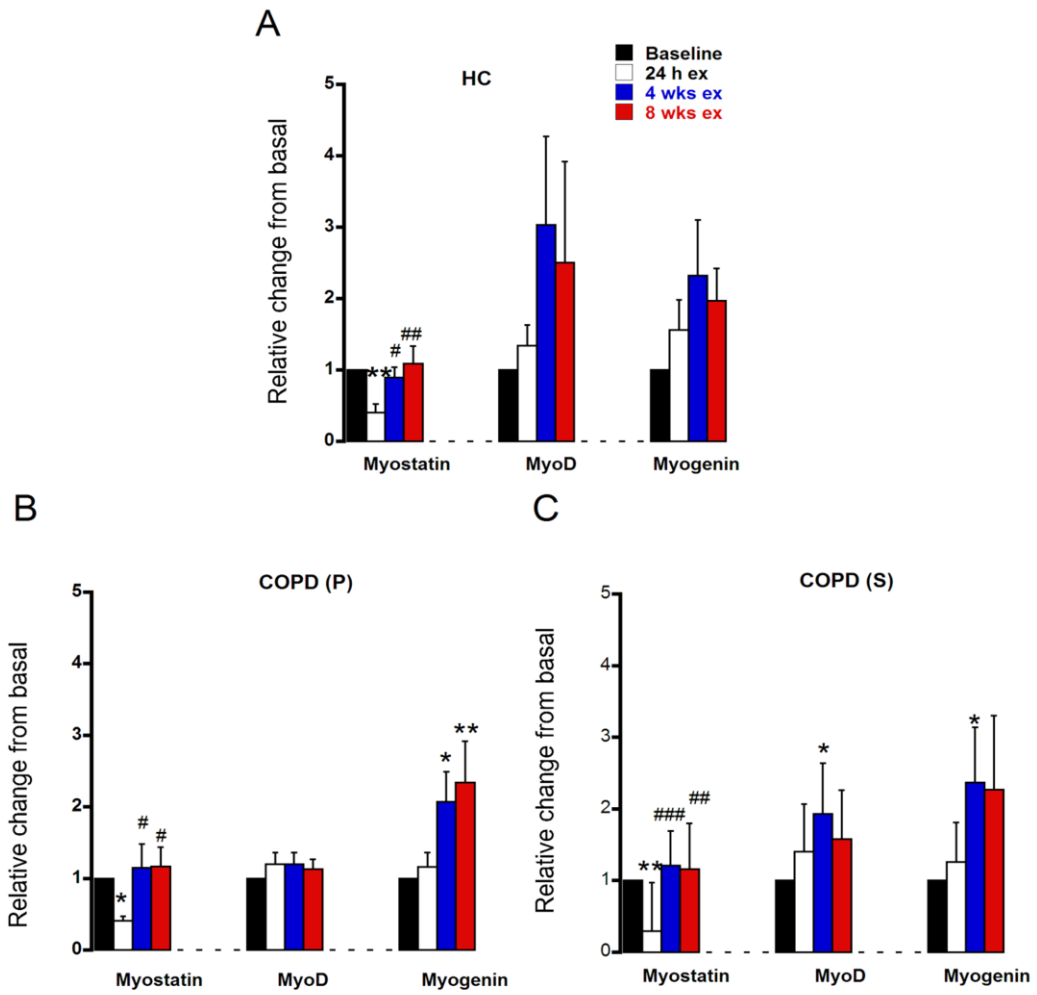


Figure 6.9 – Expression of target genes regulating myogenesis in response to training and dietary supplementation

Gene expression is represented as relative changes from basal. Values are expressed as $2^{-\Delta\Delta Ct}$ normalized to endogenous HMBS. Data represent mean ± SEM.

A: HC = Healthy Control subjects; B: COPD(P) = COPD patients receiving placebo supplements; C: COPD(S) = COPD patients receiving protein-carbohydrate supplements.

* $p < 0.05$, ** $p < 0.01$, Significantly different from baseline;

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, Significantly different from 24 hrs

Expression of muscle transcription factor mRNA

The pattern of change in mRNA expression of FOXO1 and FOXO3 was broadly similar between the HC and COPD groups **(Figure 6.10)**. RUNX1 was significantly up regulated after 24hr exercise in all groups, decreasing significantly following 4 and 8 weeks training.

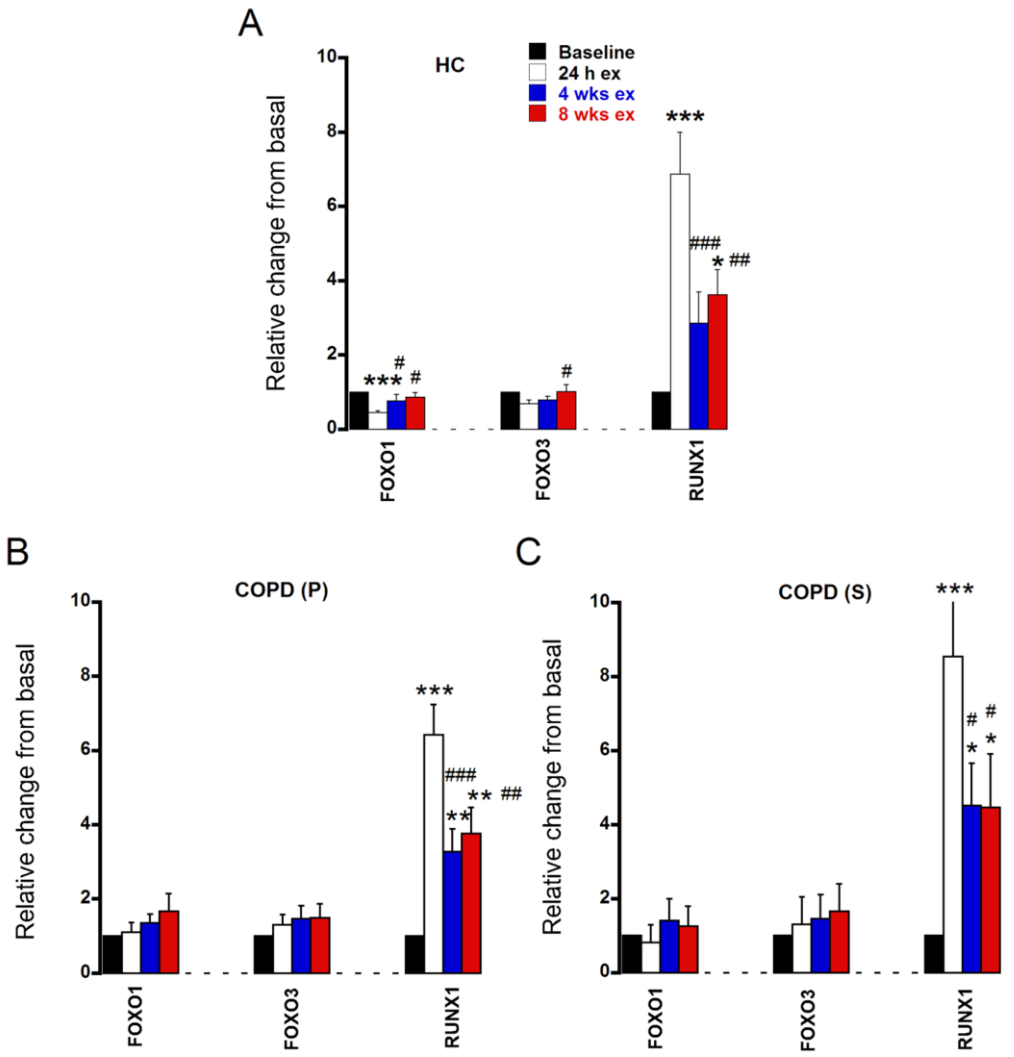


Figure 6.10 – Transcription factor gene expression in response to training and dietary intervention

Gene expression is represented as relative changes from basal. Values are expressed as $2^{-\Delta\Delta Ct}$ normalized to endogenous HMBS. Data represent mean ± SEM.

A: HC = Healthy Control subjects; B: COPD(P) = COPD patients receiving placebo supplements; C: COPD(S) = COPD patients receiving protein-carbohydrate supplements.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Significantly different from baseline

$^{\#}$ $p < 0.05$ $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, Significantly different from 24 hrs

Pro-inflammatory cytokine mRNA expression

TNF-α mRNA expression increased significantly in all 3 groups after 24hr exercise **(Figure 6.11)**. In all groups however, expression declined with further training but remained significantly different from baseline at 8 weeks in HC and COPD(S) groups. The pattern of change in muscle IL-6 mRNA expression was similar showing significant increases at 24 hrs and a subsequent decline after 4 and 8 weeks of training **(Figure 6.11)**. There were no significant differences in training induced expression of inflammatory genes between the COPD groups.

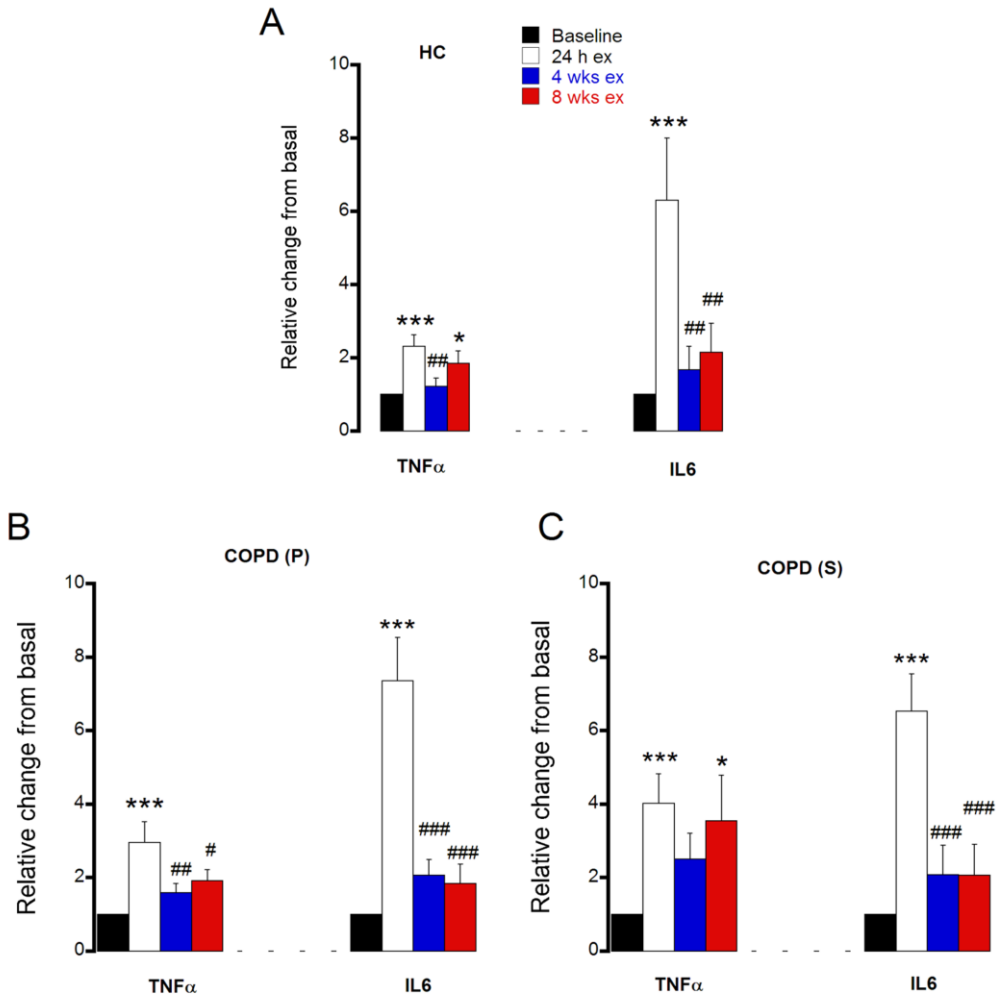


Figure 6.11 – Inflammatory cytokines gene expression in response to training and dietary intervention

Gene expression is represented as relative changes from basal. Values are expressed as $2^{-\Delta\Delta Ct}$ normalized to endogenous HMBS. Data represent mean ± SEM.

A: HC = Healthy Control subjects; B: COPD(P) = COPD patients receiving placebo supplements; C: COPD(S) = COPD patients receiving protein-carbohydrate supplements.

* $p < 0.05$, *** $p < 0.001$, Significantly different from baseline;

$^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, Significantly different from 24 hrs

Discussion

This is the first study to report detailed functional and molecular responses of skeletal muscle to RT and post-exercise protein-carbohydrate supplementation combined with RT in COPD patients and similar aged HC subjects. A major finding was that increases in thigh lean mass and knee-extensor strength over the course of 8 weeks of RT in COPD patients were similar compared to HC volunteers. Hence while baseline muscle function in COPD patients is compromised, its responsiveness to RT is preserved.

RT increased anabolic, catabolic and transcription factor protein expression (not unexpected given exercise increases muscle protein turnover) but the magnitude of increase was blunted in COPD. This was surprising given thigh lean mass and strength gains were similar, and points to a disconnection between changes in muscle protein expression and lean mass gains. There appeared to be a closer association between anabolic, catabolic and transcription factor protein expression levels and work done during RT as the latter was consistently lower in COPD patients **(Figure 6.2C)**. However, changes in myogenic protein expression with RT were similar in COPD and HC and may explain the similarity in lean mass gains. This is in line with the observation that testosterone mediated muscle hypertrophy in older people is associated with increased myogenin protein expression and satellite cell activation (Sinha-Hikim et al. 2006). Contrary to our initial hypothesis post-exercise dietary supplementation in COPD patients did not alter target gene and protein expression or thigh lean mass and functional gains when compared to training alone.

Single time-point studies comparing muscle anabolic and catabolic mRNA and protein expression levels from COPD and HC volunteers have proposed muscle atrophy in

COPD occurs as a consequence of increased ubiquitin-proteasome mediated muscle proteolysis, and that increased anabolic signalling may occur as compensatory phenomenon (Debigare et al. 2008; Doucet et al. 2007). Conversely, others report little differences in anabolic gene and protein expression levels between COPD and HC (Plant et al. 2010). MuRF1, MAFbx and nuclear FOXO1 protein expression was greater in COPD than HC at baseline in the present study **(Table 6.2)**. However, these traits were present even when thigh lean tissue mass and muscle inflammatory cytokine mRNA expression was similar between COPD and HC **(Tables 6.2 and 6.3)**, suggesting these molecular differences may be features of deconditioning, rather than increased MPB and wasting per se, which is supported by the lack of difference in proteasome protein expression at baseline. In the absence of MPB measurements it is not possible to be more conclusive. However increased muscle FOXO and MAFbx protein expression have been reported under conditions of altered muscle carbohydrate and lipid oxidation and in the absence of muscle wasting (Constantin et al. 2011). The greater muscle catabolic protein expression at baseline in COPD patients was also paralleled by greater expression of selected anabolic and myogenic signalling proteins, perhaps suggesting greater basal muscle protein turnover in COPD.

To my knowledge no study has documented the time-course of muscle functional and molecular events under conditions where muscle mass has been increased by RT in COPD patients. Muscle cytokine mRNA expression increased transiently in response to training, but the magnitude was the same in COPD and HCs **(Figure 6.11)**, and likely reflected an acute inflammatory response to unaccustomed exercise. Furthermore, this response, together with the transitory decrease in myostatin mRNA expression over the

same time-course, demonstrates short-term changes in mRNA abundance may be of limited physiological relevance in the absence of associated protein changes. This point is further substantiated by the lack of close alliance between changes in mRNA and protein abundance with training for each molecular target in the present study. Studies investigating molecular responses to RT in COPD have generally focussed on relatively few mRNA and protein targets (Troosters et al. 2010). The current study therefore extends previous reports by documenting responses of a substantially wider range of molecular targets (at both mRNA and protein level) to a training intervention that increased lean tissue mass and strength, capturing time-course changes. The disconnection between protein expression levels and muscle mass gains with RT is supported by data showing that increasing amino acid and insulin availability (thus doubling leg protein synthesis and halving leg protein breakdown in young, healthy volunteers) did not result in parallel changes in anabolic signalling activity (indicated by phosphorylation of the Akt-mTOR-P70S6k-eIF4F pathway), and more simply reflected changes in insulin availability (Greenhaff et al. 2008). Additionally, the marked decline in muscle protein synthesis observed during limb immobilization in healthy, young volunteers is not reflected by changes in expression levels of proteins comprising the mTOR signalling axis, which remained unchanged from basal (de Boer et al. 2007). A counter argument could be that measuring anabolic, catabolic and transcription factor protein expression in the resting fasted state as was done in the present study, does not provide significant insight regarding molecular responses mediated at the time of exercise. This may indeed be the case, but if so it is difficult to reconcile the numerous changes in mRNA and protein expression levels that were observed with RT in the present study. A likely explanation is that closer

association exists between changes in protein expression levels and work done during training than lean mass gains. In support of this, Burd et al recently demonstrated that the volume (not intensity) of work done during an acute bout of resistance exercise is positively associated with the magnitude of post-exercise p70S6K phosphorylation, and that this relationship exists for up to 30hrs following exercise which is considerably longer than in the present experiment (Burd et al. 2012). Furthermore, recent work by the same group demonstrates that the intensity of chronic RT does not determine the magnitude of training-induced muscle hypertrophy in young men (Mitchell et al. 2012). As this study demonstrates, this has far reaching implications for muscle rehabilitation in COPD and other wasting diseases.

Anabolic resistance of muscle to protein nutrition is a feature of ageing and has been proposed to be a causative factor in age-related sarcopenia (Cuthbertson et al. 2005; Kumar et al. 2009). It has been suggested that dietary supplementation might need to be combined with muscle contraction to facilitate muscle protein accretion in ageing (Cuthbertson et al. 2005; Dorrens and Rennie 2003). Furthermore, evidence in elderly subjects suggests that the timing of dietary supplementation in relation to exercise bouts is crucial (Esmarck et al. 2001). However, despite supplementation occurring immediately after each bout of training when the response to feeding is maximised (Esmarck et al. 2001), there was no observable effect of feeding on molecular or functional outcomes in this study. This suggests that dietary protein intake is not a major limitation to RT-induced muscle mass gains in COPD.

Controversy exists about whether impaired muscle mass and function can commonly be ascribed to COPD specific factors (e.g. systemic inflammation, hypoxia,

drug therapy) or whether it is predominantly due to physical inactivity which almost universally accompanies symptomatic disease. Results from this study showing that resistance exercise training increased thigh lean mass and knee-extensor strength substantially in COPD, and by the same magnitude as observed in control volunteers, provides further and substantial evidence that inactivity and deconditioning are key factors underpinning muscle dysfunction in COPD. Importantly, muscle mass and function increased with RT even in the face of increased catabolic mRNA and protein expression, which probably reflects exercise induced increases in muscle protein turnover. The observations from this study are limited to patients with preserved muscle mass in the stable state and who were able to undertake a demanding RT programme with repeated muscle biopsy sampling. However, the patients in this study had significant self-reported exercise limitation (MRC grades 3 to 5) demonstrating markedly impaired muscle strength and whole body aerobic capacity at baseline, suggesting the population was representative of those referred to pulmonary rehabilitation.

In conclusion, I have demonstrated that there is no disease specific barrier to increasing lean tissue mass and function through RT in COPD patients with significant lung function impairment, exercise intolerance and muscle weakness at baseline. I have also shown that increasing post-exercise dietary protein and carbohydrate intake is not a pre-requisite for achieving a normal training response in COPD. The observation that (with the exception of myogenic proteins) gains in lean mass are not tightly coupled to the magnitude of change in protein expression suggests caution is required when identifying potential targets for intervention.

CHAPTER 7

Inflammatory and Satellite Cells in the Quadriceps of Patients with COPD and the Response to Resistance Training

In this chapter I will describe the response of inflammatory and satellite cells in the quadriceps of patients and healthy controls following the RT programme. I have adapted an immunostaining technique previously used for studying inflammatory cells in bronchial biopsy specimens, and applied it to study muscle samples obtained from the quadriceps.

Introduction

Quadriceps weakness is an important extra-pulmonary manifestation of Chronic Obstructive Pulmonary Disease (COPD) (ATS/ERS 1999) that is directly linked to a number of adverse outcomes such as impaired exercise capacity (Gosselink et al. 1996), increased healthcare utilisation (Decramer et al. 1997) and reduced survival (Swallow et al.

2007). Exercise training, which is a key component of pulmonary rehabilitation, can improve various measures of quadriceps function including strength, endurance and muscle oxidative capacity (ATS/ERS 1999;Ries 2007). In healthy individuals, acute exercise leads to a skeletal muscle inflammatory cell response characterised by the infiltration of neutrophils and macrophages into muscle tissue (Tidball 2005). However the muscle cellular inflammatory response to acute and prolonged exercise in health and COPD is unknown. Given that inflammatory mechanisms have been postulated to be important in the aetiology of peripheral muscle dysfunction in COPD, it is crucial to know whether acute exercise and physical training influence local muscle inflammation in these patients with respect to magnitude, duration and inflammatory cell profile. Conversely, exercise-induced muscle cellular inflammation may be an indicator that sufficient muscle overload has occurred to induce physiological training adaptation. Given the low absolute exercise intensities that patients with COPD can achieve, the presence of such inflammation and it's modification with training might shed light on the mechanisms that underpin the RT response in COPD.

Satellite cells are undifferentiated myogenic precursor cells that play an important role in the maintenance of muscle mass and regeneration of injured muscle fibres (Morgan and Partridge 2003).These cells are usually mitotically quiescent, but become activated and proliferate rapidly in response to various stimuli such as exercise and muscle injury. Impaired muscle regeneration has been suggested as a potential mechanism for the loss of muscle mass in COPD (Hansen et al. 2006), but the distribution of satellite cells in the quadriceps of patients, and quantifying their response to RT, has not been previously studied.

The aim of this study was to examine the profile of inflammatory and satellite cell infiltration and muscle fibre composition in biopsy specimens from the vastus lateralis of COPD and similar aged healthy controls at baseline, and the response to both acute exercise and prolonged RT. The study hypothesis was that despite lower contraction intensities during RT in COPD, there would be an acute cellular intramuscular inflammatory response to exercise that might be attenuated by prolonged RT. A secondary hypothesis was that the muscular adaptation to RT will be associated with an observable increase in muscle satellite cells.

Materials and Methods

Full methodological details are provided in **chapter 4**.

Subjects

17 COPD and 10 age-matched healthy controls were included in this study. Stable outpatients who met clinical and spirometric criteria for moderate to severe COPD (FEV_1/FVC ratio <70%, FEV_1<50% predicted) with significant self-reported exercise limitation (MRC Grades 3, 4 or 5) were included in the study. Exclusion criteria were oral corticosteroid, oral anticoagulant or long term oxygen therapy. Patients with co-morbid conditions contributing to exercise limitation or preventing exercise training were also excluded. They were all participating in a larger randomised controlled trial looking at the effects of lower limb RT and dietary supplementation on skeletal muscle molecular signalling pathways. Patients were randomly allocated to receive a dietary protein-carbohydrate supplement or placebo after each bout of training. Healthy volunteers were only given placebo. Because we found no significant differences in outcomes between

patients receiving supplement or placebo, in this report we have presented data for the whole COPD cohort in comparison with healthy controls. The study was approved by the Leicestershire and Rutland Research Ethics Committee (Ref: 06/Q2501/138), and all participants provided written informed consent.

Resistance Training Protocol

Participants underwent 8 weeks of bilateral, lower limb, high-intensity isokinetic RT on an isokinetic dynamometer (Cybex II Norm, CSMi, Stoughton, MA, USA) (Jones et al. 2004). Five sets of 30 maximal isokinetic knee contractions at an angular velocity of 180°/second were performed three times per week for eight weeks. The basic measurements recorded were the peak torque in Newton-metres (Nm) and total work done in Joules (J). This training programme was chosen because high velocity muscle contraction preferentially activates type II fibres and because previous studies in healthy subjects using this protocol demonstrated significant functional and molecular adaptation at a muscle level (Jones et al. 2004).

Quadriceps Function and Exercise Performance

Functional parameters were measured at baseline and after 8 weeks. Total body lean mass was measured by DEXA (Lunar Prodigy Advance, GE Healthcare, UK), normalised for height, and expressed as the fat free mass index. Thigh lean mass (T_{dexa}) was calculated as the area between the ischeal tuberosity superiorly, and knee joint line inferiorly. Isometric (maximal static contraction with the knee at 70° flexion) and isokinetic (dynamic knee extension at a pre-set angular velocity of 60°/second) quadriceps strength were measured on the cybex. Subjects also performed a maximal, symptom-limited cycle ergometry test (Ergometric Er900; Ergoline GmbH, Bitz, Germany).

Muscle Biopsies and Immunostaining

Using a microbiopsy technique (Hayot et al. 2005), an ultrasound-guided biopsy of the dominant vastus lateralis muscle was obtained at 3 time points – baseline, 24 hours following the first bout of exercise, and after 8 weeks, 24 hours after the last exercise bout. A detailed explanation of the muscle biopsy technique is provided in **chapter 4**. Biopsies were immediately fixed in ice-cold acetone containing the protease inhibitors iodoacetamide and phenylmethylsulfonyl fluoride (PMSF), then processed into glycol methacrylate (GMA) resin as described previously (Bradding et al. 1994). Two micron sections were cut using an ultramicrotome (Leica-RM2155, Leica Microsystems, UK) and immunohistochemistry performed (Bradding et al. 1994). Aminoethylcarbazole was used as the chromogen giving a red reaction product, and sections counterstained with Mayers haematoxylin. The following mouse IgG1 primary monoclonal antibodies were used: Neutrophil elastase (NE) to neutrophils (clone NP57; 42 µg/ml; 1:1000 dilution; Dako Ltd, UK), CD163 to macrophages (clone RM3/1; 10 µg/ml; 1:10 dilution; Cambridge Bioscience Ltd, UK), Pax-7 to satellite cells (clone EE-8; 2µg/ml; 1:50 dilution; Santa Cruz Biotechnology, Inc, USA), anti-fast skeletal muscle myosin to type II fibres (clone MY-32; 5µg/ml; 1:200 dilution; Sigma-Aldrich Inc., St. Louis, USA), and anti-slow skeletal muscle myosin to type I fibres (clone NOQ7.5.4D; 5µg/ml; 1:200 dilution; Millipore UK, Ltd). Appropriate controls were performed with either the primary antibody omitted or with the addition of matched isotype controls (Negative control mouse IgG1; 1:100 dilution; Dako Ltd UK). Samples were also initially stained for T lymphocytes (CD3) and Eosinophils (MBP). However as these cells were not identified in the muscles of either controls or patients, they were subsequently removed from the staining panel. All sections were

visualized using light microscopy (Olympus BX50), and images were acquired by a digital camera (DP72, Olympus UK Ltd.) connected to the microscope.

Image Analysis and Measurements

Image processing and quantitative analyses were performed using the Cell^F software package (Version 3.2, Olympus UK Ltd.), and by an investigator blinded to the subject's identity and sampling time point. For fibre-typing and measurement of fibre cross-sectional area (CSA), four random regions were selected on each slide and photographed under low power (x10). Within each image, an area was delimited and the total number of type I and type II fibres were counted. In addition, the mean fibre CSA for each fibre type was measured by planimetry, taking care to avoid any freeze-damaged or longitudinally oriented fibres.

The slides for inflammatory cells, myonuclei and satellite cells were visualised at higher magnification (x20 or x40). Six random regions were chosen on each slide and photographed. As before, an area (TA) was delimited on each image, measured with a planimeter, and the total number of fibres (Tfib) and their CSAs (Tcsa) determined. . Cells showing positive staining for NE and CD163 were counted in each area and expressed as cells/mm^2 interstitial area **[Interstitial area = TA-(Tfib x Tcsa)]**.

Similarly pax-7+ cells and myonuclei were identified, and satellite cells were expressed as a proportion of the total number of myonuclei. i.e.

$$\frac{\textbf{Total number of pax7 cells}}{\textbf{(Total number of pax7 cells + Total Number of myonuclei)}} \times 100$$

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 5.01 for Windows (GraphPad Software Inc, California, USA) and SPSS 16.0 for Windows (SPSS Inc, Chicago, USA). Data were expressed as means (±SEM) or medians (interquartile range, IQR). Nonparametric repeated measures ANOVA (Friedman's test) and Dunn's post test was used to compare the number of inflammatory cells at various time-points. Spearman's correlation coefficient describes the relationship between measures of quadriceps muscle performance and inflammatory cell response. The relative percentage of satellite cells at various time points was normally distributed in COPD patients (based on the D'Agostino-Pearson normality test), while in healthy controls, the number of subjects were too small (n=7) to perform the normality test. Hence parametric repeated measures 1-way ANOVA with Tukey's multiple comparison post-hoc test was used to compare the relative % of pax-7+ cells at the 3 time-points. All statistical tests were two-tailed and the threshold of statistical significance was a p value <0.05.

Results

Participants

Eight participants (5 COPD and 3 healthy) were excluded, as their baseline biopsies consisted of predominantly fat tissue and therefore were unsuitable for analysis. Consequently only 19 subjects (12 COPD and 7 controls) had muscle biopsies analysed at 3 time-points, their baseline characteristics are shown in **Table 7.1**. Even when all participants (n=27) were included in the analysis, baseline subject characteristics and training induced functional outcomes were broadly similar. Subjects were well matched

for age – the majority being males, and patients had significantly worse lung function than healthy controls.

	Healthy Controls (n= 7)	COPD (n= 12)
Age, years	66.7 ± 1.9	66.7 ± 2.0
Male sex, n	5	10
FEV_1 (Litres)	2.7 ± 0.1	1.2 ± 0.1***
FEV_1 (% predicted)	103.4 ± 6.4	46.4 ± 5.9***
BMI (kg/m^2)	27.7 ± 0.9	26.1 ± 2.0
FFMI (kg/m^2)	18.8 ± 0.6	18.8 ± 0.8
T_{dexa} (g)	4562.0 ± 254.0	4420.9 ± 365.3
Quadriceps Isometric Peak Torque (Nm)	153.4 ± 16.0	134.0 ± 12.7
Quadriceps Isokinetic Concentric Torque (Nm)	117.1 ± 8.6	93.0 ± 8.8
Quadriceps Isokinetic Peak Work (J)	455.5 ± 36.9	344.8 ± 38.8
Peak VO_2 (ml/kg/min)	22.5 ± 1.8	16.1 ± 1.7*
Peak Workload (W)	126.5 ± 10.6	47.4 ± 6.4***

Table 7.1 – Baseline characteristics of study participants

Values presented Means ± SEM, except gender (absolute number)
* p<0.05, *** p<0.0001, COPD vs. Healthy

Quadriceps Function and Exercise Performance

T_{dexa} was not significantly different between patients and healthy controls at baseline **(Table 7.1)**. Isokinetic and isometric quadriceps strength, and isokinetic work output tended to be lower in the COPD group. Cycle ergometry performance at baseline was significantly better in healthy controls. T_{dexa} and isometric quadriceps strength increased significantly in both patients and controls after 8 weeks, whereas isokinetic strength and work output only increased in COPD subjects **(Table 7.2)**. Quadriceps training resulted in increased whole-body exercise performance in both groups and improved peak VO_2 in controls.

	Healthy Controls (n= 7)		**COPD (n= 12)**	
	Mean change	**p value**	**Mean change**	**p value**
T_{dexa} (g)	234.5 ± 52.9	0.004	322.4 ± 84.0	0.003
Quadriceps Isometric Peak Torque (Nm)	15.5 ± 6.2	0.046	17.7 ± 4.2	0.002
Quadriceps Isokinetic Concentric Torque (Nm)	10.1 ± 6.1	0.151	23.4 ± 5.5	0.001
Quadriceps Isokinetic Peak Work (J)	22.8 ± 23.1	0.362	104.5 ± 22.4	0.001
Peak VO_2 (ml/kg/min)	4.3 ± 1.1	0.010	0.1 ± 1.5	0.912
Peak Workload (W)	10.2 ± 2.7	0.010	9.5 ± 3.0	0.011

Table 7.2 – Training-induced changes in quadriceps function and exercise performance
Values presented as Means ± SEM

Inflammatory Cells

Table 7.3 summarises the changes to inflammatory cells and muscle fibre CSA at relevant sampling time-points during the study. NE gave intense cytoplasmic staining **(Figure 7.1d)**, while CD163 stained the periphery of cells **(Figure 7.2d)**. NE+ cells were significantly elevated in the quadriceps of patients at baseline ($p=0.03$) when compared to controls, in whom they were undetectable. CD163+ cells were detected in muscle biopsies from 2 out of 7 controls at baseline, whereas they were present in 9 out of 12 COPD patients. A significant increase in NE+ and CD163+ cells were seen at 24 hrs after the first bout of exercise in both groups **(Figures 7.1a, 7.1b, 7.2a, 7.2b)**. After 8 weeks training, NE+ and CD163+ cell counts were close to resting baseline values in spite of intensive exercise 24 hrs beforehand. The total amount of quadriceps work performed by COPD patients during the first exercise session was significantly lower when compared to the healthy controls ($p<0.05$). A positive relationship ($r =0.5$; $p=0.02$) was observed between the CD163 response measured at 24 h for the whole group, and the total amount of quadriceps work performed by the dominant thigh during the first exercise session **(Figure 7.3)**.

	Baseline	24 h	Week 8
NE (cells/mm^2 Interstitial Area)			
COPD *(n=12)*	4.1 (0.0-21.0)¶	137.9(30.1-217.9)**	0.0(0.0-15.9)$^{\#\#}$
Healthy *(n=7)*	0.0 (0.0-0.0)	153.4(13.0-305.5)*	14.2(0.0-17.3)
CD163 (cells/mm^2 Interstitial Area)			
COPD *(n=12)*	10.4(2.1-34.1)	90.3(40.9-135.7)*	26.9(12.7-72.5)
Healthy *(n=7)*	0.0(0.0-27.0)	146.7(64.3-172.2)*	0.0(0.0-59.2)$^{\#}$
Type I Fibre CSA (μm^2)			
COPD (n=12)	8433 ± 776.6	-	8618 ± 1159
Healthy (n=7)	7309 ± 288.5	-	8238 ± 477.1
Type II Fibre CSA (μm^2)			
COPD (n=12)	6606 ± 565.4	-	7843 ± 962.7^{+}
Healthy (n=7)	6549 ± 266.9	-	7570 ± 484.1^{+}

Table 7.3 – Muscle inflammatory cells and fibre CSA

Values for NE and CD163 presented as medians (IQR), and data at various time-points compared using nonparametric repeated measures ANOVA (Friedman's test) and Dunn's post test:
** p< 0.001, * p< 0.05, 24 hrs vs. baseline
$^{\#\#}$ p< 0.0001, $^{\#}$ p< 0.05, week 8 vs. 24 h
¶ p<0.05, Wilcoxon Signed Rank test; COPD vs. Healthy at baseline
Values for Fibre CSA presented as Means ± SEM. Fibre type assessments were not performed at 24 h.
$^{+}$ p < 0.05, week 8 vs. baseline

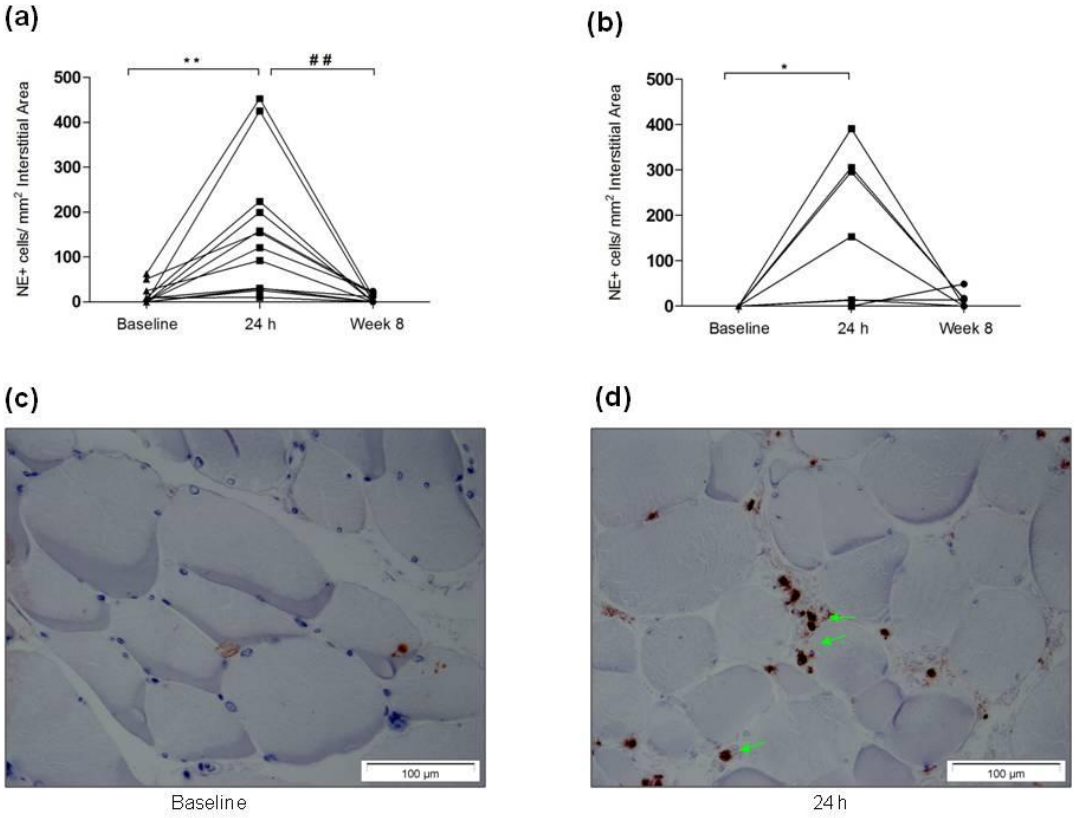


Figure 7.1 – Neutrophil counts at relevant sampling time-points in the study
(a) COPD and (b) healthy controls; muscle biopsy sections were immunostained for neutrophils using an antibody targeting neutrophil elastase, (green arrows). Representative photomicrographs are shown: (c) at rest and (d) at 24 hrs.
ANOVA ** p< 0.001, 24 hrs vs. baseline; $^{\#\#}$ p< 0.0001 week 8 vs. 24 hrs; * p< 0.05, 24 hrs vs. baseline

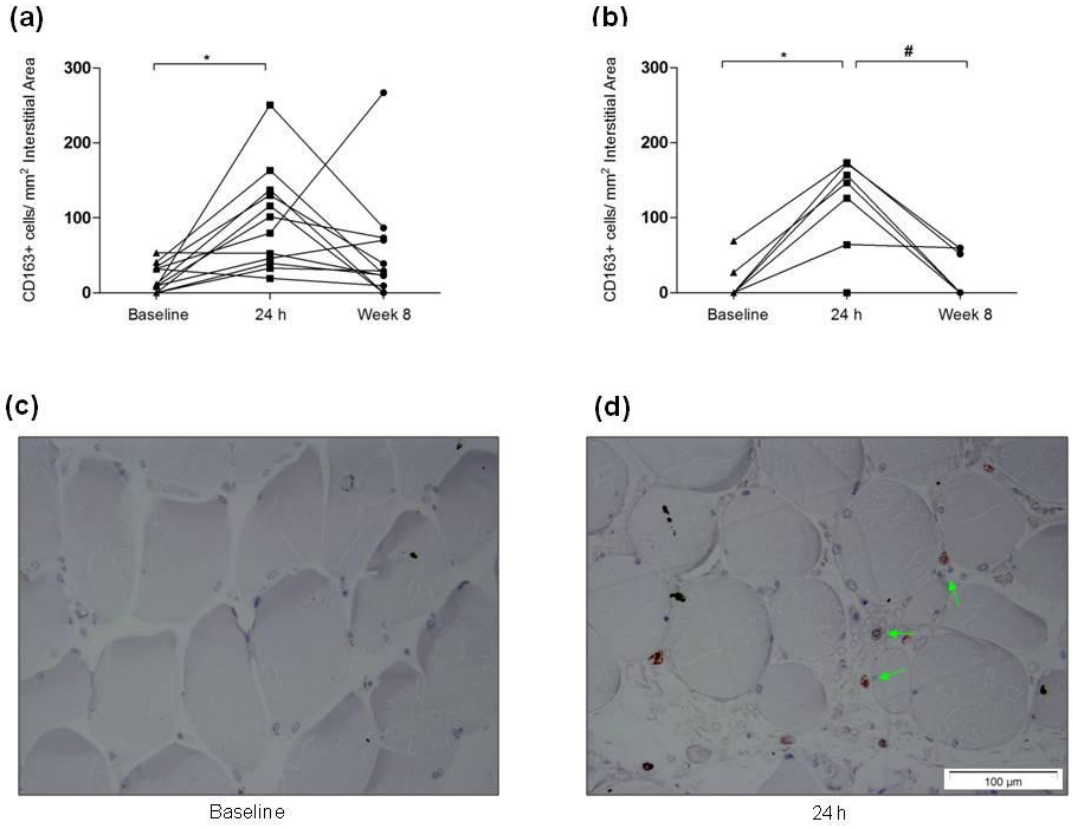


Figure 7.2 – Macrophage counts at relevant sampling time-points in the study
(a) COPD and (b) healthy controls; muscle biopsy sections were immunostained for macrophages using anti-CD163, which stains the periphery of the cells (green arrows). Representative photomicrographs are shown: (c) at rest and (d) at 24 hrs
ANOVA * $p<0.05$, 24 hrs vs. baseline; $^{\#}$ $p< 0.05$, week 8 vs. 24 hrs

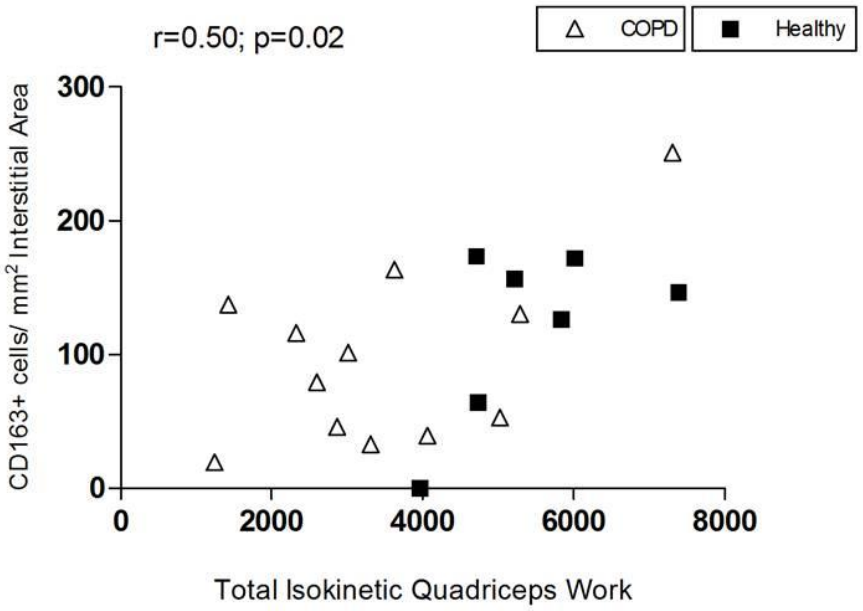


Figure 7.3 – Relationship between quadriceps work performed on the cybex during the first exercise session, and muscle macrophage response at 24 hrs
(r = 0.50; p = 0.02)

Satellite Cells

Distinct immunostaining for pax-7 was evident in satellite cells around myofibre borders (Figure 7.4d). At baseline, the proportion of pax-7+ cells was not significantly different between patients and controls There was a nonsignificant increase in the relative % of pax-7+ cells at 24 hrs in COPD patients (p=0.07). In the healthy control group, some subjects demonstrated an increase in the relative % of pax-7+ cells at 24 hrs, but overall this was not significant (p=0.18). After 8 weeks, the proportion of pax-7+ cells tended to remain above baseline in both groups **(Figures 7.4a, 7.4b)**.

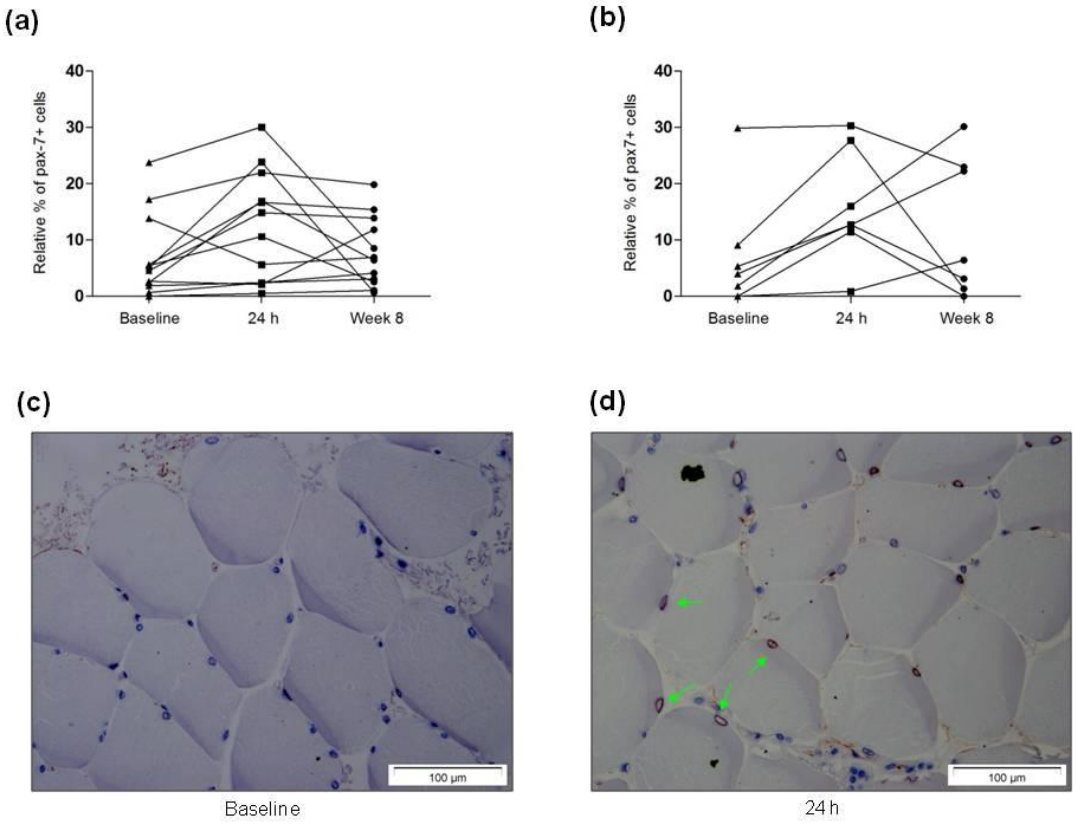


Figure 7.4 – Satellite cell numbers at relevant sampling time-points in the study
(a) COPD and (b) healthy controls; muscle biopsy sections were immunostained for satellite cells using anti- pax-7. While myonuclei stain blue due to the uptake of

haematoxylin, satellite cells stain red for pax-7 around the muscle fibre border (green arrows). Representative photomicrographs are shown: (c) at rest and (d) at 24 h.

Fibre typing and CSA

At baseline, type I and type II fibre CSAs were not significantly different between COPD patients and controls. Fibre staining was not performed on the 24 hrs muscle biopsy specimens. While the CSA of type I fibres remained unchanged post training in both groups, type II fibre CSA increased significantly in both patients (25.7%; p=0.03) and controls (15.4%; p=0.03) **(Table 7.3)**. The proportion of type II fibres at baseline tended to be higher in COPD when compared to controls (71.7 ± 3.9% vs. 60.5 ± 3.9%, p=0.07). A positive relationship was also observed between the baseline type I fibre-proportion in COPD and FEV_1 (r=0.74; p=0.005), percentage predicted FEV_1 (r=0.81; p=0.001), and peak VO_2 (r=0.62; p=0.02) **(Figure 7.5)**.

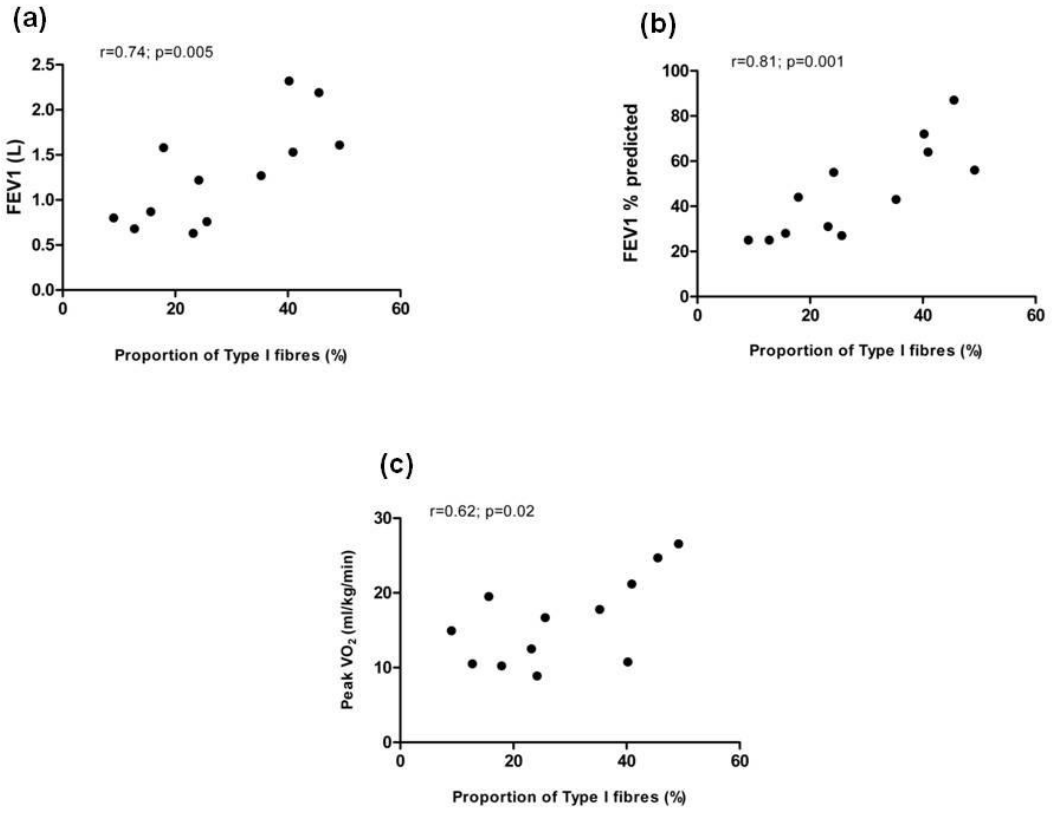


Figure 7.5 – Relationship between the proportion of type I fibres in COPD at baseline and (a) FEV_1, (b) FEV_1 percentage predicted, and (c) peak VO_2

Discussion

This study reports for the first time the intramuscular cellular response to acute and prolonged resistance exercise in healthy controls and COPD patients. The initial hypothesis was confirmed, showing that despite lower exercise intensities, there was an inflammatory response to acute resistance exercise in COPD which was attenuated by prolonged RT. However, we could not confirm the hypothesis that high intensity RT is associated with increased numbers of satellite cells, as only a trend ($p = 0.07$) towards an increase after training was observed.

When compared to controls in the resting state, there was a significant increase in the density of neutrophils in the quadriceps of COPD patients. The density of macrophages was not statistically different between the 2 groups at baseline, but they were present in most patients and undetectable in the majority of controls. Few studies have examined the distribution of inflammatory cells in the peripheral muscles of patients with COPD. While Gosker et al. showed no difference in leucocytes and macrophages in the quadriceps of patients and age-matched controls (Gosker et al. 2003), another group demonstrated increased numbers of macrophages (CD163) and T-cells (CD154) in COPD patients (Montes et al. 2005). More recently, inflammatory cell counts (CD45 and CD68) were shown to be significantly elevated in the quadriceps of patients with severe COPD when compared to controls (Barreiro et al. 2011). In line with the latter studies, this report also suggests that in patients with COPD, ongoing inflammation is occurring in the peripheral muscles at rest.

The inflammatory cell response to a bout of resistance exercise in this study consisted of an acute increase in muscle neutrophil and macrophage counts at 24 hrs in

both groups. The response was comparable between patients and controls, even though the former managed to perform significantly less quadriceps work during the first exercise session. After 8 weeks training, this inflammatory cell response to a bout of exercise was attenuated, suggesting an adaptive mechanism with regular training. It is well established that acute exercise in healthy subjects leads to a typical inflammatory cell response characterised by the infiltration of neutrophils and macrophages into the muscle (Tidball 2005). Muscle invasion by neutrophils is believed to occur within an hour of exercise, and their levels can remain elevated for up to 5 days (Fielding et al. 1993). The functional significance of these changes remains uncertain. It is likely that neutrophils are recruited acutely by factors such as chemokines released by exercising muscle, perhaps as a result of damage to muscle unaccustomed to high intensity contraction. This would suggest the primary role of both neutrophils and macrophages might be to phagocytose and remove cellular debris that accumulates. That training leads to attenuation of this inflammatory response would support this hypothesis. Interestingly, the observation that the difference in inflammatory cell infiltration evident at baseline between health and COPD was lost at 8 weeks post training raises the possibility that exercise training in COPD exerts a beneficial anti-inflammatory effect.

Neutrophils are also thought to cause muscle damage by the release of proteolytic enzymes and free radicals (Tidball 2005), and their presence in the pre-trained state in COPD suggests there is the potential to contribute to muscle dysfunction. The role of invading macrophages is less well recognised, although animal and in-vitro data suggests that they may promote both muscle injury (Nguyen and Tidball 2003) and repair (Merly et al. 1999). The current study suggests that even at lower workloads, the muscles of patients

are under sufficient stress to cause cellular inflammation. This is in keeping with previously reported data showing that metabolic stress occurs at low absolute exercise workloads in COPD (Calvert et al. 2008; Steiner et al. 2005). It is not clear whether these changes are pathological or represent part of the normal training adaptation to intense exercise; similar findings in health suggest the latter. The positive correlation between total quadriceps work and the increase in macrophage counts seen at 24 hrs indicates that the magnitude of the inflammatory cell response depends on the intensity of work performed during exercise. This again supports the view that the inflammatory response occurs in response to sub clinical muscle damage in untrained tissue.

To my knowledge the intramuscular cellular inflammatory response to either acute or prolonged exercise has not been reported in patients with COPD. Mercken et al observed increases in muscle IL-6 transcripts following acute exercise in a cohort of COPD patients but did not measure the response to prolonged training (Mercken et al. 2011) . Others have reported no up regulation of muscle IL-6 or TNFα mRNA expression in response to aerobic training (Rabinovich et al. 2003; Vogiatzis et al. 2007) but did not assess the response to acute exercise. Indeed in the study of Vogiatzis et al, baseline biopsies were obtained 24 hrs after the first training session and therefore may have been influenced by the effects of acute exercise. This highlights difficulties in comparing training studies where different training regimes, timing of tissue sampling and analytical targets have been used.

There was no significant difference in the number of satellite cells between COPD and controls at baseline. The increase in satellite cell numbers in response to acute exercise was heterogeneous, and although not statistically significant, there was a trend

towards an increase in the number of pax-7+ cells 24 hrs post-exercise in both groups. Regular exercise training appeared to maintain the increased numbers of satellite cells at 8 weeks. Although speculative, this data may be suggestive of a preserved regenerative capacity of the quadriceps in this study population. Satellite cells are a small population of stem cells that are responsible for the maintenance, growth and repair of terminally differentiated adult skeletal muscle fibres. They are anatomically located along the periphery of the myofibre, within indentations between the plasma membrane and basal lamina (Kadi et al. 2005). Satellite cells remain quiescent under normal physiological conditions, but are activated in response to stimuli such as exercise and muscle injury. Activated satellite cells undergo proliferation, and differentiate into mature muscle cells, while a small proportion return to quiescence (Zammit et al. 2004). The identification of these cells has become easier in recent years with the discovery of a number of molecular markers including pax-7, which is expressed selectively in quiescent, activating and proliferating satellite cells (Kuang and Rudnicki 2008). The distribution and activation of satellite cells has been extensively studied in various populations including the elderly (Kadi et al. 2004), in whom RT has been shown to increase the satellite cell pool (Mackey et al. 2007). In COPD, impaired muscle myogenesis has been suggested as a potential mechanism for the development of cachexia (Hansen et al. 2006), although to date this has not been substantiated. My observations are restricted to the quantity of satellite cells in muscle tissue, as the Pax-7 marker does not distinguish quiescent from activated and proliferating satellite cells. Additional analysis using markers such as MyoD and Ki-67 might allow this distinction. Interestingly, previous studies measuring the expression of

MyoD mRNA and protein have suggested training has a significant effect on satellite cell activity (Vogiatzis et al. 2007; Vogiatzis et al. 2010).

The trend towards a higher proportion of type II muscle fibres (71.7 ± 3.9%) observed in patients in this study is consistent with previous data showing a shift in fibre type from I to II in COPD (ATS/ERS 1999). Similarly, the observed association between worsening lung function and a decrease in the proportion of oxidative type I fibres has also been shown previously (Gosker et al. 2007). In addition a significant positive relationship between peak exercise capacity and type I fibre proportions has been demonstrated, which suggests that muscle fibre composition in COPD may be a marker of physical fitness. As expected from high intensity resistance exercise at a high angular velocity, training lead to a significant increase in type II fibre CSA after 8 weeks. Hence the macroscopic increase in thigh muscle mass after resistance training was mirrored at the cellular level by an increase in fibre CSA. Resistance type exercise training in healthy individuals preferentially causes hypertrophy of type II fibres (Folland & Williams 2007). There is data to show that in COPD, 10 weeks of RT, either alone, or combined with testosterone supplementation, can lead to an increase in the CSA of type II fibres (Lewis et al. 2007). The results from the current report are in keeping with this study.

The analysis of the muscle samples collected in this study has been facilitated by the use of the GMA embedding and immunostaining technique. The microbiopsy method of tissue sampling used in this study has been validated against the more traditional Bergstrom technique in COPD (Hayot et al. 2005). It is less invasive, better tolerated, and allows multiple sampling of vastus lateralis muscle. The quantity of muscle obtained by

this method may be limited; nevertheless immunostaining can be performed on these samples as the GMA technique only requires small amounts of muscle for analysis.

I acknowledge a number of limitations in this study. Muscle biopsies from several female participants were unsuitable for analysis due to infiltration of fat and connective tissue, a condition referred to as myosteatosis (Narici and Maffulli 2010). When compared to healthy controls, the COPD population in this study had similar mean thigh muscle mass, and it is possible that the inflammatory and myogenic responses might have been different in a wasted group. Data on baseline habitual physical activity was not collected, and this may have influenced the condition of the muscles at baseline and confounded the cross-sectional comparison between patients with COPD and healthy controls. The satellite cell content may be specifically reduced in type II muscle fibres in the elderly (Verdijk et al. 2007), and shown to increase with prolonged RT (Verdijk et al. 2009a). A similar fibre type-specific analysis of satellite cell content was not performed in this study. The response of the satellite cells to exercise was heterogeneous and, although there was a trend towards an increase that was not statistically significant, this study may have been underpowered to detect these differences. Similarly, the selection criteria and small sample size may limit the degree to which the findings can be generalised to the wider COPD population.

The findings from this study may have implications for the pathogenesis of skeletal muscle dysfunction in COPD and the conduct of pulmonary rehabilitation programmes. The observation of an exercise induced inflammatory “myositis” in patients with COPD at lower exercise intensities suggests that these patients are more likely to trigger this response during day to day activities. This might explain the low grade

inflammatory cell infiltrate observed at baseline in the COPD group and could theoretically be a driver of inflammation-related skeletal muscle wasting and dysfunction. The acute response and its subsequent attenuation following high intensity RT that also produced increases in muscle mass and strength indicate that this baseline inflammation does not limit the capacity for muscle remodelling in response to training in COPD, and that the inflammatory response itself can be modified by training. The findings support the positive effects of RT in COPD which is becoming a key component of pulmonary rehabilitation programmes. The role of this inflammatory response in the regulation of RT adaptation in COPD remains to be elucidated.

In summary, this study has shown that inflammatory cells are increased in the quadriceps of COPD subjects at baseline and increase further after acute resistance exercise. Regular training in both health and COPD results in muscle adaptation, whereby the acute inflammatory cell infiltrate to an exercise bout is diminished. Satellite cells have been identified for the first time in the quadriceps of COPD patients and may increase in response to exercise. Overall this study suggests that the muscle response to exercise and training is similar in both COPD and healthy controls with respect to inflammatory cell infiltration, satellite cell content, and changes in muscle fibre size. The response in patients with evidence of significant muscle wasting is an important future research question that arises from this study. Further work is also required to assess whether the increased inflammation evident at baseline in COPD is detrimental to muscle function, and whether exercise training may exert a wider anti-inflammatory effect.

CHAPTER 8

Ultrasound Assessment of Lower Limb Muscle Mass in Response to Resistance Training in COPD

Measurement of muscle mass is an important outcome measure in RT programmes. However, muscle mass measurements are frequently cumbersome as it requires radiographic techniques such as computed tomography (CT), magnetic resonance imaging (MRI) or dual energy x-ray absorptiometry (DEXA), that are not readily accessible for routine clinical use. Portable ultrasound can be used as a surrogate marker of lower limb muscle mass. A study using this technique to measure lower limb muscle mass in response to RT in COPD patients and age-matched healthy controls is described in this chapter.

Introduction

Reduced lower limb skeletal muscle mass and strength is an important systemic feature of COPD which has a significant impact on mortality, morbidity and healthcare utilisation (Decramer et al. 1997; Gosselink et al. 1996; Marquis et al. 2002; Swallow et al.

2007). Improvements in muscle mass can be achieved by lower limb RT. These improvements are restricted to the muscle group that is trained. Hence, a reliable, safe measurement of lower limb or quadriceps muscle mass which can detect the response to an intervention such as exercise training is needed.

Muscle mass can be measured using a variety of imaging techniques such as CT, MRI and DEXA. In COPD, previous studies have suggested that these techniques can be used to detect changes in lower limb muscle mass following training (Bernard et al. 1999; Casaburi et al. 2004; Kongsgaard et al. 2004) However, the equipment required for these measurements is bulky and expensive, specific expertise may be required to interpret the images, and in the case of CT and DEXA, subjects are exposed to ionizing radiation. Thus the utility of these imaging modalities as an outcome measure, where repeat testing is required, is limited. Ultrasound is an imaging technique that can determine thickness and cross-sectional areas of superficial muscles such as the rectus femoris muscle. It has the advantage of being portable, and involves no ionizing radiation. A number of studies have confirmed the reliability of this technique for measuring the size of the quadriceps muscle in health (Bemben 2002; Gruther et al. 2008; Sipila and Suominen 1991), with limited data in COPD (Seymour et al. 2009; Shrikrishna et al. 2012). Similarly, ultrasound has been previously shown to detect changes in quadriceps size in response to training interventions in healthy populations (Bemben et al. 2000; Sipila and Suominen 1996; Starkey et al. 1996). However, no comparable data exists in COPD, where muscle mass is lower and training intensities reduced. Seymour et al observed good correlation between ultrasound measurements of rectus femoris cross-sectional area and CT in a cohort of patients with

COPD but the responsiveness of this measure to an intervention that increases muscle mass has not been assessed (Seymour et al. 2009).

In this study, I compared the responsiveness of ultrasound and DEXA assessments of lower limb muscle mass in response to high intensity knee extensor RT, in patients with COPD and a similar aged healthy control group. The hypothesis was that ultrasound derived surrogates of muscle mass, namely rectus femoris cross-sectional area and quadriceps thickness, would be sensitive to changes in response to RT. In addition I assessed the inter-operator and inter-occasion reproducibility of the ultrasound technique at baseline, and compared its performance to DEXA as a means of measuring thigh muscle size.

Methods

Full methodological details are provided in **chapter 4**.

Study Subjects

45 patients with COPD and 19 age-matched controls were included in this study. They were all participating in a larger investigation of the mechanisms of adaptation to RT. Patients were recruited from outpatient clinics at Glenfield Hospital (Leicester, UK), and from those referred for pulmonary rehabilitation. Age-matched healthy controls were recruited from local advertisement. None of the subjects had been taking part in any regular exercise programs, and COPD patients who underwent pulmonary rehabilitation in the last 12 months were excluded. Other exclusion criteria included: maintenance oral corticosteroid or anticoagulant therapy, long-term oxygen therapy, diabetes or any other co morbid conditions that would prevent exercise training. The study was approved by the

Leicestershire and Rutland Research Ethics committee (Ref: 06/Q2501/138), and all participants provided written informed consent.

Resistance Training Protocol

Participants underwent 8 weeks of bilateral, knee extensor, high-intensity isokinetic RT on an isokinetic dynamometer (Cybex II Norm, CSMi, Stoughton, MA, USA). Training was fully supervised, and consisted of three half-hour sessions per week. Subjects performed 5 sets of 30 maximal knee extensions at a pre-set angular velocity (PAV) of 180°/second. The contractions were isokinetic and concentric, and each set was separated by a minutes rest. Additionally, subjects also received one-minute continuous passive movement (flexion/extension) before and after each training session to act as a warm up/ cool-down. This training protocol was chosen based upon a previous study showing that the training could produce significant increases in lower limb mass following immobilisation in healthy subjects (Jones et al. 2004). The basic measurements recorded were the peak torque in Newton-metres (Nm) and total work done in Joules (J) for each of the five sets.

Measurements Pre and Post training

Thigh Muscle Mass

DEXA

Total body lean (fat free) mass was measured by DEXA (Lunar Prodigy Advance, GE Healthcare, UK). This provides a 3-compartment model of body composition, subdividing the body into fat mass, bone-free lean mass and bone mineral mass. Using the software provided by the manufacturer, thigh lean mass (T_{dexa}) was measured from the

area delineated by the ischial tuberosity superiorly, and knee joint line inferiorly (Visser et al. 1999). The fat free mass index (FFMI) was calculated from the total body fat free mass normalised for height. Patients were deemed to be muscle wasted if the FFMI < 16kg/m^2 in men or <15kg/m^2 in women (Schols et al. 2005) .

Ultrasound

Portable ultrasound (Hitachi EUB-425, Hitachi Medical Systems, UK) was used to measure the size of the dominant quadriceps muscle similar to the method of Bemben et al (Bemben 2002). Scanning was performed in the supine position with a rolled-up towel placed in the popliteal fossa to relax the upper thigh **(Figure 8.1)**. The scanning site was identified as the mid-point of the distance from the greater trochanter to the knee joint line as previously described (De Bruin et al. 1997). A 7.5 MHz linear array transducer was placed perpendicular to the long axis of the thigh to obtain a frozen real-time cross-sectional image of the rectus femoris muscle. Using the built-in callipers, two indices of quadriceps size were measured – rectus femoris cross-sectional area – RF_{csa}, and quadriceps thickness – Q_t. The inner outline of the rectus femoris was manually traced to calculate RF_{csa} in mm^2, while Q_t was measured in mm as the vertical distance from the superficial fat-muscle interface to the underlying femur **(Figure 8.2)**. The average of three consecutive measurements (within 10% of one another) was taken as the true value. Care was taken to ensure that adequate contact gel was used and minimal pressure applied on the transducer so as to minimise distortion of underlying tissues. Scans were performed at baseline, and after weeks 4 and 8 of training. In a subset of participants, the baseline scans were repeated by 2 additional operators **(Linzy Houchen-Wolloff and Samantha Harrison)** in order to assess inter-operator reproducibility of the method. Each operator

was blind to the other's scans. In addition, a repeat baseline scan was performed by myself on a separate visit prior to the start of exercise training to determine inter-occasion reproducibility of the ultrasound technique. None of the operators had any previous ultrasound experience, but following a brief familiarisation period, competency was gained in performing the scans independently.

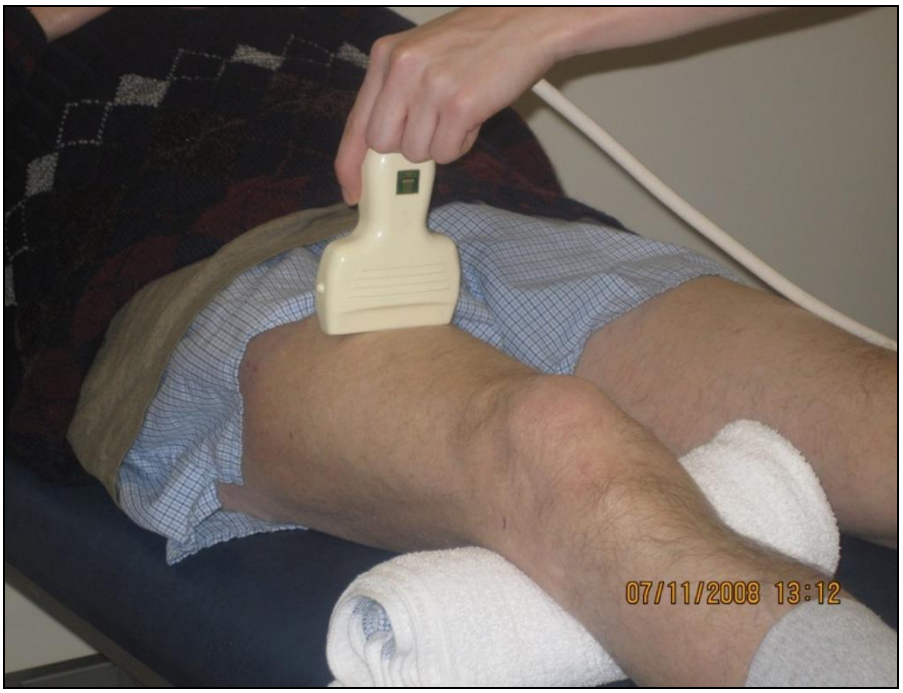


Figure 8.1 – Position of subject during ultrasound scanning

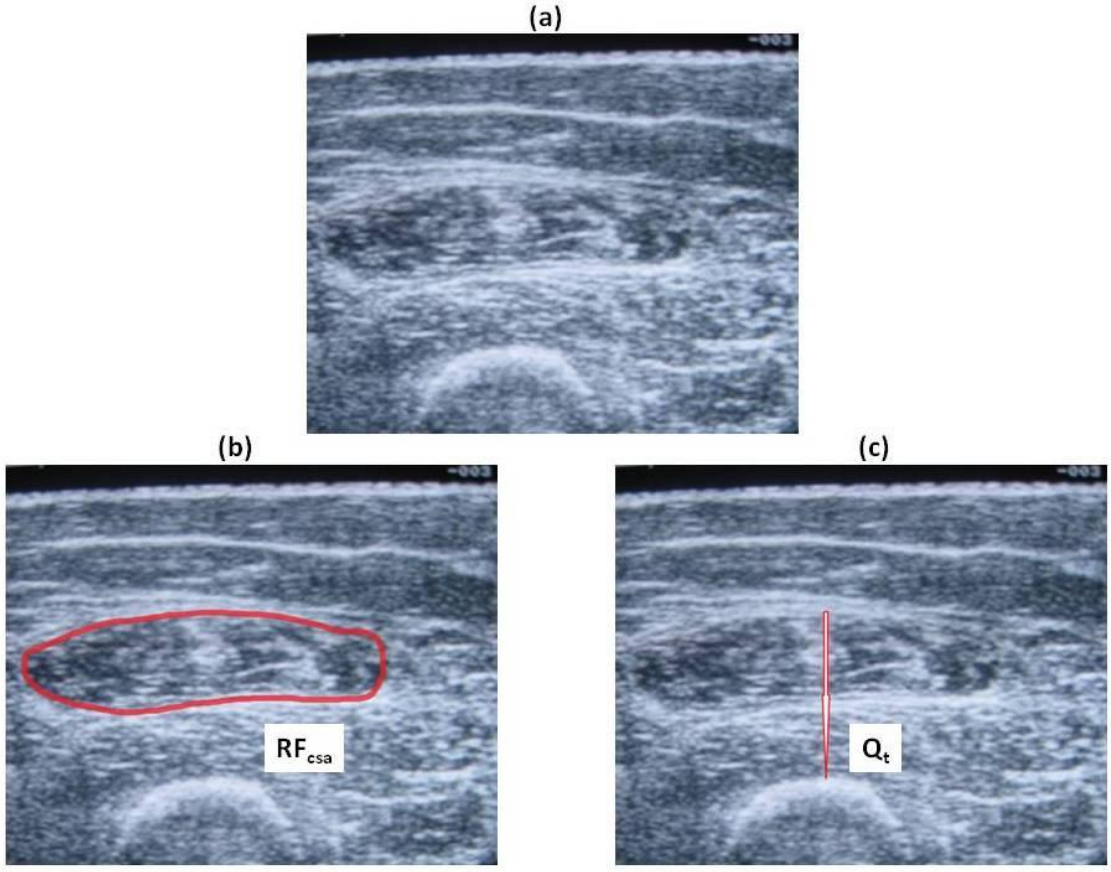


Figure 8.2 – Sample ultrasound image of the quadriceps
Sample scan of a study participant showing (a) Image at mid-thigh region, (b) RF_{csa}, and (c) Q_t

Quadriceps Strength

Quadriceps strength was measured on the cybex. After a prior familiarisation visit, quadriceps isometric maximum voluntary contraction (QMVC) of the dominant leg was measured during a maximal static contraction with the knee at 70°. QMVC was measured in Newton-metres (Nm), and the best of 6 strength measurements was taken as the true value.

Statistics

Statistical analysis was performed using GraphPad Prism Version 5.01 for Windows (GraphPad Software Inc, California, USA) and SPSS 18.0 for Windows (SPSS Inc, Chicago, USA). Parametric data were expressed as means (±SD) and non-parametric data were described as medians (interquartile range, ±IQR). Spearman's correlation was used to describe the relationship between changes in RF_{csa}, Q_t and QMVC. The reproducibility of ultrasound measurements was determined by calculating the mean differences and the intraclass correlation coefficients for repeated measurements. The effect size for changes in outcome measures after RT was calculated by dividing the mean difference by the standard deviation of the pre-training measurement. By calculating effect sizes, the magnitude of any changes can be judged according to the following criteria - small: 0.2 to 0.5; moderate: 0.5 to 0.8; large: > 0.8 (Cohen J 1988). All statistical tests were two-tailed and the threshold of statistical significance was a p value < 0.05.

Results

Baseline

Baseline subject characteristics are shown in **table 8.1**. QMVC was significantly lower in patients compared with controls, but ultrasound and DEXA indices of thigh muscle mass did not differ between the groups at baseline. There were 10 muscle wasted COPD patients. For the group as a whole, a significant linear relationship was observed between ultrasound and DEXA measured indices of quadriceps size (RF_{csa} vs. T_{dexa}: $r=0.68$, $p<0.0001$; Q_t vs. T_{dexa}: $r=0.63$, $p<0.0001$ – **Figures 8.3a and 8.3b**). Both RF_{csa} and

Q_t were significantly related to QMVC with the groups combined, (RF_{csa}: r=0.43, p< 0.0001; Q_t: r = 0.29, p= 0.01 – **Figures 8.4a and 8.4b**) and when taking into account only COPD patients [RF_{csa}: r=0.50, p= 0.0005; Q_t: r=0.33, p=0.02]. A significant but stronger correlation was observed between QMVC and T_{dexa} (r=0.68; p <0.0001 – **Figure 8.4c**).

	Healthy (n=19)	**COPD (n=45)**
Age	66.2 (5.0)	68.2 (8.2)
Gender (M:F)	8:11	27:18
Smoking (pack-years)	13.0 (22.4)	45.8 (30.5)***
FEV_1 (Litres)	2.5 (0.6)	1.1 (0.4)***
FEV_1 (% predicted)	106.6 (22.0)	47.3 (18.9)***
BMI (kg/m^2)	26.9 (2.8)	26.4 (5.3)
FFMI (kg/m^2)	17.3 (1.7)	17.5 (2.8)
QMVC (Nm)	135.7 (45.7)	109.5 (48.5)*
T_{dexa} (g)	4096.8 (849.3)	3908.5 (1104.1)
RF_{csa} (mm^2)	444.1 (98.8)	439.7 (117.9)
Q_t (mm)	21.8 (2.9)	21.5 (6.4)

Table 8.1 – Baseline characteristics of study participants
Data presented as Means (SD), except gender (absolute numbers)
* p<0.05, *** p<0.0001, COPD vs. Healthy

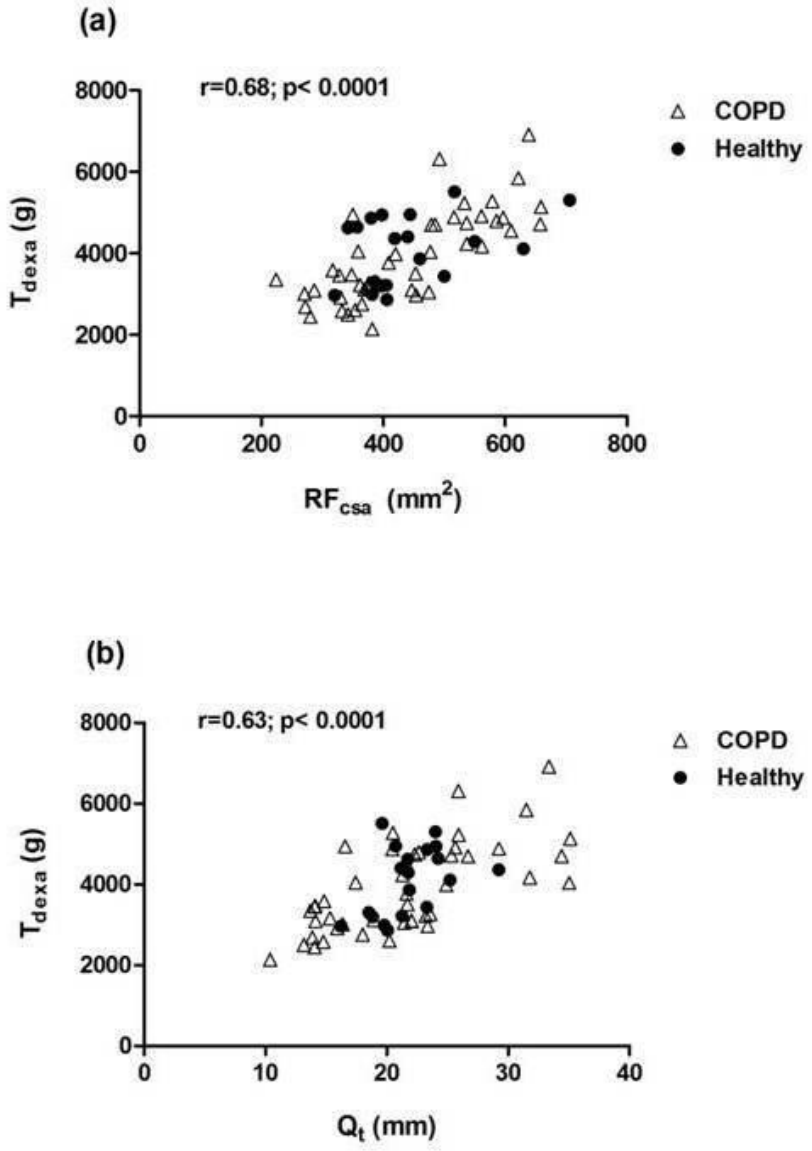


Figure 8.3 – Baseline relationships between ultrasound and DEXA measured indices of quadriceps size

(a) T_{dexa} VS. RF_{csa} and (b) T_{dexa} vs. Q_t

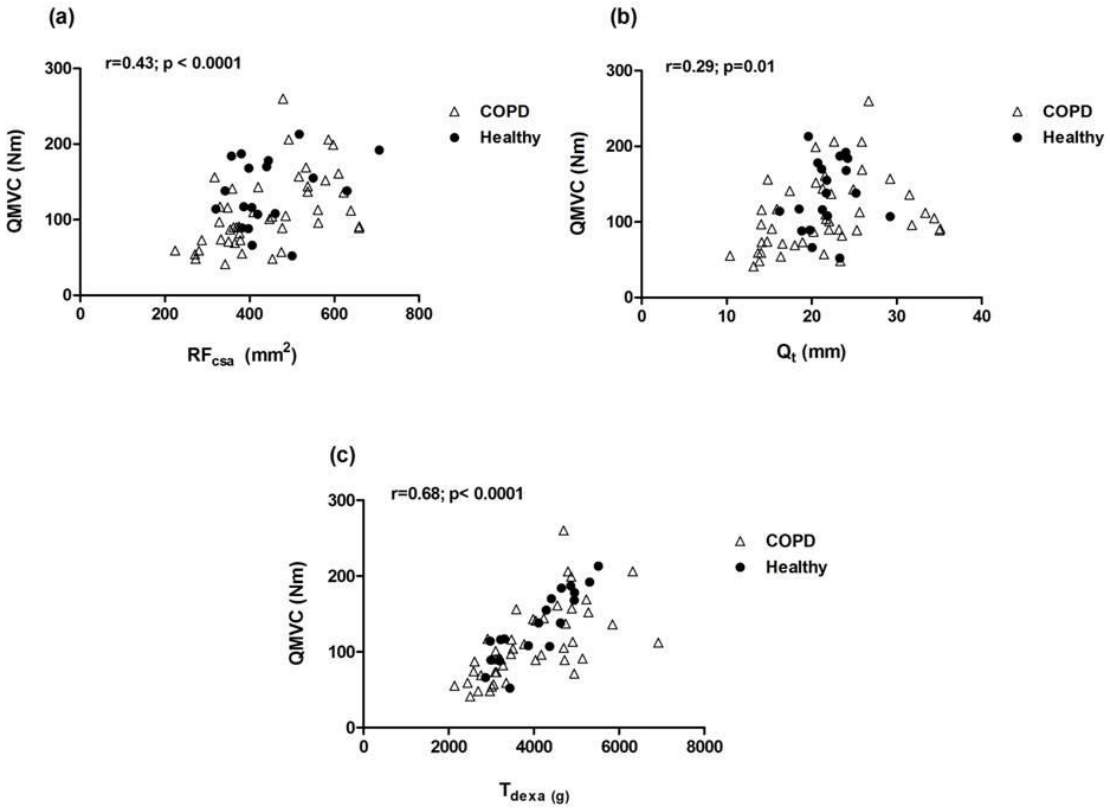


Figure 8.4 – Baseline relationships between quadriceps strength and ultrasound and DEXA measured indices of quadriceps mass
(a) QMVC vs. RF_{csa}, (b) QMVC vs. Q_t, and (c) QMVC vs. T_{dexa}

Post-Training

The mean (SD) differences and effect sizes for measures of quadriceps mass and strength after training are shown in **table 8.2**. An increase in quadriceps mass was detectable at the half-way stage (week 4) of the exercise training programme when measured by both ultrasound and DEXA. Eight weeks of RT resulted in a significant increase in T_{dexa}, RF_{csa} and Q_t [COPD: 5.7%, 21.8%, 12.1% respectively; Healthy: 5.4%, 19.5%, 10.9 respectively] (**Figures 8.5a, 8.5b**). Similarly, QMVC significantly improved after training in both groups [Mean (SD) change – COPD: 19.5 (20.6) Nm, $p<0.001$; Healthy: 15.5 (27.5) Nm, $p=0.02$]. When compared to ultrasound, the post-training change in muscle mass measured by DEXA (T_{dexa}) was more closely related to changes in muscle strength ($r=0.19$), although none of these relationships were statistically significant (**Figures 8.6a, 8.6b, 8.6c**). Similarly, training-induced changes in RF_{csa} and Q_t were not significantly correlated to changes in T_{dexa} (**Figure 8.7a, 8.7b**).

	% Change (SD)	Mean Absolute Change (SD)	95% CI	Effect Size	p value
QMVC (Nm)					
Healthy (n=19)	11.3 (19.6)	15.5 (27.5)	2.3, 28.8	0.34	0.024
COPD (n=45)	20.0 (20.4)	19.5 (20.6)	13.3, 25.7	0.40	0.000
T_{dexa} (g)					
Healthy (n=19)	5.4 (4.2)	224.7 (178.9)	138.5, 311.0	0.26	0.000
COPD (n=45)	5.7 (7.6)	213.6 (235.0)	143.0, 284.2	0.19	0.000
RF_{csa} (mm^2)					
Healthy (n=19)	19.5 (11.6)	82.4 (44.3)	61.1, 103.8	0.83	0.000
COPD (n=45)	21.8 (12.7)	91.5 (50.3)	76.3, 106.6	0.77	0.000
Q_t (mm)					
Healthy (n=19)	10.9 (7.1)	2.2 (1.5)	1.5, 3.0	0.78	0.000
COPD (n=45)	12.1 (11.2)	2.3 (2.2)	1.6, 3.0	0.36	0.000

Table 8.2 – Effect of training on quadriceps strength and mass

Percentage and Mean absolute (SD) changes in outcome measures for healthy controls and COPD patients after 8 weeks of resistance training. 95% confidence intervals for the differences are quoted. p values were calculated using paired student's t-tests.

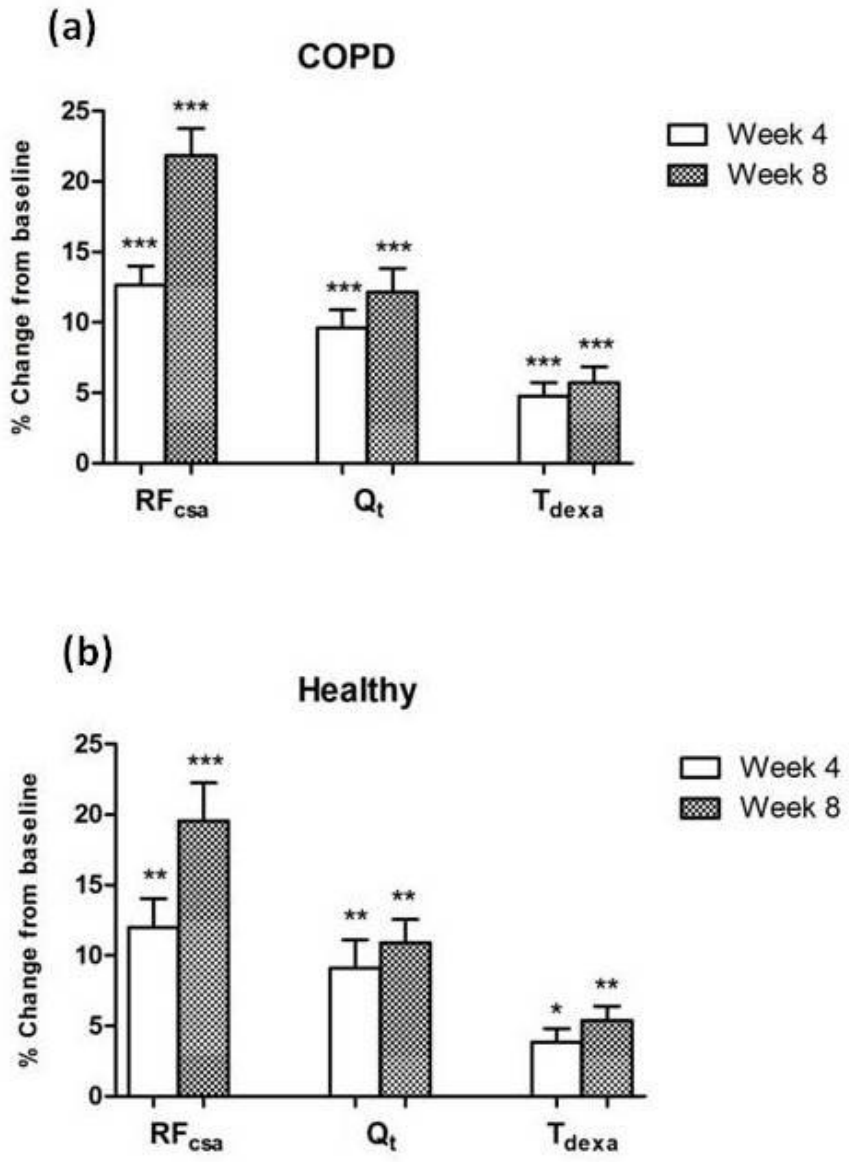


Figure 8.5 – Percentage change from baseline in ultrasound and DEXA measured indices of quadriceps mass

(a) COPD, and (b) Healthy.

Data presented as Means (SEM)

RF_{csa}: Rectus femoris cross-sectional area measured by ultrasound

Q_t: Quadriceps muscle thickness measured by ultrasound

T_{dexa}: Thigh lean mass measured by DEXA

Wilcoxon Signed rank test: *** $p \leq 0.0001$; ** $p < 0.001$; * $p < 0.01$, significantly different from baseline

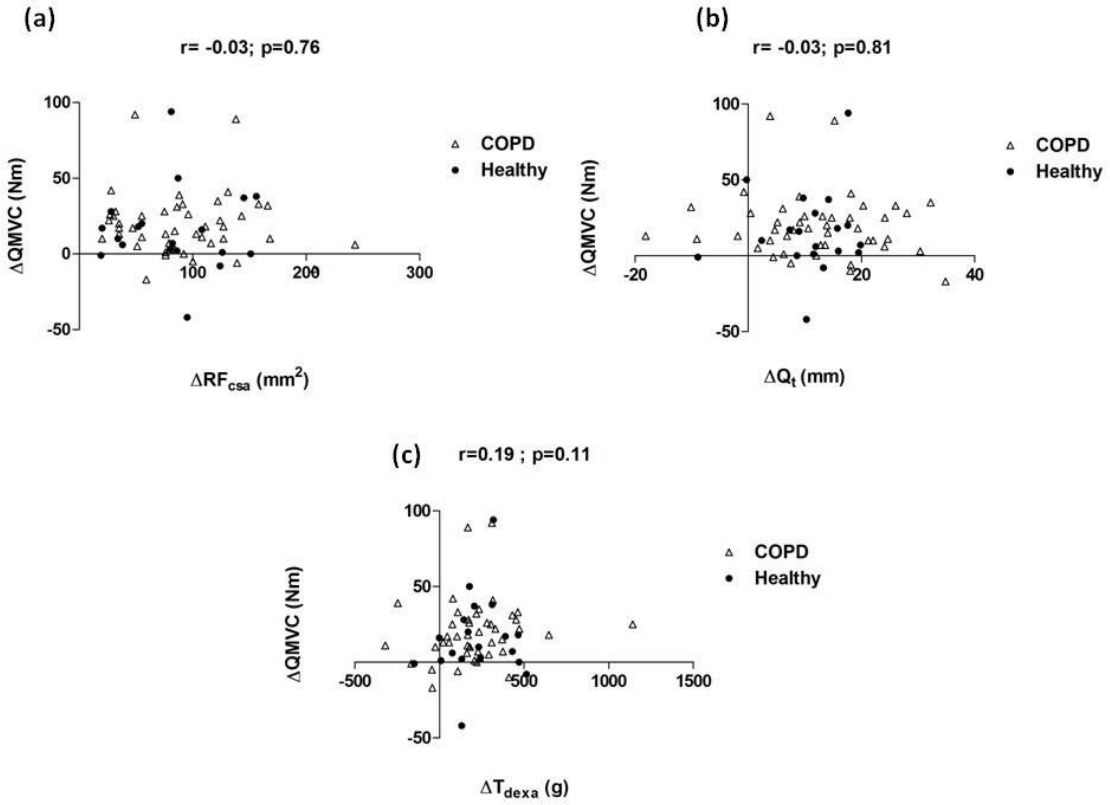


Figure 8.6 – Relationships between training-induced changes in quadriceps strength (ΔQMVC) and changes in muscle mass measured by ultrasound and DEXA
(a) ΔQMVC vs. ΔRF_{csa}, (b) ΔQMVC vs. ΔQ_t, and (c) ΔQMVC vs. ΔT_{dexa}

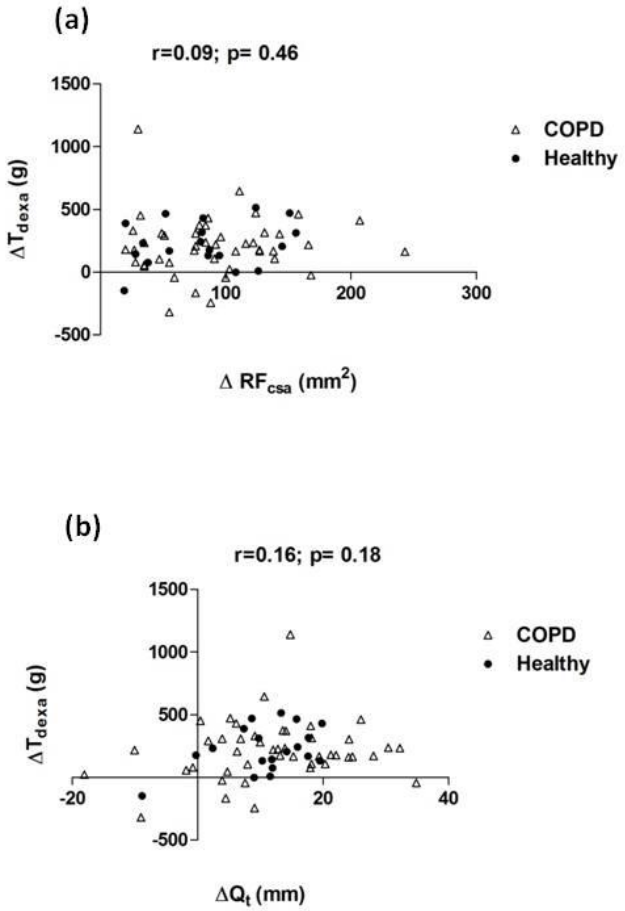


Figure 8.7 – Relationship between training-induced changes in ultrasound and DEXA indices of quadriceps mass
(a) ΔT_{dexa} vs. ΔRF_{csa}, and (b) ΔT_{dexa} vs. ΔQ_t

Muscle wasted patients

Ultrasound measured indices of quadriceps mass also improved significantly in the 10 muscle wasted COPD patients [RF_{csa}: Mean (SD) from 356.6 (61.5) mm^2 to 413.9 (76.1) mm^2, $p< 0.001$; Q_t: from 15.9 (4.0) mm to 18.1 (3.6) mm, $p< 0.01$], while nonsignificant improvements in T_{dexa} [from 2793.2 (419.3) g to 2983.9 (449.5) g, p=0.13] and QMVC [from 74.2 (34.6) Nm to 82.0 (30.8) Nm, p=0.08] were also noted.

Reproducibility of ultrasound measurements

Table 8.3 summarises the results of reproducibility studies for the ultrasound measurements. Intraclass correlation coefficients and reliability indices between the interval scans for RF_{csa} and Q_t were 0.98, indicating good inter-occasion reproducibility of the technique for the same operator (**Bland-Altman plots – Figures 8.8a, 8.8b**). Inter-operator reproducibility was assessed between 2 pairs of operators [**Operator A** (Manoj Menon) vs. **Operator B** (Linzy Houchen-Wolloff), and **Operator A** (Manoj Menon) vs. **Operator C** (Samantha Harrison)] at baseline. Intraclass correlation coefficients and indices of reliability between operators were >0.95, indicating good inter-operator reproducibility for the ultrasound measurements (**Bland-Altman plots Figure 8.9a, 8.9b, 8.9c, 8.9d**).

	Mean difference*		**SD#**		**p-value**		**ICC**	
	RF_{csa} (mm^2)	Q_t (mm)	RF_{csa} (mm^2)	Q_t (mm)	RF_{csa}	Q_t	RF_{csa}	Q_t
Inter-occasion (n=64)	1.18	0.04	21.69	1.09	0.66	0.75	0.98	0.98
Inter-operator (A vs. B; n=15)	8.73	0.20	25.39	0.88	0.20	0.38	0.95	0.95
Inter-operator (A vs. C; n=20)	5.90	0.07	15.01	0.93	0.09	0.74	0.99	0.98

Table 8.3 – Reproducibility of ultrasound measurements

*: Difference between measurements taken on 2 separate occasions at baseline (Test-Retest)
#: of mean difference
Significance was tested using paired t tests
ICC: Intraclass correlation coefficient

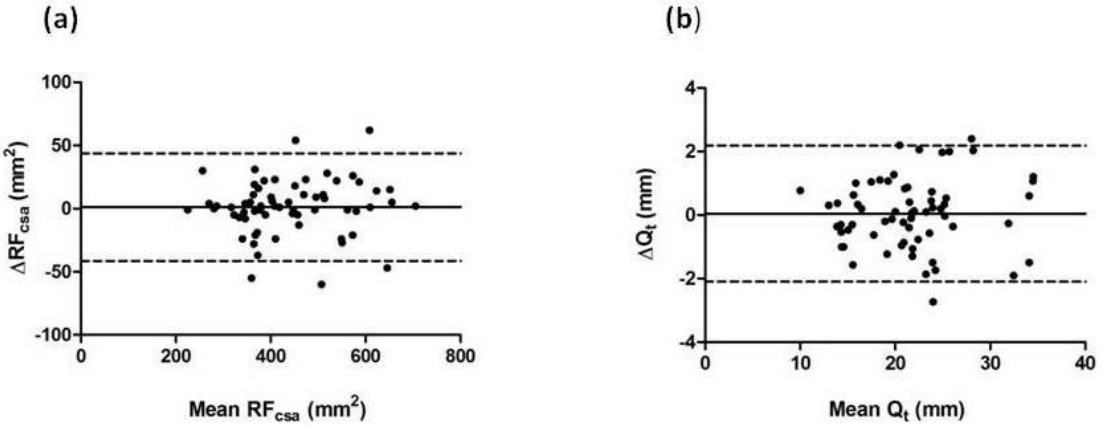


Figure 8.8 – Bland-Altman plots of the inter-occasion reproducibility of ultrasound measurements
(a) RF_{csa} and (b) Q_t

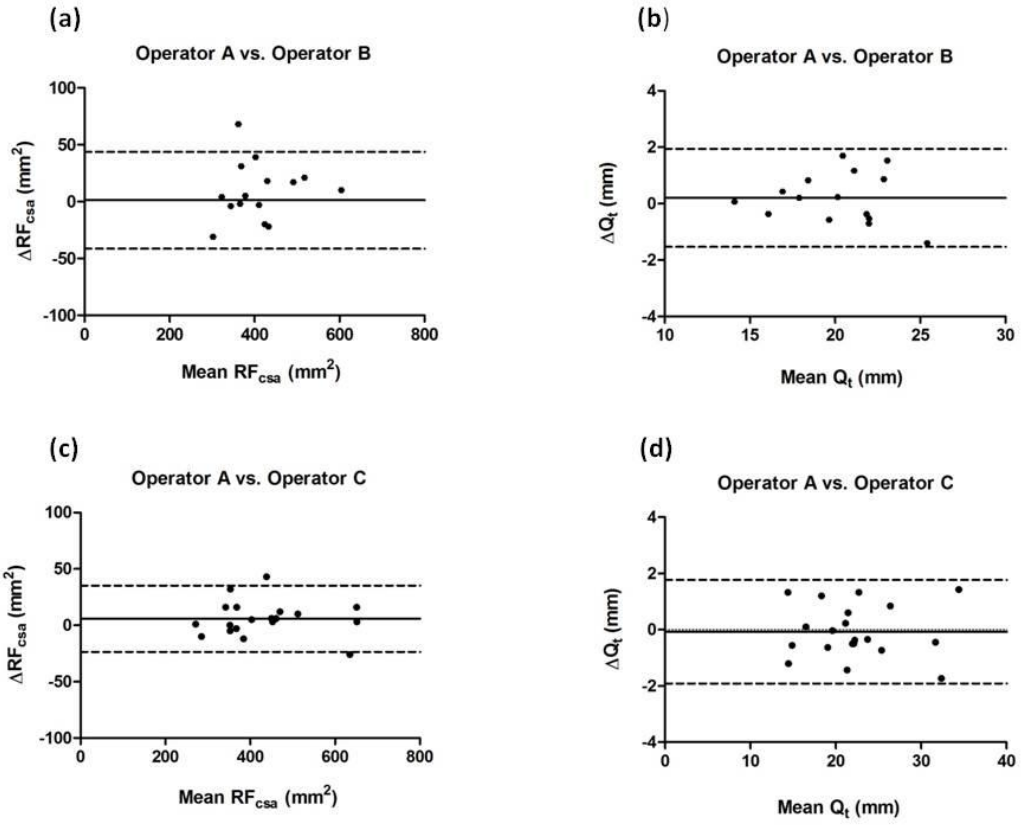


Figure 8.9 – Bland-Altman plots of the inter-operator reproducibility of ultrasound measurements

(a) RF_{csa}: Operator A vs. Operator B, (b) Q_t: Operator A vs. Operator B, (c) RF_{csa}: Operator A vs. Operator C, and (d) Q_t: Operator A vs. Operator C.

Discussion

This study shows that indices of quadriceps mass measured by ultrasound are sensitive to change in response to RT in COPD patients (including subjects with low muscle mass) and age-matched healthy controls. Compared with DEXA, effect sizes for RF_{csa} and Q_t were larger, which may suggest greater sensitivity to the intervention with ultrasound. Both RF_{csa} and Q_t demonstrated good inter-occasion and inter-operator reproducibility, and correlated well at baseline with measurements of muscle size obtained by DEXA. However the changes in measures of quadriceps muscle mass detected by ultrasound and DEXA following training were poorly correlated, suggesting that these measurement methods are not interchangeable when assessing the response to an intervention.

To my knowledge, this is the first study to assess ultrasound for quantifying the effects of RT on indices of quadriceps mass in COPD. Two measures of quadriceps muscle mass – rectus femoris cross sectional area (RF_{csa}) and quadriceps thickness (Q_t), were determined in this study. Both these ultrasound-derived measures have previously been used as surrogate markers of quadriceps size in healthy older populations (Sipila & Suominen 1991). Studies have demonstrated the sensitivity of serial ultrasound measurements to detect changes in quadriceps mass in critically ill patients (Gruther et al. 2008) and in healthy populations following exercise interventions (Sipila & Suominen 1996; Starkey et al. 1996).

In the present study, a significant increase in rectus femoris cross-sectional area and quadriceps thickness was observed after 8 weeks of knee extensor RT in patients with COPD and age-matched healthy controls. Thigh muscle mass measured by DEXA was also

increased after training in both groups. The magnitude of post-training changes in muscle mass determined by ultrasound and DEXA in this study is comparable to training data from other patient populations. Jones at al. showed that 6 weeks of lower limb isokinetic RT in healthy young volunteers resulted in approximately a 4.5% increase in DEXA-measured thigh lean mass (Jones 2004) . In older men with prostate cancer, 20 weeks of progressive RT lead to a 15.7% increase in quadriceps thickness measured by ultrasound (Galvao et al. 2006) . Similarly a 20% increase in rectus femoris cross-sectional area was observed in post menopausal women after 6 months of lower limb RT (Bemben et al. 2000) This study provides additional data to support the use of ultrasound as a bedside imaging modality for serial measurements of the quadriceps during RT in COPD patients.

There was good inter-operator and inter-occasion reproducibility of ultrasound measurements in this study. None of the operators had any previous ultrasound experience. However, after a familiarisation period of 10 to 14 days, all the operators became competent at performing the scans independently. Non clinicians can therefore be easily trained to perform the leg ultrasound scans. In addition, the reproducibility and sensitivity of both ultrasound indices of muscle mass – RF_{csa} and Q_t, were noted to be similar. Although RF_{csa} may correlate better with muscle strength (Sipila & Suominen 1991) and is less prone to operator-dependent errors, it may be difficult to measure in certain individuals, such as those with excess or very little fat in the thighs (Seymour et al. 2009). The intermuscular septae are not clearly visualised in these patients, and measuring Q_t may be more appropriate in this situation.

When compared with ultrasound, the post-training changes in muscle mass measured by DEXA were more closely related to changes in QMVC, although this was not

statistically significant. A number of factors may account for this lack of correlation between changes in QMVC and changes in muscle mass measured by both ultrasound and DEXA: (i) The rectus femoris constitutes only about 10% of the total cross-sectional area of the quadriceps muscle (Trappe et al. 2001), while measurement of muscle thickness at the mid-thigh region excludes two major muscles belonging to the quadriceps group – the vastus lateralis and vastus medialis. On the other hand, the inability of DEXA measurements to differentiate between flexor and extensor muscles within a limb is a limitation. Therefore, indices of quadriceps mass determined by DEXA and ultrasound do not measure the mass of the whole knee extensor muscle. (ii) The differential response to training between two and three dimensional measurements may be relevant. The rectus femoris has a comparatively large surface area to volume ratio as it is a small muscle. Hence small changes in mass might cause larger changes in measured cross-sectional area of the rectus femoris when compared to DEXA, which measures a larger muscle. (iii) There is data to suggest that small changes in skeletal muscle mass following RT may be undetected by DEXA (Delmonico et al. 2008), which is not the case with ultrasound (Bemben et al. 2000; Galvao et al. 2006).

The recognition of the functional and prognostic importance of reduced skeletal muscle mass has intensified interest in developing interventions to address this issue. RT has been shown to be effective in this respect but there is continuing interest in pharmacological and nutritional therapies aimed at achieving this either alone or in conjunction with training. Developing intelligence about the reproducibility and sensitivity of measurements of total and regional muscle mass is a key part of this endeavour. Muscle mass may fall rapidly during a period of acute illness such as an acute exacerbation of

COPD (Spruit et al. 2003), and tools to record these changes are needed. Ultrasound is a sensitive and reproducible test that could be used for repeat testing in clinical trials or in situations such as acute illness. It has a number of advantages in these settings including its potential use at the bedside and its lack of ionising radiation, which makes it a useful tool for performing serial measurements. The technique is relatively easy for non radiologists to learn, as its increasing use in other situations such as the insertion of intercostal chest drains for pleural disease illustrates.

I recognise that ultrasound has limitations when compared to other methods such as DEXA. Measurement errors can occur with the use of this technique as it is more operator-dependent than other imaging techniques. This can be minimised by avoiding excessive tissue compression during scanning and ensuring that the probe is always placed perpendicular to the long axis of the limb being measured. As previously discussed, visualisation of intermuscular septae may be difficult in severely obese subjects (Seymour et al. 2009) and in those with tissue depletion, hence accurate measurements of rectus femoris cross-sectional may not be obtainable (Hudash et al. 1985). DEXA measurements on the other hand are not operator dependent, and it gives information on whole-body and regional limb body composition, including bone mineral and lean tissue mass. DEXA may therefore perform better than ultrasound as a method for screening and identifying nutritional depletion (Engelen et al. 1998), whereas ultrasound may be best used as an assessment tool to measure the response to intervention. The inclusion of an untrained control group would have allowed a more robust assessment of the impact of RT on muscle mass and provided information on the longer term biological variability of the measurement. However, the objective of this study was the investigation of the sensitivity

of ultrasound in comparison with other measurement methods rather than the impact of training per se.

In conclusion, indices of quadriceps mass measured by portable ultrasound are reproducible and sensitive to change in response to knee extensor RT in COPD. This study suggests that ultrasound may have the potential to be used as a field measurement of lower limb muscle mass in this population, whilst also highlighting limitations of the technique. The differences in the response to the intervention between ultrasound and DEXA suggest these measurements reflect different anatomical characteristics of the lower limb muscles and are not interchangeable.

CHAPTER 9

Conclusions

The aim of this thesis was to explore the molecular, cellular and functional adaptations in the lower limb muscles of COPD patients when compared to healthy controls following a programme of high-intensity lower limb RT, and to determine in patients the impact of providing a protein-carbohydrate dietary supplement at the time of training. RT is an important component of PR, and this form of training allows smaller muscle groups to be trained without impinging on the ventilatory system. Therefore in subjects limited by breathlessness, RT is an attractive therapeutic option for improving the function of the peripheral muscles. Provision of nutritional supplements during RT has been shown to produce additional gains in muscle mass and strength in the healthy population, but the effect of this therapeutic combination has not been previously studied in COPD.

The main hypothesis tested in this thesis was that RT-induced increases in lower limb muscle mass and strength in COPD patients would be mediated through the expression of genes and proteins associated with muscle mass regulation in humans. The main trial outcomes are reported in **chapters 5 and 6**. In addition, a number of other sub-

studies are described, which examined questions relating to the measurement of lower limb muscle mass and muscle inflammatory and satellite cell responses to RT.

Main Findings

The impact of RT and supplementation on functional outcomes is described in **chapter 5**. RT led to significant gains in muscle mass, muscle strength and whole-body exercise performance in both the supplemented and nonsupplemented COPD groups, with no significant between-group differences. Hence it seems that protein-carbohydrate supplementation during training does not provide any additional benefits in functional outcomes over and above RT. Moreover even though COPD patients had compromised muscle function at baseline, and trained at lower absolute training intensities when compared to healthy controls, they still made comparable improvements in functional outcomes. This suggests that deconditioning may be a key factor leading to lower limb dysfunction in COPD, although we cannot be sure whether the response in patients with muscle wasting or excessive systemic inflammation would be similar.

Thigh muscle mass increased after training in all groups, which suggests that the RT programme was effective in increasing net MPS in the study participants. There was a discrepancy between the training-induced changes in muscle strength (around 20%) and muscle mass (around 5%), which implies that factors other than increased muscle mass are involved in producing muscle force. Neural adaptations are known to be responsible for increasing muscle strength during the initial stages of RT, and although not directly measured in this thesis, may have contributed to some of the improvements in muscle strength seen in the study participants.

The reasons for the apparent failure of nutritional supplementation to enhance the effects of RT are unclear. It may be that the signal from RT is so strong, that it masks any additional effects of supplementation, or that the availability of protein substrate is not a limiting factor for muscle growth in COPD. It is less likely that the quantity (or dose) of protein provided was inadequate to elicit a favourable response because the amount of protein we provided (19g) was similar to (Moore et al. 2009) and much greater (Esmarck et al. 2001) than has been given in other similar studies involving nutritional supplementation and training in healthy subjects.

The training-induced increase in lower limb function in COPD was observed to translate into improved whole body exercise performance (peak VO_2 and Peak Work). This has not been consistently shown in previous studies and could be attributed to enhanced cycling performance resulting from increased quadriceps strength. An alternative explanation is that the angular velocity (180°/sec) of the isokinetic RT protocol employed in this thesis incurred significant energy demands and therefore provoked aerobic adaptation at the muscular level.

Chapter 6 describes the molecular responses to RT and nutritional supplementation in COPD patients and healthy controls. This study represents the first detailed time course investigation of muscle signalling in response to RT in COPD. The breadth of analysis was substantially wider than previous reports and highlights the limitations of interpreting cross-sectional molecular data. Anabolic, catabolic and transcription factor protein expression increased following RT in all groups, but the magnitude of increase was blunted in COPD. This blunting was in line with lower absolute (but similar relative) workloads in patients, and one might speculate that molecular

signalling is determined by the absolute training load, while the functional response is determined by the relative training load. Apart from myogenesis, changes in the expression of molecular markers were dissociated from muscle mass and strength gains. Therefore, caution is required when identifying potential molecular targets for intervention. . Moreover, the mRNA and protein measurements were not always concordant, highlighting the limitations of previous reports that have mainly measured skeletal muscle mRNA expression in this population.

Myogenic protein expression was comparable between COPD patients and controls, and might explain the similarity in lean mass gains in the two groups. Additional data using immunostaining techniques (**described in chapter 7**) have shown that satellite cell numbers in the quadriceps of COPD are increased in response to acute and chronic RT. Hence the myogenic pathway is of potential interest for developing therapies aimed at improving muscle function in COPD. Similar to the functional response, molecular signalling was not altered by the provision of protein-carbohydrate supplementation during RT in COPD.

The work described in **chapter 7** introduced a novel immunostaining technique to study skeletal muscle structure and cellular inflammatory and satellite cell response to RT. In the resting state, inflammatory cells were increased in the quadriceps of COPD patients, with further increases seen after acute RT. In both patients and controls, muscle adaptation occurred with regular training, whereby the acute inflammatory cell infiltrate to a bout of RT was diminished. Satellite cell numbers in the quadriceps were comparable at baseline between patients and healthy controls, tended to increase with RT in both groups, and remained elevated above baseline levels in response to chronic training.

Chapter 8 demonstrated that portable ultrasound can be used to detect changes in lower limb muscle mass in response to RT in COPD and age-matched healthy controls. This technique has good reproducibility and may be more sensitive to changes in muscle mass when compared to DEXA. However there was a poor correlation between ultrasound and DEXA-measured indices of muscle mass, which suggests that these measurement methods are not interchangeable when assessing the response to an intervention.

Limitations

Some potential limitations of the work presented in this thesis have already been addressed in the discussion in each chapter. A few more general issues are given particular attention here. The main criteria for inclusion into this study were patients with COPD who had moderate to severe disease (FEV_1/FVC <70, FEV_1<50% predicted), with significant self-reported exercise limitation (MRC Dyspnoea Grades 3, 4 or 5), and able to undergo lower limb RT for 8 weeks. Although these patients had evidence of lower limb dysfunction, their muscle mass was not significantly different from healthy controls. The study conclusions were therefore based on a range of COPD patients with varying muscle mass. Moreover, the demanding nature of the exercise programme meant that patients who completed the study may have been more "healthier" than patients who generally attend PR, which limits extrapolation of these results to the wider COPD population.

We inferred effects on net muscle protein turnover by measuring changes in muscle mass, but more insight might have been provided by direct measurements of MPS and MPB. The amino acid content of the supplement used in this thesis would have been of interest as there is evidence to show that MPS is stimulated in proportion to the quantity of essential amino acids contained in intact protein. Also, when compared to intact protein,

essential amino acids double the anabolic stimulus and increase the reutilisation of amino acids which would otherwise have been excreted or wasted (Campbell et al. 1995). Therefore another limitation might be that a lack of adequate essential amino acids in the provided supplement contributed to the apparent negative effect of supplementation. Habitual diet of study participants was not measured. There is data to show that in elderly subjects who consume adequate amounts of dietary protein, RT-induced improvements in functional performance is not further enhanced by the ingestion of additional protein during training (Campbell & Leidy 2007). Moreover, older individuals may offset supplementation with a reduction in their normal food intake (Lewis et al. 1987). Therefore information about the dietary habits of participants during the study period may have enabled us to better judge the impact of the nutritional supplementation.

Pax-7 staining allows detection of satellite cells, but is not informative on their activity status. In order to distinguish quiescent from activated satellite cells, this should be combined with a MyoD staining, and in order to detect proliferating (daughter) myoblasts, this should be combined with a marker such as Ki-67. These additional co-stainings may have allowed us to improve the interpretations on satellite cell activation and proliferation. The presence of an untrained control group in the ultrasound study (Chapter 8) might have enabled an assessment of the stability and reproducibility of the technique. However there have been previous studies in both healthy individuals and COPD patients (Seymour et al. 2009) that have confirmed the reliability of ultrasound measurements. Given the limitations of DEXA for assessing quadriceps muscle per se, it would have been clinically more useful to compare ultrasound measurements with CT or MRI as the reference method.

Sample size calculations were not performed for all the specific aims in this thesis which is also a limitation. At the time of writing the study protocol, there was limited data available (Esmarck et al. 2001) to help us determine the number of subjects that would be required to show an additional effect of protein-carbohydrate supplementation on quadriceps strength, over and above the effect of RT. Hence the study in chapter 5 (Functional Responses to Resistance Training) was powered based on the expected improvements in quadriceps isometric strength following RT. We did have significantly more participants (n=38 in the supplemented group and n=33 in the placebo group) than the study by Esmarck et al. which consisted of 13 elderly men who demonstrated skeletal muscle hypertrophy in response to RT and post exercise protein supplementation (Esmarck et al. 2001). However we acknowledge that the results pertaining to the additional impact of nutritional supplementation should be treated with caution as it may not have been adequately powered for this outcome. The changes in gene and protein expression across a range of planned targets are difficult to quantify, but most human studies in this field have enrolled in the region of 10 subjects. We recruited significantly more subjects (>20 in each group) for our study (Chapter 6 – Molecular Responses to Resistance Training), and therefore believe that the findings are of relevance. In chapter 7, a convenience sample of COPD patients and healthy controls that had additional muscle biopsies was used to study the effects of RT on inflammatory and satellite cells. There is no comparable literature regarding the inflammatory and satellite cell response in the lower limbs following RT. However, the number of subjects in our study is comparable to other training studies in this field. A power calculation was also not performed for the study in chapter 8 which investigated the responsiveness of ultrasound-measured indices of lower limb muscle mass

to RT in COPD patients and healthy controls. This was also a convenience sample of subjects from the main training study who underwent additional ultrasound scanning of the lower limbs. Nevertheless, the number of participants and the results from this study are comparable to previously published data in the healthy population (Bemben et al. 2000; Starkey et al. 1996).

Future Work

A number of further questions arise as a result of work done in this thesis. It would be useful to examine the effect of protein-carbohydrate supplementation combined with RT in the subgroup of patients with reduced muscle mass. The amount of protein given, and the timing of supplementation in relation to exercise must be carefully controlled as these factors seem to influence the training response. Also, in order to maximise MPS, the protein supplement should contain adequate amount of essential amino acids. In addition, the measurement of MPS and MPB in conjunction with target gene and protein analysis would yield additional information linking the molecular responses with muscle protein turnover and the functional response to training and supplementation. The effect of providing post exercise protein-carbohydrate supplementation to healthy controls, and comparing the response to COPD patients should also be the subject of further study.

In this thesis, muscle wasting was defined by using FFMI cut-off values that have previously been shown to be predictive of mortality and morbidity in the COPD population. However by applying this criteria, only few patients with muscle wasting (n=14) were recruited. The use of arbitrary FFMI cut-off values may not reflect a condition of progressive wasting or cachexia. Hence it may be more informative to look at muscle mass as a continuous variable and observe for natural cut-offs within the spread of data,to

identify clinically relevant wasted subgroups. The effect of training and supplementation in these wasted groups will be of clinical interest. Moreover low muscle mass may be a feature of the natural body habitus in some patients, and predates the diagnosis of COPD. These individuals do not suffer from progressive muscle wasting, and thus a single cross-sectional measurement of muscle mass will not distinguish them from patients who have cachexia. This highlights the need to develop reliable biomarkers for skeletal muscle wasting, which can also preclude the use of invasive muscle biopsies.

Data presented in this thesis confirms the effectiveness of RT in improving lower limb function in COPD. However it is difficult to ascertain whether these improvements are clinically meaningful as there are no established minimal clinically important difference (MCID) values for changes in lower limb mass and strength, and whole-body exercise performance following RT. Therefore it would be useful to derive MCIDs for the various functional outcomes in response to RT in this population.

The study described in **chapter 7** looked at the inflammatory and satellite cell profile in COPD patients and controls following RT. The response in patients with muscle wasting would be of interest, in particular to see if the inflammatory cell increase seen at baseline adversely affects muscle function, and whether exercise training in general might exert a wider anti-inflammatory effect. Also, additional staining with markers such as MyoD and Ki-67 would enable identification of activated and proliferating satellite cells. In **chapter 8** I have shown that portable ultrasound is a more sensitive technique than DEXA for detecting changes in lower limb muscle mass after RT. The sensitivity of ultrasound in response to training interventions, when compared to the gold standard method of measuring muscle mass such as CT or MRI, is an area that needs further work.

In addition, it would be interesting to examine whether ultrasound can detect changes in muscle mass following generic PR, which is the most common type of training intervention currently offered for patients with COPD.

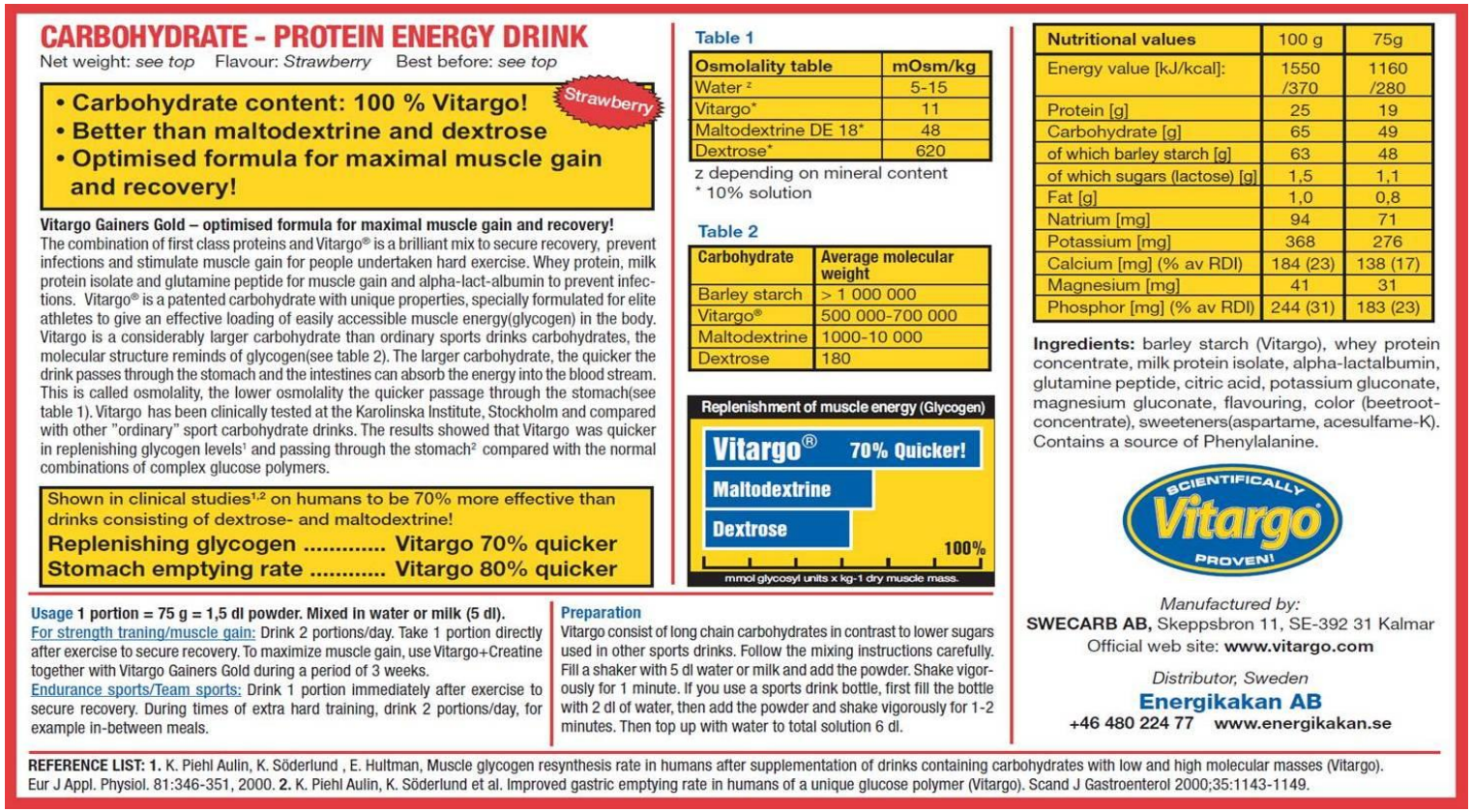
Concluding Remarks

Wasting and weakness of the lower limb muscles is a key extra pulmonary manifestation of COPD that impacts adversely on exercise performance, healthcare utilization and mortality. RT is an important intervention that can improve muscle mass and strength in these patients. Nutritional supplementation combined with RT has been shown to be beneficial in the healthy elderly. This thesis describes the first study to explore detailed functional and molecular responses of skeletal muscle to RT, and the role of protein-carbohydrate supplementation as an adjunct to training in COPD.

The main message from this thesis is that RT-induced improvements in lower limb muscle mass and strength are comparable between COPD patients and healthy controls, while the molecular responses to training seem to be uncoupled from the functional gains. Despite patients demonstrating lower muscle function at baseline, their capacity to respond to RT is preserved, which suggests that inactivity and deconditioning are major factors underpinning muscle dysfunction in this population. In addition, protein-carbohydrate supplementation provided at the time of training does not seem to confer any additional functional benefits to COPD patients, over and above RT.

Appendices

Appendix Ia. Ingredients in the Protein-Carbohydrate supplement



CARBOHYDRATE - PROTEIN ENERGY DRINK

Net weight: *see top* Flavour: *Strawberry* Best before: *see top*

Strawberry

- Carbohydrate content: 100 % Vitargo!
- Better than maltodextrine and dextrose
- Optimised formula for maximal muscle gain and recovery!

Vitargo Gainers Gold – optimised formula for maximal muscle gain and recovery!

The combination of first class proteins and Vitargo® is a brilliant mix to secure recovery, prevent infections and stimulate muscle gain for people undertaken hard exercise. Whey protein, milk protein isolate and glutamine peptide for muscle gain and alpha-lact-albumin to prevent infections. Vitargo® is a patented carbohydrate with unique properties, specially formulated for elite athletes to give an effective loading of easily accessible muscle energy(glycogen) in the body. Vitargo is a considerably larger carbohydrate than ordinary sports drinks carbohydrates, the molecular structure reminds of glycogen(see table 2). The larger carbohydrate, the quicker the drink passes through the stomach and the intestines can absorb the energy into the blood stream. This is called osmolality, the lower osmolality the quicker passage through the stomach(see table 1). Vitargo has been clinically tested at the Karolinska Institute, Stockholm and compared with other "ordinary" sport carbohydrate drinks. The results showed that Vitargo was quicker in replenishing glycogen levels[1] and passing through the stomach[2] compared with the normal combinations of complex glucose polymers.

Shown in clinical studies[1,2] on humans to be 70% more effective than drinks consisting of dextrose- and maltodextrine!

Replenishing glycogen ..... Vitargo 70% quicker
Stomach emptying rate ..... Vitargo 80% quicker

Table 1

Osmolality table	mOsm/kg
Water [z]	5-15
Vitargo*	11
Maltodextrine DE 18*	48
Dextrose*	620

z depending on mineral content
* 10% solution

Table 2

Carbohydrate	Average molecular weight
Barley starch	> 1 000 000
Vitargo®	500 000-700 000
Maltodextrine	1000-10 000
Dextrose	180

Usage 1 portion = 75 g = 1,5 dl powder. Mixed in water or milk (5 dl).
For strength traning/muscle gain: Drink 2 portions/day. Take 1 portion directly after exercise to secure recovery. To maximize muscle gain, use Vitargo+Creatine together with Vitargo Gainers Gold during a period of 3 weeks.
Endurance sports/Team sports: Drink 1 portion immediately after exercise to secure recovery. During times of extra hard training, drink 2 portions/day, for example in-between meals.

Preparation
Vitargo consist of long chain carbohydrates in contrast to lower sugars used in other sports drinks. Follow the mixing instructions carefully. Fill a shaker with 5 dl water or milk and add the powder. Shake vigorously for 1 minute. If you use a sports drink bottle, first fill the bottle with 2 dl of water, then add the powder and shake vigorously for 1-2 minutes. Then top up with water to total solution 6 dl.

Nutritional values	100 g	75g
Energy value [kJ/kcal]:	1550 /370	1160 /280
Protein [g]	25	19
Carbohydrate [g]	65	49
of which barley starch [g]	63	48
of which sugars (lactose) [g]	1,5	1,1
Fat [g]	1,0	0,8
Natrium [mg]	94	71
Potassium [mg]	368	276
Calcium [mg] (% av RDI)	184 (23)	138 (17)
Magnesium [mg]	41	31
Phosphor [mg] (% av RDI)	244 (31)	183 (23)

Ingredients: barley starch (Vitargo), whey protein concentrate, milk protein isolate, alpha-lactalbumin, glutamine peptide, citric acid, potassium gluconate, magnesium gluconate, flavouring, color (beetroot-concentrate), sweeteners(aspartame, acesulfame-K). Contains a source of Phenylalanine.

SCIENTIFICALLY Vitargo PROVEN!

Manufactured by:
SWECARB AB, Skeppsbron 11, SE-392 31 Kalmar
Official web site: **www.vitargo.com**

Distributor, Sweden
Energikakan AB
+46 480 224 77 www.energikakan.se

REFERENCE LIST: 1. K. Piehl Aulin, K. Söderlund , E. Hultman, Muscle glycogen resynthesis rate in humans after supplementation of drinks containing carbohydrates with low and high molecular masses (Vitargo). Eur J Appl. Physiol. 81:346-351, 2000. **2.** K. Piehl Aulin, K. Söderlund et al. Improved gastric emptying rate in humans of a unique glucose polymer (Vitargo). Scand J Gastroenterol 2000;35:1143-1149.

Appendix Ib – Ingredients in the placebo

Water, dextrin(starch fibre), aroma, acidifier(citric acid), xanthangum, titanium dioxide, colour (red beat powder), sweetener (aspartame, acesulfam-K).

Appendix II. Patient volunteer information sheet

PATIENT INFORMATION SHEET

Molecular Approaches to Reversing Skeletal Muscle Wasting in COPD. The Role of Resistance Training and Protein Supplementation.

Principal Investigator: **Dr Michael Steiner**

Co-Researchers: **Prof Sally Singh**
Dr. Michael Morgan
Dr Manoj Menon
Ms Linzy Houchen
Mrs Carolyn Sandland

You may contact: **Dr. Michael Steiner**
0116 256 3450

This study is sponsored by the **Medical Research Council**.

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

What is the purpose of this study?

A large number of people in the UK have long term breathing problems due to lung diseases such as Chronic Obstructive Pulmonary Disease (COPD). Patients with these conditions experience breathlessness when they try to exercise or perform physical tasks. In many patients this is made worse by weakness and wasting of the leg muscles. However, little is known about the underlying reasons for muscle wasting in patients who suffer with COPD. It is known that certain genes are involved in regulating the size and strength of the leg muscles in healthy humans. Recent research has shown that these genes are important in the increase in muscle bulk seen after exercise training in healthy people, particularly when exercise is combined with additional dietary protein intake. However, we don't know if these genes are important in muscle wasting in patients who have COPD. We know that exercise training is effective in improving symptoms for patients with COPD. However the mechanisms by which these improvements are brought about are largely unknown.

The aim of this research is to study the effects of a programme of exercise training and dietary supplementation on functioning of these genes in patients who suffer with COPD. We will compare these effects in similar aged healthy volunteers.

This knowledge will help us understand why muscle wasting happens in patients with lung diseases such as COPD. This is important because if we can understand how our genes regulate these changes in muscle bulk we may be able to develop new treatments to address this problem in patients with chronic diseases such as COPD.

Why have I been chosen?

Members of the pulmonary rehabilitation research team are conducting this study in collaboration with Prof Paul Greenhaff at the University of Nottingham. Patients with COPD who are going to attend the outpatient pulmonary rehabilitation programme or who are attending out patients at Glenfield Hospital are being invited to participate. We are also inviting similar aged healthy individuals who do not have lung disease to participate.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?

The purpose of this research study is to determine the effects of a programme of leg exercise training combined with dietary supplements. If you agree to participate, you will therefore be asked to undertake an eight-week programme of leg (quadriceps muscle) training.

Because we don't know whether taking additional dietary supplements is helpful, patients will be put into two groups and then compared. The groups are selected by a computer, which has no information about the individual – i.e. by chance. One group will receive a dietary supplement drink and the other group will receive an inactive dummy drink (called a placebo). Neither you nor your doctor will know in which treatment group you are (although, if your doctor needs to find out he/she can do so).

In order to find out what the effects of these treatments is, you will be asked to attend the hospital for additional assessments before the start of the exercise programme, in the middle of the programme and at the end. Some of these assessments will require a specific visit to hospital. Others will be performed on a day you are attending anyway as part of the exercise programme.

Your involvement in the study will last 10 to 12 weeks. The study overall will be running for around three and a half years.

Exercise Programme

You will be asked to attend Glenfield Hospital three times a week to exercise both legs on a piece of equipment called a "Cybex". This will involve extending your leg as hard as you can on the Cybex. We will ask you to do this 15 to 30 times (also called "repetitions"). There will be up to five bouts of these repetitions. You will be able to rest

between these bouts. You may be asked if we can observe your breathing during one of your training sessions in weeks 1, 4 and 8. This will involve breathing normally through a mouthpiece whilst you do your exercise.

Dietary Supplement

Depending on which group you are put in you will be asked to drink a dietary supplement or a placebo immediately after each bout of exercise during the exercise programme.

Assessment Visits

Visit 1

During this visit a doctor will discuss the study with you, take a medical history and examine you. You will also complete a questionnaire which asks about your daily physical activity. You will then be asked to perform breathing tests, which involves taking deep breaths, holding your breath and blowing down a tube. We will also measure your height and weight. We will also ask you to have a practice run at exercise on a bike and on the Cybex.

Visit 2

We will ask you to perform an exercise test on a special bicycle to establish your exercise ability. During the exercise we will record your heart and oxygen levels and you will breathe in and out of a mouthpiece. The bike will get gradually harder to pedal until you ask to stop. You will then be asked to perform a test to measure strength in your thigh muscle. This will involve 2 tests. For the first test you will sit on a chair and extend your leg (which is attached to a lever) as hard as you can for a few seconds against a resistance. The second test involves extending your leg against a pad which will be held in the researcher's palm.

You will also have a DEXA Scan. This measures the amount of fat, muscle and bone in your body. The scan is painless and requires you to lie flat for a few seconds. In addition, you will have an ultrasound scan to measure the amount of muscle in your thigh. This scan is also painless and you will be asked to lie on a couch for a few minutes. By using a small amount of gel, an ultrasound probe is placed over the front of your thigh and some pictures will be taken.

The exercise programme will start one to two weeks after visit 2. During this time you may be asked to wear an activity monitor at home for 7 days. This will count the times that you are active throughout the day and can be hidden under your clothing.

Visit 3

On the first day you attend for the exercise programme we will take a small sample of muscle from your thigh. This is called a muscle biopsy. Local anaesthesia is used to numb the area and a special needle is inserted through a small incision in the skin into the thigh muscle. We will also obtain a sample of blood from a forearm vein. Your first exercise session of the programme will be performed after these tests.

Visit 4

The day after your first exercise session we will take another muscle biopsy from your thigh. We will also take another blood test.

Visit 5

Before one of your exercise sessions in the 4^{th} or 5^{th} week of the programme we will take another muscle biopsy, blood test, thigh ultrasound and another DEXA Scan.

Visit 6

We will ask you to attend Glenfield Hospital after the exercise programme has been completed. We will take another muscle biopsy and another blood test. We will then ask you to repeat the tests of muscle strength and the cycle test. You will have a final DEXA Scan and thigh ultrasound, and be asked to complete the physical activity questionnaire again. If you wore an activity monitor before the training programme we will ask you to wear one again for another 7 days at home.

We would like to see whether the benefits of the strength training programme are maintained. We will therefore invite you to visit us 6 months after you have finished the training programme to re-test your muscle strength. This visit will be optional.

Financial Arrangements

The study involves a number of visits to hospital, you will be compensated for your time. Travel by taxi for all these visits will be provided for you if you need or wish it. Alternatively we will reimburse any travel expenses which you incur.

What will happen to samples I donate as part of this study?

Muscle and blood samples you provide during the study will be stored and analysed at Nottingham University under the supervision of Prof Paul Greenhaff. Some of the analysis will take place after the study has been completed and samples may be stored for up to five years. Any remaining tissue will be destroyed at the end of the study and will not be used for any other purpose or future research. The samples you donate will not be tested for genetic diseases or other conditions.

What do I have to do?

Other than detailed above, you will not have to make any changes to your medication or lifestyle.

What are the possible benefits of taking part?

We would expect you to benefit from improvements in muscle strength as a result of the exercise programme although this may not occur in all participants. If the dietary supplement proves beneficial you may benefit also from this if you are placed in the active treatment group.

We hope the information we get from this study will be helpful in understanding the problem of muscle wasting in patients with COPD and developing new treatments for this problem.

What are the possible disadvantages and risks of taking part?

You may find the muscle biopsy uncomfortable. Your thigh may ache for a day or two after the biopsy. If the biopsy site is uncomfortable we can provide painkillers for you. There is a very small risk that the site of biopsy could bleed or become infected.

The DEXA scans involve a very small exposure to x-rays. This is equivalent to a fraction of that involved in having a standard chest X-ray. A greater exposure would occur naturally from the environment if you were to take a two week holiday in Cornwall.

There is a very small risk of injury from performing the exercise.

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms would be available to you.

Will my taking part in this study be kept confidential?

Yes. Your personal details and all information collected about you during this study will be held in strictest confidence. People organising the trial may see your medical records but in no circumstance will personal details be made available to the public. We will inform your GP that you are taking part in the study. Any information about you that leaves the hospital will have your name and address removed so that you cannot be recognised. If the results of the study are published you will not be identified in the report.

Who has reviewed the study?

All research that involves NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be approved by an NHS Research Ethics

Committee before it goes ahead. Approval does not guarantee that you will not come to any harm if you take part. However, approval means that the committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits and that you have been given sufficient information on which to make an informed decision.

If you have any questions please do not hesitate to contact:

Dr Manoj Menon	Tel 0116 258 3652
Dr. Michael Steiner	Tel 0116 256 3450

Thank you for reading this information leaflet

Appendix III. Healthy Volunteer information Sheet

HEALTHY VOLUNTEER INFORMATION SHEET

Molecular Approaches to Reversing Skeletal Muscle Wasting in COPD. The Role of Resistance Training and Protein Supplementation.

Principal Investigator: **Dr Michael Steiner**

Co-Researchers: **Prof Sally Singh**
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Dr Manoj Menon
Ms Linzy Houchen
Mrs Carolyn Sandland

You may contact: **Dr. Michael Steiner**
0116 256 3450

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exercise training is effective in improving symptoms for patients with COPD. However the mechanisms by which these improvements are brought about are largely unknown.

The aim of this research is to study the effects of a programme of exercise training and dietary supplementation on functioning of these genes in patients who suffer with COPD. You are being asked to take part because we need to compare these effects in healthy people of a similar age.

This knowledge will help us understand why muscle wasting happens in patients with lung diseases such as COPD. This is important because if we can understand how our genes regulate these changes in muscle bulk we may be able to develop new treatments to address this problem in patients with chronic diseases such as COPD.

Why have I been chosen?

Members of the pulmonary rehabilitation research team are conducting this study in collaboration with Prof Paul Greenhaff at the University of Nottingham. You have been invited to participate as a healthy subject who does not have lung disease. Patients with COPD who are attending out- patient departments at Glenfield Hospital are also being invited to participate.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?

The purpose of this research study is to determine the effects of a programme of leg exercise training combined with dietary supplements. If you agree to participate, you will therefore be asked to undertake an eight-week programme of leg (quadriceps muscle) training. Healthy volunteers who agree to participate will not need to take the supplements.

In order to find out what the effect of the training programme is, you will be asked to attend the hospital for additional assessments before the start of the exercise programme, in the middle of the programme and at the end. Some of these assessments will require a specific visit to hospital. Others will be performed on a day you are attending anyway as part of the exercise programme.

Your involvement in the study will last 10 to 12 weeks. The study overall will be running for around three and a half years.

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If you have any questions please do not hesitate to contact:

Dr Manoj Menon Tel 0116 258 3652
Dr. Michael Steiner Tel. 0116 256 3450

Thank you for reading this information leaflet

Appendix IV. Consent form

Participant Identification Number for this trial:

CONSENT FORM

Molecular Approaches to Reversing Skeletal Muscle Wasting in COPD. The Role of Resistance Training and Protein Supplementation.

Principal Investigator: **Dr Michael Steiner**

Co-Researchers: **Prof Sally Singh**
Dr. Michael Morgan
Dr Manoj Menon
Ms Linzy Houchen
Mrs Carolyn Sandland

Please initial box

1. I confirm that I have read and understand the information sheet dated 19/05/2008 (version 7) for the above study and have had the opportunity to ask questions ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix V. Equipment calibration procedures

Isokinetic dynamometer

The isokinetic dynamometer (Cybex II Norm: CSMi, Stoughton, USA) was calibrated every month in accordance with manufacturer's guidelines. This involved placing 100lbs of weight onto the knee/hip adapter arm which was set at a specific length. The weight was then dropped and the system shows the amount of torque that it should read when 100lbs is dropped. Following this weight drop, the system adjusts its internal conversion factors. 'Success' will be displayed if the calibration is ok. If there is an error, the calibration should be repeated before contacting CSMi for technical support.

DEXA Scanner

The DEXA scanner was calibrated daily with a Quality Assurance (QA) test; as suggested by the manufacturer. This involved placing a calibration block under the laser light of the DEXA scanner arm. The block consists of tissue equivalent material with three bone-simulating chambers of known bone mineral content. The remainder of the test runs automatically; testing the detector status, bone mineral density of the block and system status. The software automatically returns to the QA screen once the test is completed. A green light indicates a pass and is a sign that the system is ready to measure patients. A red light indicates a fail. In this scenario the test should be repeated before calling the manufacturer for assistance.

Cycle ergometer

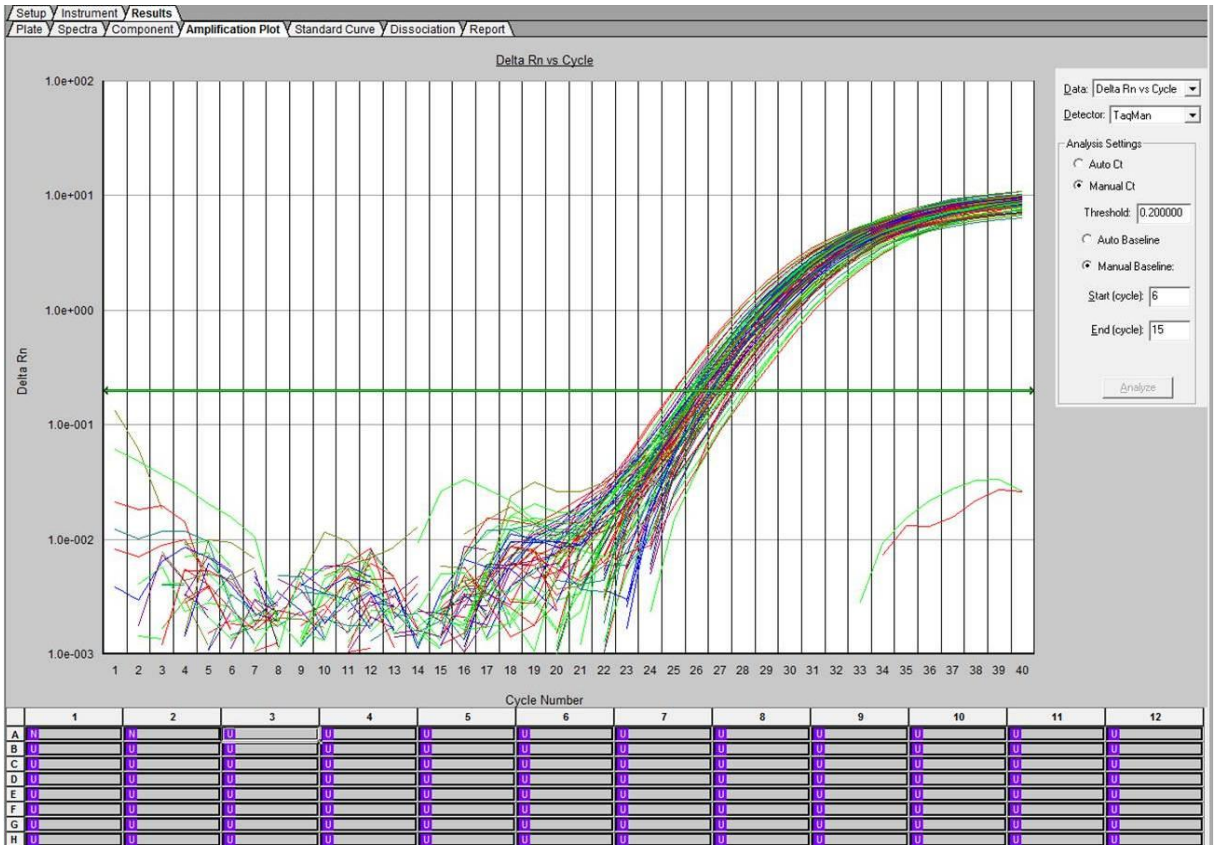
Gas calibration was performed everyday, in line with manufacturer recommendations. Prior to calibration the system had to be switched on for a minimum warm-up time of 20 minutes. The gas calibration involved opening the gas bottle and connecting the gas-suction tube to the calibration port on the front panel of the Zan 600. Gas mixture I (bottle gas) is compared to gas mixture II (ambient air). The actual values are displayed together with the expected values before the calibration is complete. Volume calibration is performed before each individual test, in accordance with manufacturer guidelines. To do this, the flow sensor is connected to a 3l calibration syringe. The piston of the syringe is moved from end to end for six strokes and the results of the calibration are shown. If the calibration volume equals the syringe volume then the calibration is successful. If the deviations are too large, the test is repeated before contacting the manufacturer for support. In addition, a biological quality control test was carried out monthly starting 1 year prior to the study start and during the study; using a young human male subject. After several practice tests, baseline tests were performed over a short period of time. All tests were then compared to these baseline values. The test involved a maximal incremental cycle ergometry test using a ramp protocol. The load was increased by 30W/min after a 1-minute warm-up (no load).

Respiratory Physiology Equipment

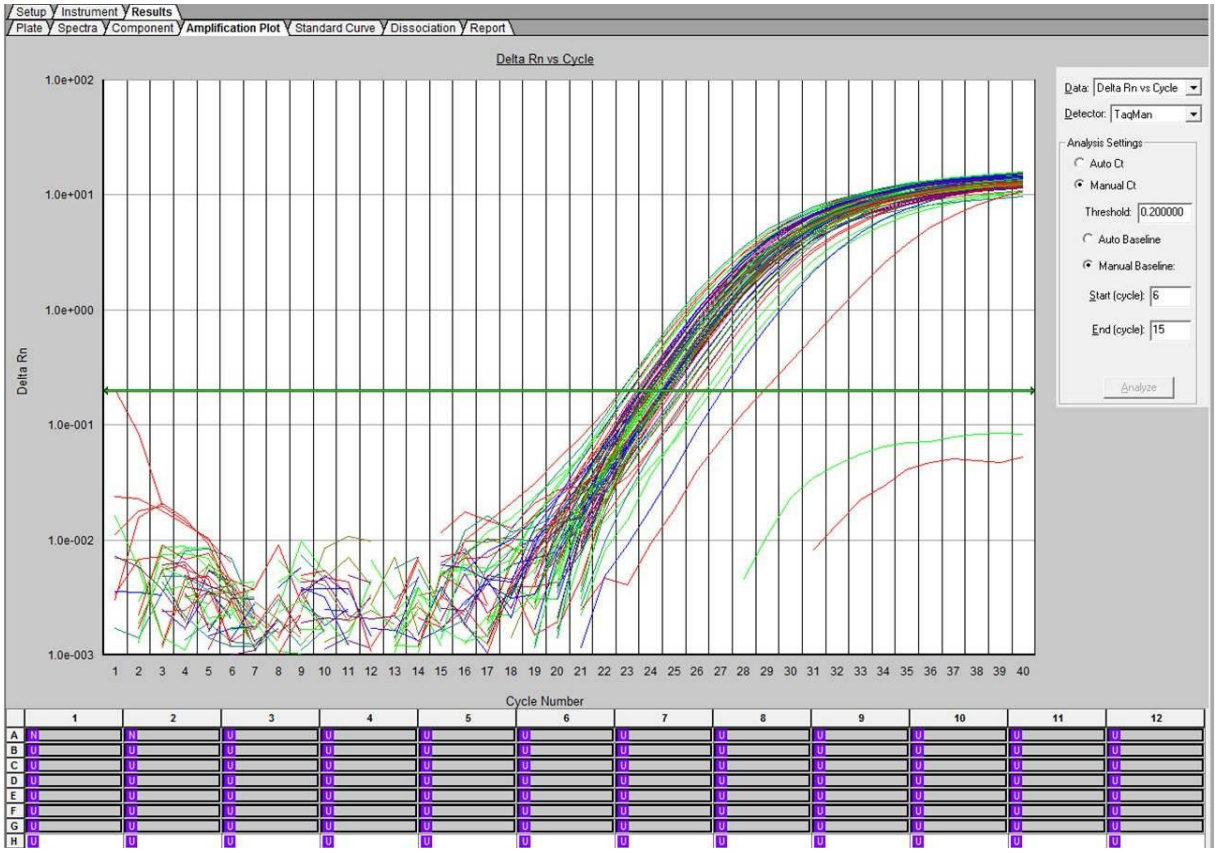
The SPIRO AIR® equipment was calibrated every morning for volume, FRC and transfer factor. A green light is displayed as a pass for all three tests. If a fail occurs, the calibration should be re-done before contacting the manufacturer for assistance. A healthy, young biological control used the equipment every 3 weeks as a further quality control measure.

The blood gas analyser uses a two point calibration to check expected and actual concentrations of O_2, carbon dioxide CO_2 and pH. This occurs automatically every two hours. A further quality control is completed every 2 weeks where 3 known levels (low – high) of O_2, CO_2 and pH are analysed by the system. These should be within 2 SD of what is expected.

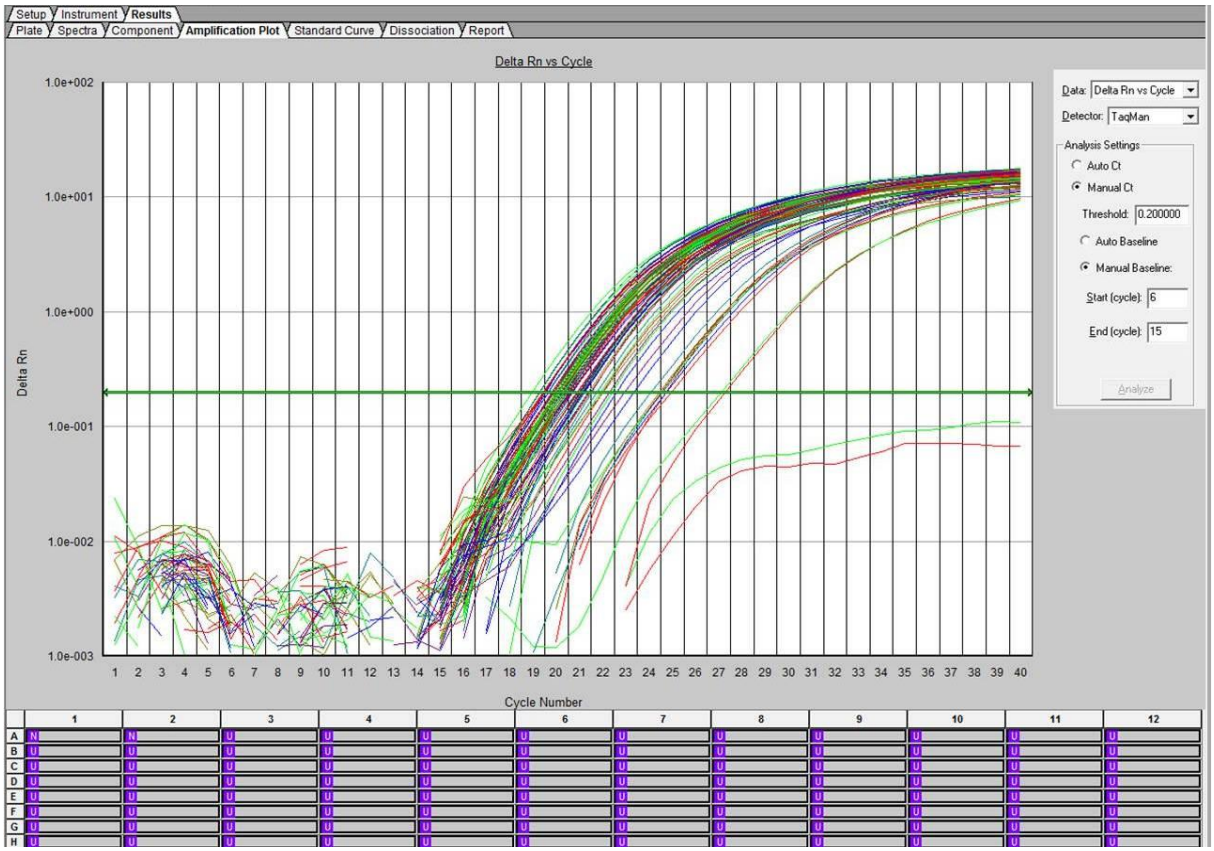
Appendix VI. Example of Amplication curves (4EBP1)



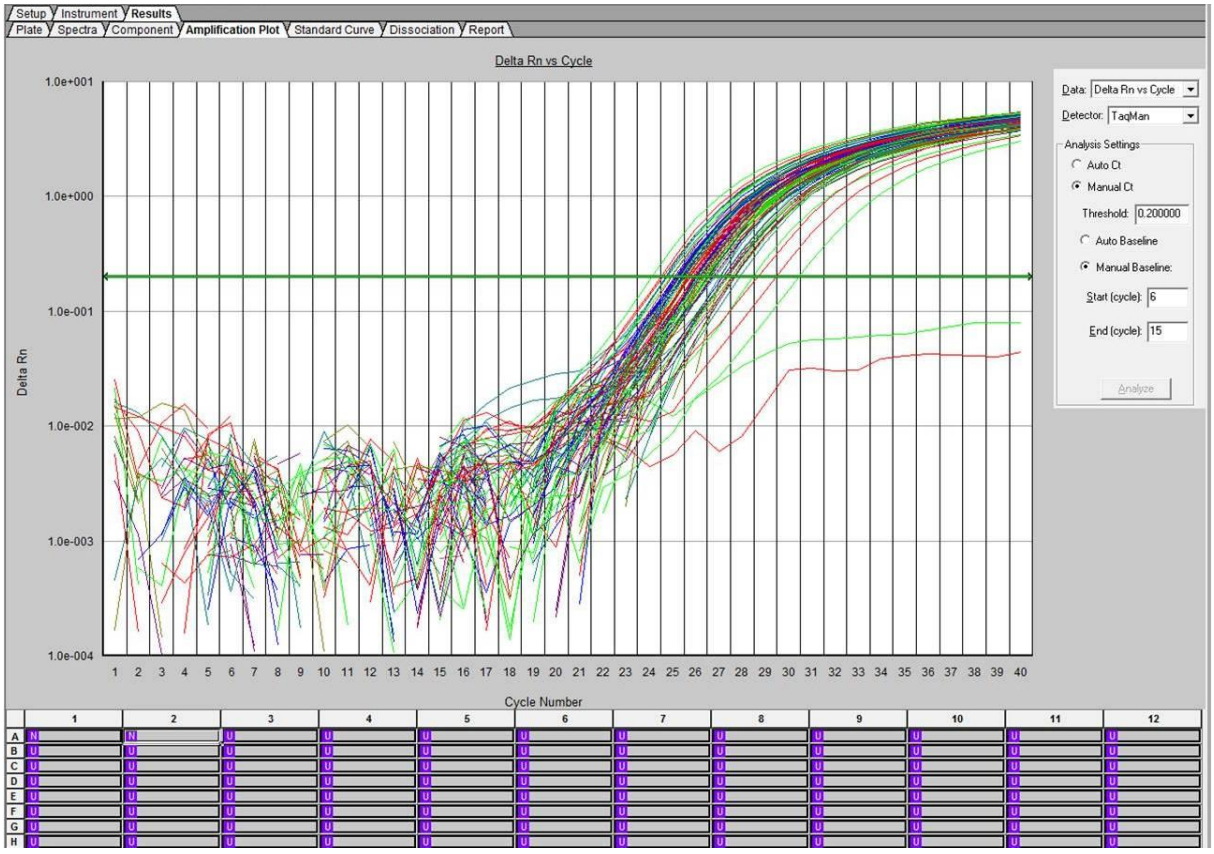
Appendix VII. Example of Amplication curves (Akt1)



Appendix VIII. Example of Amplication curves (Calpain3)



Appendix IX. Example of Amplication curves (FOXO1)



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