Cardiorespiratory and muscle molecular responses to exercise training in advanced age and chronic obstructive pulmonary disease

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Abstract

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INTRODUCTION: Maintenance of cardiorespiratory fitness is important for health in advanced age and in chronic obstructive pulmonary disease (COPD). Aerobic training increases peak oxygen uptake ($\dot{V}O_2^{PEAK}$) in health. Quadriceps weakness is common in COPD. Non-volitional neuromuscular electrical stimulation (NMES) may improve muscle mass and strength as an alternative or adjunct to voluntary resistance exercise (RE). This thesis examines the impact of aerobic exercise training and withdrawal on cardiorespiratory and skeletal muscle molecular markers of adaptation to exercise in advanced age and in COPD and the acute mRNA responses to RE and NMES in COPD.

METHODS: Young and older healthy individuals and patients with COPD performed eight weeks aerobic cycling exercise training followed by four weeks exercise withdrawal. Exercise tests and quadriceps muscle biopsies were performed at baseline, after four and eight weeks training and after four weeks exercise withdrawal. Separately, patients with COPD had quadriceps muscle biopsies before and 24 hours after a single bout of RE or NMES.

RESULTS: $\dot{V}O_2^{PEAK}$ increased similarly in young and older sedentary volunteers after training, but was unchanged in patients with COPD. Cardiorespiratory adaptations were not reversed by four weeks exercise withdrawal in healthy volunteers but $\dot{V}O_2^{PEAK}$ decreased in patients with COPD. Change in abundance of targeted mRNA transcripts in quadriceps muscle was strikingly similar in all three groups, predicted influence over the same biological functions and was maintained after exercise withdrawal. Voluntary RE influenced the abundance of a broader range of mRNA targets that NMES; a small number of transcripts were influenced similarly by both interventions.

CONCLUSIONS: Young and older sedentary volunteers and patients with COPD experienced the same muscle molecular responses to aerobic training and subsequent exercise withdrawal. Training did not increase $\dot{V}O_2^{PEAK}$ in COPD patients suggesting that the limitation to aerobic training adaptations is either downstream of muscle mRNA signalling or due to central limitations. Contractions evoked by neuromuscular electrical stimulation are sufficient to change the abundance of skeletal muscle mRNA targets but voluntary resistance exercise exerts a broader influence.

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Firstly I would like to thank my supervisors. Professor Mick Steiner gave me the opportunity to conduct this research and believed that I was capable of delivering a PhD thesis. Professor Paul Greenhaff has inspired and challenged me throughout this process. As a result of their support, patience and positivity I have achieved more than I thought possible and I hope we will continue to collaborate in the future.

The research projects described in this thesis are the result of successful teamwork. I thank everyone in Leicester and Nottingham who made this work possible. Particular thanks go Dr Neil Greening and Dr Bhavesh Popat who performed the biopsies that I relied upon for my analyses and were active in the recruitment and testing of study volunteers. Dr Linzy Houchen-Wolloff was instrumental in the delivery of the aerobic training study. I am grateful to the wider team at Nottingham, particularly Dr Despina Constantin who taught me laboratory techniques and Dr Tim Constantin for his technical support. At Leicester, the team in the Centre for Exercise and Rehabilitation Science have been a huge support.

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I am indebted to every patient and healthy volunteer who donated their time, effort and quadriceps muscle, motivated overwhelmingly by a sense of altruism and a desire to improve treatments for patients in the future.

Away from work my family have been a great support, always there for me and never asking about my thesis. To John, for the motivation, and to Donna for the encouragement to finish I thank you. I thank my husband, Richard for being there to share life's adventures, enjoy the highs and get me through the tough times. Finally, I am grateful to our daughter, Mary, whose arrival gave me a new perspective on this thesis and on life and who is a daily source of joy.

Declaration

The MATCH Study provided data for Chapters 3-6 and its successful completion resulted from a team effort. I shared the responsibility and workload for patient and healthy volunteer recruitment, physiological assessments and training supervision. Dr Bhavesh Popat conducted over 200 *vastus lateralis* biopsies and the tissue was snap-frozen and stored by Dr Despina Constantin or Dr Tim Constantin at the University of Nottingham. I conducted all subsequent tissue processing, PCR, data analysis and bioinformatics myself with guidance gratefully received from Despina and Tim. Further assistance for some study assessments and training supervision was provided by Dr Linzy Houchen-Wolloff. Physiological data presented in this thesis is from a shared dataset arising from the MATCH study. I have conducted all analysis of the data presented here.

Recruitment of volunteers to the resistance exercise trial described in Chapter 7 took place before I joined the department (conducted by Dr Lori Calvert and Dr Manoj Menon) as did the processing of LDA cards for the resistance exercise group (done by Despina Constantin). I worked alongside Dr Neil Greening in the recruitment and assessment of patients to the NMES arm of the acute study; Neil performed the biopsies and I processed the tissue and ran the LDA cards. I conducted all of the data analysis.

Work that I have conducted for this thesis has been completed during the period of my registration.

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Abbreviations

ASMI	appendicular skeletal muscle index
ATP	adenosine triphosphate
BMI	body mass index
BODE Index	Body mass index, airflow Obstruction, Dyspnoea and Exercise capacity
Borg Breath	Borg scale of breathlessness (scored 0-10)
Borg RPE	Borg Rate of Perceived Exertion (scored 6-20)
cDNA	complimentary DNA
CK	creatine kinase
COPD	chronic obstructive pulmonary disease
CRP	c reactive protein
DCA	dichloroacetate
DNA	deoxyribonucleic acid
FEV1	forced expiratory volume in 1 sec
FFMI	fat free mass index
FVC	forced vital capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HIF-1α	hypoxia inducible factor 1 alpha
HIT	high intensity training
HR	heart rate
IL-6	interleukin-6

IPA	Ingenuity Pathway Analysis®
KCO	transfer coefficient of the lung for carbon monoxide (corrected for alveolar volume)
Mn-SOD	manganese superoxide dismutase
MPB	muscle protein breakdown
MPS	muscle protein synthesis
mPTP	mitochondrial permeability transition pore
MRC Grade	Medical Research Council grade of dyspnoea
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MuRF1	muscle RING-finger protein-1
MVV	predicted maximum voluntary ventilation
NF-κB	nuclear factor kappa B
NMES	percutaneous neuromuscular electrical stimulation
O_2	oxygen
p70S6K	70 kDa ribosomal S6 kinase
Pack Years	average number of cigarette packs smoked per day x number of smoking years
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PR	pulmonary rehabilitation
QMVC	quadriceps maximal voluntary contraction

RE	resistance exercise
RER	respiratory exchange ratio
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RT-PCR	real-time reverse transcription polymerase chain reaction
RV	residual volume
SOB	shortness of breath
SpO2	peripheral capillary oxygen saturation
TLC	total lung capacity
TlCO	transfer factor for the lung of carbon monoxide
TNF-α	tumor necrosis factor-alpha
tRNA	transfer ribonucleic acid
$\dot{V}CO2$	rate of carbon dioxide exhalation
VE	minute ventilation
VEGF	vascular endothelial growth factor
$\dot{V}O_2^{MAX}$	maximal rate of oxygen uptake
VO_2^{PEAK}	symptom limited peak rate of oxygen uptake
W^{PEAK}	work rate corresponding to $\dot{V}O_2^{PEAK}$ in incremental cycle exercise test

Chapter One

Introduction

This thesis will examine the influence of advanced age and the presence of chronic obstructive pulmonary disease (COPD) on exercise capacity, the responses to exercise training and the withdrawal of exercise training. In order to understand the characteristics of exercise performance and adaptation to training in patients with COPD compared to healthy people of a similar age it is important to first understand what characteristics result from ageing itself. This introduction and literature review firstly examines what is known about the influence of age on exercise performance and subsequently the influence of COPD so as to give background to the experimental work described in subsequent chapters.

1.1 Ageing: Definition and context

Ageing has two distinct components: primary ageing and secondary ageing. Primary ageing is the inevitable intrinsic process of biological ageing with the passage of time leading to a decline in the function of all biological processes and is not modifiable. The inevitable decline in physical function with advancing age can be observed in the performance of competitive masters athletes. Records of competitive athletic performance give insight into peak physical function in individuals of varying ages. Both sprint and endurance running performance assessed by age-group world record times peak in the second or third decade of life before a gradual decrease in performance towards the eighth decade (Moore 1975) cited in (McComas 1996), despite the maintenance of dedicated physical training programmes.

Secondary ageing is influenced by environmental factors such as diet; obesity; physical activity and fitness; exposure to noxious substances (e.g. cigarette smoke or industrial chemicals); and the presence of disease. Secondary ageing influences life expectancy and a major factor that can slow or accelerate secondary ageing is physical activity or inactivity (Booth, Laye et al. 2011). In modern westernised societies the opportunities for physical inactivity are plenty.

1.2 Changes in exercise capacity with advancing age

In healthy individuals, peak oxygen uptake is influenced by the maximal rate at which oxygen can be delivered to the contracting muscle and the maximal rate of extraction by muscle tissue (Lundby, Montero et al. 2016). The capacity for oxygen delivery is determined by cardiac output and blood haemoglobin concentration. The rate at which skeletal muscle extracts oxygen from the circulation is largely determined by the capillarity of the tissue (capillary to fibre ratio) and the oxidative capacity of individual fibres (dependent on mitochondrial density). Oxygenation of blood at the lung is not rate limiting in normal healthy subjects (although in extremely highly trained endurance athletes ventilatory capacity may become a factor). The principle limitation to maximal aerobic capacity in health is cardiac output (Saltin and Calbet 2006).

Low cardiorespiratory fitness in older adults is associated with increased all-cause mortality, morbidity and reduced physical function (Blair, Kampert et al. 1996; Blair and Wei 2000; Blair 2009) (Kaminsky, Arena et al. 2013). There is some evidence that the rate of decline in peak oxygen uptake with age may be linked to physical activity status. In an eight-year longitudinal study of men in their 60's masters endurance athletes experienced lower relative and absolute reduction in $\dot{V}O_2^{MAX}$ compared to their sedentary peers (Rogers, Hagberg et al. 1990) while another study reported that sedentary older men experienced a decline in $\dot{V}O_2^{MAX}$ three times greater than regular runners, although this was over a relatively short (< 3 years) follow-up period (Dehn and Bruce 1972). Contrasting evidence has been put forward by a number of authors who have found that across sedentary, active and endurance trained populations, $\dot{V}O_2^{MAX}$ declines with age, independent of the habitual level of physical activity or aerobic training status (Rosen, Sorkin et al. 1998; Wilson and Tanaka 2000; McKendry, Breen et al. 2018). While the influence of aerobic training status on the rate of decline in $\dot{V}O_2^{MAX}$ is ambiguous, what is clear is that while reduction in aerobic capacity with advancing age is inevitable, aerobically trained individuals have a higher capacity in their youth and therefore maintain higher $\dot{V}O_2^{MAX}$ than their sedentary peers as they age. Associated with reductions in $\dot{V}O_2^{MAX}$, the exercise intensity threshold of lactic acid accumulation is reduced with advancing age (Allen, Seals et al. 1985; Evans, Davy et al. 1995) thus reducing functional capacity by limiting the absolute exercise intensity that can be

sustained for long periods. Declines in whole-body aerobic exercise capacity are underpinned by changes in the cardiovascular system and skeletal muscles.

1.2.1 Impact of age on cardiovascular function

Cardiac output (a product of stroke volume multiplied by heart rate) is known to decline with age. Fleg *et al* showed that declines in VO_2^{PEAK} are accompanied by reductions in both peak heart rate and stroke volume (quantified via the surrogate marker O_2 pulse) with advancing age. During a seven year longitudinal study of healthy individuals peak heart rate was shown to reduce by 4-6% per decade across all age groups while O_2 pulse decreased beyond age 40 years with the most rapid declines observed in those aged over 70 years (Fleg, Morrell et al. 2005). Ageing is also associated with increased resting blood pressure related to increased arterial stiffness (Franklin, Gustin et al. 1997).

1.2.2 Muscle structure and function in older adults

Some of the physiological changes that underpin decline in physical function with advancing age are at the level of the skeletal muscles. Reductions in muscle mass and strength as well as declines in aerobic potential are common features of ageing muscle.

1.2.2.1 Muscle aerobic capacity

A reduction in mitochondrial density and function has been observed with advancing age (Conley, Jubrias et al. 2000), however there is some evidence (albeit from a small sample size) that in physically active older men mitochondrial content (expressed as quantity of electron transport chain complexes I and II as well as citrate synthase activity) and respiratory capacity is preserved when compared to physically active young men (Gouspillou, Sgarioto et al. 2014) although some mitochondrial functions (respiratory uncoupling, heightened sensitivity to apoptotic signalling, impaired mitophagy) show signs of dysfunction. There is a reduction in the number of capillaries per myofibre in old age (Frontera, Meredith et al. 1990), and although fibre cross-sectional area is also reduced, the ratio of capillaries per unit fibre area, which is key for oxygen diffusion during aerobic exercise, is still lower in older compared to young healthy volunteers (Coggan, Spina et al. 1992) (data on gastrocnemius muscle).

1.2.2.2 Muscle Weakness

Cross-sectional data suggest that there is also a decline in muscle strength (Young, Stokes et al. 1985; Frontera, Hughes et al. 1991; Hurley, Rees et al. 1998) and bulk (Lexell, Taylor et al. 1988; Frontera, Hughes et al. 1991; Janssen, Heymsfield et al. 2000) in old compared to young healthy individuals. This deterioration in function seems to be associated with a reduction in muscle quality (Visser, Kritchevsky et al. 2002). The loss of muscle size is accompanied by a reduction in the cross-sectional area of individual type II fibres although type I fibre size is preserved (Janssen, Heymsfield et al. 2000).

Ageing is associated with a loss of muscle fibres (Lexell, Taylor et al. 1988) which may explain the loss of muscle mass and strength. If a motor neurone dies, the resultant denervation of muscle fibres causes them to atrophy and ultimately die. Collateral reinnervation of "orphaned" myofibres may take place (McComas 1996), so that not all fibres in a motor unit are lost when the motor neurone dies. The phenotype displayed by an individual muscle fibre is determined by the pattern of its neural activation (McComas 1996). Histological evidence for this phenomenon demonstrates the presence of hybrid fibres which express both type I and type II myosin heavy chain isoforms and fibre type grouping which may indicate that a change in the innervation of the myofibres has occurred with age (Scelsi, Marchetti et al. 1980). There is some evidence that with advancing age there is a shift towards a greater proportion of type I fibres (Scelsi, Marchetti et al. 1980) which is in opposition to that observed in COPD (Gosker, Zeegers et al. 2007). Evidence for this phenomenon in health is somewhat equivocal, with more recent evidence suggesting that there may be a shift towards a greater proportion of type II fibres (Gouzi, Maury et al. 2013) with advancing age.

1.2.3 The influence of habitual physical activity

Declines in athletic performance with advancing years suggest that no amount of physical training can maintain physical function at its youthful peak indefinitely and that primary ageing will exert its influence. However, the vast majority of the population are not highly trained masters athletes and physical inactivity is abundant in young, old, healthy and patient populations. While advancing age has an unavoidable impact on aerobic fitness, the impact of inactivity should not be underestimated as highlighted by the follow-up to the Dallas bed rest study, where three weeks of extreme inactivity in

young men had similar negative impact on cardiorespiratory fitness as the subsequent 40 years of ageing (McGavock, Hastings et al. 2009).

COPD is a disease that most commonly presents in later life. It is common practice to use age-matched healthy volunteers as controls in studies of exercise capacity and rehabilitative exercise. It is important to consider that while we use older healthy controls as a benchmark for defining exercise capacity in COPD, muscle strength and oxidative capacity function declines in older populations with associated structural modifications and that healthy older adults have reduced exercise capacity compared to young healthy adults.

1.3 COPD: Definition prevalence and aetiology

COPD is a progressive condition defined by irreversible airflow limitation. This disease of the lungs commonly causes debilitating dyspnoea in sufferers with associated declines in physical function and reduced quality of life. COPD is associated with significant mortality and morbidity with related economic and social burdens both for the individual and the population (Vestbo, Hurd et al. 2013). COPD is the third largest cause of death worldwide (Lozano, Naghavi et al. 2012). In the global population, 11.8% of men and 8.5% of women are thought to have COPD (Buist, McBurnie et al. 2007).

Diagnosis of COPD usually follows the appearance of symptoms including breathlessness, chronic cough, wheeze and chronic sputum production. International standards for diagnosis require spirometry to identify airflow obstruction with a post-bronchodilator ratio of Forced Expiratory Volume in one second (FEV_1) to Forced Vital Capacity (FVC) of <0.7 indicating airflow limitation that is not fully reversible and therefore COPD.

COPD arises as a result of maladaptive remodelling of the airways driven by a chronic inflammatory response to inhaled pathogens (GOLD). Chronic inflammation of the lung causes the small airways to narrow with an associated increase in airway resistance (obstructive bronchiolitis) and the progressive destruction of the parenchyma (emphysema) which reduces the elasticity of lung tissue (GOLD). Long term

environmental exposure to a variety of noxious gases and particles can trigger the inflammatory response which drives the pathogenesis of COPD. These include industrial pollution and the burning of biomass fuels; however in the Western world the most common risk factor for COPD is exposure to tobacco smoke (GOLD ; Hogg 2004).

COPD develops and becomes symptomatic only after many years of environmental exposure. Ageing itself is a risk factor for COPD; worldwide average life expectancy is increasing (Leon 2011) and as the global population gets older, more of us will develop COPD (GOLD). It is normal for pulmonary function to decline with advancing age (Quanjer, Stanojevic et al. 2012). The rate of lung function decline is accelerated in COPD and thus COPD is viewed by some as a model of accelerated ageing (MacNee, Rabinovich et al. 2014).

The natural progression of COPD is punctuated by acute exacerbations of the disease involving acute inflammation of the lung (Bhowmik, Seemungal et al. 2000; Saetta, Turato et al. 2001) and a worsening of symptoms. Exacerbations are most commonly triggered by bacterial or viral infection of the respiratory tract (Papi, Bellettato et al. 2006; Bafadhel, McKenna et al. 2011), cause an increased need for medication and may require hospitalisation of the patient.

1.4 Systemic manifestations of COPD

Whilst the primary pathology centres on the lung, systemic manifestations of COPD include skeletal muscle dysfunction, osteoporosis, cardiovascular disease, metabolic syndrome, anaemia, gastroesophageal reflux, and clinical anxiety and depression (GOLD; Barnes and Celli 2009; Nussbaumer-Ochsner and Rabe 2011). These extra-pulmonary morbidities contribute significantly to the burden of chronic lung disease (GOLD). Whilst there is little that can be done to reverse the pathological remodelling of lung tissue, dysfunctional skeletal muscles are an appealing therapeutic target in patients with COPD. Skeletal muscle function is important for locomotion, but is also a good prognostic indicator. Weakness of the quadriceps muscles is associated with more frequent admission to hospital for COPD patients (Decramer, Gosselink et al. 1997) and is a predictor of survival independent of either FEV_1, body mass index or age (Swallow, Reyes et al. 2007). Fat free mass index (FFMI) is a surrogate marker for muscle mass and

low FFMI is associated with higher risk for all-cause and COPD-related mortality (Vestbo, Prescott et al. 2006).

1.5 Exercise tolerance in COPD

In addition to reduced muscle strength, it is common for patients with COPD to exhibit impaired capability in endurance activities such as walking. The relative contribution of ventilatory limitation and skeletal muscle dysfunction to this impaired endurance exercise capacity varies between individuals (Vogiatzis and Zakynthinos 2012). The burden of symptoms (principally breathlessness) can drive behavioural adaptations in patients with COPD leading to avoidance of exercise and physical activities which increase dyspnoea in the short-term. Resulting physical inactivity drives deconditioning which results in further reductions in exercise tolerance which in turn lowers the threshold for dyspnoea in a cycle of decreasing physical function (Figure 1.1).

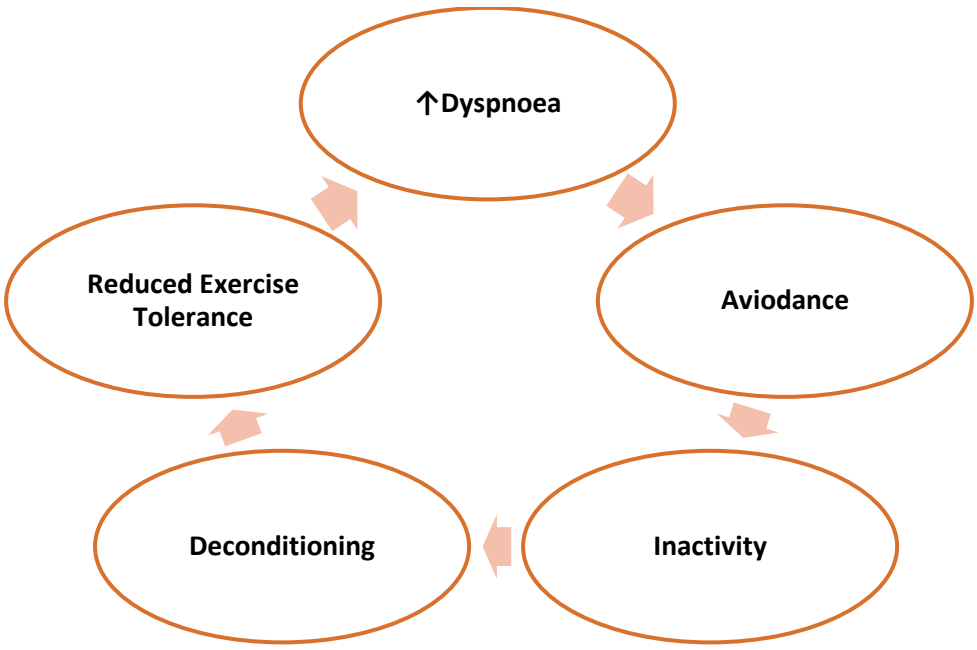


Figure 1-1. Processes contributing to skeletal muscle dysfunction and reduced physical function in COPD

Patients with COPD generally demonstrate reduced peak exercise capacity compared to healthy controls in laboratory testing. On average peak oxygen uptake is ~50% lower in patients with COPD compared to healthy controls of a similar age (Maltais, LeBlanc et al. 1996; Gosker, Lencer et al. 2003; Vaes, Wouters et al. 2011).

1.5.1 Ventilatory limitations to exercise

In health, the capacity of the respiratory system exceeds ventilatory demands during exercise at maximal aerobic intensity ($\dot{V}O_2^{MAX}$) with the cardiovascular system limiting maximal exercise (Wasserman 2012), however some patients with COPD exhibit a ventilatory limitation to exercise. Ventilatory limitation in COPD has multiple contributing factors. High airway resistance and increased lung compliance (typical of emphysema) can substantially increase the estimated energy demands of breathing for patients with COPD, by as much as 5-10 fold above that of a healthy person at rest (MacIntyre 2008). Hyperinflation (both static and dynamic) increases end expiratory lung volumes, reducing inspiratory capacity and thus limiting tidal volume (O'Donnell, Revill et al. 2001). Hyperinflation also puts the respiratory muscles at a mechanical disadvantage largely through flattening of the diaphragm (Gibson 1996) which reduces its force generating capacity (Sinderby, Spahija et al. 2001). As the highest rate of oxygen uptake that can be achieved during intense exercise is restricted by ventilatory constraints in patients with COPD it is common practice to use the term peak oxygen uptake ($\dot{V}O_2^{PEAK}$) in this population opposed to $\dot{V}O_2^{MAX}$, a term which implies that a plateau in rate of oxygen uptake has been reached despite increasing exercise intensity.

There are some therapeutic strategies for overcoming the ventilatory restrictions experienced by patients with COPD. Bronchodilation has been found to improve exercise tolerance in around 50% of studies (Liesker, Wijkstra et al. 2002) but the effects of bronchodilators are generally small compared to the effects of pulmonary rehabilitation interventions (Borel, Provencher et al. 2013). Inhalation of low density heliox in place of room air is another strategy that has been trialled aiming to reduce the work of breathing with generally positive effects on the intensity and duration of exercise that patients can sustain (Hunt, Williams et al. 2010). For a small proportion of the COPD population who have significant hyperinflation, lung volume reduction by surgical or bronchoscopic means may relieve symptoms and improve exercise tolerance

(Ferguson, Fernandez et al. 1998; Criner, Cordova et al. 2011; Kemp and Shah 2016). Whilst these interventions improve lung function and / or exercise performance in some patients, they do not restore function to the same level as healthy individuals and are only effective in the short-to-medium term.

1.5.2 Skeletal muscle dysfunction

It would be easy to assume that exercise intolerance in COPD results purely from impaired lung function and associated dyspnoea; however dysfunction of the skeletal muscles makes a significant (and sometimes dominant) contribution to premature fatigue during prolonged exercise as well as causing weakness and associated frailty. Skeletal muscle dysfunction in COPD can be classified by two functional limitations; reduced muscle strength (associated with low muscle mass) and reduced resistance to fatigue (associated with low oxidative capacity).

1.5.2.1 Muscle fatigue during endurance exercise in COPD

Premature fatigue of ambulatory muscles has long been recognised as a common cause for exercise termination for patients with COPD. The relationship between lung function and exercise performance is not tightly coupled. Indeed, the most extreme intervention for improving lung function; lung transplant can restore lung function to within the normal range, yet exercise tolerance remains impaired in these patients (Williams, Patterson et al. 1992). When asked their reasons for stopping in a maximal incremental cycle ergometer test Killian *et al* reported that only 26% of COPD patients stopped solely due to breathlessness, while 43% stopped due to leg fatigue and 31% stopped due to a combination of the two factors (Killian, Leblanc et al. 1992). Killian *et al* also reported that the degree of lung function impairment was not tightly related to dyspnoea or leg fatigue scores. Evidence that bronchodilator therapy improves exercise tolerance in some, but not all COPD patients also points to the contribution of peripheral muscle dysfunction to reduced exercise tolerance in COPD (Saey, Debigare et al. 2003); demonstrating that some patients have a ventilatory limitation to exercise while others are limited by early fatigue of the leg muscles. Endurance exercise capacity is therefore influenced by both pulmonary function and peripheral muscle function in COPD (Gosselink, Troosters et al. 1996).

1.5.2.2 Peripheral muscle weakness in COPD

In COPD weakness does not affect all muscle groups equally, with muscle dysfunction more common in the ambulatory muscles (Maltais, Decramer et al. 2014) whereas strength is relatively well preserved in the upper limbs (Bernard, LeBlanc et al. 1998; Maltais, Decramer et al. 2014). This observation is likely related to the physical activity demands placed on the muscle groups as part of an independent lifestyle: it may be possible to reduce ambulatory activity as a dyspnoea avoidance strategy, but the upper limbs are required to maintain activities of daily living such as lifting heavy shopping or cooking utensils. COPD patients exhibit reduced thigh muscle size and strength compared to healthy age-matched controls (Bernard, LeBlanc et al. 1998). Around a third of patients with COPD have quadriceps muscle weakness, with this figure rising to 43% in the most breathless patients (Seymour, Spruit et al. 2010). Compared to healthy controls, patients with COPD have a reduction in quadriceps peak force ranging from 16-18% in isokinetic (Coratella, Rinaldo et al. 2018) or isometric (Gagnon, Maltais et al. 2013) voluntary contractions with one study reporting a deficit as large as 30% in isometric quadriceps maximum voluntary contraction force in COPD (Seymour, Ward et al. 2012). There are few longitudinal studies of skeletal muscle function in COPD however it has been shown that the greatest rates of muscle loss occur in patients with the lowest lung function and those who continue to smoke (Hopkinson, Tennant et al. 2007; van den Borst, Koster et al. 2011).

1.5.3 Structural and physiological determinants of reduced aerobic capacity in COPD skeletal muscle

The ability of muscle to perform repetitive contractions over an extended period of time is dependent on its capacity for metabolising fuels by oxidative means. A reduced capacity for oxidative metabolism which is a feature of COPD (Maltais, Simard et al. 1996), reduces the absolute workload that the muscle can produce for an extended period with reliance shifting to anaerobic adenosine triphosphate (ATP) resynthesis at relatively low absolute work intensities triggering early fatigue. Consequently, early lactic acidosis during prolonged exercise is a feature of COPD (Maltais, Simard et al. 1996; Engelen, Schols et al. 2000). The reliance on anaerobic metabolism and lactic acid production is not due to reduced arterial oxygen delivery, which is maintained in COPD,

but due to dysfunctional muscle metabolism (Maltais, Jobin et al. 1998). There is evidence that unlike healthy controls, patients with COPD experience a reduction in adenosine triphosphate (ATP) availability during five minutes exercise at 80% of peak work despite exercising at a lower absolute intensity (Steiner, Evans et al. 2005) suggesting that the energy delivery pathways of oxidative phosphorylation are inadequate to meet the demands of even low intensity exercise in this patient group. Reduced oxidative capacity of skeletal muscles is underpinned by a number of structural and functional changes to the muscle.

Mitochondrial density is reduced in the quadriceps muscles of patients with COPD (Gosker, Hesselink et al. 2007) with citrate synthase activity (a proxy for mitochondrial content) estimated to be 10 – 38% lower in patients with COPD compared to healthy controls. As the mitochondria account for all oxidative metabolism of fuel, a reduced content will reduce the capacity of the muscle to generate ATP via oxidative means. There is somewhat equivocal evidence regarding the function of individual mitochondrial units in COPD. There is evidence for increased reactive oxygen species (ROS) production by mitochondria during exercise in COPD patients (Meyer, Zoll et al. 2013) and for altered respiration and function of the electron transport chain (Gifford, Trinity et al. 2018), however the capacity for ATP synthesis has not been carefully studied. One study has targeted mitochondrial function directly using a drug (dichloroacetate; DCA) which activates the pyruvate dehydrogenase complex resulting in an amelioration of the natural metabolic inertia in mitochondrial ATP production at the start of exercise. Administration of DCA reduced the reliance on anaerobic ATP regeneration (evidenced by reduced blood lactic acid concentrations) and increased peak work and $\dot{V}O_2^{PEAK}$ in stable patients with COPD (Calvert, Shelley et al. 2008). The impact of DCA on exercise performance highlights the mitochondria as a target for interventions aiming to improve exercise tolerance in COPD.

In the peripheral muscles an abnormal fibre type composition is apparent in both early (Slot, van den Borst et al. 2014) and advanced (Vogiatzis, Terzis et al. 2011) disease suggesting a preferential loss of type I oxidative fibres. The ambulatory muscles of patients with COPD thus contain a greater proportion of type II glycolytic fibres which are more prone to fatigue during prolonged exercise. The relatively high proportion of

type II fibres in the muscles of COPD patients may partially explain the relatively low mitochondrial density observed in the muscle as a whole since type II fibres generally express lower mitochondrial content than type I fibres (Gosker, Hesselink et al. 2007).

Another structural alteration to skeletal muscle in COPD is the relative reduction in capillary density in muscle tissue compared to healthy controls (Jobin, Maltais et al. 1998; Spruit, Singh et al. 2013). High capillary density is associated with high oxidative capacity of muscle as it facilitates efficient of delivery of oxygen and fuel substrates as well as improved metabolite removal from the contracting muscle fibre. Among patients with COPD those with the lowest capillary to fibre ratio are more prone to fatigue induced by intense cycling exercise (Saey, Michaud et al. 2005).

As discussed above, reduced oxidative capacity in the muscles of patients with COPD causes greater production of lactic acid compared to healthy controls exercising at a similar intensities (Maltais, Simard et al. 1996). Lactic acidosis causes muscle discomfort and fatigue, ultimately leading to exercise cessation but can also exacerbate dyspnoea. The accumulation of metabolites in skeletal muscle (e.g. hydrogen ions arising from lactic acid) causes activation of group III and IV muscle afferents which deliver feedback to the central nervous system signalling for respiratory centres to increase ventilation. The influence of group III and IV afferents has been highlighted by work that showed reduced sensations of dyspnoea, altered ventilatory mechanics and increased endurance time in patients with COPD when afferent nerve signalling was blocked pharmaceutically during steady state exercise (Amann, Blain et al. 2010; Gagnon, Bussieres et al. 2012).

The respiratory muscles of patients with COPD are required to work against increased loads (due to high airway resistance) and often with sub-optimal biomechanics (flattening of the diaphragm due to hyperinflation both at rest and in response to increased ventilation during exercise). Of note, despite the mechanical disadvantage that respiratory muscles are subject to in COPD, there is evidence that their function is maintained through compensatory mechanisms (Similowski, Yan et al. 1991), including a shift towards fatigue resistant slow twitch muscle fibre phenotype (Levine, Kaiser et al. 1997; Doucet, Debigare et al. 2004) and associated increase in mitochondrial capacity

compared to healthy controls (Levine, Gregory et al. 2002). This phenomenon is likely explained by a training effect brought about by the increased mechanical load required for ventilation in the patient population and suggests that the muscle phenotype is a product not only of the muscle's environment but of the demands placed upon it and may therefore be amenable to training.

There have been recent attempts to characterise the skeletal muscle transcriptome in COPD and identify muscle biomarkers of skeletal muscle dysfunction. The work of Tényi *et al* (Tenyi, Cano et al. 2018) identified four network modules of genes that are specific to quadriceps muscle in COPD (compared to sedentary healthy controls) and which represent atypical energy production; muscle contractility; inflammatory stress; and oxidative stress. These modules did not have significant associations with disease severity defined by FEV_1, but did have associations with measures that included an exercise component (BODE index, $\dot{V}O_2^{PEAK}$, Peak Power and blood lactate accumulation during exercise).

1.5.4 Structural and physiological determinants of muscle weakness in COPD

In health, the force generating capacity of skeletal muscle (its strength) is directly proportional to the muscle's physiological cross sectional area. There is evidence that in COPD this force-to-size ratio is maintained (Bernard, LeBlanc et al. 1998) with reduced strength being a function of reduced muscle mass. Low muscle mass can occur even in individuals of normal body weight as shown in the phenotyping study by Vanfleteren *et al* (Vanfleteren, Spruit et al. 2013) who found muscle wasting in 28% of their cohort while only 14% were underweight.

The cross sectional area of Type I, IIa, and IIb quadriceps muscle fibres is smaller in patients with COPD than in healthy age-matched controls (Whittom, Jobin et al. 1998). Even in patients with normal body mass index (BMI), around 26% have significantly reduced fat-free mass index (FFMI) and low FFMI is associated with higher risk for all-cause and COPD-related mortality (Vestbo, Prescott et al. 2006).

1.5.5 Causes of skeletal muscle dysfunction in COPD

Patients with low muscle mass are more likely to have a lung pathology characterised by static hyperinflation and low diffusion capacity (an emphysematous phenotype)

(Vanfleteren, Spruit et al. 2013) raising the possibility that there is a common mechanism underpinning the pulmonary and extra-pulmonary elements of COPD and muscle wasting. A range of individual factors including inflammation, oxidative stress, mitochondrial dysfunction, hypoxia and hypercapnia, inadequate nutrition, smoking, negative muscle protein balance, acute exacerbations and physical inactivity have been identified that contribute to reduced mass, strength and aerobic capacity of the skeletal muscles in COPD. It is likely that all of these factors contribute to varying degrees in different individuals.

1.5.5.1 Inflammation

Cross sectional studies have found associations between markers of inflammation and exercise capacity or muscle mass. COPD patients with elevated C-reactive protein (CRP; a marker of systemic inflammation) levels in their blood have been shown to produce lower peak power in an incremental cycle test and lower six minute walking distance compared to those with normal CRP (Broekhuizen, Wouters et al. 2006), while patients with low body mass have significantly higher serum tumour necrosis factor (TNF)-α levels than those with normal body mass. Circulating levels of the inflammatory cytokines interleukin-6 (IL-6) and TNF-α have also been shown to negatively correlate with quadriceps strength both in healthy older volunteers and patients with airway obstruction (Yende, Waterer et al. 2006). A murine model of pulmonary inflammation (with overexpression of TNF-α in lung tissue) has provided insight into a possible mechanistic link between systemic inflammation and skeletal muscle dysfunction. Inflammatory overspill from the lungs was apparent from raised TNF-α in the circulation and was associated with reduced body weight, reduced muscle mass and reduced myoblast proliferation in the skeletal muscles of mice. Further evidence of the negative impact of TNF-α on skeletal muscle was offered by the authors of this study who reported that myoblasts cultured with TNF-α in vitro had reduced differentiation potential characterised by lower accumulation of myosin heavy chain proteins (Langen, Schols et al. 2006). To balance the evidence that inflammation in general and TNF-α in particular exert negative effects on skeletal muscle there are data from a study of patients with COPD that suggests the opposite is true. Barreiro *et al* (Barreiro, Schols et al. 2008) reported that quadriceps TNF-α levels were lower in patients with COPD than

in age-matched healthy controls and found a positive correlation between TNF-α and quadriceps voluntary and non-volitional muscle strength. In explanation of these seemingly contradictory findings it seems likely that the location of inflammatory cytokines is important (in peripheral blood or within muscle tissue) and that their physiological role is determined by concentration (there is evidence that TNF-α exerts catabolic actions at low concentrations and anabolic actions at high concentrations related to modification of muscle protein synthesis and breakdown rates (Alvarez, Quinn et al. 2001)); and other coinciding factors for example muscle injury or exercise status (Sin and Reid 2008).

1.5.5.2 Oxidative stress

Oxidative stress in the lungs is a feature of COPD and markers of oxidative stress are elevated in the circulation of patients with COPD compared to healthy individuals. Patients with COPD also exhibit reduced antioxidant capacity the cause of which may be cigarette smoke exposure and recurrent exacerbations (Kirkham and Barnes 2013). An early study assessing oxidative stress in skeletal muscle (opposed to markers in the blood) found no difference in protein oxidation, increased lipid peroxidation, and a strong upregulation of the anti-oxidant manganese superoxide dismutase (Mn-SOD) in stable patients with COPD compared to healthy controls (Barreiro, Gea et al. 2003). In later work, Barreiro et al (Barreiro, Schols et al. 2008) found protein carbonylation in quadriceps muscle was elevated in COPD compared to healthy controls and that the degree of protein carbonylation negatively correlated with muscle strength. It has also been shown that patients with advanced COPD exhibit markers of oxidative stress in the diaphragm muscle (Barreiro, de la Puente et al. 2005). Carbonylation of the protein creatine kinase (CK) in quadriceps muscle has a negative correlation with peak oxygen uptake in patients with COPD and it is possible that carbonylation of CK reduces its activity and may contribute to impaired muscle function in COPD although the authors of this report did not find CK carbonylation to be elevated in COPD compared to a healthy control group (Barreiro, Gea et al. 2005).

Mitochondrial respiration is a source of free radical production when there is uncoupling of ATP production from oxygen consumption leading to leak of electrons from the electron transport chain (Jastroch, Divakaruni et al. 2010). The increased demand for

ATP during exercise can increase the rate of free radical production by the mitochondria as there is greater flux in the respiratory chain. Acute exercise increases markers of oxidative stress in the circulation of patients with COPD (Vina, Servera et al. 1996) and increases protein oxidation and peroxidation of lipids in quadriceps muscle (Couillard, Maltais et al. 2003). Oxidative stress is more pronounced in chronically hypoxaemic patients who exhibit greater lipid peroxidation in quadriceps muscle at rest and exhaustive exercise induces greater lipid and protein oxidation compared to normoxic patients with COPD (Koechlin, Maltais et al. 2005). Unlike healthy individuals, patients with COPD do not increase their ability to counteract oxidative stress within the exercising muscle after eight weeks high-intensity aerobic training (Rabinovich, Ardite et al. 2001).

In summary, elevated oxidative stress is apparent in resting skeletal muscle of patients with COPD who despite demonstrating some signs of elevated anti-oxidant capacity still accumulate damage to proteins and lipids through oxidative action. It is possible that in some patients the degree of oxidative stress is made worse by increased mitochondrial activity during exercise and that unlike healthy controls anti-oxidant capacity is unable to keep pace with free radical production thus contributing to impaired adaptation to exercise training in the skeletal muscles of COPD. A correlation between raised inflammatory markers in COPD and an oxidative stress response to acute exercise in COPD has been identified (Koechlin, Couillard et al. 2004) and although the authors were unable to identify a clear mechanistic link between the two processes, this finding highlights the interconnected nature of factors affecting skeletal muscle function.

1.5.5.3 Mitochondrial dysfunction

Alterations in mitochondrial function in the skeletal muscles of patients with COPD may reduce the oxidative capacity and therefore endurance of the muscle and the role of mitochondria in oxidative stress has already been discussed, however it is also possible that dysfunctional mitochondria initiate other maladaptations in skeletal muscle, in particular muscle atrophy. The autophagy protein breakdown pathway is referred to mitophagy when mitochondria are the primary target for proteolysis. Autophagy is upregulated in the skeletal muscles of patient with COPD compared to healthy controls and is associated with upregulation of muscle protein breakdown signalling and

inhibition of muscle protein synthesis signalling (Guo, Gosker et al. 2013). An upregulation of mitophagy in COPD may contribute to reduced mitochondrial content in muscle fibres if mitochondrial biogenesis does not keep pace with breakdown (Taivassalo and Hussain 2016). Mitochondria can initiate apoptosis through opening of the mitochondrial permeability transition pore (mPTP) which allows release of pro-apoptotic factors in to the cytoplasm. In COPD one small study has shown signs of elevated apoptosis in quadriceps muscle of patients with COPD and low BMI compared to healthy controls and COPD patients with normal BMI (Agusti, Sauleda et al. 2002). The contribution of the mitochondria to elevated apoptosis in low-BMI patients with COPD is unclear with one study of isolated mitochondria reporting elevated mPTP opening in mitochondria from patients with COPD (Puente-Maestu, Perez-Parra et al. 2009) while another study showed reduced opening of mPTP when studied in permeabilised fibres (Picard, Godin et al. 2008).

1.5.5.4 Hypoxia and hypercapnia

Systemic hypoxia and hypercapnia tend to be associated with advanced severity of stable COPD, and may also present during acute exacerbations (Suntharalingam, Wilkinson et al. 2017). Hypoxia in skeletal muscles may arise from inadequate oxygenation of the blood at the lung and resultant hypoxaemia, or can occur locally in exercising muscle due to inadequate delivery of oxygenated blood (due to low capillary density). Chronic hypoxaemia is associated with low body and muscle mass in patients with COPD (Schols, Soeters et al. 1993) and impaired adaptation to pulmonary rehabilitation exercise training (Costes, Gosker et al. 2015). As discussed previously, COPD patients with chronic hypoxaemia are more susceptible to oxidative stress induced by single leg exercise than patients with similar lung function but normal oxygen saturations (Koechlin, Maltais et al. 2005). When exposed to hypoxic conditions *in vitro*, cultured myotubes have been shown to atrophy and reduce maximum contraction force in a manner likened to the phenotype of skeletal muscle in COPD (Martin, Aguilar-Agon et al. 2017). Intermittent hypoxia-hypercapnia over prolonged periods has been shown to reduce the proportion of type I oxidative muscle fibres *in vitro* (Huang, Jin et al. 2016) which again mirrors the characteristics of the ambulatory muscles in patients with COPD, whereas exposure of myotubes to constant hypoxia for $\leq$ 3 days has been shown

to up-regulate the expression of slow-oxidative type I myosin via HIF-1α signalling (Slot, Schols et al. 2014). Myogenesis and satellite cell function are influenced by hypoxia with extreme hypoxia having a deleterious effect, but the possibility that mild hypoxia may enhance myogenesis (Chaillou and Lanner 2016). The influence of hypoxia on skeletal muscle function therefore is likely dependent on the duration and the severity of reduced oxygen availability.

Carbon dioxide as well as oxygen is important when considering the impact of blood gas concentration on skeletal muscle fibres. Induction of acute hypercapnia ($\leq$ 20 minutes) has been shown to reduce the force generating capacity of peripheral muscle (Mador, Wendel et al. 1997) but persistent exposure is likely to induce lasting physiological changes in muscle fibres. In animal studies, mechanistic links have been shown between hypercapnia and up-regulation of skeletal muscle atrophy signalling (Jaitovich, Angulo et al. 2015).

1.5.5.5 Muscle protein balance

Muscle protein balance is maintained when muscle protein synthesis (MPS) matches rates of muscle protein breakdown (MPB). Negative muscle protein balance occurs when MPS is reduced or MPB is increased and leads to net loss of muscle mass. An early study to suggest reduced MPS was by Morrison *et al* using a stable isotopic tracer technique (Morrison, Gibson et al. 1988) and reported lower resting (16 hour fasted) whole-body muscle protein synthesis in COPD patients compared to healthy controls, although controls were younger and not matched for habitual physical activity (it is also not clear whether these data were corrected or the lower muscle mass of patients in the COPD group). In a comparison of key signalling molecules impacting on muscle mass in the quadriceps and diaphragm of patients with COPD Doucet *et al* (Doucet, Dube et al. 2010) found an imbalance towards up-regulation of Atrogin-1 and MuRF1 mRNA expression and relative down-regulation of activated proteins of the Akt / mTOR pathway indicating a shift towards atrophy and away from hypertrophy signalling in the quadriceps of patients with COPD. Another study reported that expression of the muscle catabolic signalling proteins (MAFbx and MuRF1) was higher in the quadriceps of patients with COPD than healthy controls, however in this sample certain proteins associated with anabolic and myogenic signalling (phosphorylated-P70s6k, Redd1,

MyoD and myogenein) were also higher in COPD perhaps suggesting greater muscle protein turnover in COPD (although that was not measured directly) (Constantin, Menon et al. 2013). Regulators of the ubiquitin proteasome system (including MuRF1) have been shown to be elevated in patients with COPD and reduced muscle mass compared to healthy controls (Plant, Brooks et al. 2010). Adding to this evidence, Guo *et al* (Guo, Gosker et al. 2013) showed an upregulation of autophagosome formation as well as inhibition of the Akt / mTOR pathway. Combined with reductions in muscle protein synthetic rates, evidence of up-regulation in the lysosomal autophagy system as well as the ubiquitin-proteasome system of muscle protein breakdown suggests a shift towards a catabolic state in COPD which is borne out by the common observation of low muscle mass in individuals with COPD.

1.5.5.6 Nutritional considerations

Some patients with COPD experience weight loss as a result of energy expenditure exceeding nutritional energy intake. There is evidence that energy expenditure is higher in COPD than in health, possibly due to the increased work of breathing (Creutzberg, Schols et al. 1998). In addition to increased energy expenditure at rest, some patients with COPD experience reduced appetite due to dyspnoea when eating or dysregulated hormonal control of appetite (which may be influenced by systemic inflammation) which contributes to undernutrition (Rawal and Yadav 2015). Nutritional supplementation in stable patients with COPD my not benefit body composition or exercise / functional capacity (Ferreira, Brooks et al. 2000). Dietary supplementation in addition to an exercise stimulus has had mixed effects. One study found that a nutritional supplement during rehabilitation led to gains in fat mass rather than lean mass (Schols, Soeters et al. 1995) whilst another showed supplementation prevented loss of fat mass during pulmonary rehabilitation but did not influence lean mass (Steiner, Barton et al. 2003). Carbohydrate supplementation, which might reasonably be expected to influence muscle protein synthesis through activation of the insulin signalling pathway in muscle, did not augment increases in thigh lean mass and strength following a quadriceps resistance exercise training programme in normal weight patients with COPD (Constantin, Menon et al. 2013). An interesting observation from a sub-group analysis in one study is that while patients with low body mass (BMI < 19

kg/m^2) are considered to be nutritionally depleted it was those with BMI > 19 kg/m^2 whose incremental shuttle walking test performance benefitted from the consuming a drink containing carbohydrate, fat and protein regularly throughout a PR programme (Steiner, Barton et al. 2003).

1.5.5.7 Smoking

Exposure to cigarette smoke is the principal risk factor for the development of COPD (Hogg 2004), but it also has direct influence on muscle function. Continued cigarette smoking is associated with greater rates of skeletal muscle loss in patients with COPD (Hopkinson, Tennant et al. 2007; van den Borst, Koster et al. 2011). Smokers show reduced rates of muscle protein synthesis at rest than non-smoking controls (Petersen, Magkos et al. 2007) and mice exposed to cigarette smoke show decreased PGC-1α signalling and a loss of oxidative phenotype in classically oxidative muscle fibres (soleus muscle) (Tang, Wagner et al. 2010) as well as reduced muscle mass. Further evidence shows that mice exposed to cigarette smoke have reduced capillarity in soleus muscle, reduced *ex vivo* muscle fatigue resistance and slow uptake of calcium ions to the sarcoplasmic reticulum associated with slow relaxation at the end of contraction (Nogueira, Trisko et al. 2018). *In vitro*, myofibres exposed to cigarette smoke exhibit signs of NF-κB mediated protein loss (Kaisari, Rom et al. 2013). Exposure to cigarette smoke in healthy non-smokers acutely impacts on the ability of mitochondria extracted from blood cells to utilise oxygen *ex vivo* (Alonso, Cardellach et al. 2004) suggesting the potential impact of smoking on skeletal muscle aerobic capacity. In human studies, assessment of skeletal muscle function in smokers *vs* non-smokers can sometimes be confounded by lower physical activity levels in the smoking group, however there is evidence of the direct effect of cigarette smoke on skeletal muscle in reducing muscle strength and oxidative capacity (Degens, Gayan-Ramirez et al. 2015).

1.5.5.8 Deconditioning

Muscle contraction triggers a cascade of signalling events within muscle fibres causing acute responses to a single exercise bout, or physiological adaptation to chronic exercise training. The absence of this stimulus due to muscular inactivity or immobilisation leads to deconditioning and loss of force generating or oxidative potential. Patients with COPD are known to have low levels of habitual physical activity even in the stable state which

is associated with low quadriceps mass (Shrikrishna, Patel et al. 2012). The impact of physical inactivity will be discussed in detail below.

1.5.5.9 Acute exacerbations

Acute exacerbations of COPD are associated with loss of skeletal muscle mass and strength and patients who suffer frequent exacerbations experience a greater rate of decline in muscle strength than those who have longer periods of disease stability (Hopkinson, Tennant et al. 2007). During a period of acute illness the skeletal muscles are subject to an onslaught of challenges including increased systemic inflammation and oxidative stress; hypoxia and hypercapnia; systemic corticosteroid therapy; negative nutritional energy balance; and extreme inactivity of the ambulatory muscles or mechanical overloading of the respiratory muscles (Gayan-Ramirez and Decramer 2013). Systemic administration of corticosteroids impacts on muscle function and may induce atrophy and muscle weakness (van Balkom, van der Heijden et al. 1994). The impact of steroids on skeletal muscle is likely dose dependent and whilst the highest doses of steroid are administered around times of acute exacerbation, the use of low-dose maintenance steroids is associate with a greater rate of quadriceps muscle strength decline (Hopkinson, Tennant et al. 2007).

1.5.6 Physical activity in COPD

Physical activity has been defined as “any bodily movement produced by skeletal muscles that results in energy expenditure” and should not be confused with exercise which is “a subset of physical activity that is planned, structured, and repetitive and has as a final or an intermediate objective the improvement or maintenance of physical fitness” (Caspersen, Powell et al. 1985). It is well established that habitual physical activity levels decrease with advancing disease severity (Watz, Waschki et al. 2009) and sedentary time is a predictor of mortality in COPD (Furlanetto, Donaria et al. 2017). Patients with COPD do less physical activity than healthy age-matched controls (Pitta, Troosters et al. 2005), spending around half as much time engaged in moderate intensity physical activity than their healthy peers (van Remoortel, Camillo et al. 2013) and have been characterised as taking as few as 2,200 steps per day (Tudor-Locke, Washington et al. 2009). However, in relation to skeletal muscle dysfunction, it is unclear whether reduced physical activity is a driver or a consequence (see Figure 1.1). It has even been

suggested that physical inactivity may drive the development of COPD lung pathology via mediators such as oxidative stress and systemic inflammation (Hopkinson and Polkey 2010). Numerous studies have found significant correlations between exercise capacity in a self-paced walking test and physical activity status (Watz, Pitta et al. 2014) and a weak correlation has been shown with peak oxygen uptake (Pitta, Troosters et al. 2005) suggesting that habitual physical activity may play a role in determining exercise capacity, although formal exercise is likely to have a greater impact.

1.6 Strategies for improving muscle function and exercise tolerance in healthy ageing and COPD

As previously discussed (Chapter 1.3) poor lung function is largely irreversible in COPD, making the skeletal muscles an appealing therapeutic target. The capacity for improvements in the physical function of patients with COPD is demonstrated by the efficacy of pulmonary rehabilitation (which has a heavy emphasis on exercise training) in improving functional exercise capacity (Lacasse, Goldstein et al. 2006). Indeed, pulmonary rehabilitation has been shown to offer greater improvements in exercise tolerance and health-related quality of life than pharmaceutical interventions (Lacasse, Wong et al. 1996) and is effective across the spectrum of disease severity (Vogiatzis, Terzis et al. 2011). In this section the normal adaptive responses to aerobic exercise training in healthy young and older individuals will be discussed as will the impact of aerobic training on patients with COPD. Additionally the impact of resistance exercise and neuromuscular stimulation on muscle mass and strength in COPD will be discussed.

1.6.1 Aerobic exercise

Aerobic exercise training is a proven strategy for increasing peak oxygen uptake in healthy individuals. Studies employing walking, running or cycling exercise regimes have demonstrated increases in $\dot{V}O_2^{MAX}$ in old and young men and women ranging from 10 – 24% compared to pre-training values (Kohrt, Malley et al. 1991; Wibom, Hultman et al. 1992; Houmard, Tyndall et al. 1996; American College of Sports, Chodzko-Zajko et al. 2009). A meta-analysis including 723 healthy young to middle-aged participants suggests that endurance training improves $\dot{V}O_2^{MAX}$ by 4.9 ml/kg/min compared to

untrained controls (Milanovic, Sporis et al. 2015). In addition to continuous aerobic exercise training (at a single exercise resistance or intensity), low-volume high intensity training (HIT) has been shown to be effective in promoting central and peripheral aerobic training adaptations (Gibala, Little et al. 2012) in healthy individuals and may be more appealing to individuals with busy lifestyles due to the relatively short duration of sessions. Perhaps unsurprisingly it seems that individuals with lower baseline fitness make larger gains in $\dot{V}O_2^{MAX}$ and that longer training interventions confer greater benefit than short exercise training programmes (Milanovic, Sporis et al. 2015).

In health, improvements in peak oxygen uptake with aerobic training are underpinned by cardiovascular adaptation (increased cardiac output) and peripheral adaptation (increased oxygen extraction at the exercising muscle) (Murias, Kowalchuk et al. 2010; Murias, Kowalchuk et al. 2010). The time course of these adaptations may differ in young and older individuals. Early adaptations to an aerobic training programme in young volunteers are centred on improved oxygen extraction in the periphery with increases in cardiac output coming later; conversely, in older volunteers cardiac output increases in the first three weeks of a training regime with later adaptations apparent in peripheral oxygen extraction capacity (Murias, Kowalchuk et al. 2010; Murias, Kowalchuk et al. 2010; Murias, Edwards et al. 2016). Indeed, it may take as long as 12 weeks of aerobic exercise training for training-induced differences in arteriovenous oxygen extraction at peak exercise to become apparent in older individuals (Montero and Díaz-Cañestro 2016). The importance of peripheral muscle adaptation for improvements in peak oxygen uptake in older individuals irrespective of its time course has however been acknowledged since the 1980s (Seals, Hagberg et al. 1984).

Aerobic exercise training has recognised benefits and is recommended for patients with COPD (Bolton, Bevan-Smith et al. 2013). High intensity aerobic exercise training has been shown to increase peak work rate and six-minute walking distance in patients with COPD, accompanied by increases in muscle capillary-to-fibre ratio and fibre cross-sectional area in the quadriceps in both patients with normal body composition and those with muscle wasting (Vogiatzis, Simoes et al. 2010). The magnitude of improvements in peak performance ($\dot{V}O_2^{PEAK}$) may be limited by the ventilatory capacity of some individuals (Plankeel, McMullen et al. 2005), however even where peak

performance is not improved, submaximal endurance capacity can be enhanced by aerobic training. It is important to note that exercise training elicits improvements in physical performance without improving lung function (Spruit, Gosselink et al. 2002).

A problem when employing aerobic exercise training in patients with COPD is the question of how to perform exercise of sufficient intensity to stimulate skeletal muscle adaptation within the bounds of tolerable dyspnoea for the patient. Completing continuous exercise training of sufficient intensity is particularly problematic for patients with significant ventilatory limitation (Maltais, LeBlanc et al. 1997). One strategy to overcome this is interval training, where the training session is divided into periods of high intensity work interspersed with periods of low load or rest. Vogiatzis *et al* showed that interval training on a static bicycle as part of a comprehensive pulmonary rehabilitation programme elicited lower sensations of dyspnoea and leg discomfort than work-matched continuous cycling yet prompted similar increases in peak power, lactate threshold, and quadriceps capillary-to-fibre ratio and citrate synthase activity (Vogiatzis, Terzis et al. 2005). Another mode of training that aims to maximise the intensity of training stimulus delivered to the muscle is single leg training. By exercising one leg at a time each limb has access to the full cardiovascular and ventilatory reserve and in COPD it has been shown that patients can reach the same $\dot{V}O_2^{PEAK}$ during one-legged cycling as they can during two-legged cycling (Dolmage and Goldstein 2006). During single leg cycle training each leg can perform exercise at greater than 50% of the two-legged maximum meaning that the physiological training stimulus on each limb is greater than during two-legged cycling. Single-leg cycling training has been shown to be superior to two-legged cycling for increasing $\dot{V}O_2^{PEAK}$ in patients with COPD (Dolmage and Goldstein 2008; Bjorgen, Hoff et al. 2009) and can feasibly be incorporated into a pulmonary rehabilitation programme (Evans, Dolmage et al. 2015).

There have been relatively few studies examining the comparative response of patients with COPD and age-matched healthy controls to aerobic training, particularly where the training intensity has been carefully matched in both groups. A study by Rodriguez *et al* (Rodriguez, Kalko et al. 2012) observed increases in peak oxygen uptake in both COPD patients and healthy controls after eight weeks of interval style aerobic training while Guzun *et al* (Guzun, Aguilaniu et al. 2012) observed increased $\dot{V}O_2^{PEAK}$ in patients with

COPD, but not healthy controls, however neither of these studies matched the training load between the two groups making the relative training effects had to interpret. When the relative training intensity of patients with COPD and healthy controls is matched patients appear to make smaller gains in $\dot{V}O_2^{PEAK}$ than healthy controls (Sala, Roca et al. 1999; Rabinovich, Ardite et al. 2001; Radom-Aizik, Kaminski et al. 2007; Gouzi, Prefaut et al. 2013). Of the studies that matched relative training load, only one, by Gouzi et al, also matched patients and controls for habitual physical activity and found that COPD patients made significantly smaller increases in $\dot{V}O_2^{PEAK}$ and had blunted skeletal muscle architectural adaptations in response to and aerobic training regime incorporated into a multicomponent pulmonary rehabilitation programme compared to healthy controls (Gouzi, Prefaut et al. 2013).

1.6.2 Resistance exercise

There is evidence for the efficacy of resistance training in improving muscle strength in young as well as older healthy individuals; although the relative gains may be diminished in older adults (Welle, Totterman et al. 1996), it is still possible to improve muscle strength and size well into old-age (Fiatarone, Marks et al. 1990). There is an interesting cross-over effect in that resistance training can improve the aerobic capacity of the quadriceps in older individuals (Frontera, Meredith et al. 1990) and aerobic training can increase muscle size (Harber, Konopka et al. 2012). In reality, for a sedentary individual, it is likely that an increase in muscular work of any modality will prompt general improvements in muscle function.

The effectiveness of resistance or weightlifting training in patients with COPD has been known for some decades (Simpson, Killian et al. 1992) with consistently positive results demonstrated across a range of lung function impairment in a number of studies (Clark, Cochrane et al. 1996; Casaburi, Bhasin et al. 2004; Hoff, Tjonna et al. 2007). Quadriceps strength has been shown to increase by at least 16% in studies employing resistance training in COPD (Bolton, Bevan-Smith et al. 2013). There have been various studies of interventions intended to augment the impact of resistance training as part of pulmonary rehabilitation in COPD. Testosterone supplementation has been shown to increase lean mass and leg strength as a standalone intervention in male patients with COPD who had low baseline testosterone levels as did resistance exercise training but

the combination of the two interventions had the largest impact (Casaburi, Bhasin et al. 2004). Nutritional supplementation with creatine has been shown to improve muscle mass gains with resistance training in young and older healthy individuals but was found not to benefit patients with COPD undertaking a comprehensive programme of Pulmonary Rehabilitation (Deacon, Vincent et al. 2008).

1.6.3 Alternative methods

In the field of pulmonary rehabilitation aerobic and resistance exercise training are the gold standard exercise interventions with proven benefits for exercise capacity and quality of life in patients with COPD. Other interventions including inspiratory muscle training; anabolic steroids; nutritional supplements; non-invasive ventilation; breathing low-density heliox instead of room air; and oxygen supplementation have all been trialled with the aim of improving exercise performance or relieving symptom burden but have thus far failed to generate sufficiently compelling evidence for inclusion in guidelines for the delivery of PR (Bolton, Bevan-Smith et al. 2013). Another intervention that currently does not meet the criteria for inclusion into national pulmonary rehabilitation guidelines, but which shows some promise particularly for very weak or deconditioned patients is neuromuscular electrical stimulation (NMES).

NMES is a non-volitional means of activating skeletal muscle contraction. Electrodes placed on the skin surface deliver an electrical current to the motor neurones in the muscle causing depolarisation and muscle fibre contraction. High frequency ($\geq$50 Hz) NMES is thought to mimic the neuromuscular action of high force muscle contractions with activation of type II muscle fibres similar to the muscular activity during resistance exercise. NMES has been proposed as a means of training the muscles of patients with COPD and it is an appealing method for use in breathless populations because compared to conventional exercise it does not induce significant dyspnoea or exert high demand on the cardiorespiratory system (Sillen, Janssen et al. 2008; Sillen, Franssen et al. 2014). For patients who are very breathless, hospitalised or severely deconditioned and weak NMES may be a viable alternative to conventional resistance exercise (Neder, Sword et al. 2002; Dirks, Hansen et al. 2015) and it is possible to perform NMES during inpatient hospitalisation for an acute exacerbation of COPD (Chaplin, Houchen et al. 2013), a time when physical function normally declines and where conventional exercise is often not

well tolerated by the patient. Trials of NMES in COPD have shown increases in muscle mass and strength (Sillen, Franssen et al. 2013; Sillen, Franssen et al. 2014) after an eight-week programme of high-frequency quadriceps NMES. NMES has been shown to prompt gains in muscle strength that are comparable to the effects of resistance exercise (Sillen, Franssen et al. 2014) despite eliciting contractile forces that are typically less than 15% of maximum voluntary force production (Newham, Mills et al. 1983). The mechanisms behind adaptation to training with NMES are not fully understood but it has been shown to influence phosphorylation of p70S6K (a marker of activation of the AKT / mTOR pathway which upregulates muscle protein synthesis), (Vivodtzev, Debigare et al. 2012) and to increase muscle fibre cross-sectional area (Dal Corso, Napolis et al. 2007), suggesting that repeated bouts of NMES in COPD promote muscle fibre adaptation.

1.7 Skeletal muscle molecular responses to exercise training

There is a chain of signalling and regulatory events that starts with exercise and muscle contraction and ends with physiological adaptation (for example increased capillary density in muscle or altered contractile protein composition in muscle fibres). Exercise initiates biochemical signals leading to regulation of gene transcription, translation into protein, post-translational modification and ultimately adaptation. The maximal athletic potential of individuals is likely heavily influenced by the genome they inherited, however what proportion of that potential they reach is down to environmental factors including exercise training and nutrition. We cannot (yet) influence the genomic cards that we are dealt at birth, but through exercise we can alter the expression of our genes and adapt our physiology to suit our requirements, be it the ability to cycle up mountains or produce bursts of power and speed. The abundance of mRNA is one step in the transduction of signals from muscle contraction to physiological adaptation and the abundance of proteins is another, both of which can be measured in samples of skeletal muscle. The study of mRNA and protein abundance in response to a challenge (e.g. acute or chronic exercise) in different populations or in response to different exercise interventions allows us greater understanding of the processes underpinning physiological adaptation.

Most of the approximately 25,000 genes in the human genome contain a code which determines in what order amino acids should be bound so as to produce a protein which will go on to perform a physiological role (e.g. as a structural protein or part of an enzyme complex). Figure 1.2 gives a simplified overview of the processes of transcription of mRNA and translation into protein and the basic principles of these processes will be discussed here. Deoxyribonucleic acid (DNA) is a very large molecule made up of two strands of nucleotides; adenine (A), thymine (T), cytosine (T) and guanine (G) bound to a deoxyribose backbone in a double helix formation. Nucleotides in the double helix form pairs with the opposite strand with A binding to T and G binding to C. Instructions for the assembly of individual proteins are stored in DNA as specific sequences of nucleotides called genes. The beginning and end of each gene is marked by a specific pattern of nucleotides called a start codon or a stop codon. Transcription is the process by which single stranded mRNA is constructed as a complimentary molecule to one strand of the DNA double helix. In order for transcription to occur, a section of the DNA double helix is unzipped and an RNA polymerase molecule synthesises a strand of mRNA made up of the complimentary nucleotides to DNA (in mRNA the nucleotide T is replaced by uracil, U). Each mRNA molecule bears the information carried by a single gene, encoded by the order of nucleotide bases and is small enough to leave the nucleus. The number of mRNA molecules carrying the code for specific genes can be quantified in tissue samples. Every nucleated cell in an organism carries identical DNA (the genome), however the mRNA molecules that are present within a cell (the transcriptome) will vary between cell types and within the same cell type dependant on the stimuli applied to it. Characterisation of mRNA abundance in sampled tissue tells us which of the genetic instructions are being read at any one time and how many copies are available.

Translation is the process by which proteins are synthesised on ribosomes using mRNA as a template. Amino acids are the building blocks of proteins with each amino acid represented by a three-nucleotide sequence in mRNA called a codon. At one end of a tRNA molecule there is an anti-codon to compliment the codon on the mRNA molecule. Amino acid molecules are bound to the other end of the tRNA molecule. Molecules of tRNA pair with their complimentary codons in order on the mRNA strand thereby delivering amino acids in the correct order to form a specific polypeptide. rRNA

molecules that make up the ribosome catalyse the formation of covalent peptide bonds between amino acids to form a polypeptide chain. Once formed, the polypeptide chain is subjected to post-translational modifications including folding and the binding of other molecules for example phosphorylation or glycosylation or incorporation into large protein complexes. The abundance of specific proteins can also be quantified in tissue samples giving an indication of the abundance of a functional component of interest in a tissue at a certain time.

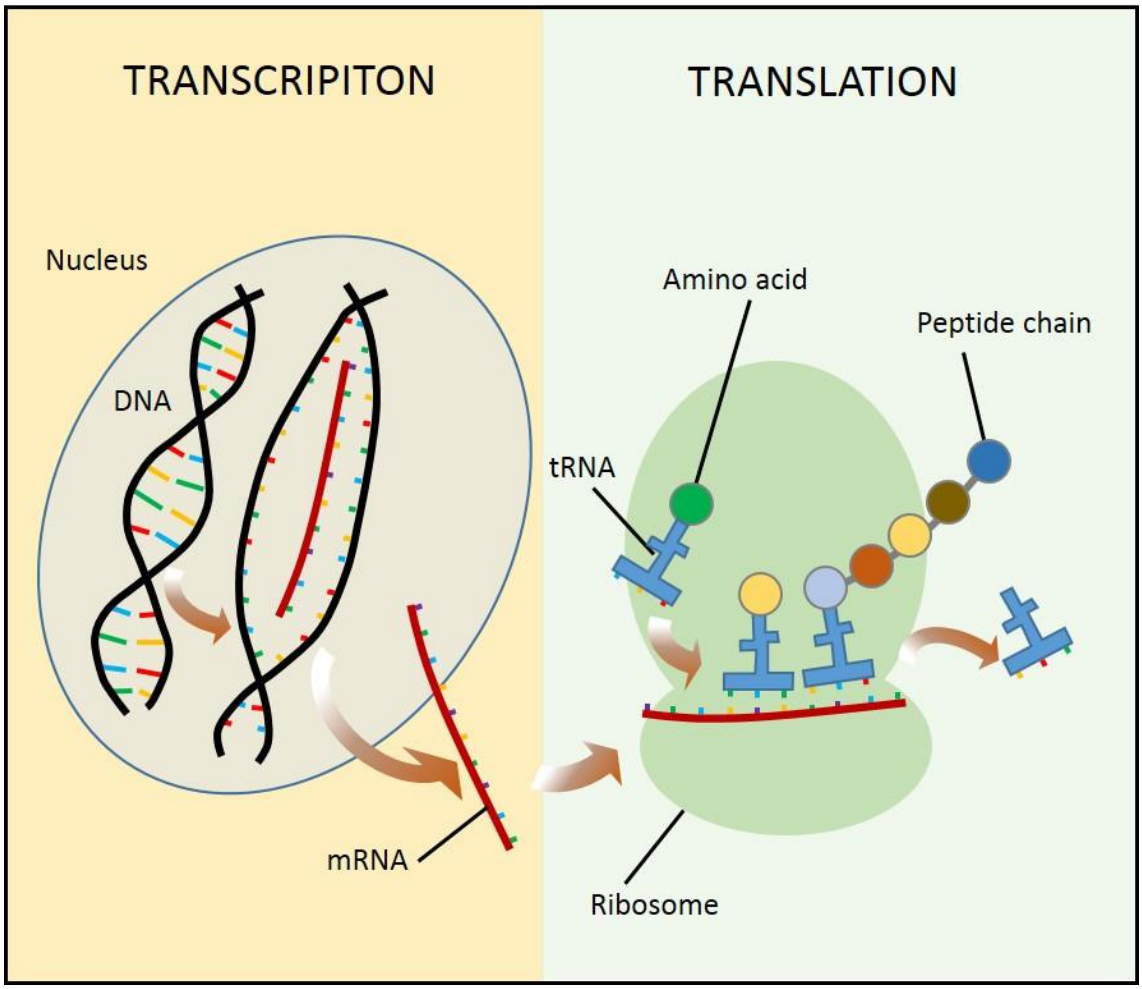


Figure 1-2. Overview of transcription and translation.

Biopsy studies have given insight into the mRNA and protein responses that occur in skeletal muscle after a single bout or prolonged programme of exercise training. The timing of tissue sampling relative to exercise is important, particularly in acute studies of the impact of exercise; for example one study of the acute responses to exercise in the muscles of young untrained healthy individuals showed that abundance of selected mRNAs with functions linked to fuel metabolism or myogenesis peak 8 to 12 hours after an acute bout of running or resistance exercise before returning to baseline by 24 hours whereas other mRNAs with similar functions are unchanged in abundance over the same time period (Yang, Creer et al. 2005). As well as acute responses to exercise, more stable alterations in the expression of mRNA and proteins have been identified after a prolonged period of training. For example, one microarray analysis of skeletal muscle biopsies revealed a broad variety of mRNA transcripts that are up- or down-regulated following a 20 week aerobic exercise training programme in healthy volunteers and found differences in mRNA profiles between groups defined by their insulin sensitivity responses (Teran-Garcia, Rankinen et al. 2005). There is a broad literature of studies examining the skeletal muscle molecular responses to different interventions in various populations but in this chapter the focus will be on reviewing the influence of advanced age and of COPD on muscle mRNA and protein abundance in the context of exercise.

1.7.1 Ageing

The abundance of some skeletal muscle mRNA transcripts is influenced by age itself but work by Phillips *et al* suggests that these genes are distinct from ones that are responsive to physical exercise in healthy individuals across a range of ages (Phillips, Williams et al. 2013). Robinson *et al* also identified differences in muscle gene expression in untrained young and older volunteers (although habitual physical activity levels in the two groups were not closely controlled). In a subsequent twelve week study of resistance or high intensity interval training Robinson *et al* concluded that the exercise modality was more influential than participant age in determining the mRNA response to exercise training (Robinson, Dasari et al. 2017). An apparent blunting in the mRNA response to resistance exercise training has been described in older compared to young volunteers with the abundance of fewer mRNA transcripts influenced in older groups (Jozsi, Dupont-Versteegden et al. 2000; Melov, Tarnopolsky et al. 2007; Raue, Trappe et al. 2012).

Aerobic exercise has been shown to influence the abundance of a wide variety of muscle mRNA transcripts in young healthy individuals in a number of studies (Teran-Garcia, Rankinen et al. 2005; Riedl, Yoshioka et al. 2010; Timmons, Knudsen et al. 2010) but there have been few studies of the comparative response of older and younger volunteers to this training modality. In the study of Robinson *et al* high-intensity interval training (which places a significant anaerobic energy demand on muscle fibres) was shown to change the abundance of both mRNA transcripts and proteins with functions relating to the mitochondrial in both young and older volunteers (Robinson, Dasari et al. 2017) but the magnitude and time course of muscle molecular responses to a classic aerobic training programme has not been investigated in older compared to young volunteers.

1.7.2 COPD

In patients with COPD, aerobic training has been shown to influence the expression of genes associated with hypertrophy and myogenesis signalling (Vogiatzis, Stratakos et al. 2007). Patients with low muscle mass however demonstrate different molecular responses to rehabilitative exercise training including dampened myogenesis signalling (associated with smaller gains in muscle mass) compared to patients with preserved lean mass at baseline (Vogiatzis, Simoes et al. 2010). When compared to healthy individuals, patients with COPD appear to have a blunted muscle molecular response to aerobic training. A study that focussed on mRNA abundance of inflammatory genes acutely influenced by a single bout of aerobic exercise found that NFκB signalling was blunted in patients with COPD compared to healthy controls (Mercken, Hageman et al. 2011). In one microarray study the abundance of double the number of mRNA transcripts was influenced in health compared to COPD after an aerobic training programme and whereas healthy volunteers experienced upregulation of transcripts related to energy production pathways, patients with COPD experienced a different response including upregulation of oxidative stress and muscle protein breakdown pathways (Radom-Aizik, Kaminski et al. 2007). Turan *et al* performed microarray analysis of skeletal muscle sampled at rest, before and after an eight week endurance training programme. COPD patients with normal fat-free mass experienced a narrower mRNA response than healthy controls (around a quarter of the number of mRNAs influenced in health were

changed in abundance in COPD) but the transcripts that were influenced by exercise in the COPD group fitted into the same functional pathways that were influenced in health including muscle bioenergetics, ribosome biogenesis, VEGF and pro-inflammatory signalling (Turan, Kalko et al. 2011). Similar to the findings of Vogiatzis *et al*, Turan and colleagues observed different mRNA responses to exercise training in muscle-wasted patients with COPD who in contrast to patients with normal fat-free mass did not experience a significant transcriptional response to training. Further weight to the argument that patients with COPD experience abnormal skeletal muscle molecular responses to exercise comes from the work of Marin de Mas *et al* (Marín de Mas, Fanchon et al. 2016) who employed a computational modelling technique to analyse data from three previous gene expression experiments (Sala, Roca et al. 1999; Turan, Kalko et al. 2011; Rodriguez, Kalko et al. 2012), concluding that functional adaptations in the muscle of patients with COPD may be undermined by blunted mRNA responses, particularly relating to tissue remodelling and energy metabolism pathways. Further analysis of data from studies using the same eight week aerobic training programme analysed by Marin de Mas *et al* has recently been performed by Tényi *et al* who conclude that pathways relating to oxidative phosphorylation and bioenergetics were uninfluenced in COPD while pathways relating to skeletal muscle remodelling were influenced in COPD but not in health with the most abnormal mRNA responses apparent in patients with low fat-free mass (Tenyi, Cano et al. 2018). Impaired muscle mRNA responses to exercise training in patients with COPD, particularly in those with low muscle mass, may be a feature of the underlying disease but it may also be attributable to the absolute exercise intensity that patients are able to perform. As discussed earlier in this chapter the ventilatory limitation to exercise experienced by some patients will limit the muscle-specific training intensity that can be achieved and advanced deconditioning and weakness in patients with low muscle mass may further confound attempts to compare muscle responses to whole-body exercise.

Measuring the abundance of mRNA transcripts and proteins allows characterisation of the response to acute and chronic muscle contraction as part of the adaptive response to exercise. The abundance of mRNA and proteins in skeletal muscle by no means make up the whole picture of the muscle adaptive response to exercise with multiple signalling

and control points influencing translation of mRNA into protein and post-translational processes governing the formation of secondary and tertiary protein structures and the aggregation of multiple polypeptides into complex and functional multi-protein structures (Hoffman, Parker et al. 2015). By measuring the abundance of mRNA and protein however, it is possible to look for differential responses in distinct groups of volunteers or in response to distinct interventions and to start to build part of the picture of a fully integrated whole-body physiological response to exercise.

When interpreting results in published literature or designing a study it is important to consider the method chosen for assessing mRNA abundance. Real-time reverse transcription polymerase chain reaction (RT-PCR) employing sequence-specific DNA probes linked to a fluorescent reporter dye allows the quantitative assessment of cDNA reverse transcribed from mRNA relative to a housekeeping gene. RT-PCR is highly accurate and allows quantification of abundance over a large range of values but each target mRNA is assessed separately which can be time-consuming and limit the number of targets measured. High-throughput microarray techniques allow the examination of large numbers of targets (20,000 plus) using a microchip pre-loaded with probes designed to detect specific DNA sequences. cDNA samples loaded onto microarrays are labelled with a dye or fluorophore and the abundance of targets is assessed via the intensity of the fluorescence signal detected by a digital scanner. The advantage of the microarray technique is the breadth of targets examined with the possibility of examining the expression of the whole genome. The disadvantage of microarrays is that they are semi-quantitative and relatively insensitive compared to RT-PCR meaning that small but biologically relevant differences in expression may be missed. Microarrays are often used to identify targets of interest which are then assessed using RT-PCR in order to quantify differences in abundance between samples. Low density arrays (LDA) allows the simultaneous quantification of up to 384 mRNA targets using RT-PCR. Selection of candidate genes of interest (and that will answer the scientific question posed) must be based on prior knowledge either from microarray data, published data or database searches. The assessments of mRNA abundance in this thesis are performed using a medium-throughput LDA approach with gene targets selected based on previous work within the research group, searches of the published literature and searches of a

commercial database (Ingenuity Knowledge Base; QIAGEN, USA). This approach allows quantification of a large number of targets in a large number of samples with both accuracy and a large range of expression values.

1.8 Cessation of exercise training

In both COPD and healthy populations there has been a heavy emphasis on the effects of exercise training but less focus on the effects of exercise withdrawal. What happens after a period of regular exercise when individuals go back to their previous habitual physical activity levels: are training adaptations sustained or reversed, and over what time period?

The effects of extreme reductions in habitual physical activity have been examined using bed rest studies. The landmark study by Saltin *et al* (Saltin, Blomqvist et al. 1968) recruited three sedentary and two physically active (exercise trained) young men and imposed a 20 day period of strict bed rest with out-of-bed time limited to 10 min per day and no standing permitted. During this period of bed rest $\dot{V}O_2^{MAX}$ decreased by an average of 28% and was closely associated with maximal cardiac output which decreased by 26% over the same period. This study showed that in healthy young individuals extreme inactivity has a dramatic influence on aerobic capacity. More recently Coker *et al* (Coker, Hays et al. 2014) showed that ten days bed rest prompted a reduction in peak oxygen uptake of 13% in a group of 19 older individuals (14 females) of average age 70 years as well as declines in muscle strength, muscle mass and performance on functional tests (e.g. sit-to-stand). Acute bed rest in the absence of chronic or acute illness therefore has significant deleterious effects on aerobic fitness in both young and older individuals.

There is a further body of literature examining the effects on deconditioning associated with the withdrawal of exercise in otherwise free-living populations. Much of this "detraining" literature has been conducted in athletic populations with the intervention being one of training load reduction (e.g. end of season break or interruption of training due to injury), or interventions aimed at enhancing athletic performance (training taper) pre-competition in chronically trained individuals (Mujika and Padilla 2000; Mujika and

Padilla 2003). There are some studies focussing on the effects of short-term exercise withdrawal (2-8 weeks) in individuals who had been trained only recently. One report on the effects of eight weeks training withdrawal following eight weeks aerobic exercise training in healthy men (Klausen, Andersen et al. 1981) describes a reversal of training adaptations to near pre-training levels for measures of $\dot{V}O_2^{MAX}$, quadriceps capillary density, activity of oxidative enzymes and muscle fibre cross sectional area. A similar pattern was reported by Wibom *et al* (Wibom, Hultman et al. 1992) who observed a 10% increase in $\dot{V}O_2^{MAX}$ after 6 weeks aerobic training in previously inactive young men which then declined by 6% after just 3 weeks exercise withdrawal. Wibom *et al* also observed significant training related increases in oxidative enzyme activity and maximal mitochondrial ATP production rate in the quadriceps which were reversed after exercise withdrawal. In contrast to this, another study of previously sedentary individuals did not observe a reduction in $\dot{V}O_2^{MAX}$ during three weeks of exercise cessation despite the preceding seven week training programme having prompted a 24% increase in maximal oxygen uptake (Moore, Thacker et al. 1987); however in this study there were signs of physiological detraining evidenced by significant increases in RER during submaximal testing and a decrease in citrate synthase activity after three weeks exercise withdrawal. Short-term (2 week) exercise withdrawal did not influence $\dot{V}O_2^{MAX}$ in one study of 10 middle-aged men and women, however this group had not experienced a significant increase in $\dot{V}O_2^{MAX}$ during the preceding 12 weeks of training and the main focus of the report was insulin sensitivity which did decrease with exercise cessation (Houmard, Tyndall et al. 1996). Thus the evidence in healthy individuals suggests that physiological adaptations elicited by aerobic exercise training programmes in previously sedentary individuals are reversed in the weeks following exercise withdrawal but there is some uncertainty regarding the rate of reversal (in particular for $\dot{V}O_2^{MAX}$).

Evidence relating to the maintenance of training adaptations after a period of supervised exercise training in COPD is relatively sparse. Pulmonary rehabilitation (PR) programmes incorporate aerobic and resistance exercise training alongside educational components, nutritional intervention and psychosocial support. It is known that the benefits of PR decline over time, although due to the multidisciplinary nature of the PR intervention outcome measures used in studies focus on symptoms, quality of life and

functional measures as well as maximal or submaximal exercise performance. Foglio *et al* (Foglio, Bianchi et al. 1999) observed increases in $\dot{V}O_2^{PEAK}$ and Peak Work after an 8-10 week PR programme but found that these had been reversed after 12 months despite encouragement for patients to maintain their PR independently. Over a shorter exercise withdrawal period Otsuka *et al* found that the 6% increase in peak oxygen uptake observed in 11 patients with COPD after eight weeks exercise training was undiminished after five months exercise withdrawal (Otsuka, Kurihara et al. 1997). Ries *et al* did not observe an increase in $\dot{V}O_2^{PEAK}$ with PR while treadmill endurance increased but returned to baseline levels at 12 month; improvements in maximum treadmill workload however were sustained at 12 months (though they returned to baseline after two years) (Ries, Kaplan et al. 1995). There are two significant difficulties when trying to gauge the effect of exercise withdrawal on both healthy individuals and those with COPD. Firstly, the training interventions vary in exercise mode, intensity and duration; secondly the duration of the exercise period varies between studies and crucially there is no objective measure of habitual physical activity during exercise withdrawal. There is currently no evidence examining the impact of advanced age or of COPD on the response to exercise withdrawal subsequent to a carefully controlled aerobic training programme.

The skeletal muscle molecular responses to exercise withdrawal have not been widely studied. The study by St-Amand *et al* (St-Amand, Yoshioka et al. 2012) examined data from a genome-wide analysis of pooled mRNA from young healthy untrained men before and after 12 weeks of aerobic training and after 12 weeks of subsequent detraining. The training intervention changed the abundance of 243 mRNA transcripts, most of which returned to baseline values after exercise withdrawal, however 33% of these transcripts (representing those with the largest magnitude of fold change with training) were still influenced in abundance after the period of exercise withdrawal. The transcripts that remained elevated in skeletal muscle despite a reversal of gains in $\dot{V}O_2^{MAX}$ after exercise withdrawal code for mitochondrial components, contractile proteins and the apparatus of protein synthesis. This work suggests that some of the molecular markers of training adaptation in skeletal muscle may persist for some months after the training stimulus is withdrawn. The impact of aerobic exercise training withdrawal on skeletal muscle molecular markers in COPD remains to be established.

1.9 Aims of this thesis

There are currently gaps in our understanding of the influence of advanced age and the presence of COPD on whole-body cardiorespiratory and skeletal muscle molecular responses to aerobic exercise training and exercise withdrawal. Specifically, the time course and magnitude of changes in $\dot{V}O_2^{PEAK}$ in response to supervised aerobic training and subsequent exercise withdrawal in young compared to older individuals and in patients with COPD compared to age-matched controls remains unknown. Likewise, the comparative molecular responses of mRNA and protein abundance in the quadriceps throughout the course of aerobic training and exercise withdrawal have not been characterised in in young and older volunteers or patients with COPD. There are also questions remaining to be answered regarding the response of quadriceps muscle to an acute bout of NMES in COPD; with the relative changes in mRNA abundance that occur after a bout of NMES compared to a bout of resistance exercise currently unknown.

The aim of this thesis is to investigate limitations to exercise capacity associated with healthy ageing and with COPD, and to examine whole-body physiological adaptations and skeletal muscle molecular responses to chronic training or acute muscle contraction. To this end it will characterise whole body exercise limitations in the untrained state and the cardiorespiratory responses to moderate intensity aerobic cycle ergometer training and subsequent exercise withdrawal in young compared to older healthy volunteers, and in patients with COPD compared to age-matched healthy controls. Detailed time course examination of gene and protein expression in quadriceps muscle will be described in order to identify any deficits associated with ageing or COPD both in the untrained state and in response to exercise training. In addition to exploring the influence of aerobic training on quadriceps gene expression, two further modes of muscle contraction will be examined for their acute influence on muscle mRNA abundance in patients with COPD, the first is the definitive strength training intervention voluntary resistance exercise; and the second, non-volitional neuromuscular electrical stimulation.

1.10 Research questions

1) The first research question focusses on the influence of age on the response to aerobic training and subsequent exercise withdrawal in healthy volunteers.
    a) Firstly, do young and older sedentary volunteers make similar physiological adaptations (primarily defined by change in peak oxygen uptake) in response to an aerobic training programme at the same relative moderate intensity; and does a subsequent exercise withdrawal period affect both groups equally?
    b) Second, does age influence the expression of targeted mRNA transcripts with functions linked to aerobic training and are patterns of change in gene expression in response to aerobic training and exercise withdrawal different for young and older volunteers? Finally, led by results of the gene expression analysis and focussing on proteins whose genes are most markedly influenced by training and exercise withdrawal, are there measurable changes in protein expression, and do such changes correlate with the change in mRNA abundance in quadriceps muscle?
2) Having examined the effect of age, the second research question centres on the impact of COPD on responses to aerobic training and exercise withdrawal compared to an age-matched healthy control group.
    a) First, does the presence of COPD influence the physiological responses to aerobic training at matched relative intensity and exercise withdrawal in age-matched groups of patients and healthy volunteers?
    b) Secondly, is baseline expression and response to training and exercise withdrawal of targeted mRNA transcripts different in the quadriceps of COPD patients compared to age-matched controls? Finally, does the abundance of targeted proteins vary in response to training and exercise withdrawal in COPD and health, and is there a relationship between gene and protein expression in the quadriceps?
3) The third question that this thesis will address focusses on the acute gene expression response to a single bout of volitional or non-volitional muscle contraction.
    a) In patients with COPD, does a bout of neuromuscular electrical stimulation training influence the abundance of mRNAs known to be responsive to resistance exercise in health?

b) Does the mRNA response following a bout of electrically stimulated contractions differ to that elicited by a bout of voluntary resistance exercise?

1.11 Thesis outline

This thesis is designed to address the research questions outlined. A detailed description of the methodology used to collect and analyse data is presented in Chapter 2, followed by five chapters describing experimental data. Chapter 3 describes experimental data comparing the characteristics of young and older healthy volunteers in the untrained state including anthropometric measures, pulmonary function, maximal and sub-maximal exercise performance and aerobic capacity before examining the influence on these parameters of an aerobic training programme of cycling exercise and subsequent exercise withdrawal. Chapter 4 examines the influence of age in the same healthy volunteers on the response of gene and protein targets to the aerobic training and exercise withdrawal intervention providing mechanistic insight into the adaptive processes occurring in quadriceps muscle. Chapters 5 and 6 report on exercise outcomes and muscle molecular responses to the same aerobic training and exercise withdrawal intervention in patients with COPD using the older healthy volunteers from chapters 3 and 4 as age-matched controls. Chapter 7 is the final experimental chapter and scrutinises the acute influence on skeletal muscle mRNA abundance of two interventions known to enhance muscle mass and strength in COPD; voluntary resistance exercise and neuromuscular electrical stimulation. The relative influence of the two contraction modalities on quadriceps mRNA abundance is examined in a stable COPD population following a single bout of muscle contraction.

Chapter Two

Materials and Methods

2.1 Ethics

Ethical approval for studies presented in this thesis was granted by the NHS National Research Ethics Service. Local approval and sponsorship of studies was gained from the host institution for each site. Details of the studies were placed on a publically accessible international clinical trials register prior to commencement of the trial (Table 2.1).

2.1 Funding

The MATCH Study was funded by the COPDMAP Consortium. COPDMAP is a collaboration between the Medical Research Council and the Association of the British Pharmaceutical Industry as part of the Inflammation and Immunology Initiative. The Neuromuscular Electrical Stimulation Study was funded by the National Institute for Health Research (NIHR) Collaboration for Leadership in Applied Health Research and Care with additional funding for the gene expression portion of the study from the NIHR Leicester Respiratory Biomedical Research Unit. The study of acute impact of resistance exercise on quadriceps gene expression in COPD was funded by a grant from Remedi: Enabling Research in Rehabilitation (www.remedi.org.uk, a UK registered charity 1063359).

2.2 Volunteers

Patients with an existing diagnosis of COPD were recruited from Pulmonary Rehabilitation waiting lists, databases of patients who have previously expressed an interest in becoming a research volunteer, responders to poster advertisement within the research hospital and through recommendation from outpatient clinics. Healthy volunteers were recruited from existing research volunteer databases, newspaper advertisement, mailshot and poster advertisement. Criteria for the eligibility of volunteers for each study is detailed in Table 2.2 (MATCH Study) and Table 2.3 (Acute mRNA expression study). Before the start of study investigations or procedures all

volunteers provided informed written consent. Participants were free to withdraw their consent at any time without giving a reason.

Table 2-1 Summary of ethical approvals for work undertaken

Study	**Ethical Approval**	**Local Approval**	**Clinical Trials Register**
Mitochondrial Adaptations to Aerobic Training in COPD and Health: The MATCH Study	NRES Committee West Midlands – Coventry & Warwickshire, Reference: 13/WM/0075. UK CRN ID: 14080	CLRN 119870 (Leicester)	ISRCTN10906292
The effects of unilateral neuromuscular stimulation on quadriceps muscle morphology, architecture and function in chronic obstructive pulmonary disease.	West Midlands Research Ethics Committee, Reference: 10/H1208/73	CLRN 38011	ISRCTN87439020
Acute impact of resistance exercise on quadriceps gene expression in COPD: The Remedi Study	Leicestershire, Northamptonshire and Rutland REC, reference 05/Q2502/131	N/A	Study commenced before 2010, therefore not registered.

2.3 Study Protocols

2.3.1 Aerobic Training Study

There is limited evidence in the literature regarding the impact of advanced age on the whole-body cardiorespiratory and skeletal muscle molecular responses to aerobic exercise training. Furthermore the influence of COPD on exercise training adaptations in groups matched for age and physical activity status is unknown. Additionally the influence of advanced age and of COPD on the effects of exercise withdrawal have not been reported in the literature. The MATCH (Mitochondrial Adaptations to Aerobic Training in COPD and Health) study was designed to allow careful assessment of whole-body cardiorespiratory and skeletal muscle molecular responses to aerobic exercise training and exercise withdrawal in young and older healthy sedentary volunteers and patients with COPD. The rational for this study was that by selecting participants with similar habitual physical activity status it would be possible to discern the influence of advanced age and in a separate comparison the effect of the presence of COPD in age-matched cohorts on the response to aerobic exercise training and subsequent withdrawal. The MATCH Study was a parallel cohort interventional design. An overview of the protocol can be seen in Figure 2.1.

2.3.1.1 Aerobic Training Study Subjects

Subjects were recruited at three sites: University Hospitals of Leicester NHS Trust, Glenfield Hospital; Nottingham University Hospitals Trust; and The University of Nottingham. Groups were defined as patients with COPD and Young and Older Healthy Controls. Only sedentary healthy volunteers were recruited in order to match as closely as possible the habitual physical activity level of patients with COPD. Inclusion criteria were designed to select untrained / sedentary healthy volunteers, therefore volunteers who were engaged in a regular exercise regime or who reached the government target of performing 150 min of vigorous exercise per week (Department of Health 2011) were excluded (Table 2.2).

Study volunteers for all groups were recruited from existing research databases and poster advertisement. Additionally patients with COPD were identified from secondary care clinics and healthy volunteers were identified from newspaper advertisement and

a mail shot using a Royal Mail postal service. All participants were provided with information about the trial and responded to the researchers if they were interested in participation. A diagram describing participant flow through the trial can be found in Appendix 1.

Table 2-2. Inclusion / Exclusion Criteria for Participation in the MATCH Study

	Inclusion	**Exclusion**
Young Healthy Adults	Age ≥18 & ≤35	
	Not engaged in regular exercise programme	Exceeding 150 min / week moderate intensity exercise
	Normal lung function: FEV_1 >80% predicted, FEV_1 / FVC ≥70%	Any respiratory diagnosis
Older Healthy Adults	Age ≥60 & ≤80	
	Not engaged in regular exercise programme	Exceeding 150 min / week moderate intensity exercise
	Normal lung function: FEV_1 >80% predicted, FEV_1 / FVC ≥70%	Any respiratory diagnosis
COPD	Age ≥60 & ≤80	
	Clinical diagnosis of COPD and obstructive spirometry: FEV_1 <80% predicted, FEV_1 / FVC <70%	
	Not engaged in regular exercise programme	Attended PR within last 12 months
	MRC grade ≥3	
	Clinically stable	Exacerbation within last 4 weeks
All Groups	Ability to give informed consent	Any medical condition associated with metabolic disturbance (e.g. type II diabetes), inflammation (e.g. rheumatoid arthritis, inflammatory bowel disease), impaired muscle function or one which affects the ability to perform exercise testing and training (e.g. cardiovascular disease, significant osteoarthritis)
		Receiving systemic corticosteroid medication
		Receiving anticoagulation therapy or condition causing impaired clotting / platelet dysfunction
		Current smoker (ex-smokers > 1 year were acceptable)

2.3.1.2 Aerobic Training Study Intervention

Volunteers performed eight weeks of supervised aerobic exercise training on a cycle ergometer. Training consisted of 30 min of continuous cycling, performed three times per week at an intensity corresponding to 65% of the peak work rate achieved in the baseline test. If an individual was unable to complete 30 min continuously, they were allowed a short break (< 5 min) before resuming until a total of 30 min was achieved. Training intensity was reset after testing at four weeks. A figure showing the training load performed by each group when training commenced and after being reset at four weeks can be found in Appendix 2. At the end of week eight supervised exercise ceased and subjects were instructed to resume their habitual physical activity levels.

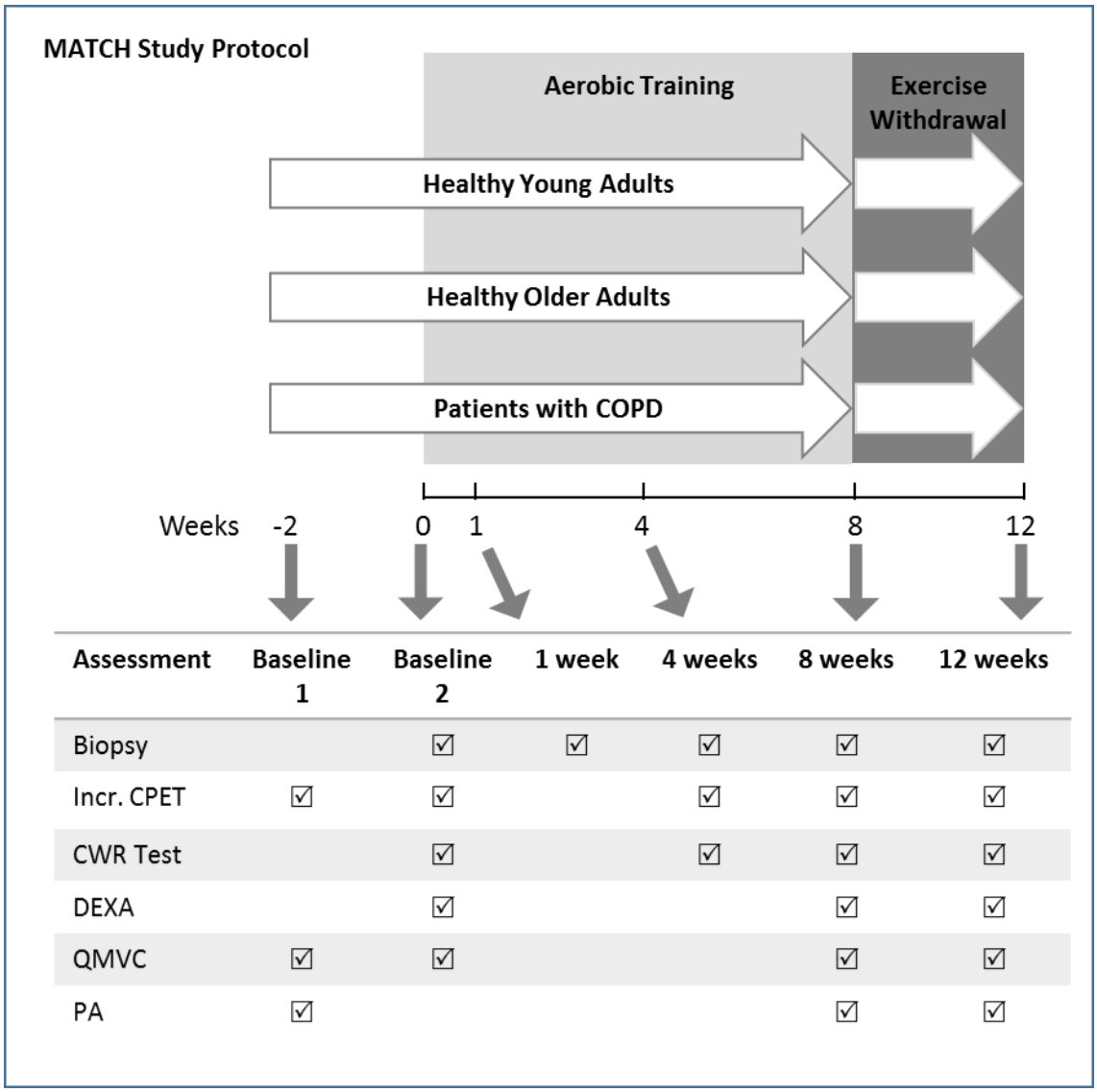
2.3.1.3 Aerobic Training Study Measures

All volunteers attended Glenfield Hospital in Leicester for physiological assessments. Baseline testing was split over two visits (separated by $\geq$ 2 days). Written informed consent was obtained before collection of demographic and medical history information, followed by pulmonary function testing. Incremental cardiopulmonary exercise testing (CPET) familiarisation and quadriceps strength (QMVC) testing familiarisation was performed on the first visit. On the second visit incremental CPET and strength testing were repeated. In addition, a submaximal CPET at 65% of the work load corresponding to $\dot{V}O_2^{PEAK}$ and body composition assessment by DEXA were performed on the second visit. Submaximal exercise testing was performed at 65% of the work rate corresponding with $\dot{V}O_2^{PEAK}$ from the incremental test. While an intensity of 65% is too low to permit assessment of time to exhaustion as an outcome measure (an intensity of 75% is commonly used for this purpose) it has the advantage of matching the intensity of the training sessions and therefore provides insight into the physiological demands of the exercise training intervention. Exercise tests were separated by a minimum of 30 min recovery time. It is possible that the results of the second exercise test may have been influenced by fatigue caused by the first test on the same day, however, in line with guidelines for exercise testing in COPD (Holland, Spruit et al. 2014), this possibility was minimised by the 30 minute rest period which was strictly enforced and it was ensured that heart rate, ventilation and respiratory exchange ratio had returned to baseline resting values before commencing recording of the second test.

Data from the incremental CPET of longest duration out of the two baseline tests and the highest QMVC value are used as the baseline value. Physical activity monitoring was performed for the seven days following baseline physiological assessments.

A minimum of seven days following baseline exercise testing, subjects attended the Greenfield Exercise Physiology Unit at the University of Nottingham where a resting, fasted *vastus lateralis* muscle biopsy was performed on the dominant leg. After a light meal and a period of resting, subjects performed their first exercise bout of the training period.

Vastus lateralis muscle biopsies were performed at rest in the fasted state at baseline, after 1, 4, and 8 weeks training and at 12 weeks, after the detraining period. Biopsies during the training period of the study were performed 24 hours after the preceding training session. Time points for incremental and constant work rate cardiopulmonary exercise testing; DEXA scans; QMVC; and physical activity monitoring can be found in Figure 2.1 and were designed to capture the effect and time course of adaptations to training and the effect of detraining.



Assessment	Baseline 1	Baseline 2	1 week	4 weeks	8 weeks	12 weeks
Biopsy		☑	☑	☑	☑	☑
Incr. CPET	☑	☑		☑	☑	☑
CWR Test		☑		☑	☑	☑
DEXA		☑			☑	☑
QMVC	☑	☑			☑	☑
PA	☑				☑	☑

Figure 2-1. Aerobic Training Study Protocol.Biopsy, vastus lateralis tissue sample; Incr. CPET, incremental cardiopulmonary exercise test; CWR Test, submaximal constant work rate test; DEXA, dual-energy x-ray absorptiometry; QMVC, quadriceps maximal voluntary contraction; PA, physical activity monitoring.

2.3.2 Acute mRNA expression studies

Voluntary resistance exercise increases muscle mass and strength in patients with COPD who are able to perform it at a sufficient intensity (Simpson, Killian, et al. 1992) and a bout of RE influences the abundance of range of mRNA transcripts in stable COPD (Constantin, Menon et al. 2013). There is evidence that NMES may increase skeletal muscle mass and strength in COPD (Sillen, Franssen et al. 2013; Sillen, Franssen et al. 2014) but to date there have been no reports of the transcriptional impact of NMES in COPD. This study was therefore devised to directly compare the transcriptional response

of muscle to a bout of RE or NMES in COPD. The data in Chapter 7 are sourced from two studies of the acute response of mRNA expression in the *vastus lateralis* of patients with COPD in the 24 hr following a single bout of muscle contraction. The effects of unilateral neuromuscular stimulation on quadriceps muscle morphology, architecture and function in chronic obstructive pulmonary disease Study (NMES Study) examined the effects of quadriceps NMES. The Remedi Study examined the acute impact of resistance exercise on quadriceps gene expression in COPD. Other than the intervention, the studies had identical experimental designs.

2.3.2.1 Acute mRNA Expression Study Subjects

Matched cohorts of patients with COPD undertaking NMES and RE were included in the study. Groups were matched based on lung function and body composition. Selection criteria are found in Table 2.3.

2.3.2.2 Acute mRNA Expression Study Intervention

The NMES protocol employed a hand-held, battery powered device (Empi 300PV, Minnesota, USA) connected to two skin surface gel electrodes (70 x 40 mm each) placed over the quadriceps. The negative electrode was placed proximal to the patella, covering the *vastus lateralis* and *vastus medialis* motor points with the positive electrode placed longitudinally over the *rectus femoris* motor point (see Appendix 3 for an example of electrode placement). The leg to be stimulated was selected by random allocation so that an equal number of patients trained their dominant or non-dominant leg. The stimulation protocol consisted of 30 minutes at 50 Hz, with a 300 µs pulse duration and a duty cycle consisted of 15 s on (including 2 s ramp up and 2 s ramp down) and 5 s off, with the intensity (mA) selected by the patient from a possible range of 1 to 100 mA. Patients self-selected stimulation intensity and were encouraged to set the current at the limit of tolerability. During familiarisation, patients underwent stimulation starting at minimum intensity (1 mA) and progressing to the self-determined limit of tolerance during a session lasting no longer than 10 min.

RE consisted of 5 sets of 30 maximal isokinetic knee extensions at 180°/s with 1 min rest between sets performed on an isokinetic dynamometer (Cybex NORM II, CSMi, Stoughton, USA). Patients were seated with a hip flexion angle of 90°. The starting

position for the knee extension was at a knee flexion angle of 90° and ended at full knee extension. This protocol has previously been shown to change quadriceps mRNA abundance in COPD patients 24 h following exercise (Constantin, Menon et al. 2013).

Table 2-3. Inclusion / exclusion criteria for participation in acute mRNA expression studies

Inclusion	**Exclusion**
Age ≥40 years	
Clinical diagnosis of COPD and obstructive spirometry: FEV_1 <80% predicted, FEV_1 / FVC <70%	
Not engaged in regular exercise programme	Attended PR within last 12 months
MRC grade ≥3	
Clinically stable	Exacerbation within last 4 weeks
Ability to give informed consent	Any medical condition associated with metabolic disturbance (e.g. type II diabetes), inflammation (e.g. rheumatoid arthritis, inflammatory bowel disease), impaired muscle function or one which affects the ability to perform exercise testing and training (e.g. cardiovascular disease, significant osteoarthritis)
	Receiving systemic corticosteroid medication
	Receiving anticoagulation therapy or condition causing impaired clotting / platelet dysfunction

2.3.2.3 Acute mRNA Expression Study Measures

Patients attended baseline assessments to perform spirometry, undergo body composition measures and perform NMES or RE familiarisation a minimum of 1 week before the first biopsy visit. Resting biopsies were performed on the *vastus lateralis* muscle using the micro-biopsy technique previously used in our laboratory (Menon, Houchen et al. 2012). Tissue was snap frozen in liquid nitrogen and stored for later analysis by RT-PCR. After tissue acquisition a light dressing was applied to the biopsy site and the exercise bout (either transcutaneous NMES or voluntary RE of the quadriceps) was performed. Twenty-four hr later, a second resting biopsy was performed at least 2.5 cm from the previous biopsy site, thereby minimising confounding changes in mRNA abundance due to tissue sampling (Lundby, Nordsborg et al. 2005; Constantin, Menon et al. 2013; Murton, Billeter et al. 2014).

2.4 Lung Function

Spirometry for the NMES and Remedi Studies was performed using a portable spirometer (Microlab; CareFusion, Basingstoke, United Kingdom) in order to establish forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC). Participants in the MATCH Study underwent full pulmonary function testing in the Respiratory Physiology Department at Glenfield Hospital in accordance with BTS guidelines. In addition to standard spirometry, gas transfer factor (T_LCO) and measurements of lung volumes by plethysmography (Jaeger Body Box; CareFusion, Basingstoke, United Kingdom) were performed by a respiratory physiologist accredited by the Association for Respiratory Technology & Physiology (ARTP). All lung function testing was performed in accordance with ARTP / British Thoracic Society (BTS) guidelines (1994). Predicted maximum voluntary ventilation (MVV) was calculated as:

$$MVV\ (l/min) = FEV_1\ (l) \times 37.5$$

2.5 Body Size and Habitus

Body mass was measured to the nearest 0.1 kg using digital scales with the subject wearing light clothing and shoes removed. Height was measured in stocking feet to the nearest 0.01 m using a portable, freestanding stadiometer. Body mass index (BMI) was calculated accordingly:

BMI (kg/m^2) = body mass (kg) / height squared (m^2)

Body composition was measured using Dual Energy X-ray Absorptiometry (DEXA) scan (Lunar Prodigy; GE Healthcare, Buckinghamshire, United Kingdom) and analysed using enCORE software. DEXA scans discriminate between fat, lean, and bone mineral mass according to their relative absorbance of low-energy radiation. The low dose of radiation associated with a single whole-body DEXA scan (approximately 40 µSv) permits repeated measures over the course of a study with minimal risk of harm. Fat free mass (FFM) was calculated as lean mass plus bone mineral mass. Fat free mass index was calculated accordingly:

FFMI (kg/m^2) = FFM (kg) / height squared (m^2)

The appendicular skeletal muscle mass index (ASMI) was calculated using arm and leg muscle mass values from the DEXA scan (Figure 2.1 a&b) (Cruz-Jentoft, Baeyens et al. 2010; van de Bool, Rutten et al. 2015).

$$ASMI\ (kg/m^2) = \frac{\text{Muscle mass (kg) left leg + right leg + left arm + right arm}}{\text{height squared } (m^2)}$$

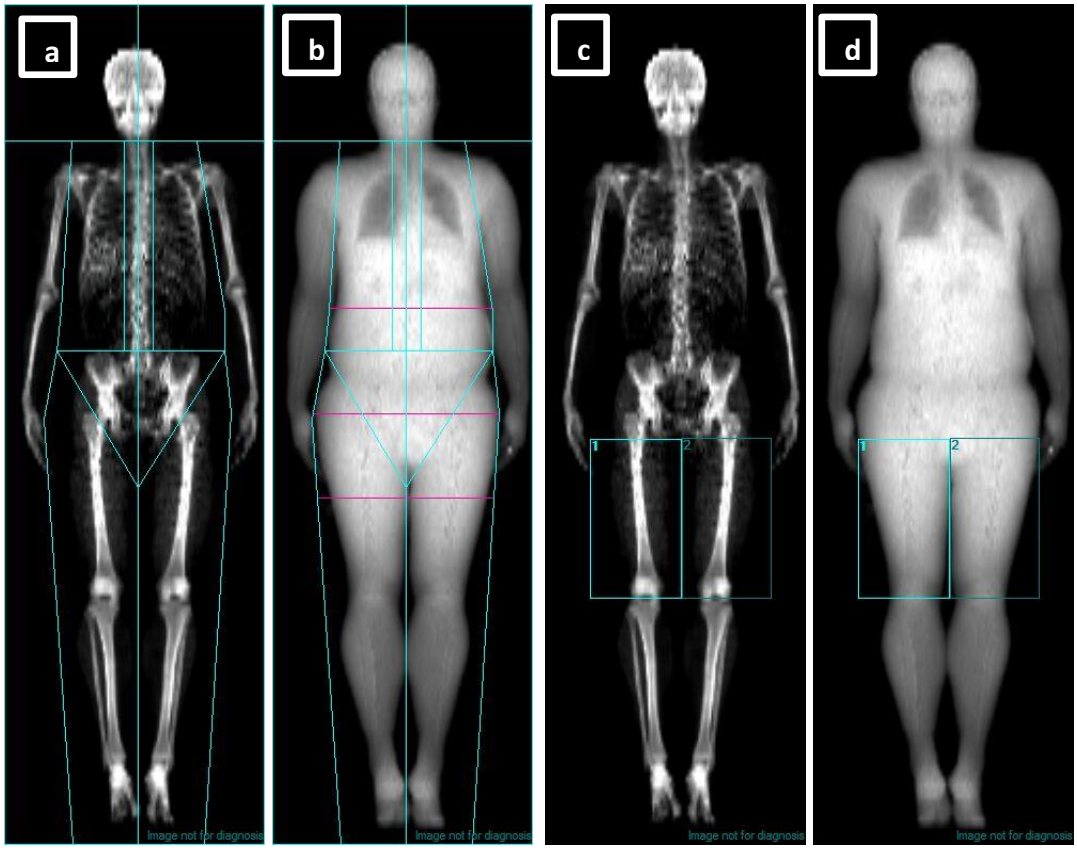


Figure 2-2. DEXA scan regions of interest. Appendicular limb boundaries are shown relative to skeletal (a) and soft tissue (b) landmarks. Thigh region of interest is shown relative to skeletal (c) and soft tissue (d) landmarks.

A custom region of interest around the thigh was created on DEXA scan images by delineating a region of interest encapsulating all tissue between the ischial tuberosity to the knee joint midline of each leg so as to capture the lean mass of the quadriceps and hamstrings (Figure 2.2 c&d).

2.6 Exercise Capacity

2.6.1 Quadriceps Maximal Voluntary Contraction (QMVC)

The strength of the quadriceps was assessed in all studies that form part of this thesis. For the NMES study participants were seated on a rigid rig with knee and hip flexion of 90°. The leg to be tested rested in a brace positioned immediately proximal to the malleoli and was secured in place with an inflexible, broad nylon strap. The leg brace

was attached to a digital load cell (Applied Measurements Ltd; Aldermaston, Reading, United Kingdom) the signal from which was amplified through a data acquisition unit (1401, Cambridge Electronic Designs; Cambridge, United Kingdom) and recorded and analysed using Spike2 software (Cambridge Electronic Designs, Cambridge, United Kingdom). A seatbelt was secured around the hips to prevent undesired movement and participants were instructed to sit upright and place their hands in their lap, or fold their arms (Figure 2.3, a). Participants were instructed to attempt to extend their leg against the resistance of the ankle strap and the force produced was measures by the load cell. The quadriceps maximal voluntary contraction (QMVC) testing protocol consisted of four warm-up effort at 50% of maximal effort, four at 75% of maximal effort followed by six efforts lasting a minimum of three seconds separated by 30 seconds rest. Verbal encouragement was given throughout. QMVC was defined as the instantaneous peak in force (N) produced during the maximal contraction.

For the MATCH Study quadriceps strength was recorded using an isokinetic dynamometer (Cybex NORM II; CSMi, Stoughton, USA; Figure 2.3, b). Participants were seated with knee and hip flexion of 90° and secured with a shoulder and waste harness as well as a Velcro strap over the knee. The leg was attached to the input arm of the dynamometer by placing the shin against a cushioned pad, immediately proximal to the malleoli and secured with a Velcro strap. Force applied to the input arm is translated to a moment about the centre point of the dynamometer and is recorded as a torque (Nm) by HUMAC software (CSMi, Stoughton, USA). The isometric QMVC testing procedure was the same as that performed for the NMES Study with the addition of 30 seconds of continuous passive movement before and after the procedure. Isokinetic QMVC at a rotational velocity of 60°/s was assessed immediately following isometric QMVC. One set of three repetitions was performed at 50% effort to re-familiarise the participant with the pace of knee extension followed by two sets of three maximal repetitions separated by 30 seconds recovery. The highest value for peak torque was recorded as isokinetic QMVC.

Figure 2-3. Quadriceps strength testing apparatus. a) Isometric QMVC rig utilised during testing for the NMES Study. b) Cybex Isokinetic Dynamometer during isokinetic quadriceps manoeuvre.

2.6.2 Cardiopulmonary Exercise Testing (CPET)

2.6.3 CPET Equipment

Volunteers were seated on an electrically braked cycle ergometer (Ergoselect 100; Ergoline, Bitz, Germany; NMES Study); or Lode Corival, Groningen, The Netherlands; MATCH Study) and monitored using a CPET cart (Ergocard Professional; Medisoft, Sorinnes, Belgium). Continuous cardiac monitoring was achieved using 12 lead electrocardiogram (ECG), oxygen saturation levels were monitored using finger pulse-oximetry (Nonin Medical B.V. Europe, Amsterdam, The Netherlands) and blood pressure was monitored using an automatic sphygmomanometer all of which systems fed live data to the computer coordinating the CPET. Ventilation, oxygen uptake and carbon dioxide exhalation were measured breath-by-breath using an online gas analyser comprising a non-dispersive infrared CO_2 sensor (response time +/- 80 ms, accuracy approximately 0.01%, range 0-10% CO_2) and an laser spectrometer O_2 sensor (response time approximately 60 ms, resolution 0.015%, range 5-100% O_2) with flow volumes determined by means of a pneumotachograph (range 0.03 – 15 L/sec, bandwidth 0-35 Hz, error <3%, dead space <13 ml) attached to a face mask (Hans Rudolph, Kansas, USA).

The accuracy of the CPET equipment was verified before every test. Volumes from the pneumotachograph were tested using a 3 l syringe with an error of <3% deemed acceptable. The gas analyser was tested using alternatively room air and a calibration gas mixture composed of 16% oxygen and 4% carbon dioxide.

2.6.4 Maximal Aerobic Exercise Capacity

Peak exercise capacity was measured using incremental cycle ergometry to exhaustion. Precise testing protocols may be found in Appendix 4. After instrumentation, data acquisition commenced and volunteers were monitored at rest and then during unloaded pedalling at 60 revolutions per minute (rpm). Following the unloaded period, the pedalling resistance was progressively increased by increments of 1 Watt. Ramp protocols varied from 5 W / minute (i.e. an increase of 1 W every 12 seconds) to 30 W / min (1 W every 2 s). A change in the pedalling load of 1 W is virtually imperceptible and the subject experiences a smooth transition to higher workloads, thus the protocol is considered to be a ramp protocol rather than an incremental one. Volunteers were monitored throughout the test and exercise terminated if oxygen saturation, blood pressure or ECG deviated from clinically acceptable parameters. Volunteers were instructed prior to the test and verbally encouraged throughout to maintain a pedalling frequency of 60 rpm until leg fatigue or dyspnoea prevented them from continuing. The test was terminated by the investigators if the subject was unable to maintain a cadence of 60 rpm.

Peak oxygen uptake ($\dot{V}O_2$ peak) was defined as the highest oxygen uptake (L / min) during loaded pedalling. Peak work rate (W^{PEAK}) was defined as the highest value out of the pedalling power in W coinciding with $\dot{V}O_2$ peak, or as the maximal work rate achieved before termination of the test.

2.6.5 Sub-Maximal Cycle Ergometry

In the MATCH Study, volunteers performed sub-maximal exercise testing. The work rate for this test was 65% of the work rate corresponding to $\dot{V}O_2^{PEAK}$. Instrumentation was the same as for maximal exercise testing. The test protocol began with 3 minutes of resting recordings and 3 minutes of unloaded pedalling. The work rate then ramped over the period of one minute from 0W to the target intensity with volunteers instructed to maintain a cadence of 60 rpm throughout. Physiological measures were taken during the test and averages calculated which exclude the first 3 minutes of pedalling at the test workload in order to produce data representative of steady state exercise. The test

was terminated by the investigators if the participant reached 30 minutes of pedalling under load. Data from baseline and follow-up tests were averaged over a duration corresponding to that of the shortest test so that data from repeated measures represent the cardiorespiratory responses to exactly the same exercise challenge (both duration and workload) at repeated time points.

2.7 Physical Activity Monitoring

Habitual physical activity levels were monitored in volunteers for the MATCH study at baseline and for two periods during the detraining phase which followed the exercise intervention. On each occasion the volunteers were asked to wear a SenseWear tri-axial accelerometer (BodyMedia, Pittsburgh, USA) during waking hours for 7 consecutive days, removing the monitor only for bathing or during any activity where it would be exposed to water. Physical activity was not monitored on days that the volunteer attended for study related testing or training. The monitor is a small, battery powered device worn next to the skin on the back of the right arm and held in place by an elasticated Velcro strap (Figure 2.4). The accelerometer component of the device provides data on body motion and steps; two electrodes that contact the skin measure galvanic skin responses by measuring the electrical conductivity of the skin; and temperature sensors in contact with the skin and on the side of the monitor are used to estimate heat flux from the body (the rate of heat dissipation from the body). Data are recorded on the device's internal memory and downloaded for analysis using the proprietary SenseWear software. To be included in the analysis, a minimum of 8 hrs of data per day was required. In order to reduce variation between subjects who had worn the monitor for differing lengths of time during each day (e.g. 8 hrs *vs* 18 hrs) only the first 8 hours of data after waking were included in the analysis. The mean step count and mean energy expenditure for each individual was calculated and used for statistical analysis.

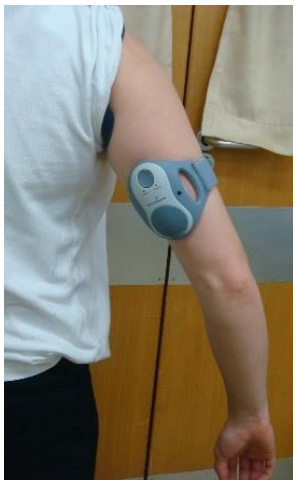


Figure 2-4. Positioning of physical activity monitor.

2.8 Skeletal Muscle Biopsy Procedure

Tissue from the *vastus lateralis* muscle was sampled using a sterile micro-biopsy technique at mid-thigh level. Briefly, skin was sterilised with a Betadine solution and local anaesthetic (lignocaine) injected subcutaneously and to the depth of the fascia. A small incision (5 mm) was made in the skin and any subcutaneous adipose through which a 12 g micro-biopsy needle was inserted (Bard Magnum, Arizona, USA). Four passes were performed each obtaining approximately 20 mg of tissue. Tissue for molecular biology analysis was immediately dissected free of visible adipose and connective tissue and snap frozen in liquid nitrogen. Tissue was stored in liquid nitrogen until analysis. The biopsy site was dressed with a butterfly closure and a waterproof sterile dressing. A compression bandage was used to apply light pressure to the biopsy site for the remainder of that day in an attempt to minimise the risk of bleeding or bruising. Subsequent biopsies were performed approximately 2 cm from the preceding incision site.

2.9 Gene Expression Analysis

2.9.1 RNA Extraction

RNA was extracted using TRI Reagent Solution (Applied Biosystems / Life Technologies, Paisley, UK) which contains phenol and guanidine thiocyanate to inhibit RNase activity. The full protocol can be found in Appendix 5**,** however briefly, tissue was transferred

directly from liquid nitrogen to ice cooled TRI Reagent Solution and homogenised using a PowerGen 1000 homogeniser (Fisher scientific, Loughborough, UK). Chloroform was then used to separate the homogenate into aqueous-, inter- and organic-phases. Isopropanol facilitates the precipitation of RNA from the aqueous phase. The RNA pellet formed by centrifugation of the solution was washed with ethanol and dissolved in water ready for storage at -80 ˚C.

2.9.2 RNA Quantification

RNA quantification was performed using 1.5 µl of the RNA suspension solution, loaded onto a Nanodrop 2000 Spectrophotometer (Thermoscientific, Waltham, MA, USA). The sample was recovered after quantification. Concentration of RNA in solution, expressed in ng/µl was established by measuring the sample's absorbance of 260nm light. Purity of the sample was measured by measuring the ratio of absorbance at 260 nm compared to 230 nm and 280 nm respectively. A 260/280 nm absorbance ratio of 2.0 is considered pure for an RNA sample. A 260/230 nm absorbance ratio of 2.0-2.2 is considered pure for nucleic acids.

2.9.3 Reverse Transcription

Complementary DNA (cDNA) was synthesised using a reverse transcriptase (RT) enzyme. The full protocol can be found in Appendix 5. A volume of RNA suspension equivalent to 1 µg of RNA per sample was incubated with random hexamers which act as primers for the reverse transription and dNTPs (deoxynucleotide triphosphates) as a source of nucleotides for cDNA synthesis. This first incubation uses a thermal cycler to heat the sample to 65˚C for 5 minutes in order to degrade the RNA structure such that the primers can bind. Subsequently a master mix containing DTT (Dithiothreitol) to aid activation of the reverse transcriptase enzyme, RNase inhibitor, and 5X first-strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$) was added to the RNA suspension. Finally the reverse transcriptase (SuperScript III, Life Technologies / Invitrogen) was added prior to a final incubation in the thermal cycler at 25 ˚C for 5 minutes, 50 ˚C for 60 minutes and finally 70 ˚C for 15 minutes during which time primers are annealed to the RNA and the RT reaction extends. Following reverse transcription samples were stored at -80 ˚C.

2.9.4 Real-time PCR

2.9.4.1 cDNA Amplification

The expression of target genes, plus housekeeping genes was assessed using quantitative real-time PCR. mRNA abundance was measured using TaqMan 384 well custom low density array (LDA) microfluidic cards (Life Technologies / Applied Biosystems) processed on a TaqMan 7900HT Real-Time PCR Instrument (Applied Biosystems, Paisley, UK) using the $\Delta\Delta C_T$ (Relative Quantitation; RQ) protocol for 384 Well TaqMan Low Density Array, under the control of SDS (Sequence Detection System) 2.3 software. The design of the cards used in the studies described in this thesis is described in later chapters with a full list of target genes available in Appendix 6. Each of the card's wells contained a specific probe for the identification of a single gene and reporter / quencher dyes (FAM / MGB-NFQ) for quantification.

A quantity of 100 ng cDNA in solution with TaqMan Gene Expression Master Mix (Life Technologies / Applied Biosystems) was loaded onto each of the card's 8 channels. Care was taken to ensure that the concentration of cDNA in the reaction mixture for each sample was constant.

The master mix contains a DNA polymerase enzyme (AmpliTaq Gold DNA Polymerase, Ultra Pure) which becomes active at a temperature greater than that required to fully denature the cDNA. Also in the master mix is Uracil-DNA Glycosylase (UDG, also known as uracil-N-glycosylase) which removes any contaminating uracil nucleotides that have been carried forward from RNA to cDNA. The final element of the master mix is a passive reference dye (ROX Passive Reference; Life Technologies / Applied Biosystems) which acts as an internal reference against which signal from the probe's reporter-dye can be compared and normalised to correct for any variation in volume or concentration. The passive reference does not participate in the PCR reaction, so the amount of signal it emits remains constant throughout the PCR cycles.

When the samples were placed in the real-time PCR instrument / thermal cycler the first step was to heat the plate to 50 $^{\circ}$C for two minutes. This step causes activation of the UDG enzyme and subsequent removal of unwanted uracil nucleotides. The second step is a 10 minute hold at 95 $^{\circ}$C which allows full activation of the DNA polymerase enzyme.

Following these two steps the PCR cycles begin. At the end of the protocol data files were saved for later analysis.

During each of the 40 cycles of PCR the temperature fluctuates between 97.0 ˚C for 30 seconds (denaturing phase), and 59.7˚C for 1:00 minute (extension / annealing phase). If the target sequence is present in the sample, the probe will hybridise to it. TaqMan probes are labelled with a reporter dye and a quencher dye. When the reporter dye (at the 5' end of the probe) is in close proximity to the quencher dye (at the 3' end of the probe) the fluorescence of the reporter dye is suppressed.

Once the hybridization has occurred, the DNA polymerase enzyme will cleave the probe between the reporter and quencher dyes (releasing inhibition of the reporter dye). The remains of the probe are then removed from the target and the process of polymerisation of the cDNA strand continues in order that the cDNA product may accumulate exponentially. There is a block on the 3' end of the probe to prevent extension of the probe itself. It is a key assumption of subsequent calculations that each cycle of PCR is 100% efficient, leading to a doubling of cDNA product.

In order for the fluorescence signal to increase it is necessary that the probe and the target sequence are complimentary and that amplification occurs during the cycles of PCR, thus, non-specific amplification will not result in increased fluorescence.

2.9.4.2 Normalisation

The intensity of fluorescence emitted by the reporter dye is normalised to the intensity of the ROX Passive Reference dye which is present in the Master Mix. The normalised reporter ratio (R_n) is calculated in this manner:

$$R_n = \frac{\textit{Reporter Dye Emission Intensity}}{\textit{ROX Passive Reference Emission Intensity}}$$

The fluorescence intensity for each well, containing all components is R_n^+. The value R_n^- represents the fluorescence of the unreacted sample and is obtained from early cycles of PCR. ΔR_n gives the magnitude of the fluorescence signal generated by the PCR.

$$\Delta R_n = (R_n^+) - (R_n^-)$$

2.9.4.3 Threshold Cycle

The threshold cycle (C_T) is defined as the cycle number at which the fluorescence signal (ΔR_n) increases above the statistically significant threshold above background fluorescence, see Figure 2.5.

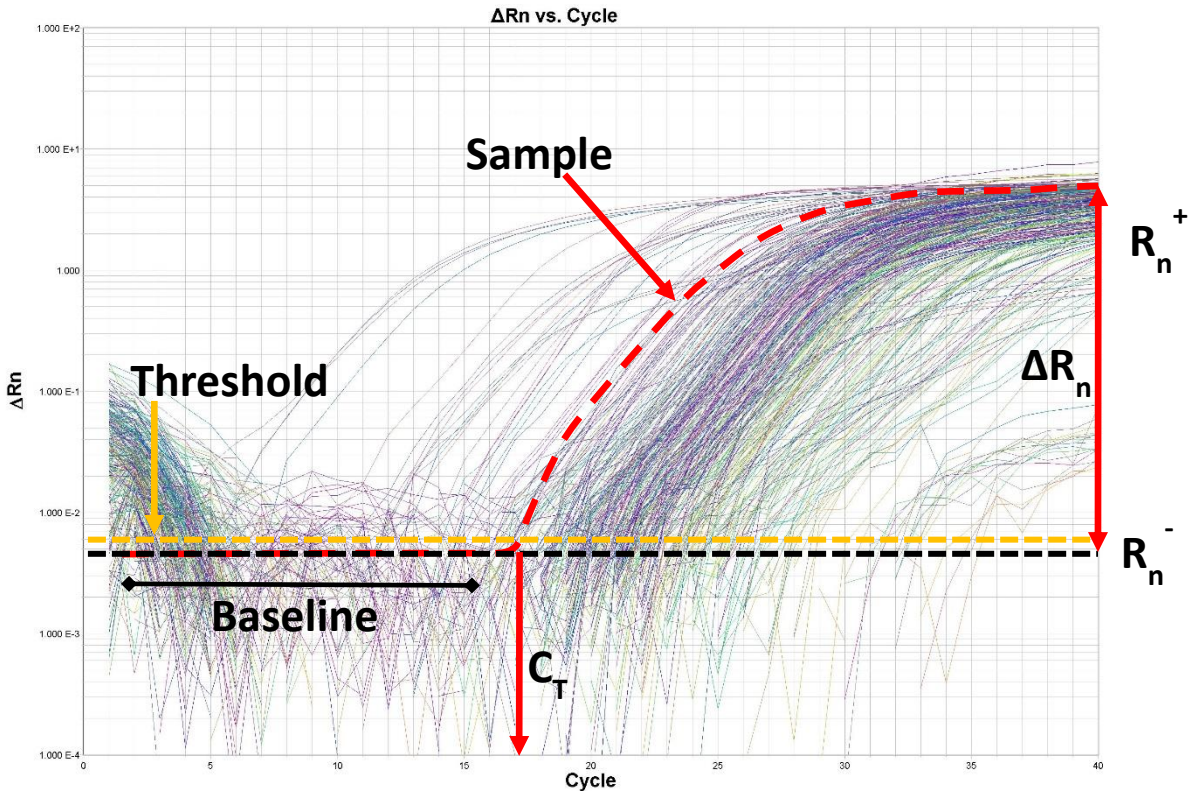


Figure 2-5. Threshold Cycle

Figure 2.5 demonstrates a deflection in ΔR_n for a given sample above the threshold (broken red line). This identifies the threshold cycle (C_T) for that sample. Annotations are overlaid on an example plot generated during a study for this thesis. Note in the example plot there is significant noise in ΔR_n during the baseline period. The cycle at which a significant increase in ΔR_n is first detected is identified by ABI proprietary software (SDS) using a validated algorithm.

2.9.4.4 Results Analysis

Individual amplification plots were analysed by SDS software which automatically generates a C_T value for each target on the 384-well plate. Data were extracted for analysis using the comparative C_T method ($\Delta\Delta C_T$) using the Relative Quantitation (RQ) Manager software. The comparative C_T method permits relative quantitation of the

target gene against an internal control (i.e. one of the housekeeping genes chosen in the card design). For this work, ΔC_T for each sample was calculated for individual genes using the endogenous control HMBS, according to the following formula:

$$\Delta C_T = C_T \text{ target gene} - C_T \text{ endogenous control gene}$$

HMBS was selected as its raw C_T values were similar to the raw C_T values of the target genes and there was no significant change in HMBS C_T values between baseline and stimulated samples.

$\Delta\Delta C_T$ was calculated by deducting baseline ΔC_T from stimulated ΔC_T:

$$\Delta\Delta C_T = \Delta C_T \text{ stimulated} - \Delta C_T \text{ baseline}$$

The relative expression of individual genes was expressed as a fold-change where:

$$\text{Fold-change} = 2^{-\Delta\Delta CT}$$

2.9.5 Gene Expression (NMES Study)

The microfluidic LDA cards were custom-designed to target families of genes whose functions include metabolic processes, mitochondria, diabetes, cell cycle/growth/differentiation, inflammation and immune responses. Target selection was directed by data from two studies that employed a similar isokinetic RE protocol to that used in the current study. The first study reported an Affymetrix based analysis of tissue sampled at rest pre- and 24 hrs-post resistance exercise in healthy volunteers (Murton, Billeter et al. 2014). The second study highlighted gene expression changes following a similar bout of RE in young healthy volunteers who had undergone a period of immobilisation (Jones, Hill et al. 2004).

Change in gene expression was tested for significance using the R Statistical Package (R Version 3.0.0, 2013-04-03, The R Foundation for Statistical Computing). Paired t-tests were performed on ΔC_T baseline and ΔC_T stimulated. In order to correct for multiple comparisons the False Discovery Rate (FDR) (Benjamini and Hochberg 1995) adjustment was applied to the level of significance. Differential expression between time points was defined as FDR <5%.

In the case where a gene was not expressed, or gene expression was lower than the limit of detection with 40 cycles of PCR, a missing value occurred. Any genes with values missing for >2 subjects were excluded from the analysis, meaning that all genes were measured on at least n=11 subjects. Data are presented as fold change with statistical significance expressed as FDR.

2.9.6 Gene Expression (MATCH Study)

Targets for gene expression analysis were selected using data generated within the research group for mRNA abundance changes (targeted RT-PCR and Affymetrix gene chips) observed in quadriceps muscle biopsy samples from healthy young and older volunteers in response to a single bout of acute exercise and several weeks of limb immobilisation (data unpublished). In addition, searches of SA Biosciences and IPA databases were performed. The list of 96 mRNA transcripts quantified (including 2 housekeeping genes) can be found in Appendix 6.

The abundance of mRNA was quantified for each group (within group comparison) using the comparative C_T method to calculate fold change from baseline and using HMBS as the internal control. Data were quality checked, excluding data points arising from a C_T value > 36. Fold change data show high inter-subject variation; it is therefore important to maximise sample size when calculating an average value so that extreme values in a small data set do not unduly influence results. In order to make data as robust as possible, values from $\geq$ 70% of subjects in each group were required for data from that transcript and time point to be included in the analysis.

2.9.6.1 Network Analysis

Gene expression data from the MATCH Study were interrogated using Ingenuity Pathway Analysis (IPA®; QIAGEN Redwood City, USA, www.qiagen.com/ingenuity) in order to identify biological functions significantly influenced by the intervention. IPA is a web-based analysis tool that groups data about the expression of multiple genes into functional networks. This is not a statistical clustering technique; rather it takes gene

expression values from the experimental dataset (expressed as fold change in the number of transcript copies from baseline) and models these into networks of genes that have a known biological function that has been published in the literature. IPA generates histograms describing the likelihood that a function (e.g. carbohydrate metabolism) is significantly different to the baseline biopsy determined by Fisher's exact test.

The strength of using a network-based approach to analysing gene expression data is that it allows the influence of multiple genes acting in synchrony to be analysed with respect to a meaningful biological function. This is more powerful than considering the change in expression of a single transcript over time.

2.10 Protein Expression

Proteins of interest were identified following analysis of gene expression data for the MATCH Study and quantified in *vastus lateralis* tissue using western blotting with infrared detection. Portions of muscle tissue (approx. 30 mg per sample) were reserved for this purpose and stored in liquid nitrogen until the time of analysis. Protein extractions and western blotting were kindly performed by Dr Despina Constantin at the University of Nottingham, who provided me with raw optical density values for target proteins and internal controls. All subsequent analysis of western blot data was performed by me.

2.10.1 Protein extraction

Extraction of nuclear and cytosolic proteins was performed using a method adapted from Blough et al (Blough, Dineen et al. 1999). Approximately 30 mg of frozen wet tissue was homogenised in Tris buffer (50mmol / l Tris-HCl, 1 mmol / l EDTA, 1mmol / l EGTA, 1% (v/v) Triton-X100 and 0.1% (v/v) 2-mercaptoethanol (pH 7.5) with 10 µl of phosphatase and protease inhibitor added for every 1 ml of homogenisation buffer (P2850, Sigma-Aldrich, Poole, UK). The homogenate was centrifuged at 10,000 g for 5 min at 4°C and the supernatant containing cytosolic proteins stored at -80°C. The resulting pellet was re-suspended in ice cold buffer containing 20 mmol / l HEPES (pH 7.9), 25% (v/v) glycerol, 500 mmol / l NaCl, 1.5 mmol / l MgCl2 and 0.2 mmol / l EDTA

(pH 8.0). More protease and phosphatase inhibitors were added at a concentration of 10 µl / ml before centrifugation at 3,000 g for 5 min. The resulting supernatant containing nuclear proteins was stored at -80°C until Western blotting was performed.

2.10.2 Immunoblotting

The Bradford assay was used to quantify protein concentration in the supernatant (Bradford 1976) prior to Western blotting. Pre-cast 4-12% Bis-Tris polyacrylamide gels were used to run samples for 2 hr at 200 V before transfer to a polyvinylidene difluoride (PVDF) membrane in ice-cold buffer at 4°C overnight at 100 mA. Ponceau S red was used as a stain to visualize the protein loading onto the PVDF membrane. The membrane was then blocked in BSA-TBS-Tween at room temperature for 1 hr. Primary antibodies were applied overnight at 4°C. Membranes were subsequently washed in TBS-Tween, before application of the secondary antibody. Secondary antibodies linked to fluorescent dyes from the near-infrared spectrum were used for detection (IRDye 800 anti-rabbit and IRDye 680 anti-mouse; LI-COR, Cambridge, UK). Bands were detected using digital image capture (Odyssey IR Imaging System; LI-COR) and quantified using Image Studio software (LI-COR). Compared to chemiluminescence techniques, near-infrared fluorescence imaging has high signal-to-noise ratios due to low background auto fluorescence in the near-infrared region; and has a superior dynamic linear range. The Western blotting laboratory protocol can be found in Appendix 7.

2.11 Statistical Analyses

Tests within this thesis are considered to have reached statistical significance with a probability of $\alpha < 0.05$). For analyses involving groups of 10 subjects or fewer, as in the case of the MATCH Study, non-parametric statistical tests were employed as the small sample size is unlikely to meet the criteria of normality required for parametric testing.

2.11.1 Aerobic training Study (MATCH)

Cross-sectional baseline comparisons of continuous and ordinal data for two groups were performed using the Mann-Whitney U test for independent samples and reported as median values. Within group change over time was assessed with Wilcoxon signed rank or Friedman's ANOVA.

Retrospective power calculations were performed after data collection using mean and standard deviation values for observed $\dot{V}O_2^{PEAK}$ (ml/min/kg lean-mass) within-group change from baseline to post-training (8 weeks) for the Young, Older and COPD groups. To detect the magnitude of change in $\dot{V}O_2^{PEAK}$ observed following training in the Young and Older heathy groups with 80% power and significance set at $p < 0.05$ a sample size of n = 6 per group was required. Data from the COPD group (where no significant change in $\dot{V}O_2^{PEAK}$ was observed in this study) was used to calculate the effect size necessary to detect a significant within-group change with a 5% type I error rate and 80% power. It was calculated that the MATCH study sample size (n = 19) was sufficient to detect a 2.13 ml/min/kg lean-mass change in $\dot{V}O_2^{PEAK}$ after 8 weeks of training, which would represent an 8.7% increase, thus the COPD group was adequately powered to detect a clinically meaningful change in peak oxygen uptake.

2.11.2 NMES vs RE Acute Study

Baseline between group differences in physiological variables were tested by t-test, Mann-Whitney U test (ordinal data), or Pearson X^2 test (categorical data) using SPSS statistical analysis software. Paired t-tests were used to identify significant change in expression of the target gene relative to the housekeeping control gene (ΔCT) from baseline to 24 hrs. The *p* value relating to pairwise comparisons was adjusted to reduce the risk of type 1 error arising from multiple comparisons using the False Discovery Rate method described by Benjamini & Hochberg (Benjamini and Hochberg 1995). This method was chosen as a compromise between unadjusted probabilities and the overly conservative Bonferroni multiplier adjustment which increases the risk of type 2 error. Analysis of ΔCT data and generation of heatmaps was performed using the R Statistical Package (R Version 3.0.0, 2013-04-03, The R Foundation for Statistical Computing). Gene functions were determined from the results of literature searches (search terms: "<<gene name>>" AND "mRNA" AND "skeletal muscle" Filters: humans) and cross referenced with the PubMed (NCBI) gene database https://www.ncbi.nlm.nih.gov/gene.

Chapter Three

Influence of age on the responses to aerobic training and subsequent detraining: cardiorespiratory responses to exercise

3.1 Introduction

Low aerobic fitness is a predictor of all-cause mortality, morbidity and reduced physical function in young and older adults (Blair, Kampert et al. 1996; Blair and Wei 2000; Blair 2009). In health, the factors limiting peak oxygen uptake are thought to be oxygen delivery to exercising muscle (limited mainly by cardiac output) and the rate of oxygen extraction at the periphery (Saltin and Calbet 2006; Lundby, Montero et al. 2016) which itself is determined by muscle oxidative capacity. In the absence of disease, habitual physical activity levels are a key determinant of aerobic capacity in both young and old (Dehn and Bruce 1972), however peak oxygen uptake declines with advancing age even in highly active older people, albeit at a slower rate than in sedentary populations (Rogers, Hagberg et al. 1990; Pollock, Carter et al. 2015).

It is known that both young and older sedentary adults are capable of improving aerobic capacity by 10-24% with the appropriate exercise training (Kohrt, Malley et al. 1991; Wibom, Hultman et al. 1992; Houmard, Tyndall et al. 1996; Milanovic, Sporis et al. 2015) however few studies have been designed to directly compare the relative response of young and older adults to an aerobic training programme and even fewer have examined the influence of exercise withdrawal or detraining following training. In this context, periods of enforced inactivity (for example illness or musculoskeletal injury) contribute to the acceleration of deconditioning in elderly populations and may hasten the onset of frailty and impaired quality of life often associated with advancing age (Coker, Hays et al. 2014). Young adults too are susceptible to deconditioning during periods of inactivity (Saltin, Blomqvist et al. 1968). It is unknown whether a period of exercise withdrawal (prompting a detraining / deconditioning effect) will affect young and old equally.

The principal hypothesis tested in this chapter is that young and older sedentary volunteers will experience an increase in $\dot{V}O_2^{PEAK}$ in response to eight weeks aerobic exercise training. Additional research objectives are to address the following questions:

1. Are the whole-body cardiorespiratory adaptations (change in $\dot{V}O_2^{PEAK}$ and responses to sub-maximal exercise testing) in response to aerobic exercise training at the same relative workload similar in young and older volunteers?
2. Does exercise withdrawal affect young and older volunteers equally? What are the effects on $\dot{V}O_2^{PEAK}$ and other cardiorespiratory measures?

In order to address this hypothesis and objectives, data are presented comparing sedentary young and older volunteers recruited as part of the MATCH Study. After characterisation of body composition, physical activity status and whole-body aerobic capacity at baseline, the response to an eight week aerobic training programme and subsequent four week period of exercise withdrawal is reported.

3.2 Methods

3.2.1 Participants

Subjects were defined as young healthy (aged 18 – 35 years) referred to in this chapter as “Young Group” or older healthy (aged 60 – 80 years) referred to in this chapter as “Older Group”. Inclusion criteria (Chapter 2.4.1) were designed to select sedentary but otherwise healthy volunteers.

3.2.2 Study Design

An overview of the protocol can be seen in Chapter 2.4.1. Briefly, volunteers performed supervised aerobic exercise training three times per week for eight weeks on a cycle ergometer. Training sessions consisted of 30 min of continuous cycling, at an intensity corresponding to 65% of the work rate coinciding with $\dot{V}O_2^{PEAK}$ achieved in the baseline test incremental cycle exercise test. Training intensity was reset after repeat incremental exercise testing at four weeks. Assessments were conducted at baseline and after four and eight week’s training. Supervised exercise ceased after eight weeks

and subjects were instructed to resume their habitual physical activity levels before repeat testing four weeks after exercise withdrawal.

3.2.3 Measures

Characteristics of participants at baseline include lung function, average step count, body composition by DEXA and isometric quadriceps strength. Cardiopulmonary exercise testing (CPET) provided exercise outcome measures for incremental ($\dot{V}O_2^{PEAK}$; Peak Workload; and RER, VE and HR at $\dot{V}O_2^{PEAK}$) and sub-maximal testing at 65% W^{PEAK} (RER, VE and HR) with tests performed on a cycle ergometer. Full testing procedures are found in Chapter 2.5 – 2.8.

3.2.4 Data Analysis

Due to the relatively small sample size (n = 10 per group) non-normal data distribution was assumed and therefore non-parametric statistical tests were employed. Between group comparisons were assessed using the Mann-Whitney U test for continuous or Pearson Chi-Square test for categorical variables; within group change with Friedman's ANOVA or Wilcoxon Signed Rank test.

3.3 Results

3.3.1 Subject Characteristics

Subject characteristics for the 10 Young and 10 Older adults who completed the intervention are presented in Table 3.1. Groups were well matched for gender, height, total body mass and accordingly, BMI (between group differences all $p > 0.05$). Older volunteers had significantly lower absolute FEV_1 than Young volunteers, however there was no significant difference in FEV_1 expressed relative to predicted normal values. The FEV_1 / FVC ratio was higher in Young than in Older volunteers however both groups were within the normal healthy range both for FEV_1 / FVC and all other measures of lung function (including total lung volume, residual volume and gas transfer, data not shown).

There were no significant differences between groups for strength or body composition measures at baseline (Table 3.1). When muscle mass and quadriceps strength were re-measured after 8 weeks training and then after 4 weeks detraining there were no

significant within-group changes ($p > 0.05$ for all measures and all time points, data not shown).

Table 3-1. Baseline Subject Characteristics of Young & Older groups

	Older Group		Young Group		
	Mean	SD	Mean	SD	p
Demographics					
Gender (male : female) *	5 : 5		4 : 6		NS
Age (years)	71	5	28	5	< 0.001
Height (m)	1.65	0.09	1.68	0.11	NS
Body mass (kg)	78.4	13.7	74.6	27.6	NS
BMI (kg/m^2)	28.5	3.3	26.0	7.6	NS
Lung Function					
FEV_1 (L)	2.57	0.38	3.47	0.92	0.029
FEV_1 (% predicted)	113	21	98	9	NS
FVC (L)	3.5	0.6	4.3	1.4	NS
FVC (% predicted)	120	21	104	16	NS
FEV_1 / FVC ratio	75	4	82	9	0.043
Smoking history (Current : Never : Ex) *	0 : 6 : 4		0 : 10 : 0		0.025
Pack-year history	18.3	21.5	-	-	-
MRC Grade $	1	1 - 1	1	1 - 1	NS
Body Composition & Muscle Function					
Whole Body Lean Mass (kg)	47.7	8.2	44.6	12.6	NS
FFMI (kg/m^2)	18.2	1.5	16.5	2.8	NS
ASMI (kg/m^2)	7.3	0.9	7.0	1.7	NS
Thigh Lean Mass	9.42	3.28	8.99	2.13	NS
Isometric QMVC (Nm)	130	36.8	163	72.5	NS

Between group differences tested by Mann-Whitney U test for ordinal and continuous data. Categorical variables (*) tested with Pearson Chi-Square test. NS = $p > 0.05$. $ Median, IQR.

3.3.2 Habitual Physical Activity

The average number of days for which 8 hours of baseline data was available was 6.2 days for the Young group and 7.2 days for the Older group.

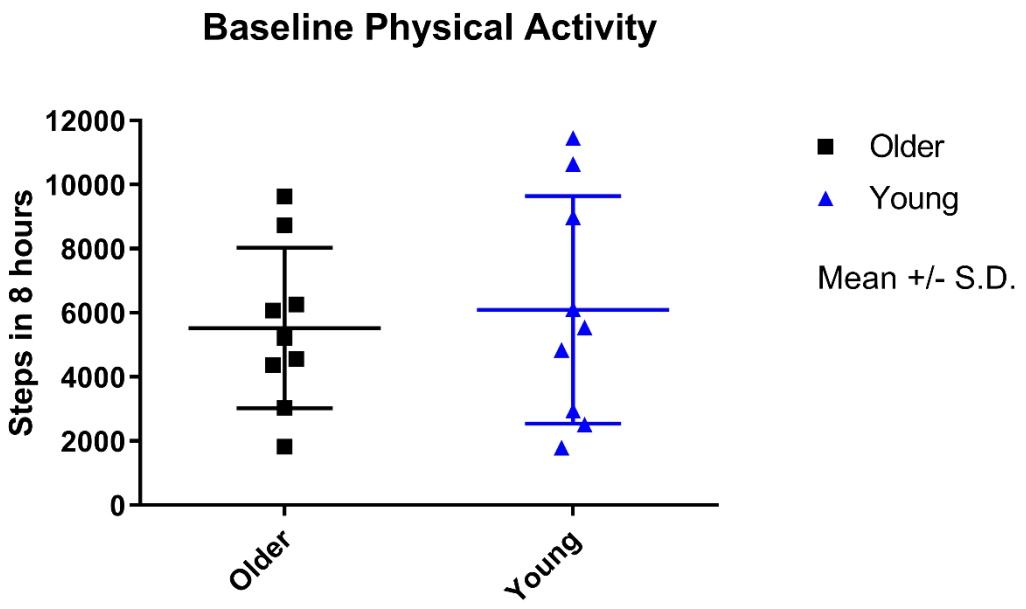


Figure 3-1. Habitual physical activity at baseline in Young & Older groups. Individual data points represent the mean step count for all days recorded for the individual. Line indicates group mean ± S.D.

There was large inter-subject variation in step count in both groups and no difference between groups (Figure 3.1). Habitual physical activity did not change during the trial period for either group. Within-group comparison of step-count measured in the 9th and 12th week of the study (first and last week of the exercise withdrawal period) did not differ significantly for either Young or Older volunteers (all $p > 0.05$, Appendix 8).

3.3.3 Exercise Capacity and Cardiorespiratory Responses to Incremental Exercise

3.3.3.1 Baseline

Cardiorespiratory measures from the baseline incremental exercise test (CPET) are shown in Table 3.2.

Table 3-2. Baseline Incremental Exercise Test for Young & Older groups.

	Older Group		Young Group		p
	Mean	SD	Mean	SD	
$\dot{V}O_2^{PEAK}$ (l/min)	1.41	0.36	2.00	0.68	0.052
$\dot{V}O_2^{PEAK}$ (ml/min/kg lean mass)	29.7	4.0	44.7	5.7	<0.001
Peak $\dot{V}CO_2$ (L/min)	1.69	0.45	2.47	0.84	0.035
RER at $\dot{V}O_2^{PEAK}$	1.20	0.09	1.24	0.10	NS
W^{PEAK} (W / kg Lean Mass)	2.4	0.2	3.8	0.6	0.005
Peak $\dot{V}E$ (L/min)	62.85	16.39	82.94	25.49	NS
% MVV at peak exercise	66	16	66	17	NS
HR at Peak Exercise (beats/min)	141	14	186	12	<0.001
% Estimated Max HR	94	10	97	5	NS
Borg Breath Pre-Test $	0	0 - 0	0	0 - 0	NS
Borg Breath End-Test $	4	3.25 - 4.75	6	4 - 7	NS
Borg RPE Pre-Test $	6	6 - 6	6	6 - 6	NS
Borg RPE End-Test $	16	13 - 17	17	17 - 18	NS
SpO_2 Pre-Test	97.0	1.5	97.8	1.3	NS
SpO_2 End-Test	96.4	1.3	97.1	1.8	NS
Change in SpO_2	-0.6	2.2	-0.4	1.3	NS
Reason for Stopping (SOB : Leg Fatigue : SOB = Legs : Other) *	2 : 5 : 2 : 1		2 : 8 : 0 : 0		NS
Between group comparisons by Mann-Whitney U test for ordinal and continuous data. Categorical variables (*) tested with Pearson Chi-Square test. $, median IQR.					

The Young Group had greater $\dot{V}O_2^{PEAK}$ (corrected for lean mass), Peak $\dot{V}CO_2$ and W^{PEAK} than the Older group (all $p < 0.05$). Heart rate (beats / min) was significantly greater in the Young compared to the Older group, however when expressed as proportion of predicted maximum heart rate there was no difference between groups ($p > 0.05$).

3.3.4 Influence of Aerobic Training on Cardiorespiratory Measures at Peak Exercise Intensity

$\dot{V}O_2^{PEAK}$ (ml / min / kg lean mass) increased significantly from baseline during the 8 week training intervention in both Young (mean increase 21%, $p < 0.001$) and Older (mean

increase 18%, p = 0.001) groups (Figure 3.2a). Similarly, both Young and Older groups increased W^{PEAK} after 8 weeks training (Figure 3.2b) with Young volunteers increasing W^{PEAK} by a mean of 23% ($p < 0.001$) and Older volunteers increasing by 24% ($p < 0.001$). The relative changes in $\dot{V}O_2^{PEAK}$ and W^{PEAK} from baseline to 8 weeks were not significantly different between the groups (p = 0.190, and p = 0.123 respectively).

After 8 weeks training, minute ventilation at $\dot{V}O_2^{PEAK}$ had increased by 26% in the Older group (p = 0.012) and by 14% in the Young group ($p < 0.001$) compared to baseline. There was no significant change in the maximum heart rate achieved or the RER at $\dot{V}O_2^{PEAK}$ in either group in follow-up tests (all $p > 0.05$).

3.3.5 Influence of Exercise Withdrawal on Cardiorespiratory Measures at Peak Exercise Intensity

There was no effect of four weeks of exercise training withdrawal on $\dot{V}O_2^{PEAK}$ in the Older group (p = 0.575; Figure 3.2a). In the Young group there was a trend towards a decrease in $\dot{V}O_2^{PEAK}$, however this did not reach statistical significance (p = 0.059; Figure 3.2a). W^{PEAK} (Figure 3.2b) declined in the Young (p = 0.005) and Older groups (p = 0.011). The percentage changes in $\dot{V}O_2^{PEAK}$ and W^{PEAK} during four weeks of exercise withdrawal were not significantly different between the groups (p = 0.190, and p = 0.123 respectively).

After four weeks of exercise training withdrawal minute ventilation at $\dot{V}O_2^{PEAK}$ was significantly reduced compared to the end of training in the Young group (p = 0.005) and there was a trend towards a reduction in the Older group (p = 0.059). Heart rate at $\dot{V}O_2^{PEAK}$ was not influenced by the period of exercise withdrawal in either group ($p >$ 0.05). There was a significant decrease between the end of training and the end of the exercise withdrawal period in RER at $\dot{V}O_2^{PEAK}$ in the Older group (p = 0.028) but not in the Young group (p = 0.114).

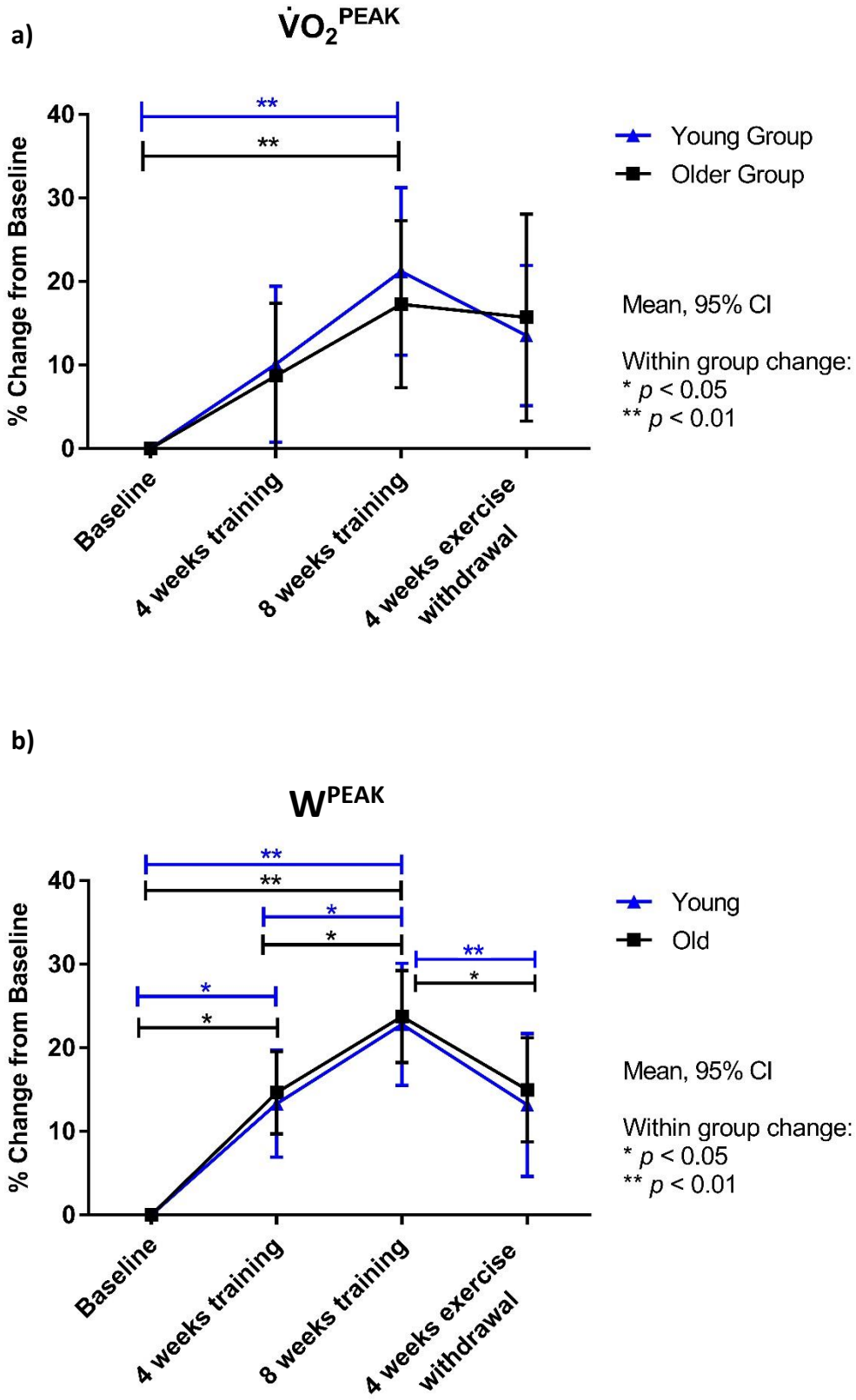


Figure 3-2. $\dot{V}O_2^{PEAK}$ (a) and W^{PEAK} at exhaustion in incremental CPET (b) with training and exercise withdrawal in Young & Older Groups

3.3.6 Submaximal Exercise Testing

The exercise resistance for each subject in the constant work-rate test was 65% of the W^{PEAK} achieved in the baseline incremental test (Table 3.2); accordingly, there was a significant between-group difference in absolute work-rate ($p < 0.004$) with the Young group highest (mean 110 $\pm$ 25.8 W) and Older group working at a mean of 76 $\pm$ 17.5 W (Table 3.3).

3.3.6.1 Baseline Sub-maximal Exercise Testing

Absolute values for minute ventilation and heart rate ($\dot{V}E$, L / min; and HR, beats / min were significantly higher in the Young compared to the Older group ($p < 0.05$), but there was no difference between groups when reported as percentage of maximum (both $p > 0.05$). There was a non-significant trend towards a small difference in RER (Table 3.3) with the Young group having a higher mean value ($p = 0.052$) and no difference in blood oxygen saturations between groups ($p > 0.05$). Physiological variables reported are averages over the duration of the test (excluding the first 3 minutes); there was no significant difference in the duration of the test between Young and Older groups (p > 0.05, data not shown).

Table 3-3. Sub-maximal, constant load exercise test at baseline for Young & Older groups

	Older Group		Younger Group		*p*
	Mean	SD	Mean	SD	
Exercise workload (W)	76	17.5	110	25.8	0.004
RER	1.04	0.03	1.07	0.04	0.052
$\dot{V}E$ (L / min)	52.20	11.43	66.25	16.90	0.015
% MVV	53.8	11.9	53.4	15.9	NS
HR (beats / min)	130	16.2	178	9.9	<0.001
HR (% max)	86.8	11.9	92.6	3.9	NS
SpO_2 (%)	95.7	1.1	94.9	1.9	NS

Values represent the steady state period of the test; excluding the first 3 minutes at workload. Between group comparisons by Mann-Whitney U test for ordinal and continuous data.

3.3.6.2 Influence of Aerobic Training on Responses to Sub-maximal Constant Load Exercise

3.3.6.3 The percentage change in cardiorespiratory responses to sub-maximal constant load exercise were examined after 4 and 8 weeks training (Figure 3.3). Minute ventilation during constant load cycling was reduced by 20% in the Older group after 8 weeks training (p = 0.002) and reduced by 17% in the Young group (p = 0.01). Heart rate was reduced by 13% in the Older group (p > 0.001) and by 14% in the Young group (p = 0.001) after 8 weeks training. There were significant reductions in RER in the Older (8%, p = 0.002) and Young (9%, p = 0.001) groups after the eight week training period.

3.3.6.4

3.3.6.5 Influence of Exercise Withdrawal on Sub-maximal Constant Load Exercise Testing

After four weeks of exercise training withdrawal there were trends for an increase in minute ventilation and heart rate in both Young and Older groups during the same sub-maximal physiological challenge (Figure 3.3 a-b), however these trends did not reach statistical significance (all p > 0.05). There was no change in RER during sub-maximal testing during the detraining period in either group (both p > 0.05, Figure 3.3 c).

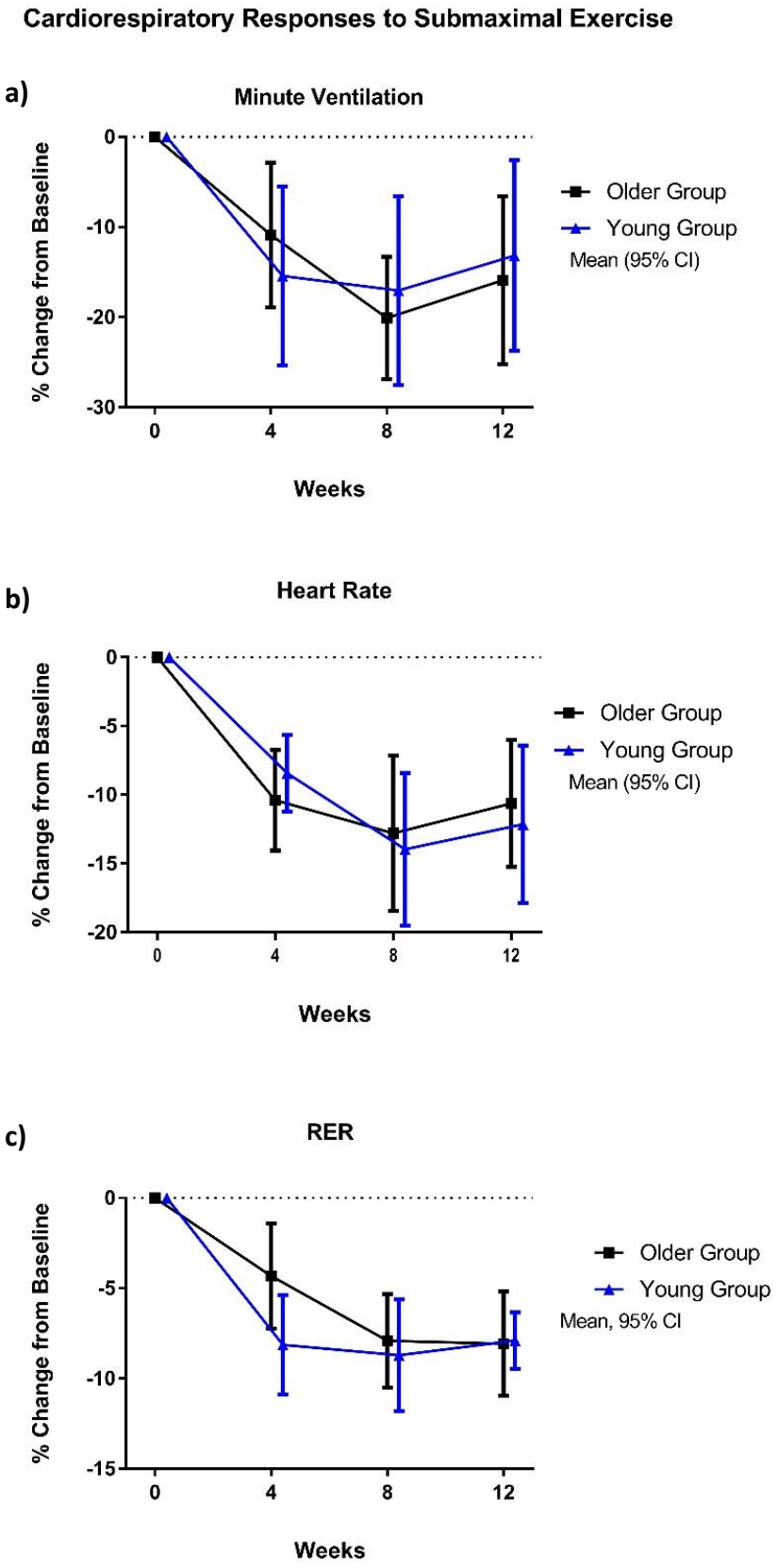


Figure 3-3. Physiological responses to sub-maximal exercise in Young & Older Groups *at the same absolute load with training and detraining*

3.4 Discussion

Older sedentary volunteers had lower maximal aerobic exercise capacity compared to Young volunteers with similar body composition and habitual physical activity levels. Despite this difference at baseline, both Young and Older volunteers experienced clear training adaptations to eight weeks of aerobic exercise training evidenced by increases in $\dot{V}O_2^{PEAK}$ and W^{PEAK} in an incremental cycling test as well as reduced heart rate, ventilation and RER during sub-maximal exercise testing at isowork. Importantly these aerobic training adaptations were of similar relative magnitude in both Young and Older groups. Four weeks of exercise withdrawal prompted a reduction in peak work in both groups however trends towards a partial reversal of cardiorespiratory adaptations were not significant. There was no clear difference in the relative magnitude of any detraining effects between the Young and Older groups.

The relative increase in peak oxygen uptake and peak work output in Older adults was preserved compared to the Young adults in this study. The mechanisms underlying improvements in peak oxygen uptake with aerobic training are known to be increased cardiac output (cardiovascular adaptation) and increased oxygen extraction at the exercising muscle (peripheral adaptation) (Murias, Kowalchuk et al. 2010; Murias, Kowalchuk et al. 2010). For both Young and Older adults, the reduction in HR, RER and VE during submaximal constant load testing in the trained state suggests central and peripheral physiological adaptations to aerobic training. The reduction in heart rate during sub-maximal testing is likely a result of reduced sympathetic activation in the trained state (Carter, Banister et al. 2003) with cardiac output maintained by increased stroke volume (Spina, Ogawa et al. 1993). The reduction in minute ventilation and RER at isowork in the trained state are indirect signs of peripheral adaptation in the trained muscle. Lipid oxidation at a given submaximal workload is increased by endurance training, along with a reduction in carbohydrate oxidation probably as a consequence of an increase in mitochondrial density. Lower carbohydrate oxidation also results in a better matching of glycolytic and pyruvate dehydrogenase complex flux at submaximal exercise workloads, resulting in less lactic acid production compared to the untrained state (Maughan, Gleeson et al. 1997). These adaptations are evidenced by reduced RER. These adaptations also partly contribute to reduced minute ventilation through

decreased CO_2 mediated activation of central mechanisms of respiratory drive and reduced direct activation of type III and IV muscle afferents by hydrogen ions arising from lactic acid production (Amann, Blain et al. 2010). Diminished muscle afferent feedback further reduces stimulation of respiratory drive and also influences the observed reduction in heart rate. The influence of training and detraining on skeletal muscle molecular markers (targeted gene expression) in the Young and Older volunteers from this current study are presented in Chapter 4 giving insight into peripheral adaptations to training.

It has previously been shown in highly trained athletic populations that peak oxygen uptake declines with advancing age (Pollock, 2015) with a suggestion that VO_2^{PEAK} is an objective marker of biological ageing. In the untrained state, sedentary Older volunteers in the current study had lower exercise capacity ($\dot{V}O_2^{PEAK}$ and W^{PEAK}) compared to Young volunteers despite being well matched in their body composition, quadriceps strength, lung function and average daily step count. Factors such as individual genetic differences, inter-generational environmental factors and the primary ageing process itself may all combine to cause the lower aerobic capacity in the Older group at baseline (Holloszy 2000; Booth, Laye et al. 2011), however the data presented here support the contention that deconditioning is a major influence on aerobic capacity at any age. Improvements in VO_2^{PEAK} in Young and Older subjects of 21% and 18% respectively show that in a sedentary population increasing physical activity by implementing an aerobic training programme has substantial benefit on aerobic capacity irrespective of age. This study is unusual in the manner in which Young and Older adults who were well-matched for habitual physical activity at baseline were trained in parallel at carefully matched relative intensities. It supports findings of previous studies of sedentary populations (Houmard, Tyndall et al. 1996; Fujimoto, Prasad et al. 2010; Milanovic, Sporis et al. 2015) and indicates no blunting of the adaptive response to training in Older individuals. In contrast to the work of Pollock et al (2015) which studied highly trained individuals, VO_2^{PEAK} cannot be considered a reliable marker of ageing for the sedentary volunteers studied here as large gains in peak oxygen uptake were achieved by sedentary elderly individuals after a relatively short (eight week) aerobic training intervention therefore undermining the correlation between age and VO_2^{PEAK}. Some previous studies have

shown considerable heterogeneity in the response of healthy individuals to aerobic training (Timmons, Knudsen et al. 2010) with up to 50% of the potential for gains in aerobic capacity with training thought to be determined by genetic inheritance (Bouchard, Daw et al. 1998), however in the current study training adaptations were consistently positive across our sample in both Young and Older subjects with all subjects benefiting from training.

The influence of four weeks exercise training withdrawal on whole-body responses to exercise was not as clear as the effects of training in either group, possibly due to the shorter duration. Neither Young nor Older volunteers experience a significant reduction in $\dot{V}O_2^{PEAK}$ after exercise withdrawal, although a trend was apparent, particularly in the Young group. Physical activity monitoring showed no change in step counts from the baseline values during the first and fourth week of the detraining period, it is therefore unlikely that the training intervention had prompted behavioural adaptations and the adoption of more active lifestyles in the study volunteers.

Short-term detraining (up to 4 weeks) has previously been shown to reverse adaptations to aerobic training (Mujika and Padilla 2000) but the effects seem to be more pronounced in highly (chronically) trained athletes who lose a greater proportion of their $\dot{V}O_2$ max than recently trained (previously sedentary) individuals over a similar timeframe. Studies where a period of exercise withdrawal has followed an aerobic training programme have previously shown that the training induced increase in $\dot{V}O_2$ max (elicited by 12 weeks training) is not reversed within two (Houmard, Tyndall et al. 1996) or three (Moore, Thacker et al. 1987) weeks of training cessation and that benefits of 16 weeks aerobic training are not completely lost during a 10 week detraining period (Sforzo, McManis et al. 1995). This study is novel in its finding that aerobic training adaptations are not fully reversed in Young and Older volunteers when habitual physical activity has been carefully monitored and found to be unchanged during the exercise withdrawal period.

3.4.1 Limitations

Whilst this study has yielded statistically significant and important results it is acknowledged that the sample size (n = 10 per group) is relatively small which limits the

generalisability of results to broader populations. The data presented in this chapter should be viewed in light of the skeletal muscle molecular analysis to follow in Chapter 4. It is also acknowledged that despite training at the same relative intensity, the Older group trained at a significantly lower absolute power than the Young group.

3.4.2 Implications

It has previously been shown that in chronically trained individuals $\dot{V}O_2^{PEAK}$ declines with advancing age with the contention being that $\dot{V}O_2^{PEAK}$ is true marker of primary ageing (Pollock, Carter et al. 2015). Whilst that may be true for highly trained athletic populations, thehe data presented in this chapter show that for previously sedentary individuals aerobic training increases $\dot{V}O_2^{PEAK}$ in older as well as young individuals and to the same relative extent. This suggests that for untrained individuals advanced age should not be seen as a barrier for improving aerobic exercise capacity through training.

Higher whole body aerobic capacity is associated with reduced risk of morbidity and all-cause mortality (Blair, Kampert et al. 1996; Blair and Wei 2000; Kaminsky, Arena et al. 2013). This study shows that sedentary older adults benefit equally from moderate intensity training when compared to sedentary young adults in terms of increased aerobic capacity. Whilst older adults made similar percentage gains in peak oxygen uptake it is worth noting that in absolute terms the gains made were smaller in the older group; an observation which may be attributable to their advanced age, or perhaps more likely the lower absolute training intensity performed by the older group. Similarly, if the training programme's duration was extended both young and older adults would eventually reach a plateau in their aerobic capacity but this is likely to be at a lower absolute value in older compared to younger individuals (Pollock, Carter et al. 2015). There is observational evidence that previously unfit individuals who improve their aerobic fitness reduce their risk of all-cause and cardiovascular disease mortality (Blair, Kohl et al. 1995), therefore it is an important finding that age is not a barrier to improving aerobic capacity with training. There were no significant between group differences in the effects of exercise training withdrawal suggesting that advanced age does not increase susceptibility to physiological detraining. Trends towards a detraining effect with exercise withdrawal cautiously reinforce the message that exercise training should be continued and integrated into an individual's lifestyle, irrespective of their age, in

order to maintain the benefits of training. However, the incomplete reversal of training gains after four weeks of relative inactivity suggest that a short-to-medium term cessation of an exercise routine does not have catastrophic effects on physical fitness. Further work is required to discover the volume of aerobic training that it is necessary to perform long-term to maintain the benefits of a relatively short training intervention.

3.4.3 Conclusion

Sedentary older adults have lower aerobic capacity compared to young sedentary adults who are well matched for habitual physical activity and body composition. Despite this lower aerobic capacity, older adults exhibit no impairment in the response to eight weeks endurance exercise training, making similar gains in aerobic capacity and similar adaptations to the cardiorespiratory responses to sub-maximal constant work rate exercise. Neither young nor older adults were particularly susceptible to the effects of four weeks exercise training withdrawal with neither group showing a significant reversal of cardiorespiratory adaptations. For sedentary adults, improved aerobic fitness is known to be associated with reduced risk of morbidity and mortality; this chapter has shown that advanced age should not be considered a barrier to improving aerobic fitness.

This chapter has described the impact of endurance training and exercise withdrawal on whole-body cardiorespiratory adaptations in young and older adults. In Chapter 4 the investigation will focus on skeletal muscle adaptations to the same intervention at an mRNA and protein level. Subsequent chapters will address the influence of COPD on responses to this intervention in comparison with the older healthy group presented here.

Chapter Four

Influence of age on the responses to aerobic training and subsequent detraining: skeletal muscle molecular responses

4.1 Introduction

Few studies have directly compared the relative magnitude and time-course of the response of young and older adults to an aerobic training programme either at a whole-body level or at a molecular level in skeletal muscle. In Chapter 3, evidence is presented showing that young and older adults matched for habitual physical activity and body composition experience similar relative adaptation in peak oxygen uptake when trained at the same relative exercise intensity. There is some evidence that the time course of central (cardiovascular) and peripheral (skeletal muscle) physiological adaptations to aerobic training may differ between young and older adults (Murias, Kowalchuk et al. 2010; Murias, Kowalchuk et al. 2010) but to date studies have not focussed on the comparative muscle molecular responses to endurance exercise training in terms of the magnitude and time-course of responses or on the relative effects of exercise withdrawal.

There are skeletal muscle mRNA transcripts the abundance of which correlate with age but which are distinct from groups of transcripts whose abundance is influenced by physical exercise in healthy individuals (Phillips, Williams et al. 2013). A comparison of changes in mRNA abundance in skeletal muscle in response to resistance training has previously been reported in old and young volunteers (Raue, Trappe et al. 2012) showing a differential response with an apparent blunting of transcript responses to resistance exercise in the older group. In the skeletal muscle of young, healthy individuals the abundance of a variety of genes is altered following aerobic exercise training (Teran-Garcia, Rankinen et al. 2005; Riedl, Yoshioka et al. 2010; Timmons, Knudsen et al. 2010). It is not known whether older individuals will demonstrate similar or different skeletal muscle mRNA responses measured quantitatively over a wide range of expression values for targeted genes that have previously been shown to respond to aerobic exercise training in young individuals.

In the skeletal muscle of young adults some of the changes in mRNA abundance prompted by aerobic training persist even after a prolonged period of detraining (St-Amand, Yoshioka et al. 2012). In Chapter 3 it is shown that 4 weeks of exercise withdrawal did not prompt significant changes $\dot{V}O_2^{PEAK}$. There have been no studies published describing the influence of exercise withdrawal on muscle molecular biology in the context of ageing.

The translation of mRNAs to their protein products is an important stage in the regulation of skeletal muscle adaptation to training. The relationship between changes in mRNA and protein abundance in response to training and subsequent detraining is not clearly understood, particularly when considering multiple targets, and the influence of advanced age on this relationship is unknown.

This chapter will address the hypothesis that mRNA responses to aerobic exercise training and subsequent withdrawal are similar in young and older sedentary volunteers and will relate to changes in the abundance of protein products. A full examination of the data aims to meet the following objectives:

1. To compare in young compared to older sedentary volunteers, whether the abundance of targeted mRNA transcripts with functions linked to aerobic training differs in the untrained state.
2. To examine whether the magnitude and time-course of change in mRNA abundance in response to an eight week aerobic training intervention and a subsequent four-week exercise withdrawal period differs between young and older volunteers.
3. Focussing on those genes found to have the largest magnitude of change in mRNA abundance, to investigate any measurable changes in the abundance of their protein products in response to aerobic training and exercise withdrawal.

In order to meet these objectives, this chapter describes data from an analysis of mRNA and protein abundance in tissue sampled from the *vastus lateralis* of young and older volunteers over the course of eight weeks of aerobic cycling training and subsequent four week exercise withdrawal period.

4.2 Methods

4.2.1 Participants

Data reported in this chapter arise from analysis of quadriceps muscle biopsy samples obtained from the Young and Older subjects whose characteristics are presented in Chapter 3.3.

4.2.2 Study Design

An overview of the protocol can be seen in Chapter 2.4.1. Skeletal muscle biopsies were performed at baseline; after 1, 4 and 8 weeks of training; and after 4 weeks exercise withdrawal. Biopsies were performed on the *vastus lateralis* muscle of the dominant leg in the resting state (24 hours after exercise during training period in order to avoid any confounding influence of acute exercise) and following a fast of $\geq$ 4 hours (see Chapter 2).

4.2.3 Measures

Abundance of mRNA was assessed using a fully quantitative RT-PCR technique (Chapter 2.10). Targets for gene expression analysis were selected using data generated within the research group for mRNA abundance changes (targeted RT-PCR and Affymetrix gene chips) observed in quadriceps muscle biopsy samples from healthy young and older volunteers in response to a single bout of acute exercise and several weeks of limb immobilisation (data unpublished). In addition, searches of SA Biosciences and IPA databases were performed. The list of 96 mRNA transcripts quantified (including 2 housekeeping genes) can be found in Appendix 6.

Protein targets were chosen by ranking gene transcripts in order of magnitude of fold-change from baseline (up- or down-regulation) and selecting those with the largest fold-change. Data from all time points in the full MATCH Study dataset, including data from a group of patients with COPD were used to select protein targets. One protein target (IL6) was selected based on mRNA expression in the COPD group, even though mRNA expression was below the lower limit of detection in Young and Older groups at all but one time point. Antibody optimisation and Western blot analysis were performed by an experienced post-doctoral fellow in the research team with all calculations and data analysis performed myself. Nine separate protein targets, plus two phosphorylated

isoforms were quantified. The Western blot protocol and antibody specifications can be found in Appendix 7.

4.2.4 Data Analysis

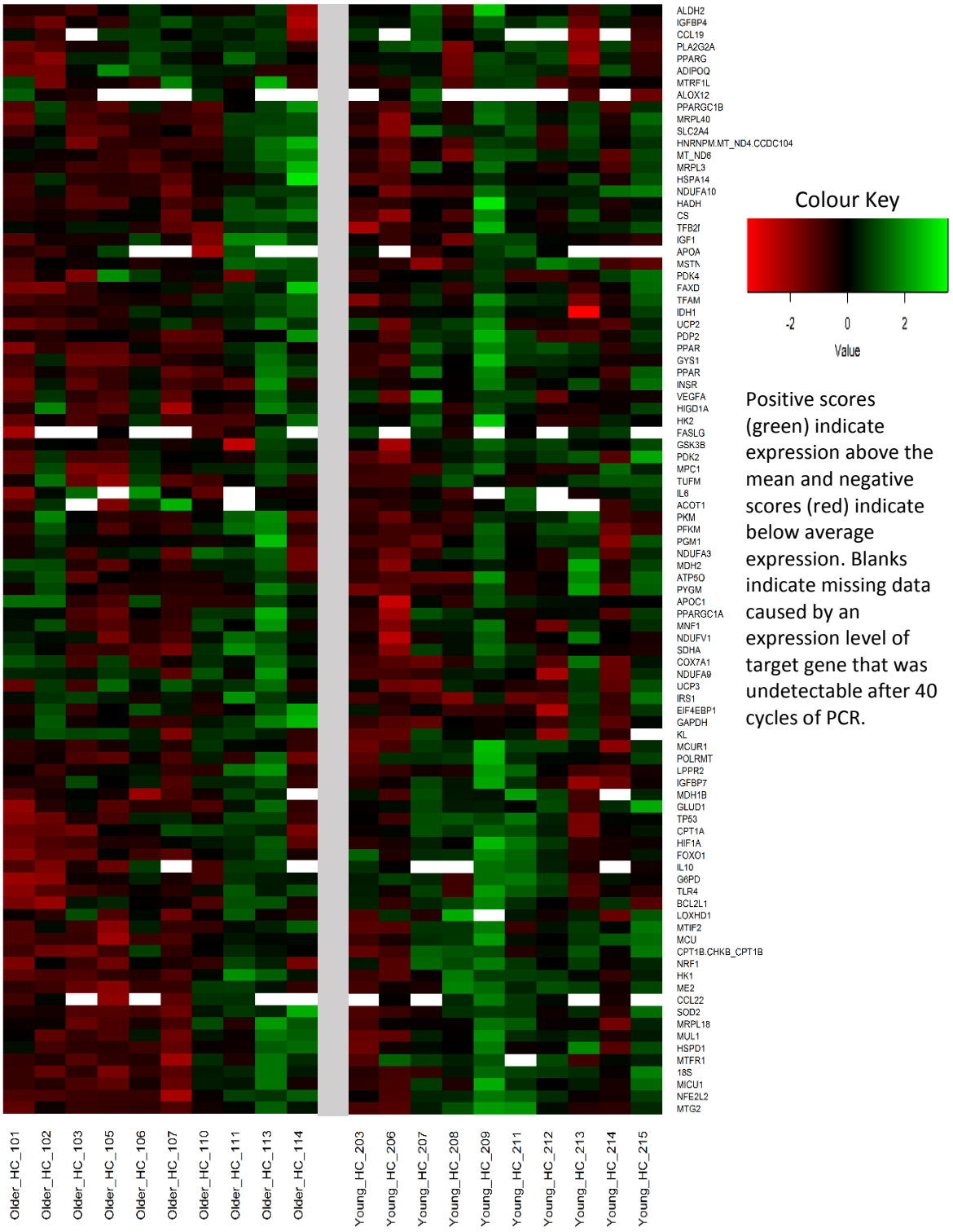
Baseline mRNA abundance was compared between Young and Older groups both qualitatively using heatmaps and quantitatively using unpaired t-tests on Δ_{CT} values with the FDR adjustment applied to control for multiple comparisons. Change in mRNA abundance over time is expressed as fold-change from baseline calculated using the $\Delta\Delta_{CT}$ method (Livak and Schmittgen 2001). Bioinformatics analysis was performed using Ingenuity Pathway Analysis (IPA®; QIAGEN Redwood City, USA, www.qiagen.com/ingenuity) in order to identify networks of genes that represent discreet biological functions. Protein abundance at baseline is corrected for abundance of the housekeeping protein (actin for cytoplasmic or lamin for nuclear proteins) and reported as arbitrary units. Change in protein abundance with time is expressed as fold change from baseline with statistical tests performed on absolute values (arbitrary units). In order to reduce the number of statistical comparisons, protein fold-change data were analysed at baseline, eight weeks (i.e. pre- and post-training) and twelve weeks (after exercise withdrawal) only.

4.3 Results

4.3.1 mRNA Abundance

4.3.1.1 Baseline

The baseline abundance of mRNA transcripts for each subject is shown in Figure 4.1. There is considerable variability in the abundance of transcripts between individuals. Qualitatively, there appears to be a trend for lower abundance of transcripts in the Older compared to the Young group (indicated by red hue). Some genes were not expressed or were expressed at a level below the lower limit of detection in more than 30% of subjects at baseline including, ALOX12, APOA1, and CCL19, CCL22, FASLG, IGF2BP1, PLD4 and UCP1. Quantitative comparison of Δ_{CT} scores did not reveal statistically significant differences between groups for any of the mRNA targets (all $p > 0.05$, see Appendix 9 for results).



Positive scores (green) indicate expression above the mean and negative scores (red) indicate below average expression. Blanks indicate missing data caused by an expression level of target gene that was undetectable after 40 cycles of PCR.

Figure 4-1. mRNA abundance in vastus lateralis tissue from Young & Older groups. Values on the colour key indicate ΔC_T values (normalised to HMBS housekeeping gene) transformed to z-scores (i.e. number of standard deviations above or below the pooled group mean). X axis displays individual mRNA transcripts, Y axis displays individual subjects arranged by group.

4.3.1.2 Influence of Exercise Training and Exercise Withdrawal on mRNA Abundance

Change in mRNA abundance from baseline in quadriceps samples collected at 1, 4 and 8 weeks into the training intervention and after four weeks of exercise withdrawal (12 weeks) were calculated. Table 4.1 shows how many of the 94 target transcripts were up- or down-regulated in each group at each time point relative to the baseline sample. Not all genes were expressed in ≥70% of subjects (the threshold for data inclusion) at each time point. Change in mRNA abundance from baseline is shown in Figures 4.2 to 4.5. Red arrows highlight the transcripts which were subsequently quantified at the protein level.

Table 4-1. Number of transcripts up- or down-regulated throughout training and exercise withdrawal, relative to baseline in Young & Older groups

		Number of transcripts			
		Total up-regulated	Total down-regulated	> 2 fold up-regulated	< 0.5 fold down-regulated
Older Group	1 week training	46	37	14	3
	4 weeks training	47	38	6	1
	8 weeks training	52	34	15	0
	4 week exercise withdrawal	52	34	7	0
Young Group	1 week training	60	22	12	3
	4 weeks training	36	48	6	2
	8 weeks training	49	36	4	1
	4 week exercise withdrawal	58	26	12	0

Number of transcripts for which the group median indicates up-regulation ($2^{-\Delta\Delta CT} > 1$) or down-regulation ($2^{-\Delta\Delta CT} < 1$) at that time point relative to the baseline sample. Up-regulation of > 2 fold or < 0.5 fold change indicate the number of transcripts which more than doubled or halved in abundance. N.B. Some transcripts were not expressed at all time points.

In general terms, transcripts that were up-regulated at 1 week ($2^{-\Delta\Delta CT} > 1$) remained elevated relative to the baseline sample at all subsequent training time points and also after 4 weeks of exercise withdrawal. The magnitude of fold change in mRNA abundance for down-regulated transcripts ($2^{-\Delta\Delta CT} < 1$) qualitatively appears smaller than the magnitude of up-regulation in transcripts that increased in abundance. Transcripts with reduced abundance at 1 week tended to remain reduced relative to baseline throughout training and after the exercise withdrawal period, though again this effect is less pronounced than with up-regulated transcripts.

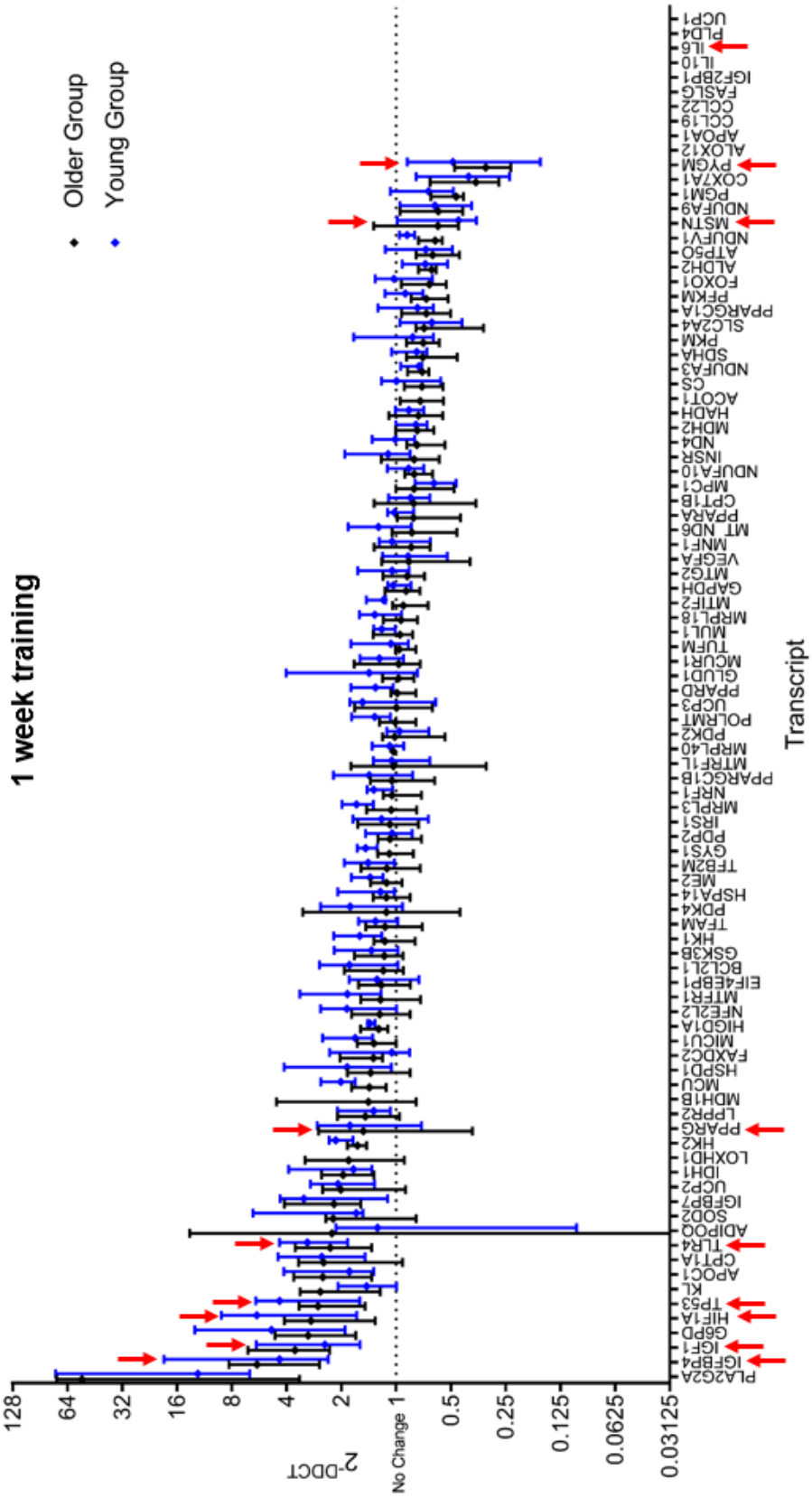


Figure 4-2. Change in mRNA abundance in Young & Older groups after 1 week aerobic training relative to baseline. Data are median, IQR. Targets with missing data were below the lower limit of detection. Arrows highlight transcripts whose protein products were subsequently quantified by Western blotting.

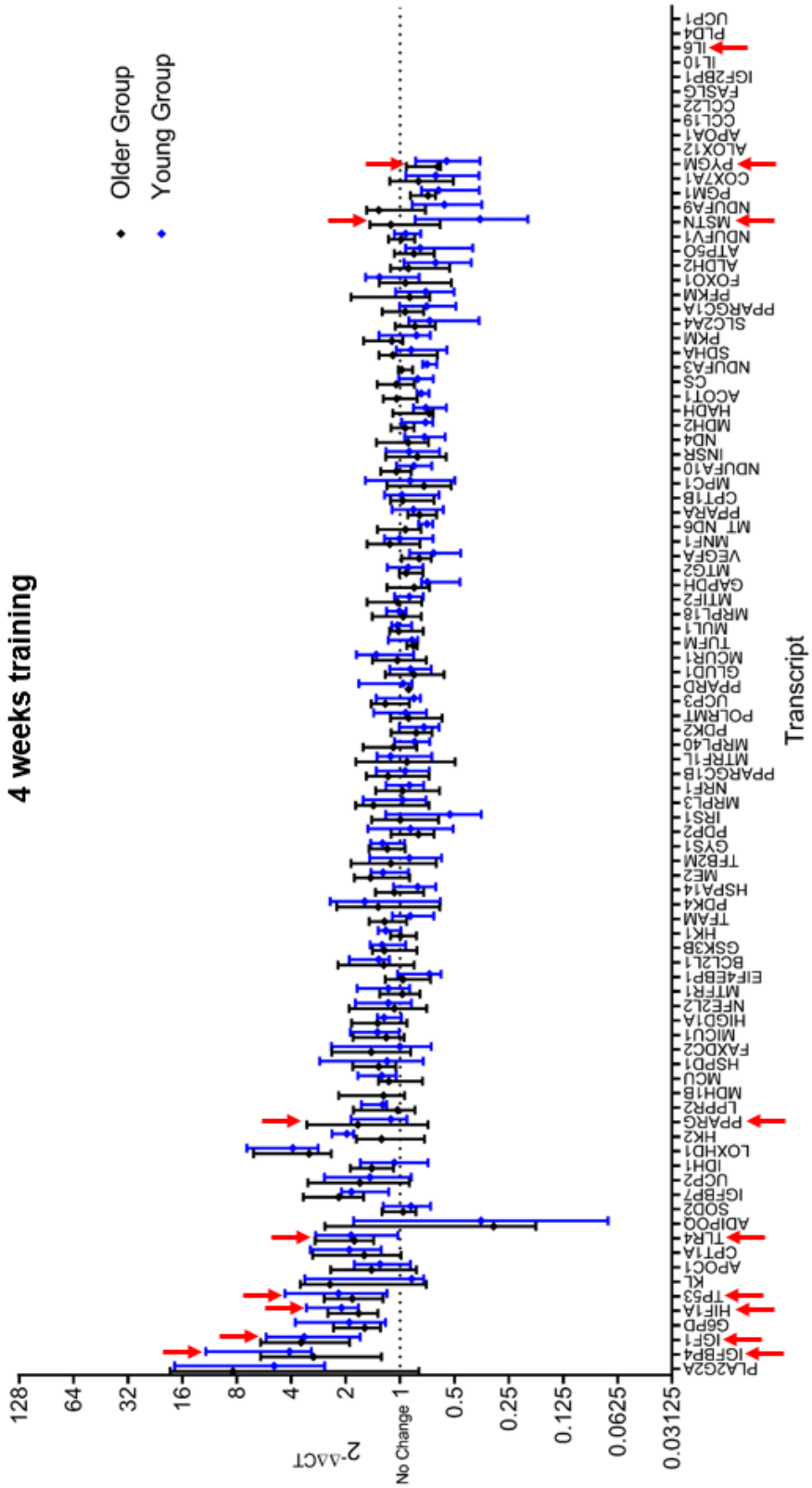


Figure 4-3. Change in mRNA abundance in Young & Older groups after 4 weeks aerobic training relative to baseline. Data are median, IQR. Targets with missing data were below the lower limit of detection. Arrows highlight transcripts whose protein products were subsequently quantified by Western blotting.

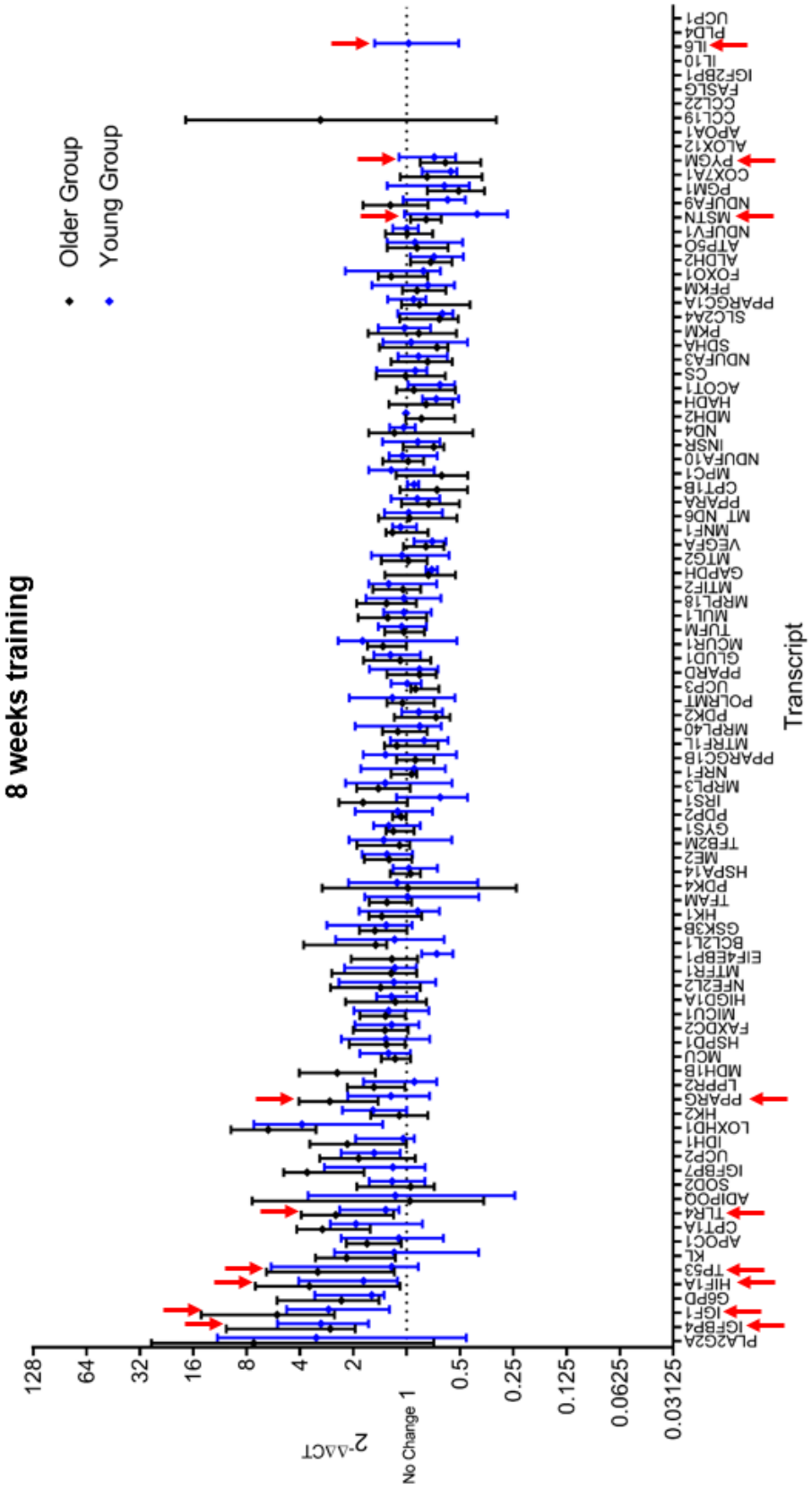


Figure 4-4. Change in mRNA abundance in Young & Older groups after 8 weeks aerobic training relative to baseline. Data are median, IQR. Targets with missing data were below the lower limit of detection. Arrows highlight transcripts whose protein products were subsequently quantified by Western blotting.

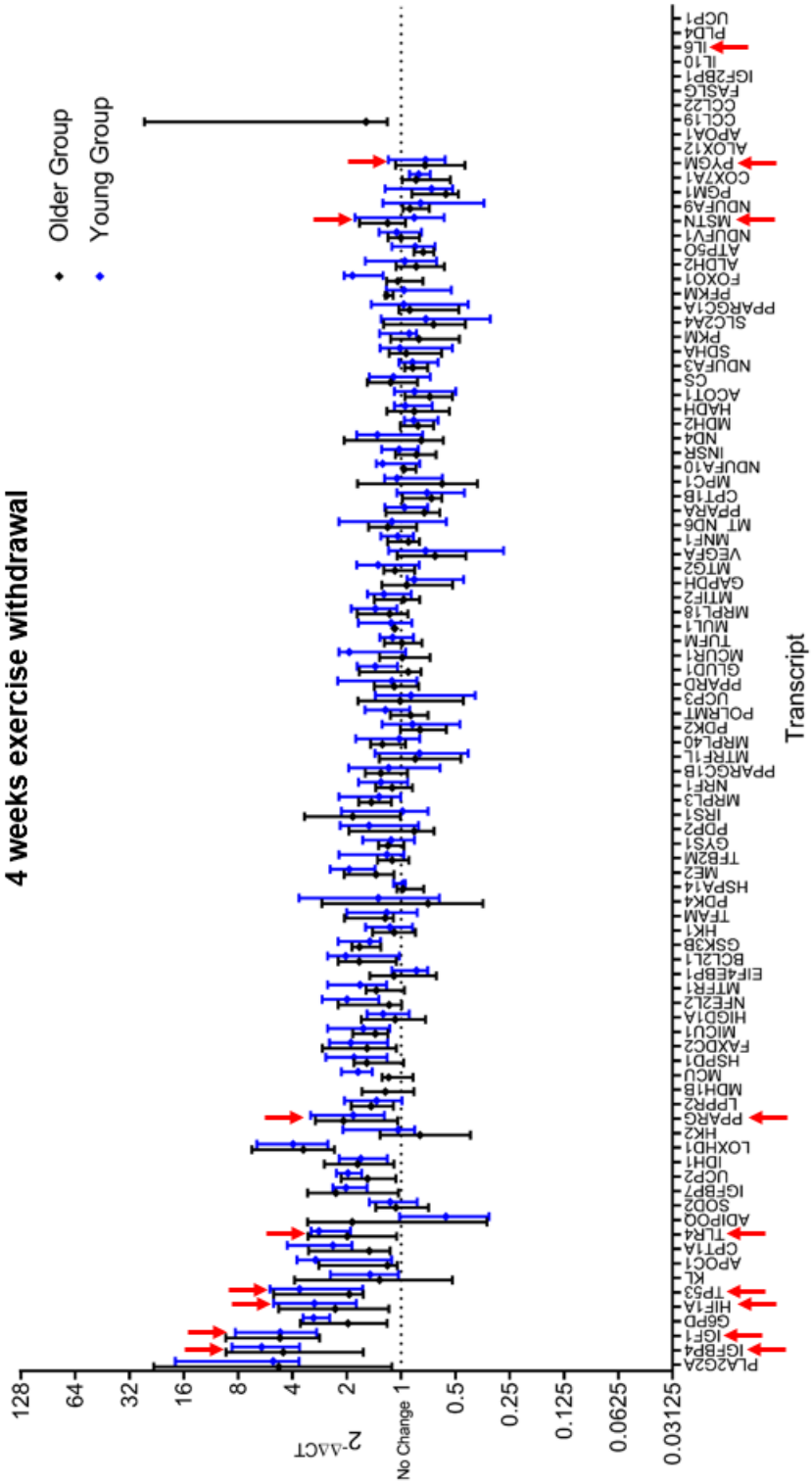


Figure 4-5. Change in mRNA abundance in Young & Older groups after 4 weeks exercise withdrawal (subsequent to 8 weeks aerobic training) relative to baseline. Data are median, IQR. Targets with missing data were below the lower limit of detection. Arrows highlight transcripts whose protein products were subsequently quantified by Western blotting.

Interrogation of mRNA abundance data relative to the baseline biopsy using IPA revealed that the same eight biological functions were significantly influenced in both groups after one, four and eight week's training and after exercise withdrawal. Functional networks were: *Energy Production*; *Lipid Metabolism*; *Connective Tissue Development and Function*; *Free Radical Scavenging*; *Carbohydrate Metabolism*; *Skeletal and Muscular System Development and Function*; *Skeletal and Muscular Disorders*; and *Inflammatory Response* (Figure 4.6). The probability of each function being significantly influenced at each time point is shown in Figure 4.6 a (Older Group) and 4.6 b (Young Group). All eight functions reach a high level of significance (well above the threshold for a significant result) at all time points indicating a strong likelihood that the biological function was influenced by changes in gene expression. The threshold of statistical significance is a -log(p-value) of 1.3 which is equivalent to $p = 0.05$.

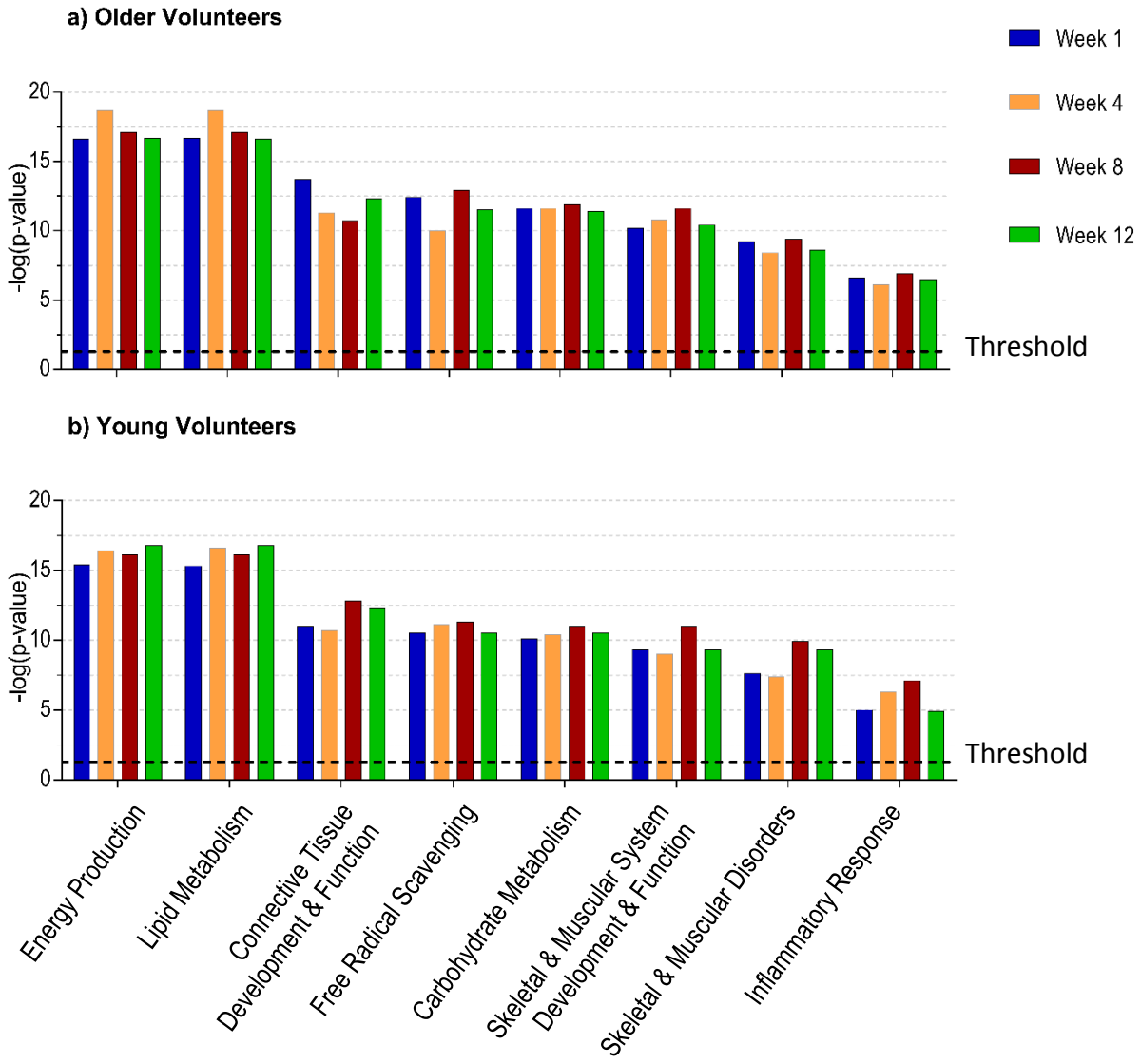


Figure 4-6. Muscle biological functions significantly altered from baseline by exercise over the course of an 8 week endurance cycling training intervention and subsequent 4 week detraining period (week 12) as identified by IPA in a) Older and b) Young Volunteers. Y axis indicates the probability that a biological function is significantly altered from baseline. Dashed line denotes threshold of statistical significance (-log(p-value) of 1.3 is equivalent to p = 0.05).

4.3.2 Protein Expression

4.3.2.1 Baseline Protein Expression

Protein expression at baseline corrected for abundance of the housekeeping protein and reported as arbitrary units is presented in Table 4.2. Abundance of IGF1 was significantly greater in the Young compared to the Older group (p = 0.015) at baseline in the rested, fasted *vastus lateralis*.

Table 4-2. Baseline protein expression in resting, fasted vastus lateralis tissue in Young & Older groups.

Protein	Older Group		Young Group		*p*
	Mean	SD	Mean	SD	
HIF1 α	0.01	0.01	0.04	0.04	NS
IGF1	0.05	0.04	0.22	0.28	0.015
p53	0.05	0.04	0.06	0.08	NS
IGFBP4	1.59	1.17	0.70	0.61	NS
(p)IGF1	0.15	0.14	0.33	0.30	NS
(p)p53	0.10	0.11	0.07	0.06	NS
IL6	0.22	0.19	0.25	0.32	NS
TLR4	0.09	0.11	0.16	0.17	NS
PPARγ	0.28	0.38	0.18	0.11	NS
PYGM	1.51	1.21	0.55	0.32	NS
MSTN *	2.28	1.23	2.29	2.91	NS

Protein content normalised for actin / lamin. Arbitrary units: Optical Density (OD) target protein / OD actin. HIF1 α, hypoxia inducible factor 1 α; IGF1, insulin-like growth factor 1; p53, (tumour) protein 53; (p)p53, phosphorylated p53; IGFBP4, insulin-like growth factor binding protein 4; (p)IGF1, phosphorylated IGF1 receptor; IL6, interleukin 6; TLR4, toll-like receptor 4; PPARγ, peroxisome proliferator activated receptor γ; PYGM, glycogen phosphorylase (muscle); MSTN, myostatin. *MSTN normalised to lamin. Between group differences tested with Mann-Whitney U test. NS, $p > 0.05$.

4.3.2.2 Influence of Exercise Training and Exercise Withdrawal on Protein Expression

There was a small but statistically significant ($p < 0.05$) decrease from baseline in the abundance of phosphorylated-IGF1 after eight weeks training in the Young Group, and this change was significantly different (Figure 4.7, e; $p < 0.05$) to the response in the

Older group (who demonstrated a non-significant increase in mean abundance). There was a significant increase in PPARγ protein expression with exercise withdrawal in the Young group ($p < 0.05$) with abundance increasing from the post-training value in all subjects (Figure 4.7, h). There was a small but significant reduction in the abundance of myostatin protein in the Older Group in response to training (Figure 4.7, k; $p < 0.05$), which was not seen in the Young group.

In order to increase statistical power, change in protein abundance was re-analysed with Young and Older groups collapsed. In the collapsed groups, there was a significant increase in IGF-1 after eight weeks of training followed by a significant decrease in the exercise withdrawal period; p53 increased during the training period; and PPARγ increased during the exercise withdrawal period (all $p < 0.05$). The individual responses of protein expression to training and exercise withdrawal expressed as fold-change values were highly variable (Figure 4.7). To determine whether individual variability in fold-change data were influenced by the individual baseline starting value, protein response graphs were also plotted using absolute values (arbitrary units) at each time point (Appendix 10). This technique did not reduce the variability in the data.

In an attempt to identify the source of the inter-subject variability in protein expression, groups were dichotomised into sub-groups who had experienced either up- or down-regulation of a given protein at any time point. These sub-groups were then compared with the aim of identifying defining characteristics. The dichotomised groups were compared for age and BMI; groups with up- or down-regulation of proteins during exercise training were compared for baseline $\dot{V}O_2^{PEAK}$ and Peak Work; training intensity and metabolic stress during training (training intensity, W; baseline sub-maximal test RER; HR; and $\dot{V}E$ %MVV) as well as $\Delta\dot{V}O_2^{PEAK}$; groups defined by up- or down-regulation of proteins during exercise withdrawal were contrasted for $\Delta\dot{V}O_2^{PEAK}$ during that period. There were no differences between sub-groups when Young and Older groups were analysed separately, nor did any defining characteristics emerge when the age groups were collapsed into one to increase statistical power (all tests $p > 0.05$).

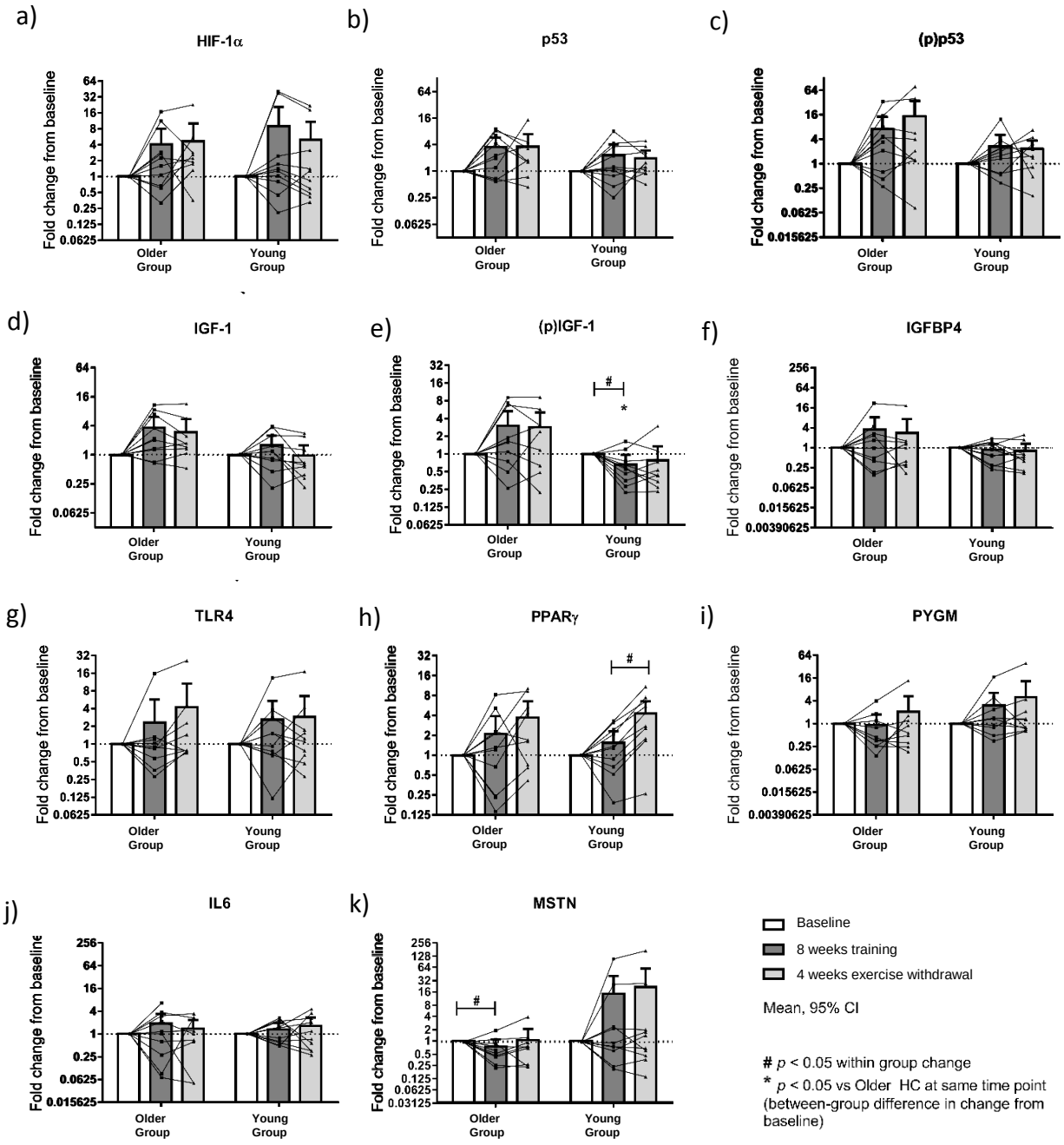


Figure 4-7. Change in muscle protein expression with exercise training and exercise withdrawal in Young & Older groups. HIF-1α, hypoxia inducible factor 1 α; p53, (tumour) protein 53; (p)p53, phosphorylated p53; IGF1, insulin-like growth factor 1; (p)IGF1, phosphorylated IGF1 receptor; IGFBP4, insulin-like growth factor binding protein 4; TLR4, toll-like receptor 4; PPARγ, peroxisome proliferator activated receptor γ; PYGM, glycogen phosphorylase (muscle); IL6, interleukin 6; MSTN, myostatin. Bars represent group mean (95% CI), lines represent individual subject responses.

4.3.3 Gene – Protein Associations

Scatter plots were drawn and correlation analysis performed to investigate the relationship between change in gene expression and change in protein abundance.

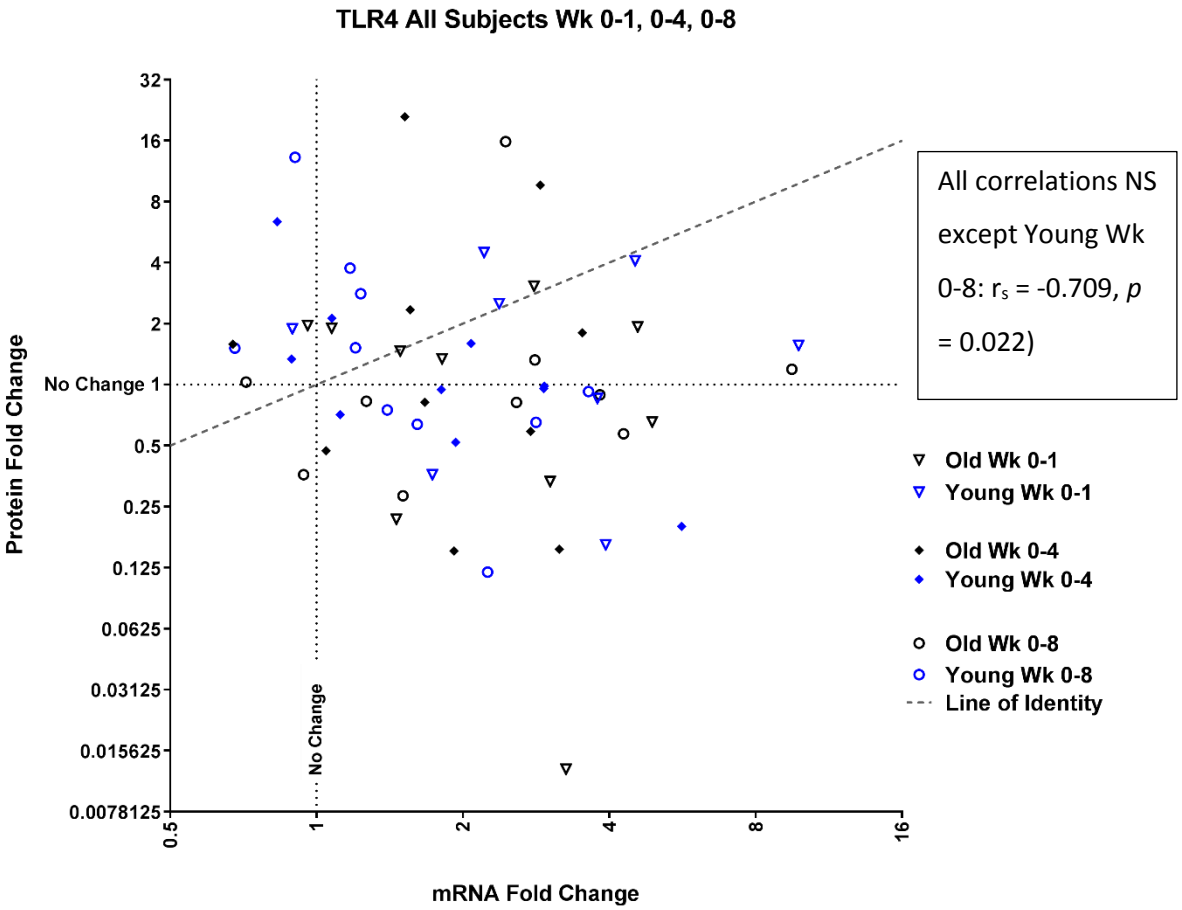


Figure 4-8. Muscle mRNA and protein expression for TLR4, toll-like receptor 4 in Young & Older groups.

For the majority of targets there was no correlation between the change in mRNA abundance and the change in protein abundance at any training time point or during exercise withdrawal (p > 0.05). Change in TLR4 mRNA abundance was negatively correlated with change in TLR4 protein abundance during 8 weeks training in the Young group (r_s = -0.709, p = 0.022; blue open circles, Figure 4.8). There was a negative correlation between mRNA and protein fold change of p53 during the exercise withdrawal period in the Young group (r_s = -0.758, p = 0.011, Appendix 11).

When Young and Older groups were collapsed, IL6 mRNA and protein fold-change values were negatively correlated (Appendix 11) during the exercise withdrawal period (r_s = 0.518, p = 0.048).

4.4 Discussion

For Young and Older adults, there were striking similarities in the magnitude and time course of changes in quadriceps mRNA abundance and the biological functions predicted to be influenced in response to an aerobic training programme. Change in $\dot{V}O_2^{PEAK}$ with aerobic training was also the same (Chapter 3), suggesting that change in abundance of targeted mRNA transcripts is a good predictor of whole-body adaptation to training. Biological functions of genes influenced by training relating to fuel selection (e.g. *Energy Production, Lipid Metabolism*) relate to the lower reliance on carbohydrate metabolism in the trained state evidenced by reduced RER at isowork in the trained state (Chapter 3). In parallel with whole-body physiological measures ($\dot{V}O_2^{PEAK}$) the influence on mRNA abundance and biological functions prompted by training was undiminished by a period of exercise withdrawal.

Protein quantification revealed a decrease in p-IGF1 abundance in the Young group and lower myostatin abundance in the Older group after the training period. During the exercise withdrawal period PPARγ increased in abundance in the Young group. Pooling of protein expression data from the two groups revealed an increase in IGF-1 during training with a subsequent decrease during exercise withdrawal; p53 increased with training; and PPARγ increased during exercise withdrawal.

4.4.1 mRNA responses to training

The qualitative assessment of mRNA abundance in the Young relative to the Older groups at baseline (Figure 4.1) shows substantial variation between individuals in both groups. No individual transcripts were significantly over-expressed in one group compared to the other at baseline although the study was likely underpowered for this type of analysis (comparison of 94 genes with n = 10 per group). Qualitative assessment of heat maps however suggests that there may be a somewhat lower abundance of the target transcripts in the Older group compared to the Young group (indicated by a red

hue in Figure 4.1). Older volunteers had a significantly lower exercise capacity ($\dot{V}O_2^{PEAK}$) than Young volunteers at baseline however the difference is not pronounced and this study was not designed to tease out small differences in mRNA abundance at baseline. Given that further analyses are based on relative change in abundance from baseline any subtle differences between the groups in mRNA abundance at baseline is unlikely to be influential.

The abundance of mRNA transcripts in quadriceps muscle and the impact of acute and chronic resistance exercise has previously been studied with genome-wide microarray data four hours after a bout of resistance exercise (RE; Raue, Trappe et al. 2012) and muscle specific microarray data on resting samples before and after a RE training programme (Jozsi, Dupont-Versteegden et al. 2000; Melov, Tarnopolsky et al. 2007) showing different patterns of change in mRNA abundance in old and young with fewer transcripts influenced in elderly compared to young volunteers. The relative responses of muscle gene expression to an aerobic training intervention in older and young individuals had not previously been reported; this is the first study to carefully examine quantitative time-course muscle mRNA responses in young and older volunteers to aerobic training and subsequent exercise withdrawal. The data in this chapter suggest that for Young and Older volunteers there is no difference in the muscle mRNA response to an aerobic training intervention for genes previously found to be influenced by endurance training in young volunteers, nor does a difference emerge after a period of exercise withdrawal. Biological functions of genes influenced by aerobic training were the same in the Young and Older groups despite differences in absolute exercise training intensity (110 W vs 76 W respectively) suggesting that either relative training intensity (which was matched) determines the change in abundance of these gene transcripts or that the lower absolute training intensity of the Older group was sufficient to stimulate the same molecular response as the Young group. There is evidence from an acute study of resistance exercise in young and older volunteers that the magnitude of muscle molecular responses (in this case muscle protein synthesis) are related to the relative intensity of the exercise bout (Kumar, Selby et al. 2009), adding weight to the contention that it is the relative intensity that is important, however in the resistance exercise study muscle protein synthesis was higher in young compared to older volunteers across a

range of intensities whereas the mRNA response to aerobic exercise is similar in this study.

The characteristics of the gene expression response to training in Young and Older groups relate closely to physiological adaptations observed at a whole-body level in Chapter 3. *Lipid Metabolism* was one of the most significantly influenced functions identified by IPA analysis (Figure 4.6). Both Young and Older groups displayed an increased contribution of fat oxidation to energy production during steady-state sub-maximal exercise in the trained state compared to baseline, evidenced by a reduction in RER at iso-work (Chapter 3, Figure 3.3), an observation that is considered to be a hallmark of endurance training (van Loon, Jeukendrup et al. 1999). A mechanism for this change in fuel utilisation is likely to be altered mitochondrial fuel selection after endurance training which has been observed before in healthy populations (Wibom, Hultman et al. 1992) and distinguishes trained from untrained individuals (Jong-Yeon, Hickner et al. 2002). There is further evidence from this study to support that contention; as part of the broader MATCH aerobic training study, an *ex vivo* analysis of quadriceps maximal mitochondrial ATP production rate in response to a variety of substrates was performed using the method described by Wibom *et al* (Wibom and Hultman 1990; Wibom, Hultman et al. 1992). This mitochondrial analysis was performed by colleagues, however some of the data are of relevance to this work with reference to lipid oxidation. When maximally stimulated with ADP and the substrate palmitoyl-CoA (activated form of palmitate fatty acid) the maximal ATP production rate increased significantly in Young and Older Healthy volunteers with aerobic training before a significant decrease during the exercise withdrawal period (Figure 4.9). This mitochondrial adaptation mirrors the whole-body evidence for enhanced fat metabolism during sub-maximal exercise after the training period (reduced RER) and corresponds with the modification of the *Lipid Metabolism* functional network identified from this gene expression analysis during the eight week aerobic training period. The *Lipid Metabolism* function remains significantly influenced during the exercise withdrawal period whereas there is a decline in maximal ATP production rate utilising palmitoyl-CoA indicating that additional factors independent of mRNA abundance are required to influence mitochondrial fuel selection. It is likely that the discrepancy

between maximal mitochondrial ATP production rate (which was measured on isolated mitochondria, *ex vivo*) and mRNA abundance changes during the exercise withdrawal period is influenced by one or a combination of other factors known to impact mitochondrial fuel selection *in vivo* such as intramuscular lipid content, intramyocellular location of lipid droplets and mobilisation of adipose and muscle lipid stores (Chee, Shannon et al. 2016).

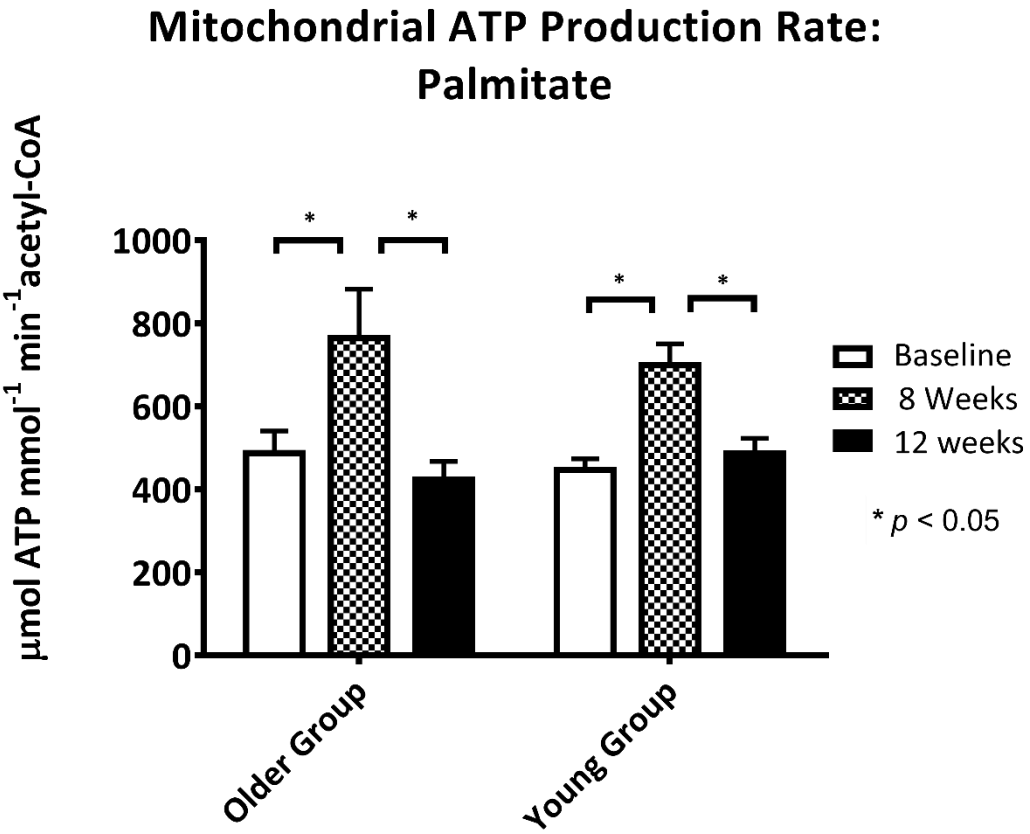


Figure 4-9. Maximal mitochondrial ATP production rate (reproduced from unpublished work by B Popat) in Young & Older groups

The *Energy Production* function closely mirrors the response of *Lipid Metabolism* in both groups of volunteers and *Carbohydrate Metabolism* was also highly significantly modulated in Young and Older volunteers by training. The influence on these three biological functions from as early as one week into the training period demonstrates that change in mRNA abundance is matching, and indeed preceding the adaptations in

fuel metabolism that occur in the quadriceps of healthy volunteers in response to training.

The influence of training on the biological functions *Connective Tissue Development and Function* and *Skeletal and Muscular System Development and Function* and *Skeletal and Muscular Disorders* sign-post towards structural adaptations in muscle tissue. In this study, only a gross measure of muscle structure (lean mass by DEXA) was made and did not change with training in either group. *Skeletal and Muscular System Development and Function* has previously been identified by St-Amand *et al* as the top ranked network identified by IPA for both aerobic training and detraining in young healthy, previously untrained males (St-Amand, Yoshioka et al. 2012). This network also appears in the top eight biological functions in the MATCH study for both training and detraining despite the targeted selection of 94 genes compared to the global mRNA analysis approach used by St-Amand *et al*.

During exercise there may be a transient increase in free radical production in muscle fibres caused by leak from the respiratory chain during mitochondrial respiration. The influence on the function *Free Radical Scavenging* at one week and throughout the intervention and exercise withdrawal period may have been triggered by enhanced mitochondrial respiratory chain activity in previously sedentary populations. Both young and older trained individuals have greater capacity for managing oxidative stress post-exercise compared to sedentary controls (Radak, Bori et al. 2011), so the *Free Radical Scavenging* function may be related to an adaptive process for increasing anti-oxidant capacity. Similarly, part of the normal response, recovery and adaptation from exercise is an intramuscular inflammatory reaction (Chazaud 2016; Rowlands, Nelson et al. 2016). The influence on the *Inflammatory Response* function may be a reaction to this phenomenon initiated by the training intervention.

4.4.2 mRNA responses to exercise withdrawal

It was apparent in both groups that the influence of 8 weeks aerobic training on mRNA abundance persisted throughout the exercise withdrawal period (Figures 4.5 & 4.6). This phenomenon has previously been observed by others: St-Amand *et al* (St-Amand, Yoshioka et al. 2012) found that some genes influenced by aerobic training had not

returned to baseline values even after 12 weeks detraining. Genes that were still changed in expression after detraining in the St-Amand study code for sub-units of NADH dehydrogenase, ATP synthase, cytochrome c oxidase and ribosomal proteins. Genes coding for similar products were also measured in this study, however none formed part of the eight networks of biological functions that have been identified in this chapter for either Young or Older volunteers. In line with the stability of mRNA abundance, there were no significant detraining effects in either Young or Older volunteers for whole-body physiological measures (e.g. $\dot{V}O_2^{PEAK}$ and RER at isowork; see Chapter 3) during the four-week exercise withdrawal period. RER during submaximal isowork exercise represents physiological function at the level of the muscle (fuel selection) and the stability of RER aligns with the stability of mRNA abundance during the exercise withdrawal period suggesting that mRNA abundance predicts muscle function in the context of reduced contractile activity.

4.4.3 Targeted protein analysis

As part of the IGF-Akt-mTOR pathway, IGF1 is a positive regulator of skeletal muscle mass and its abundance was significantly higher in the baseline samples of the Young compared to the Older groups in this study. Reduced activity of IGF1 and growth hormone has previously been described in the elderly (Heinemeier, Mackey et al. 2012) but links to reduced muscle mass are not completely clear. In this study there was no difference between Young and Older groups for gross measures of muscle mass at baseline (DEXA; Chapter 3), nor was there a difference in the abundance of activated (p)IGF1 (Table 4.2). After 8 weeks training (p)IGF1 significantly decreased in the Young group which was a significantly different effect to that observed in the Older group who trended towards increased abundance. Pooled data from the Young and Older groups showed a significant decrease in abundance of IGF1 during four weeks of exercise withdrawal. After the aerobic training period the Older group exhibited a significant decrease in abundance of another regulator of muscle mass: myostatin. Similar effects have previously been reported at an mRNA level where myostatin abundance was reduced during rehabilitation employing dynamic exercise after limb immobilisation (Jones, Hill et al. 2004) and at a protein level following an aerobic training programme

in middle-aged men where reduced abundance was associated with improved insulin sensitivity (Hittel, Axelson et al. 2010). In this context reduced myostatin abundance may be involved in regulation of fuel selection. Differences in abundance of IGF1, its activated form (p)IGF1 or of myostatin were not related to muscle mass which was unaltered in both groups across the training and exercise withdrawal periods (Chapter 3). Although not measured in this study it would be of interest to know whether more detailed measures of muscle structure (e.g. fibre cross sectional area) were influenced by exercise training or withdrawal in these groups.

During the exercise withdrawal period (but not during the training period itself) PPARγ protein levels increased in the Young group. PPARγ is a transcription factor with downstream effects including sensitising skeletal muscle to the action of insulin. While there was considerable inter-subject variability in protein abundance for most targets, all 10 Young volunteers in this study demonstrated an increase in PPARγ abundance during exercise withdrawal, perhaps signposting towards alterations in glucose metabolism. Combined data from collapsed groups showed an increase in the abundance of p53 during the training period. p53 plays a role in regulating mitochondrial biogenesis so again, so this observation may relate to adaptations in fuel selection within the skeletal muscle, although the activated (p)p53 protein levels were unchanged in either group. These observations at a protein level combined with the impact of aerobic training on the *Energy Production*, *Lipid Metabolism* and *Carbohydrate Metabolism* biological functions from the mRNA analysis are suggestive of mechanisms relating to altered fuel selection in trained muscle in both Young and Older groups.

4.4.4 Relationship between mRNA and protein abundance

There were correlations between the change in mRNA abundance and the change in the corresponding protein product for two of the nine targets measured. Change in abundance of TLR4 mRNA had a negative association with change in TLR4 protein during the training intervention period and there was a negative correlation between the change in p53 mRNA and protein abundance during the exercise withdrawal period. This result suggests that increased abundance of mRNA is associated with decreased abundance of its protein product – an observation that may initially seem counterintuitive. The disconnect between mRNA and protein abundance is not out of

keeping with the results of other studies, as it is acknowledged that change in transcription is not always mirrored by change in translation (Gygi, Rochon et al. 1999; Griffin, Gygi et al. 2002; Maier, Güell et al. 2009) and that the abundance of protein measured by Western Blotting is not tightly linked to its physiological function in vivo (i.e. the activity of a molecule is important as well as its abundance). Indeed the balance of protein synthesis is in a constant state of flux within cells and protein abundance, signalling molecule phosphorylation status and mRNA abundance can sometimes appear disconnected (Greenhaff, Karagounis et al. 2008). It is also plausible that samples taken 24 h post-exercise during the intervention period were well timed for identifying change in mRNA, but not protein abundance as the time course of change in mRNA (Bickel, Slade et al. 2005; Mahoney, Parise et al. 2005; Louis, Raue et al. 2007; Murton, Billeter et al. 2014) and protein (Egan, Dowling et al. 2011) abundance varies between targets following exercise.

It was not possible to characterise individuals who experienced either up- or down-regulation of protein targets by variables such as age, BMI, $\dot{V}O_2^{PEAK}$ or $\Delta\dot{V}O_2^{PEAK}$ with training or exercise withdrawal (using data presented in Chapter 4.3). With the data collected for this study it is therefore not possible to identify factors that predict the response of protein expression to training or withdrawal. A broad range of factors such as availability of translational apparatus (ribosomes, tRNAs and translational factors) (Chaillou, Kirby et al. 2014), translation initiation (Murton, Billeter et al. 2014), and post translational control by non-coding RNAs (particularly microRNA (Braun and Gautel 2011)) influence the quantity of protein transcribed so it is perhaps unsurprising that gross physiological measures were not clearly linked to changes in abundance of single protein molecules.

4.4.5 Limitations

It is acknowledged that the mRNA analysis was not genome-wide and it is not possible to make assumptions about targets that were not measured in this study. The choice of a RT-PCR technique however has the significant advantage of being a highly sensitive quantification method across a wide range of expression values. This study was

therefore capable of detecting subtle differences in mRNA abundance between age groups for genes that were already know to be important in the response to aerobic training.

The study was adequately powered to detect influence over biological functions at an mRNA level. It is unlikely that the relatively few significant findings from the protein analysis are due to lack of power; individual data points (Figure 4.7) do not suggest non-significant trends. It would be of great interest to assess the physiological activity of key proteins in muscle samples using a metabolomics approach to add greater understanding of function rather than mere abundance. The selection of target proteins was conducted using fold-change data from the broader MATCH study, including a group of COPD patients. An alternative method for this analysis might have been to select targets whose genes were influential within the biological functions predicted to be altered by IPA within the Young and Older groups specifically.

4.4.6 Implications

The impact of aerobic training on skeletal muscle mRNA abundance was very similar for young and older adults across a range of targets known to be important in the response to endurance training; an observation that is in line with the similar whole-body physiological adaptations to training observed in Chapter 3. Gene functions relating to fuel selection correspond with observed changes in function at a muscle and whole-body level during the training period highlighting the importance of molecular adaption for whole-body and muscle training adaptations. Changes in gene expression were maintained after exercise withdrawal which despite some changes in mitochondrial ATP production capacity (Figure 4.9) are allied with the findings in Chapter 3 which showed that there is no significant detraining effect ($\dot{V}O_2^{PEAK}$) in the four weeks following an eight week training intervention in young or older adults. These data would strongly suggest that at both a whole-body and muscle molecular level older individuals can achieve the same relative adaptations to exercise training as young individuals.

4.4.7 Conclusion

Young and older sedentary adults experience strikingly similar mRNA responses to an eight week aerobic training programme. Biological functions of genes influenced in abundance relating to fuel selection align with the substantial skeletal muscle and cardiorespiratory physiological adaptations (principally increased $\dot{V}O_2^{PEAK}$ and reduced RER at submaximal workloads) experienced equally by both groups. Alterations in mRNA abundance persist even after four weeks of exercise withdrawal. mRNA changes predict influence over biological functions relating to muscle fuel selection and remodelling and the proteins influenced in abundance also have functions relating to muscle growth and fuel selection. Having established the similarity in response to aerobic training and subsequent exercise in withdrawal in young and older sedentary adults both in terms of gene expression and whole body measures, Chapter 5 and Chapter 6 will explore the impact of COPD on the ability of individuals to adapt to training in terms of whole-body physiology and the muscle molecular response.

Chapter Five

The Influence of COPD on the responses to aerobic training and subsequent exercise withdrawal: cardiorespiratory responses to exercise

5.1 Introduction

As demonstrated in Chapter 3, sedentary older adults have lower aerobic capacity than young adults who are matched for habitual physical activity levels. In patients with COPD low cardiorespiratory fitness is known to be considerably more pronounced, with peak oxygen uptake typically around 50% lower than that in healthy age-matched controls (Maltais, LeBlanc et al. 1996; Gosker, Lencer et al. 2003; Vaes, Wouters et al. 2011). There is evidence that some patients with COPD can increase their $\dot{V}O_2^{PEAK}$ following aerobic training of sufficiently high intensity and duration (Casaburi, Patessio et al. 1991; Maltais, LeBlanc et al. 1996; Gosker, Schrauwen et al. 2006; Vogiatzis, Stratakos et al. 2007; Bronstad, Rognmo et al. 2012; Costes, Gosker et al. 2015). Some patients with COPD who have a ventilatory limitation to peak exercise capacity struggle to increase $\dot{V}O_2^{PEAK}$ (Maltais, LeBlanc et al. 1997) because the training intensity they can achieve may be too low to prompt physiological adaptation (Troosters, Gosselink et al. 2001; Burtin, Saey et al. 2012). Aerobic exercise training is a recommended component of pulmonary rehabilitation programmes (Bolton, Bevan-Smith et al. 2013; Spruit, Singh et al. 2013), a clinical intervention which is known to have a positive impact on maximal exercise capacity (McCarthy, Casey et al. 2015) in the general COPD population. Relatively few studies have examined the comparative effects of aerobic exercise training in patients with COPD and healthy age-matched controls. Of the studies that have compared responses to aerobic training in COPD and health, some did not match the relative training intensities of COPD patients and healthy controls making the relative response difficult to interpret (Guzun, Aguilaniu et al. 2012; Rodriguez, Kalko et al. 2012). When training intensity was matched COPD patients seemed to make smaller gains in $\dot{V}O_2^{PEAK}$ than healthy controls (Sala, Roca et al. 1999; Rabinovich, Ardite et al. 2001; Radom-Aizik, Kaminski et al. 2007; Gouzi, Prefaut et al. 2013). Only one of the training studies that matched training intensity also attempted to match groups for the

potential confounding variable of habitual physical activity measured objectively using accelerometry (Gouzi, Prefaut et al. 2013).

One of the main drivers of impaired exercise capacity and skeletal muscle dysfunction in COPD is thought to be deconditioning (physiological changes arising from low physical activity levels) (Watz, Waschki et al. 2009) which probably contributes to the downward spiral of dyspnoea and disability experienced by many patients (Eisner, Iribarren et al. 2011). In healthy older adults, deliberate restriction of physical activity negatively impacts on aerobic fitness and physical function (Coker, Hays et al. 2014). In the long term low cardiorespiratory fitness is associated with heightened risk of low functional capacity, morbidity and mortality in otherwise healthy individuals (Blair, Kampert et al. 1996). It is unknown whether patients with COPD are more susceptible than healthy age-matched controls to the metabolic and physiological decline that accompanies exercise withdrawal. The influence of exercise withdrawal is relatively unstudied in COPD but is relevant for understanding the sustainability of the benefits of pulmonary rehabilitation (Bolton, Bevan-Smith et al. 2013) as well as the contribution of reduced muscular activity to the acute deconditioning that occurs during acute exacerbation of COPD.

This chapter tests the principal hypothesis that patients with COPD will exhibit smaller changes in in $\dot{V}O_2^{PEAK}$ compared to healthy sedentary volunteers of a similar age in response to equivalent moderate intensity aerobic training. Additional research objectives will:

1. Examine cardiorespiratory responses to sub-maximal exercise testing at isowork in patients with COPD and age-matched sedentary volunteers before and after eight weeks aerobic exercise training.
2. Assess susceptibility to physiological deconditioning (change in $\dot{V}O_2^{PEAK}$ and cardiorespiratory responses to sub-maximal exercise testing) after four weeks exercise withdrawal in patients with COPD compared to healthy controls.

The detailed analysis of cardiorespiratory adaptations to aerobic training and subsequent exercise withdrawal will also give context to the subsequent chapter which investigates skeletal muscle molecular responses to the same intervention. Data in this

chapter are from the MATCH aerobic training study. The COPD group are compared to an age-matched healthy control group, made up of the same individuals as the Older Group in Chapters 3 & 4.

5.2 Methods

5.2.1 Participants

Patients with COPD aged 60 – 80 years are compared to age-matched, sedentary healthy controls. Inclusion criteria are in Chapter 2.4.1.

5.2.2 Study Design

The protocol followed by both groups is the same as that reported in Chapter 3 and described in detail in Chapter 2.4.1. An aerobic training intervention comprised of supervised cycle ergometer training three times per week for eight weeks at 65% of work rate that corresponded to $\dot{V}O_2^{PEAK}$ at baseline was followed by four weeks exercise withdrawal and follow-up.

5.2.3 Measures

Baseline participant testing included full pulmonary function testing, DEXA body composition assessment and quadriceps strength assessment. Outcome measures for incremental cycling exercise ($\dot{V}O_2^{PEAK}$; W^{PEAK}; and RER, $\dot{V}E$ and HR at $\dot{V}O_2^{PEAK}$) and sub-maximal exercise at 65% of peak aerobic power (RER, $\dot{V}E$ and HR) were recorded at baseline, after four and eight weeks training and after four weeks exercise withdrawal (testing procedures in Chapter 2.7).

5.2.4 Data Analysis

Between group comparisons were assessed using the Mann-Whitney U test for continuous or Pearson Chi-Square test for categorical variables; within group change was tested with Friedman's ANOVA or Wilcoxon Signed Rank test.

5.3 Results

5.3.1 Subject Characteristics

Data for the COPD (n = 19) and Older Healthy Control (HC) (n = 10) participants who completed the study are presented in Table 5.1. Groups were well matched for age and body size and although there was a greater proportion of female participants in the

COPD group, the gender balance was not significantly different between the two groups (all $p > 0.05$).

As determined by the inclusion criteria, the Older HC group had normal lung function whereas the COPD group demonstrated obstructive spirometry. Total lung capacity was the only parameter of lung function on which the two groups were similar ($p > 0.05$), however COPD patients were on average markedly hyperinflated (RV 157% predicted) and had reduced transfer factors. All COPD patients were ex-smokers, making the group significantly more likely to have smoked than the Older HC group. For those subjects who had a smoking history, Older HCs averaged 18 pack-years compared to 38 pack-years in the COPD group, however the low number of ex-smokers in the Older HC group (n = 4) and large variation makes the difference non-significant.

Measures of body composition were not significantly different but QMVC was lower in the COPD group.

Table 5-1. Baseline subject characteristics of COPD & Older HC groups

	Older HC		COPD		
	Mean	SD	Mean	SD	*p*
Demographics					
Gender (male : female)*	5 : 5		6 : 13		NS
Age (years)	71	5	70	6	NS
Height (m)	1.65	0.09	1.62	0.10	NS
Body mass (kg)	78.4	13.7	75.7	18.1	NS
BMI (kg/m^2)	28.5	3.3	28.9	6.5	NS
Lung Function					
FEV_1 (L)	2.57	0.38	1.21	0.34	< 0.001
FEV_1 (% predicted)	113	21	57	16	< 0.001
FVC (L)	3.5	0.6	2.8	0.6	0.012
FVC (% predicted)	120	21	101	24	0.019
FEV_1 / FVC ratio	75	4	45	13	< 0.001
RV (L)	2.25	0.56	3.39	1.14	0.001
RV (% predicted)	98	19	157	46	< 0.001
TLC (L)	5.83	0.97	6.30	1.39	NS
TLC (% predicted)	104	12	122	19	0.035
RV / TLC ratio	38	4	53	9	< 0.001
TlCo (% predicted)	98	13	62	19	< 0.001
KCO (% predicted)	116	20	81	21	< 0.001
Smoking history (Current : Never : Ex)*	0 : 6 : 4		0 : 0 : 19		<0.001
Pack-year history	18.3	21.5	38	15.7	NS
MRC Grade $	1	1 - 1	3	3 - 3	<0.001
Body Composition & Muscle Strength					
Whole body lean mass (kg)	47.7	8.2	43.0	10.1	NS
FFMI (kg/m^2)	18.2	1.5	17.2	2.8	NS
ASMI (kg/m^2)	7.3	0.9	6.6	1.3	NS
Thigh lean mass (kg)	9.0	2.1	7.6	2.2	NS
Isometric QMVC (Nm)	130	36.8	98	37.0	0.035

Pack years, average number of cigarette packs smoked per day x number of smoking years, for subjects with a smoking history only. Between group differences tested by Mann-Whitney U test for ordinal and continuous data. Categorical variables (*) tested with Pearson Chi-Square test. NS = $p > 0.05$. $ Median, IQR.

5.3.2 Habitual Physical Activity

Baseline physical activity was measured over an average of 7.1 days in the COPD group and 7.2 days in the Older HC group.

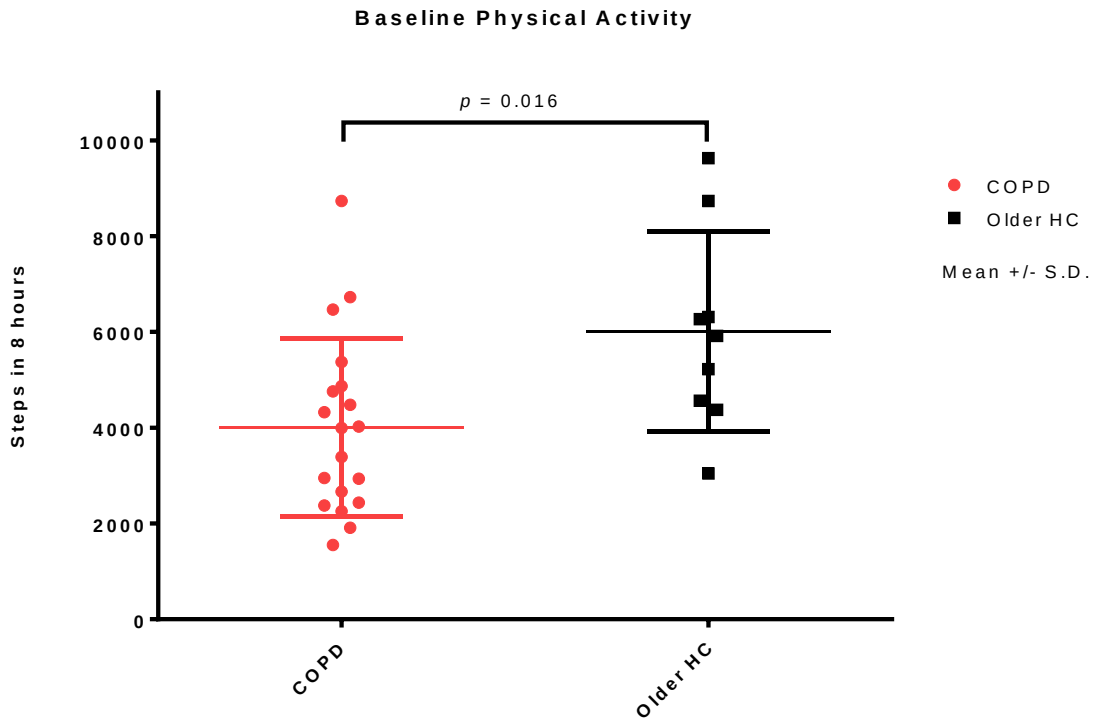


Figure 5-1. Habitual physical activity in COPD & Older HC groups.. Individual data points represent the mean step count for all days recorded for the individual. Line indicates group mean

Baseline mean step count over 8 hours was significantly lower in the COPD group (4012 ± 1861) than in the Older HC group (6007 ± 2088), p = 0.016 (Figure 5.1). Baseline estimated energy expenditure over 8 hours was 3774 ± 857 kJ in the COPD group and 3999 ± 813 kJ in the Older HC group (not a significant difference, $p > 0.05$). Step count and energy expenditure assessed during the 9th and 12th week of the study did not differ from baseline in either group ($p > 0.05$, see Appendix 8). There was no significant change in isometric QMVC in the Older group ($p > 0.05$) or COPD group ($p > 0.05$). Mean (±SD) isometric QMVC after eight weeks training and four weeks subsequent exercise

withdrawal was 142 ±45.9 Nm and 141 ±51.7 Nm respectively in the Older HC group and was 101 ± 37.8 and 96 ±38.4 Nm respectively in the COPD group. FFMI was unchanged after training and exercise withdrawal respectively in the Older HC group (18.0 ±1.7 and 18.0 ± 1.7 kg/m^2, $p > 0.05$) or in the COPD group (17.2 ±2.8 and 17.2 ± 2.8 kg/m^2, $p > 0.05$).

5.3.3 Exercise Capacity and Cardiorespiratory Responses at Peak Exercise

5.3.3.1 Baseline

Table 5.2 shows cardiorespiratory measures from the baseline incremental exercise test (CPET).

Table 5-2. Baseline Incremental Exercise Test in COPD & Older HC groups

	Older HC		COPD		*p*
	Mean	SD	Mean	SD	
$\dot{V}O_2^{PEAK}$ (l/min)	1.41	0.36	1.05	0.40	0.014
$\dot{V}O_2^{PEAK}$ (ml/min/kg lean mass)	29.7	4.0	24.4	7.4	0.014
Peak $\dot{V}CO_2$ (L/min)	1.69	0.45	1.11	0.50	0.003
RER at $\dot{V}O_2^{PEAK}$	1.20	0.09	1.05	0.09	<0.001
W^{PEAK} (W / kg Lean Mass)	2.4	0.2	1.7	0.6	0.001
Peak $\dot{V}E$ (L/min)	62.8	16.4	45.6	16.7	0.019
% MVV at peak exercise	66	16	103	24	<0.001
HR at Peak Exercise (beats/min)	141	14	129	20	NS
% Peak HR	94	10	86	12	0.062
Borg Breath Pre-Test $	0	0 - 0	0	0 - 0	NS
Borg Breath End-Test $	4	3.25 - 4.75	5	4 - 7	NS
Borg RPE Pre-Test $	6	6 - 6	6	6 - 6	NS
Borg RPE End-Test $	16	13 - 17	15	13 - 17	NS
SpO_2 Pre-Test	97.0	1.5	95.9	2.4	NS
SpO_2 End-Test	96.4	1.3	93.1	3.6	0.003
Change in SpO_2	-0.6	2.2	-2.9	2.4	0.019
Reason for Stopping (SOB : Leg Fatigue : SOB = Legs : Other) *	2 : 5 : 2 : 1		7 : 3 : 8 : 0		NS
NS = $p > 0.05$. $ Median, IQR.					

Compared to Older HC, the COPD group had lower $\dot{V}O_2^{PEAK}$, W^{PEAK}, $\dot{V}CO_2$ and RER in the baseline test (all $p < 0.05$). Minute ventilation as an absolute value was lower in the COPD group compared to Older HCs ($p < 0.05$) however this represented a significantly greater proportion of the COPD group's predicted maximum voluntary ventilation ($p < 0.05$). There was a trend ($p = 0.062$) towards the COPD group reaching a lower proportion of their predicted maximum heart rate at peak exercise compared to the Older HC group.

Patient perceptions of dyspnoea and exertion at end exercise were not significantly different between the groups ($p > 0.05$), and although there was a trend towards COPD patients being more likely to attribute their reason for stopping to shortness of breath (opposed to purely leg fatigue) the responses from the two groups were not significantly different ($p > 0.05$). The pre- to post-exercise change in peripheral oxygen saturations was significantly greater in the COPD compared to the Older HC group ($p < 0.05$).

5.3.3.2 Influence of Aerobic Training on Cardiorespiratory Measures at Peak Exercise Intensity

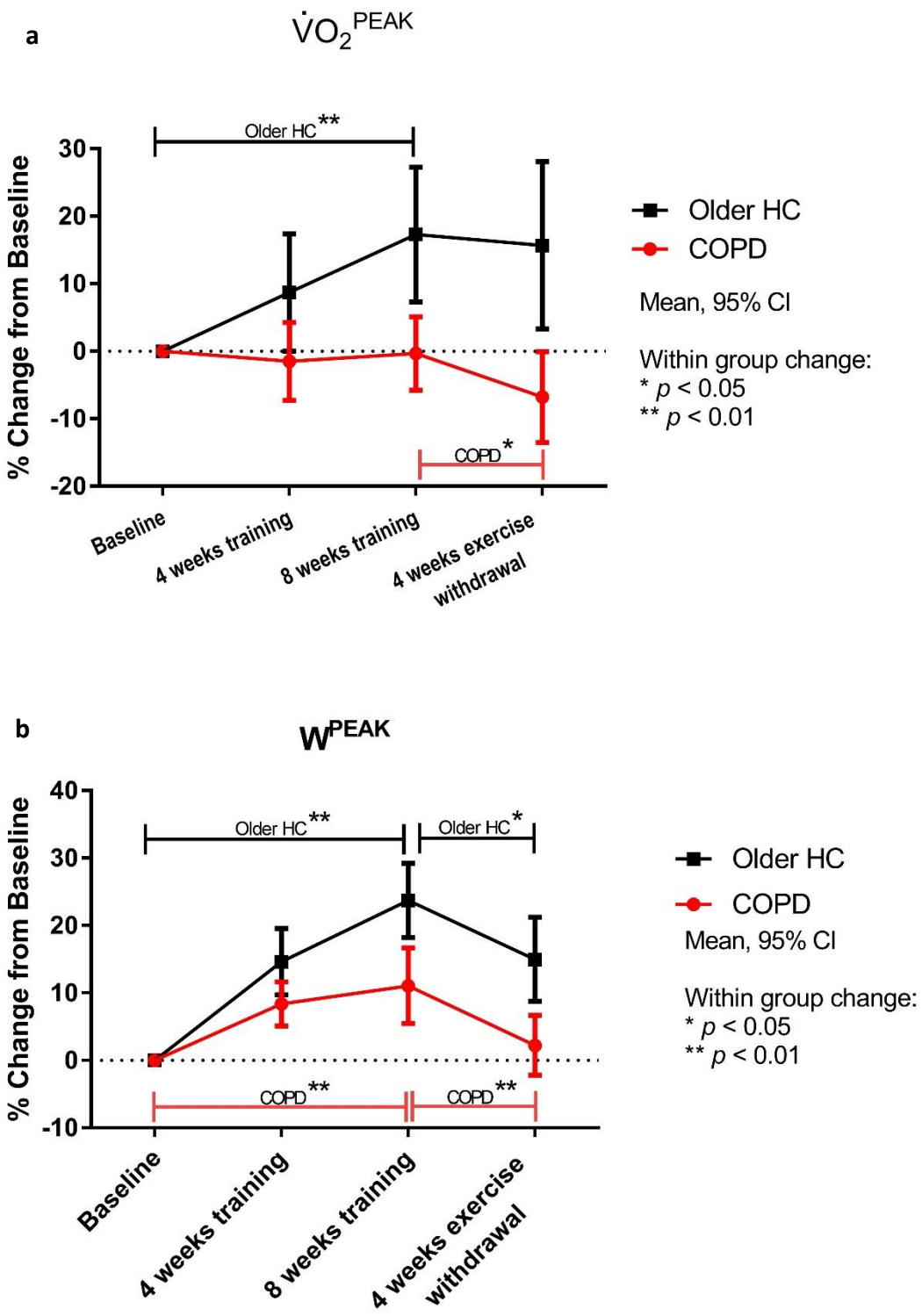


Figure 5-2. $\dot{V}O_2^{PEAK}$ (a) and W^{PEAK} (b) at exhaustion in incremental CPET after aerobic training and exercise withdrawal in COPD & Older HC groups

Change in $\dot{V}O_2^{PEAK}$ during the 8 week training intervention (Figure 5.2 a) was significantly different between groups ($p < 0.01$), with Older HCs increasing $\dot{V}O_2^{PEAK}$ (ml / min / kg lean mass) by an average of 18% ($p = 0.001$) whereas $\dot{V}O_2^{PEAK}$ was unaltered from baseline in the COPD group (within group change $p > 0.05$). W^{PEAK} (Figure 5.2 b) increased significantly in both groups (Older HC 24%, $p < 0.001$; COPD 11%, $p < 0.001$), with the magnitude of gain significantly greater in the Older HC group ($p < 0.001$).

Minute ventilation at $\dot{V}O_2^{PEAK}$ (Figure 5.3 a) increased by 26% ($p < 0.05$) in the Older HC group but was unchanged from baseline in the COPD group ($p > 0.05$) after 8 weeks training. There was no significant change in the maximum heart rate achieved (Figure 5.3 b) or the RER (Figure 5.3 c) at $\dot{V}O_2^{PEAK}$ in either group in post-training tests (all $p > 0.05$). Subject-reported Borg ratings of perceived breathlessness and rate of perceived exertion at end exercise in the incremental test were not significantly changed from baseline at any time point in either group (see Appendix 12).

5.3.3.3 Influence of Exercise Withdrawal on Cardiorespiratory Measures at Peak Exercise

$\dot{V}O_2^{PEAK}$ decreased significantly ($p < 0.01$) in the COPD group during the exercise withdrawal period (Figure 5.2) and remained unchanged ($p > 0.05$) in the Older HC group, although the changes that occurred during exercise withdrawal were not significantly different between the groups ($p > 0.05$). W^{PEAK} reduced significantly in the COPD ($p < 0.01$) and Older HC ($p < 0.05$) groups during the exercise withdrawal period (Figure 5.3) and the reduction was of a similar magnitude (no significant between group difference, $p > 0.05$).

After 4 weeks of exercise withdrawal, minute ventilation at $\dot{V}O_2^{PEAK}$ was significantly reduced in the COPD group ($p < 0.05$, Figure 5.4 a) and there was also a non-significant trend for reduced minute ventilation in the Older HC group ($p = 0.059$).

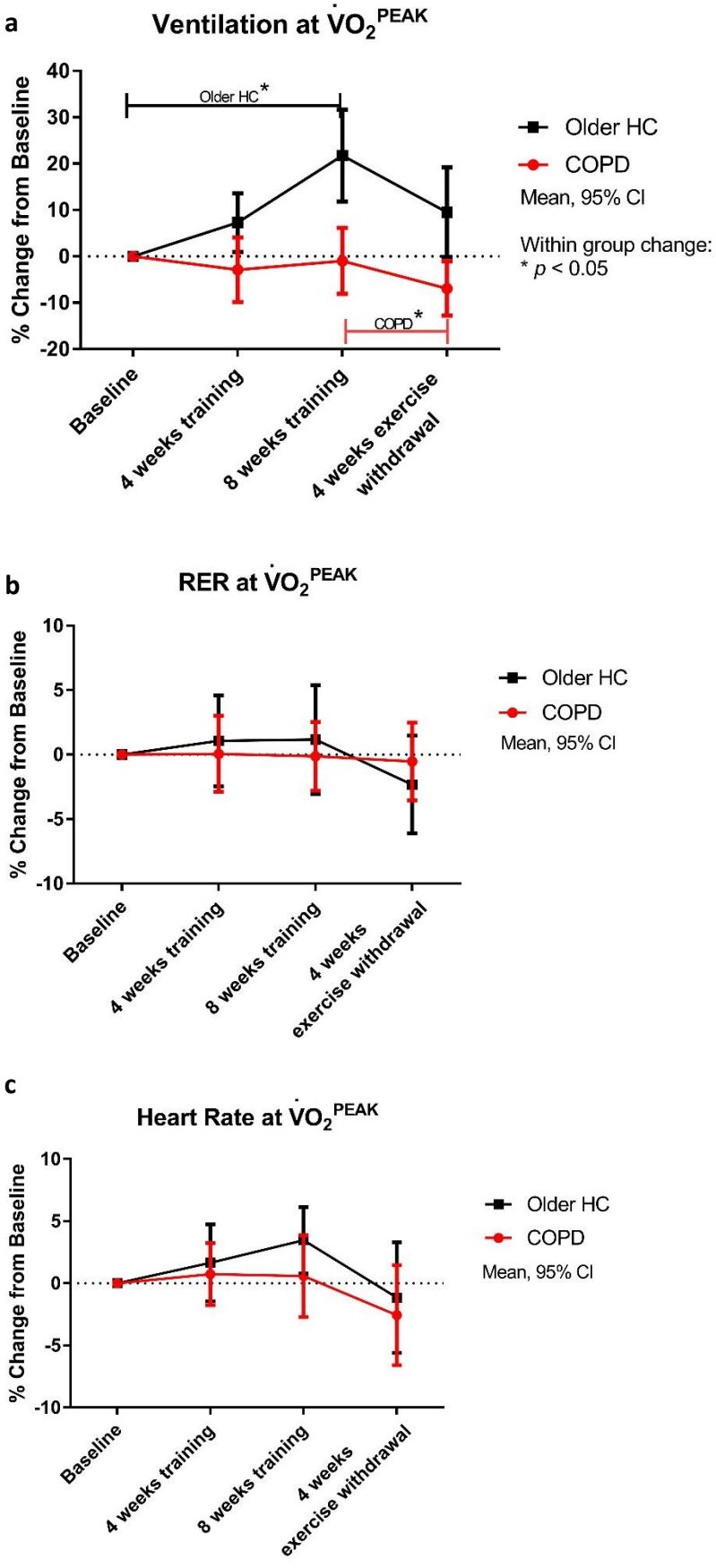


Figure 5-3. Cardiorespiratory responses to exercise at $\dot{V}O_2^{PEAK}$ in COPD & Older HC

5.3.4 Submaximal Exercise Testing

Dictated by the results of the baseline incremental test, the endurance constant work-rate test at 65% W^{PEAK} was performed at a significantly lower absolute intensity in the COPD compared to the Older HC group (47 ± 21 vs 76 ± 18 W respectively, $p < 0.01$, Table 5.3).

5.3.4.1 Baseline Submaximal Exercise Testing

Reflecting the higher absolute workload performed by the Older HC group, average $\dot{V}O_2$ and $\dot{V}CO_2$ throughout the test were significantly higher in the healthy controls compared to the COPD group (both $p < 0.01$, Table 5.3). RER was significantly higher in the Older HCs during submaximal testing ($p < 0.01$). The ventilatory response to exercise at 65% W^{PEAK} (Table 5.3) was higher in absolute terms in the Older HCs ($p < 0.001$) however the COPD patients were utilising a significantly greater proportion of their maximal ventilatory capacity ($p < 0.05$). Average peripheral oxygen saturation was lower in the COPD patients during the submaximal test than in the Older HCs ($p < 0.001$).

Table 5-3. Sub-maximal constant load exercise test at baseline in COPD & Older HC groups

	Older HC		COPD		*p*
	Mean	SD	Mean	SD	
Exercise workload (W)	76	18	47	21	0.002
RER	1.04	0.03	0.98	0.05	0.009
$\dot{V}E$ (l / min)	52.20	11.43	36.26	11.72	<0.001
% MVV	53.8	11.9	82.5	18.7	0.027
HR (beats / min)	130	16.2	118.8	20.2	NS
HR (% max)	86.8	11.9	79.5	12.4	NS
SpO_2 (%)	95.7	1.1	91.8	2.8	<0.001
Values represent the steady state period of the test; excluding the first 3 minutes at workload.					

5.3.4.2 Influence of Aerobic Training on Submaximal Exercise Testing

Cardiorespiratory responses to exercise at 65% W^{PEAK} are shown in Figure 5.4. In the trained state (at 8 weeks) Older HCs had significantly lower minute ventilation, heart rate and RER responses during the submaximal test compared to when they performed an identical intensity and duration of work at baseline (all $p < 0.01$). In the COPD group, there was a significant reduction in heart rate ($p < 0.01$) and RER ($p < 0.05$) at 8 weeks compared to baseline. There was also a trend towards lower minute ventilation in patients with COPD, but this did not reach statistical significance ($p > 0.05$).

5.3.4.3 Influence of Exercise Withdrawal on Submaximal Exercise Testing

Minute ventilation, heart rate and RER during the submaximal test did not change significantly during the exercise withdrawal period (8 – 12 weeks) in either group (all $p > 0.05$, Figure 5.4).

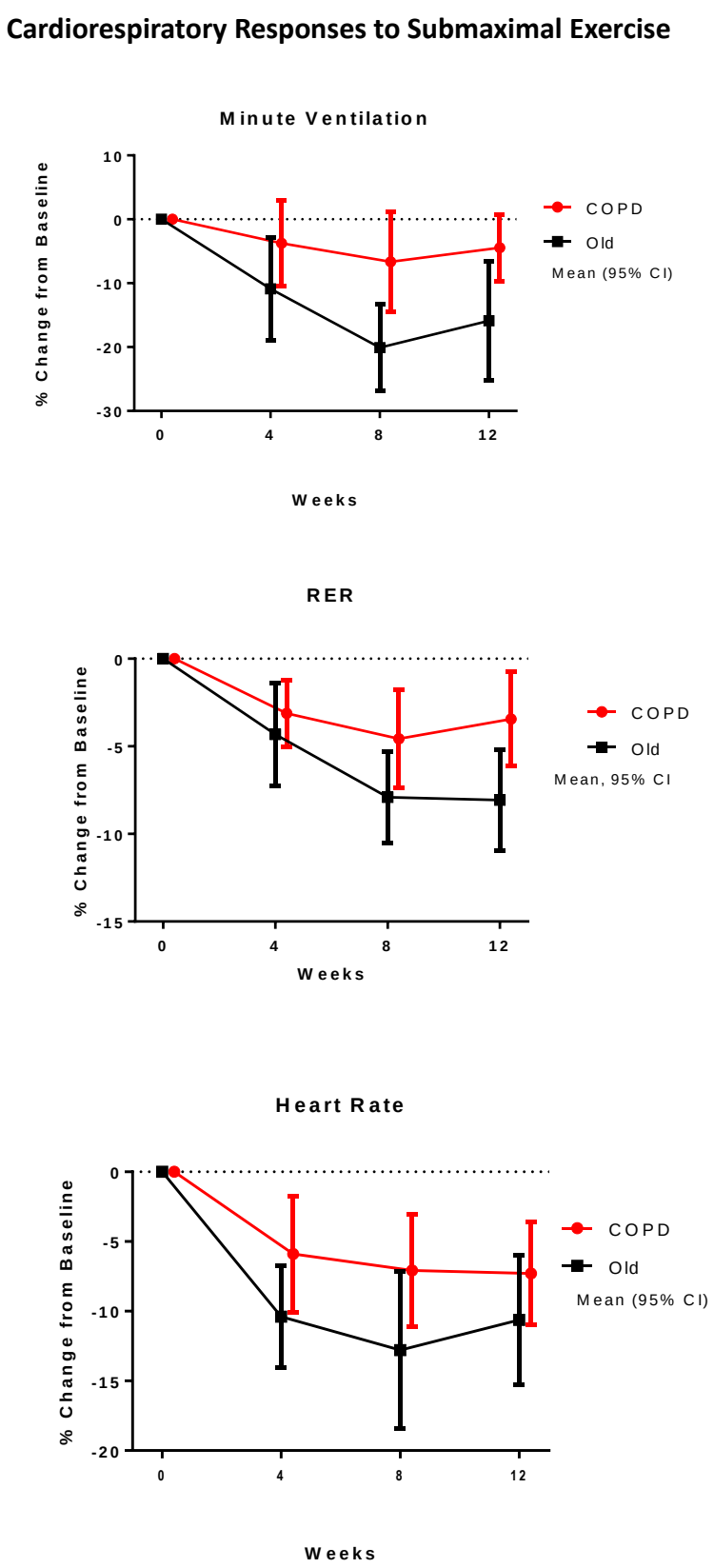


Figure 5-4. Cardiorespiratory responses to submaximal exercise with training and exercise withdrawal in COPD & Older HC groups.

5.4 Discussion

Patients with COPD had lower peak oxygen uptake than healthy age-matched controls in the untrained state and saw no increase in response to eight weeks moderate intensity aerobic cycling training whereas healthy older volunteers increased peak oxygen uptake by an average of 18%. After training W^{PEAK} in the incremental exercise test increased in patients with COPD but by a smaller magnitude than that of healthy controls (11 vs 24% respectively) and physiological adaptation was apparent with both groups exhibiting reduced heart rate and RER during submaximal testing in the trained state. After four weeks of subsequent exercise withdrawal W^{PEAK} in incremental exercise testing was reduced in both COPD and healthy older groups but only the COPD group demonstrated a reduction in $\dot{V}O_2{}^{PEAK}$.

Peak oxygen uptake in the untrained state was significantly lower in the COPD group compared to the healthy control group in this study (26% lower expressed in litres, or 18% lower when normalised for lean mass) but not as low as the 50% reduction in $\dot{V}O_2{}^{PEAK}$ compared to healthy age-matched controls commonly cited (Maltais, Simard et al. 1996; Vaes, Wouters et al. 2011). The values for COPD patients are in fact very similar to those previously described (Vaes et al (Vaes, Wouters et al. 2011) report $\dot{V}O_2{}^{PEAK}$ of 24.6 compared to 24.4 ml / min / kg lean mass in this study) and it is in fact the healthy control groups that differ between this study and the previously published literature. The comparatively low cardiorespiratory fitness of the HC group in this study is likely a consequence of the deliberate selection of less active individuals as controls.

Physical activity measured by step count was relatively low (Tudor-Locke, Craig et al. 2011) in the HC group (around 6000 steps) and the inclusion criteria for the study ensured that no participants were engaged in an exercise regime. A review of physical activity monitoring studies found that patients with COPD typically spend 56% less time engaged in physical activity than healthy age-matched controls (Vorrink, Kort et al. 2011). In this study, the daily step count was on average 33% lower in patients with COPD compared to the control group, and estimated energy expenditure was similar over the same time period, suggesting that the physical activity characteristics of the groups were more similar than for some previous studies where habitual physical activity has not been controlled through the inclusion criteria. Whilst the lower step

count in the COPD group may have influenced baseline exercise capacity, the main focus of this work is the response to a tightly supervised training programme which was of sufficient intensity and duration to overwhelm the influence of habitual physical activity and prompted large changes in aerobic capacity in healthy volunteers. It is a strength of the study that habitual physical activity was measured at baseline and again during the exercise withdrawal period showing no changes in activity behaviour to confound interpretation of the influence of the training intervention.

A key determinant of the $\dot{V}O_2^{PEAK}$ of patients with COPD in this study is ventilatory capacity. In the baseline incremental test average minute ventilation at $\dot{V}O_2^{PEAK}$ was 103% of MVV (predicted from FEV_1) in the COPD group, whereas Older HCs reached an average of 66% MVV. Symptom limited termination of exercise coincided with utilisation of a large proportion of predicted ventilatory capacity suggesting a ventilatory limitation to exercise related to dyspnoea in the COPD group whereas healthy controls had a substantial ventilatory reserve.

The most prominent observation of this study is that healthy controls increased their peak oxygen uptake with eight weeks exercise training whereas patients with COPD did not. Lack of improvement in aerobic capacity in COPD has been demonstrated before in response to moderate intensity training (Casaburi, Patessio et al. 1991) however modest improvements in $\dot{V}O_2^{PEAK}$ have previously been observed in some studies employing high intensity training (Rabinovich, Ardite et al. 2001; Radom-Aizik, Kaminski et al. 2007; Gouzi, Prefaut et al. 2013). An aerobic training study by Gelinas *et al* (Gelinas, Lewis et al. 2017) compared the responses of patients with COPD and healthy controls to eight weeks of periodised aerobic training of varying intensities and found increases in $\dot{V}O_2^{PEAK}$ in both groups but healthy volunteers made significantly larger gains (17% *vs*. 8%). In this study patients with COPD were likely limited by their impaired maximal ventilatory capacity, a variable which is not modifiable with training (Spruit, Singh et al. 2013). The COPD group utilised all of their predicted ventilatory reserves in the baseline incremental test and in contrast to healthy controls (whose minute ventilation at peak exercise in the trained state was 26% higher than baseline) were therefore unable to increase minute ventilation further during testing in the trained state (Figure 5.3 a). In the Gelinas study the COPD group which increased average $\dot{V}O_2^{PEAK}$ in response to

aerobic training had utilised a smaller proportion of its ventilatory reserve in baseline testing.

The ventilatory limitation to exercise in the COPD group may also have influenced the prescription of training intensity compared to the HC groups who had a cardiovascular limitation to exercise. Older HC's reached 94% of their predicted maximum heart rate on average (compared to 86% in the COPD group) suggesting that they reached a true maximal $\dot{V}O_2$ in the baseline test. As the peak exercise capacity of the COPD patients was restricted by ventilatory limitation, the training intensity prescribed as 65% of the work rate at $\dot{V}O_2^{PEAK}$ may have also have been restricted, resulting in a relatively lower load on the muscles and cardiovascular system during training in the COPD group (Dolmage and Goldstein 2006). In health, adaptations in the cardiovascular system contribute to increases in $\dot{V}O_2^{PEAK}$ after aerobic exercise training, with increased cardiac output an expected outcome (Murias, Kowalchuk et al. 2010). There is evidence that peak cardiac output may increase in patients with COPD after rehabilitative exercise training (inferred from peak oxygen pulse and heart data; Gelinas, Lewis et al. 2017) as well as measured (Bronstad, Tjonna et al. 2013) and oxygen pulse inferred (Lan, Yang et al. 2018) stroke volume. However, in order to prompt cardiovascular adaptation the training intensity must be sufficiently high. Evidence from the baseline submaximal test (performed at the same workload as the training sessions) shows that COPD patients were working at significantly lower RER and trended towards lower heart rate compared to the HC group (Table 5.3) which raises the possibility that the COPD patients were unable to stress their cardiovascular system sufficiently to elicit adaptation. There is evidence that patients with COPD need to train at high intensities in order to increase peak oxygen uptake (Casaburi, Patessio et al. 1991; Maltais, LeBlanc et al. 1996) with pulmonary rehabilitation guidelines suggesting walking intensity at 85% of peak (Bolton, Bevan-Smith et al. 2013) compared to the 65% intensity used in this study. The decision to choose a training intensity corresponding to 65% of W^{PEAK} in the incremental test was pragmatic and accounted for the intensity of training that was achievable for the healthy groups (for whom a higher intensity would have been unmanageable). It is a strength of the study that a consistent strategy for training load prescription was used and training was well controlled (all sessions were supervised).

Importantly, patients with COPD did show some evidence of a positive adaptation to exercise training, with physiological adaptations to training apparent in submaximal testing. There was a small but significant increase in W^{PEAK} in the incremental test post-training in the COPD group and physiological adaptation was apparent during exercise at isowork with reduced heart rate and RER and a trend towards reduced minute ventilation. These changes in cardiorespiratory responses to submaximal exercise mirror those in the Older HC group, albeit of smaller magnitude and are indicative of training adaptations in the muscle. A shift towards lipid and away from carbohydrate oxidation during submaximal exercise would be in agreement with previous studies (Casaburi, Patessio et al. 1991), account for the reduction in RER at isowork in the trained state and would be the logical result of increased mitochondrial density and altered mitochondrial fuel selection after training but would not necessarily be associated with an increase in $\dot{V}O_2^{PEAK}$ which is known to be limited by oxygen delivery and extraction (Saltin and Calbet 2006; Lundby, Montero et al. 2016). A reduction in lactic acid production for the same cycling power output would also reduce muscle afferent activation which influences respiratory drive and heart rate as discussed in Chapter 3 (Carter, Banister et al. 2003; Amann, Blain et al. 2010). The increase in W^{PEAK} in the COPD group after training while $\dot{V}O_2^{PEAK}$ remained unchanged is likely a result of greater metabolic efficiency in the muscle (e.g. altered mitochondrial fuel selection, as discussed above) and may also be related to improved neuromuscular coordination. Smooth and coordinated pedalling is a motor skill and as such may have improved through practice over the course of the eight week training intervention which would result in a more efficient transfer of muscular work to external power delivered through the cycle ergometer. Exercise withdrawal prompted a significant decrease in $\dot{V}O_2^{PEAK}$ in patients with COPD that was not apparent in Older HCs. This is somewhat surprising given that there was no significant increase in peak oxygen uptake during the training period in the COPD group and the objectively monitored resumption of pre-training physical activity levels during the exercise withdrawal period. This observation has more than one possible explanation: firstly, this may be evidence of accelerated deconditioning following exercise withdrawal; secondly, despite identical testing conditions the reduced $\dot{V}O_2^{PEAK}$ in testing at 12 weeks coincides with significantly reduced $\dot{V}E$ and a trend towards reduced HR possibly indicating insufficient effort in the final exercise test by some

participants; thirdly, the significant within-group change may result from type II experimental error due to multiple testing and it should be noted that there was no between-group difference in the change in $\dot{V}O_2^{PEAK}$ during the exercise withdrawal period. Both COPD and HC groups demonstrated reduced W^{PEAK} in the incremental test after the exercise withdrawal period. Exercise withdrawal has not been extensively studied in patients with COPD despite its importance in the context of maintaining benefits of pulmonary rehabilitation and understanding the effects of enforced inactivity during periods of acute exacerbation of COPD. In contrast to the data from the study presented in this chapter, one study of nine patients reported a significant increase in $\dot{V}O_2^{PEAK}$ with eight weeks high intensity aerobic training but no decrease in $\dot{V}O_2^{PEAK}$ during a five month exercise withdrawal period (Otsuka, Kurihara et al. 1997). A potential reason for the contrast between that study and the data presented here is that there was no measure of physical activity during the exercise withdrawal period in the study by Otsuka *et al*, therefore subjects may have changed their behaviour during the prolonged follow-up. There was no significant change in ventilation, HR or RER during submaximal exercise at isowork after the four week exercise withdrawal period in either group signifying that any detraining effect suggested by reduced $\dot{V}O_2^{PEAK}$ in the COPD group did not represent a full reversal of the physiological adaptations achieved over the preceding eight week training period.

5.4.1 Limitations

There is evidence that some patients in the COPD group experienced a ventilatory limitation to exercise. It is a limitation of the study that the sample size was not large enough to permit a sub-group analysis of patients with and without a ventilatory limitation. It would also have been of interest to measure dynamic hyperinflation during exercise testing to better characterise the ventilatory characteristics of the COPD group (who were on average hyperinflated at rest), however technical limitations of the cardiopulmonary exercise testing equipment used did not allow this.

5.4.2 Implications

Patients with COPD did not increase $\dot{V}O_2^{PEAK}$ in response to eight weeks aerobic exercise training, possibly because the absolute training intensity (65% W^{PEAK}) performed by patients was too low. It is likely that the COPD group would need to exercise at a higher intensity, which may be difficult to tolerate, in order to prompt a rise in $\dot{V}O_2^{PEAK}$. Adaptation to training was however apparent in patients with COPD in the responses to sub-maximal exercise (reduced RER and HR) which has implications for the choice of outcome measure employed in studies of this sort. It is possible that a sub-maximal test of cycling performance assessing time to fatigue (akin to the Endurance Shuttle Walking Test (Revill, Morgan et al. 1999)) may have shown functional benefit of the training intervention in the COPD group. Although peak oxygen uptake was unaltered by training in the COPD group decreased RER during submaximal testing suggests that there were some training adaptations at the level of the muscle relating to fuel selection although possibly of smaller magnitude than in the healthy group. In light of the whole-body responses described here, the impact of this intervention at the level of the muscle will be explored in Chapter 6, where an investigation of mRNA and protein abundance in quadriceps muscle will be presented.

5.4.3 Conclusion

Patients with COPD had significantly impaired exercise capacity compared to healthy age-matched volunteers, but the magnitude of the deficit is smaller than that previously reported in the literature possibly due to better matching of the control group for physical activity status. Moderate intensity aerobic exercise for eight weeks increased $\dot{V}O_2^{PEAK}$ in healthy older volunteers but not in patients with COPD although physiological adaptation was apparent in the COPD group, particularly in response to submaximal exercise. This is likely due to limits imposed on the training intensity caused by constrained ventilatory capacity which restricts the training load applied to the exercising muscle. Training adaptations were not fully reversed by four weeks exercise cessation in the healthy group whereas patients with COPD achieved a lower $\dot{V}O_2^{PEAK}$ in incremental testing after exercise withdrawal.

Evidence presented in this chapter highlights differing cardiorespiratory adaptations to training and exercise withdrawal in patients with COPD compared to healthy controls.

Chapter 6 will investigate the influence of aerobic training and subsequent exercise withdrawal on mRNA and protein abundance in quadriceps muscle. Given the differences in response at a whole-body level it will be interesting to discover what influence the presence of COPD has on the muscle molecular responses to the same intervention.

Chapter Six

Influence of COPD on the responses to aerobic training and subsequent detraining: skeletal muscle molecular responses

6.1 Introduction

In Chapters 3 & 4 evidence is presented demonstrating that young and older healthy volunteers make similar whole-body physiological and skeletal muscle molecular adaptations to aerobic training. Data presented in Chapter 5 show that the whole-body response to aerobic exercise training is blunted in patients with COPD compared to healthy people of a similar age. Skeletal muscle dysfunction is a common feature of COPD (Maltais, Simard et al. 1996; Bernard, LeBlanc et al. 1998; Barnes and Celli 2009) and there is some evidence that changes in mRNA abundance in response to acute exercise or prolonged training may be altered in patients compared to healthy controls (Radom-Aizik, Kaminski et al. 2007; Mercken, Hageman et al. 2011; Turan, Kalko et al. 2011). Molecular responses to high-intensity resistance exercise training have been shown to be blunted in COPD compared to healthy controls, associated with lower absolute training intensities performed by patients with COPD (Constantin, Menon et al. 2013). There have previously been no studies examining the skeletal muscle molecular response to aerobic training at matched relative intensities in COPD and sedentary healthy individuals of a similar age and the time course of muscle molecular responses in unknown. Given that patients with COPD did not increase $\dot{V}O_2^{PEAK}$ in response to moderate intensity aerobic training (described in Chapter 5) it is of interest to discover whether the changes in abundance of mRNA and protein targets in quadriceps muscle during the same training period are blunted, or are perhaps as in the case of the resistance exercise training study by Constantin *et al*, related to the absolute training intensity.

There have previously been no investigations of the influence of exercise withdrawal on muscle molecular responses in COPD. It is of interest to discover whether detraining affects mRNA abundance in the muscles of patients with COPD in a similar manner to

healthy individuals, or whether there is a more pronounced effect in COPD. The molecular response of skeletal muscle to inactivity is relevant in the context of maintaining the effects of pulmonary rehabilitation and in the context of acute deconditioning during periods of exacerbation of COPD (Bolton, Bevan-Smith et al. 2013).

In Chapter 5 patients with COPD demonstrated no increase in peak oxygen uptake after eight weeks of moderate intensity aerobic exercise training in contrast to the significant increases observed in the age-matched healthy control group. The hypothesis tested in this chapter is that mirroring whole-body physiological changes, the magnitude of change in abundance of targeted skeletal muscle mRNA transcripts in response to aerobic exercise training will be less in patients with COPD compared to healthy controls. Further research objectives are to examine the following:

1. Whether any changes in abundance of mRNA transcripts during the eight week aerobic training programme follow the same time course of response and predict the same changes in biological functions in patients with COPD compared to those observed in the healthy controls.
2. Assess whether any differences between patients with COPD and healthy older adults in the biological functions influenced by exercise training associate with differences in whole-body physiological adaptations.
3. Compare the effects of exercise withdrawal on muscle mRNA abundance in patients with COPD and healthy age-matched volunteers.
4. In Chapter 4, highly up- and down-regulated mRNA transcripts were found to relate poorly to changes in abundance of their protein products in healthy older adults in response to exercise training and withdrawal. This chapter will determine whether there is a relationship between mRNA and protein abundance in patients with COPD.

In order to address this hypothesis and research objectives, data are reported from mRNA and protein analyses of muscle tissue samples obtained from patients with COPD and healthy age-matched volunteers (Older HC Group) who undertook an eight week aerobic cycle exercise training programme and subsequent four week exercise

withdrawal period. Data relating to the whole-body physiological outcomes for these subjects are reported in Chapter 5 and mRNA and protein data for the older heathy control group have been discussed previously in comparison to young healthy volunteers in Chapter 6.

6.2 Methods

6.2.1 Participants

These data arise from analysis of *vastus lateralis* muscle samples obtained from patients with COPD and age-matched healthy volunteers (all aged between 60 and 80 years) undergoing the MATCH Study of aerobic training and subsequent detraining. Subject characteristics and whole-body exercise outcomes are described in Chapter 5.

6.2.2 Study Design

An overview of the protocol is found in Chapter 2.4.1. Skeletal muscle biopsies were performed on the *vastus lateralis* muscle of the dominant leg in the resting state (24 h after exercise during training period) and following a fast of $\geq$ 4 hours (see Chapter 2). Biopsies were performed at baseline; after 1, 4 and 8 weeks of training; and after 4 weeks exercise withdrawal.

6.2.3 Measures

Abundance of mRNA was quantified using a RT-PCR technique (Chapter 2.10). Targets for gene expression analysis were the same as those described in Chapter 4.2.3 having been selected based on data generated within the research group for mRNA abundance changes (targeted RT-PCR and Affymetrix gene chips) observed in quadriceps muscle biopsy samples from healthy young and older volunteers in response to a single bout of acute exercise and several weeks of limb immobilisation (data unpublished). Additionally, searches of SA Biosciences and IPA databases were performed. The list of 96 mRNA transcripts quantified (including 2 housekeeping genes) can be found in Appendix 6.

As for the ageing comparison described in Chapter 4, protein targets were chosen by ranking gene transcripts in order of magnitude of fold-change from baseline (for both

up- and down-regulation) and selecting those with the largest fold-change. Data from all time points in the full MATCH Study dataset, including young healthy volunteers were used to select protein targets. Antibody optimisation and Western blot analysis were performed by an experienced post-doctoral fellow in the research team with all calculations and data analysis performed myself. Nine separate protein targets, plus two phosphorylated isoforms were quantified. The Western blot protocol and antibody specifications can be found in Appendix 7.

6.2.4 Data Analysis

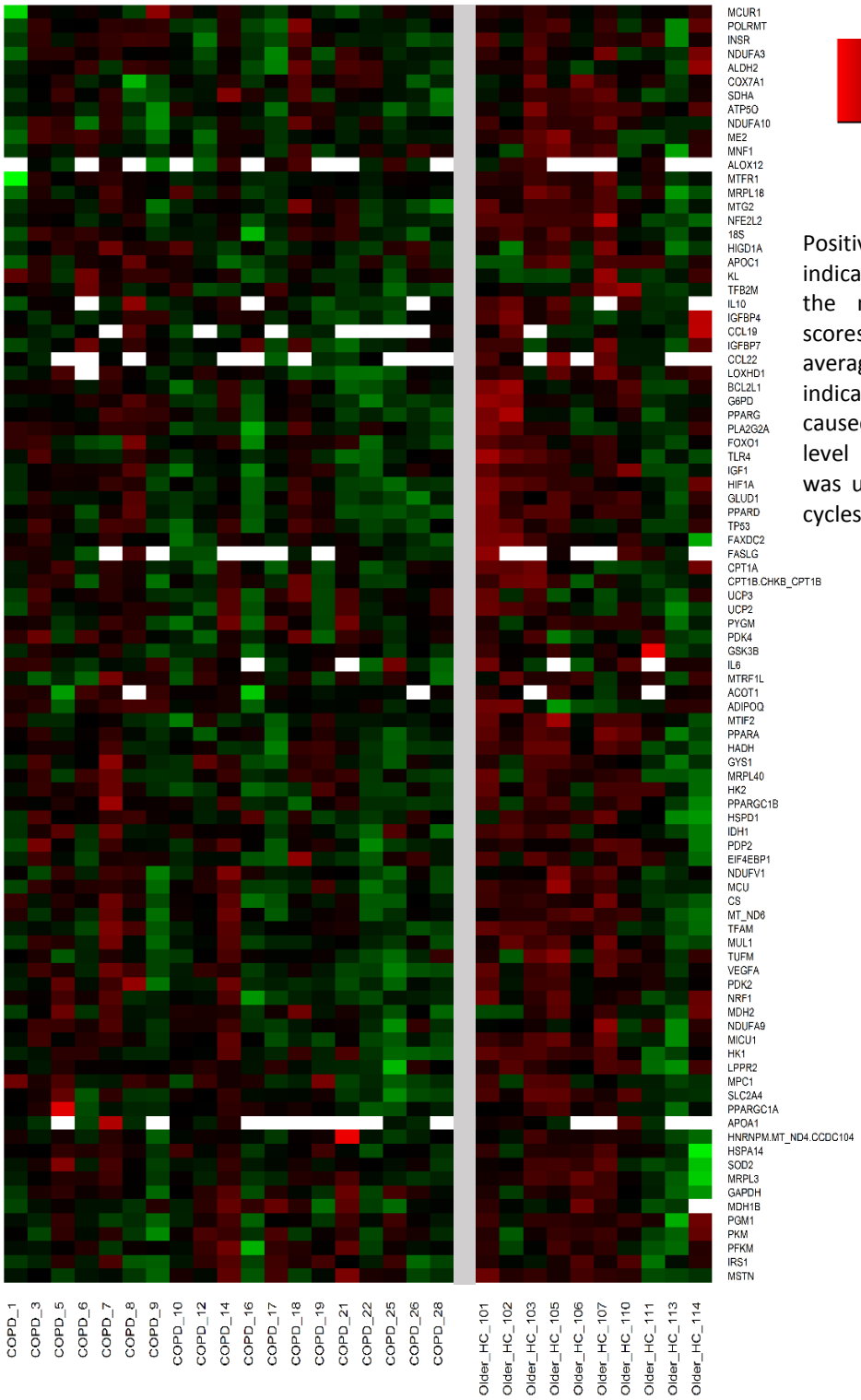
As for Chapter 4, baseline mRNA abundance was compared between COPD and Older groups both qualitatively using heatmaps and quantitatively using unpaired t-tests on Δ_{CT} values with the FDR adjustment applied to control for multiple comparisons. Change in mRNA abundance over time is expressed as fold-change from baseline calculated using the $\Delta\Delta_{CT}$ method (Livak and Schmittgen 2001). Bioinformatics analysis was performed using Ingenuity Pathway Analysis (IPA®; QIAGEN Redwood City, USA, www.qiagen.com/ingenuity) in order to identify networks of genes that represent discreet biological functions. Protein abundance at baseline is corrected for abundance of the housekeeping protein (actin for cytoplasmic or lamin for nuclear proteins) and reported as arbitrary units. Change in protein abundance with time is expressed as fold change from baseline with statistical tests performed on absolute values (arbitrary units). In order to reduce the number of statistical comparisons, protein fold-change data were analysed at baseline, eight weeks (i.e. pre- and post-training) and twelve weeks (after exercise withdrawal) only.

6.3 Results

6.3.1 mRNA Abundance

6.3.1.1 Baseline

Quantitative comparison of baseline of Δ_{CT} scores did not reveal a statistically significant difference for any of the mRNA targets (all $p > 0.05$, see Appendix 13) for results of statistical analysis. Baseline abundance of mRNA transcripts is shown in Figure 6.1, and demonstrates considerable inter-subject variability in the abundance of transcripts. Transcript abundance lower than the pooled mean for all subjects is indicated by a red hue. Visual inspection of the heatmaps (Figure 6.1) suggests that for the majority of transcripts, a greater proportion of Older HCs appear to have below average abundance of mRNA transcripts compared to patients with COPD. mRNA transcripts that were expressed at a level below the lower limit of detection (shown as a white block in the heatmap) in > 30% participants include ALOX12, CCL22, FASLG and APOA1.



Positive scores (green) indicate expression above the mean and negative scores (red) indicate below average expression. Blanks indicate missing data caused by an expression level of target gene that was undetectable after 40 cycles of PCR.

Figure 6-1. Muscle mRNA abundance in vastus lateralis tissue from COPD & Older HC groups at baseline. Values on the colour key indicate Δ_{CT} values (normalised to HMBS housekeeping gene) transformed to z-scores (i.e. number of standard deviations above or below the pooled group mean). X axis displays individual mRNA transcripts, Y axis displays individual subjects arranged by group.

6.3.1.2 Influence of Exercise Training and Exercise Withdrawal on mRNA Abundance

The number of transcripts up-regulated or down-regulated compared to baseline in quadriceps biopsy samples at 1, 4 and eight weeks into the training programme and after four weeks of exercise withdrawal are shown in Table 6.1. The magnitude of change in abundance at the same time points is shown in Figures 6.2 to 6.5. Changes in the abundance of mRNA targets from baseline at one, four and eight weeks into the aerobic training intervention and after four weeks exercise withdrawal (12 week time point) is shown in Figures 6.2 to 6.5 with blue arrows highlighting the transcripts which were subsequently quantified at the protein level.

In both patients with COPD and Older HCs change in abundance was apparent after one week of aerobic training. Transcripts that were upregulated at one week broadly remained so at all subsequent time points, including after the exercise withdrawal period although there is considerable inter-subject variation. Increase in abundance ($2^{-\Delta\Delta CT} > 1$) was more pronounced than decrease in abundance ($2^{-\Delta\Delta CT} < 1$). The response in COPD and Older HC groups was broadly similar in terms of the direction and timing of change in abundance of individual targets.

Table 6-1. Number of transcripts up- or down-regulated throughout training and exercise withdrawal, relative to baseline in COPD & Older HC groups

		Number of transcripts			
		Total up-regulated	Total down-regulated	> 2 fold up-regulated	< 0.5 fold down-regulated
Older Group	1 week training	46	37	14	3
	4 weeks training	47	38	6	1
	8 weeks training	52	34	15	0
	4 week exercise withdrawal	52	34	7	0
COPD Group	1 week training	44	40	8	1
	4 weeks training	38	47	7	0
	8 weeks training	66	20	4	0
	4 week exercise withdrawal	69	18	18	0

Number of transcripts for which the group median indicates up-regulation ($2^{-\Delta\Delta CT} > 1$) or down-regulation ($2^{-\Delta\Delta CT} < 1$) at that time point relative to the baseline sample. Up-regulation of > 2 fold or < 0.5 fold change indicate the number of transcripts which more than doubled or halved in abundance. N.B. Some transcripts were not expressed at all time points.

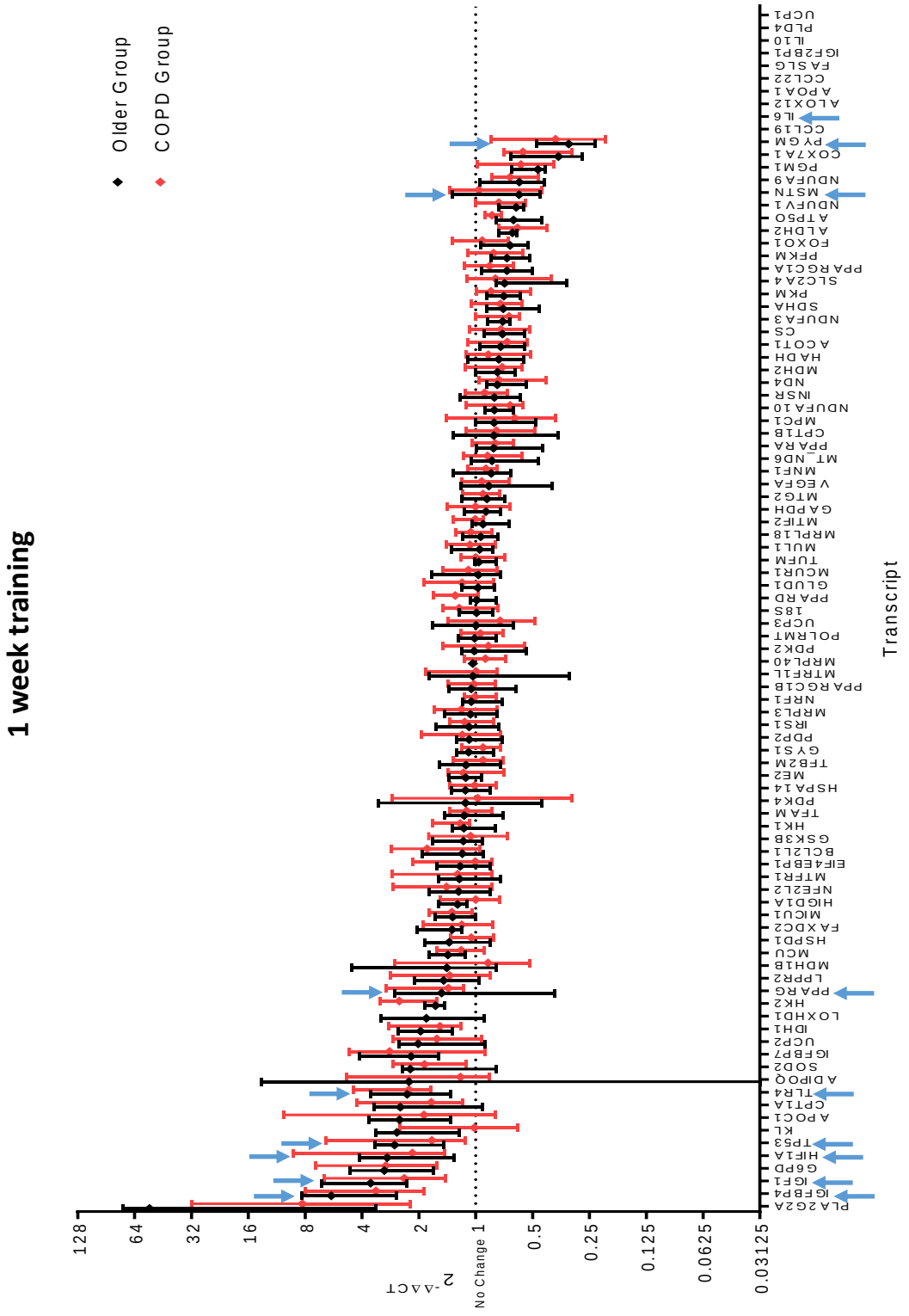


Figure 6-2. Change in muscle mRNA abundance in COPD & Older HC groups after 1 week of aerobic training relative to baseline. Data are median, IQR. Targets with missing data were below the lower limit of detection. Arrows highlight transcripts whose protein products were subsequently quantified by Western blotting.

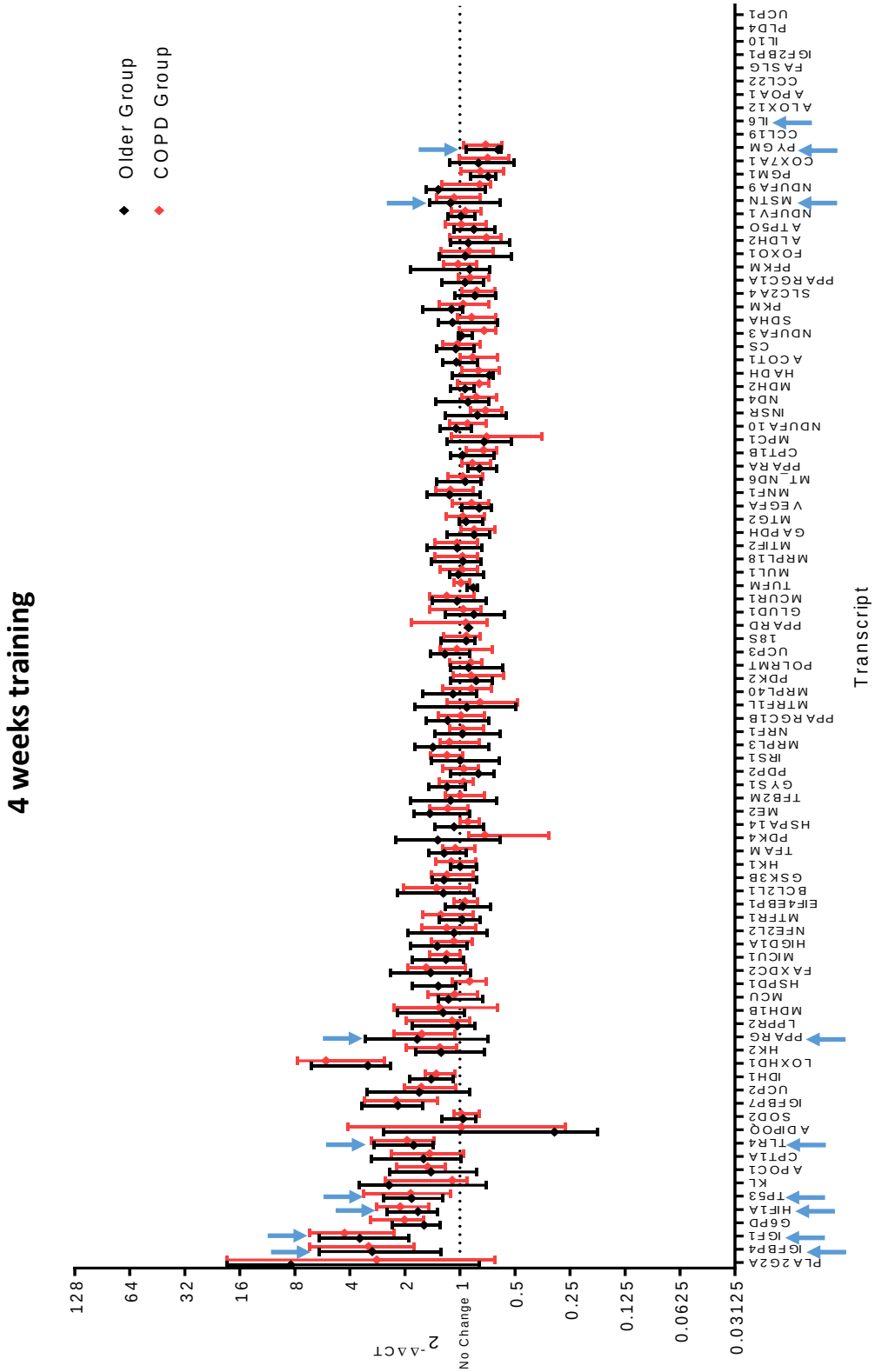


Figure 6-3. Change in muscle mRNA abundance in COPD & Older HC groups after 4 weeks of aerobic training relative to baseline. Data are median, IQR. Targets with missing data were below the lower limit of detection. Arrows highlight transcripts whose protein products were subsequently quantified by Western blotting.

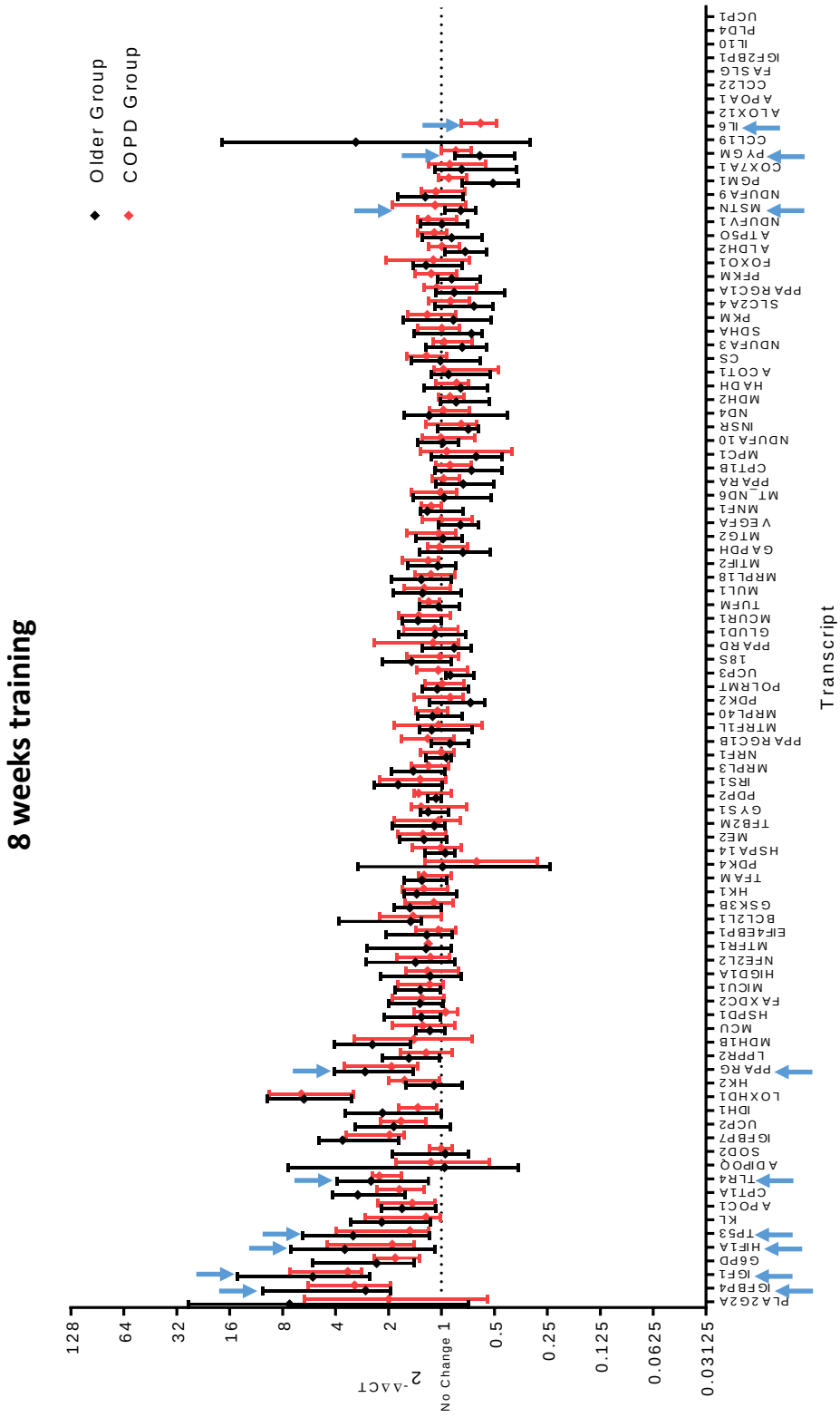


Figure 6-4. Change in muscle mRNA abundance in COPD & Older HC groups after 8 weeks of aerobic training relative to baseline. Data are median, IQR. Targets with missing data were below the lower limit of detection. Arrows highlight transcripts whose protein products were subsequently quantified by Western blotting.

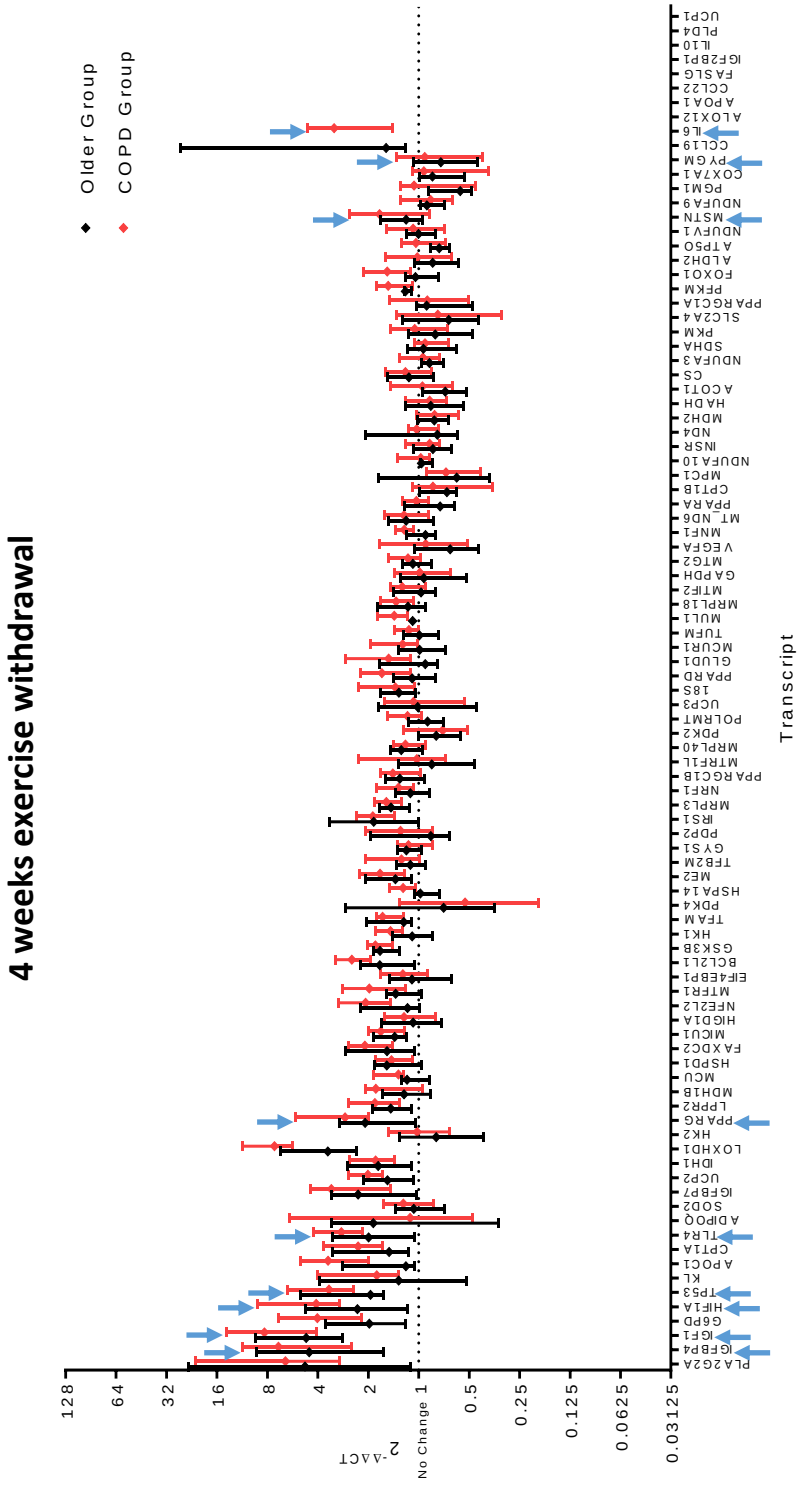


Figure 6-5. Change in muscle mRNA abundance in COPD & Older HC groups after 4 weeks exercise withdrawal (subsequent to 8 weeks aerobic training) relative to baseline. Data are median, IQR. Targets with missing data were below the lower limit of detection. Arrows highlight transcripts whose protein products were subsequently quantified by Western blotting

Bioinformatics analysis using IPA revealed that the same eight biological functions were significantly influenced in COPD as had previously been identified in the Older HC group and described in Chapter 4. These functional networks were: *Energy Production*; *Lipid Metabolism*; *Connective Tissue Development and Function*; *Free Radical Scavenging*; *Carbohydrate Metabolism*; *Skeletal and Muscular System Development and Function*; *Skeletal and Muscular Disorders*; and *Inflammatory Response* (Figure 6.6). All of the identified biological functions were highly significant (–log(p-value) > 1.3 is equivalent to $p < 0.05$) indicating a strong likelihood of a biologically meaningful change in function. The influence of exercise training was apparent early (after one week) and was not diminished by exercise withdrawal. The pattern of influence was very similar in COPD and Older HC groups (Figure 6.6 a & b).

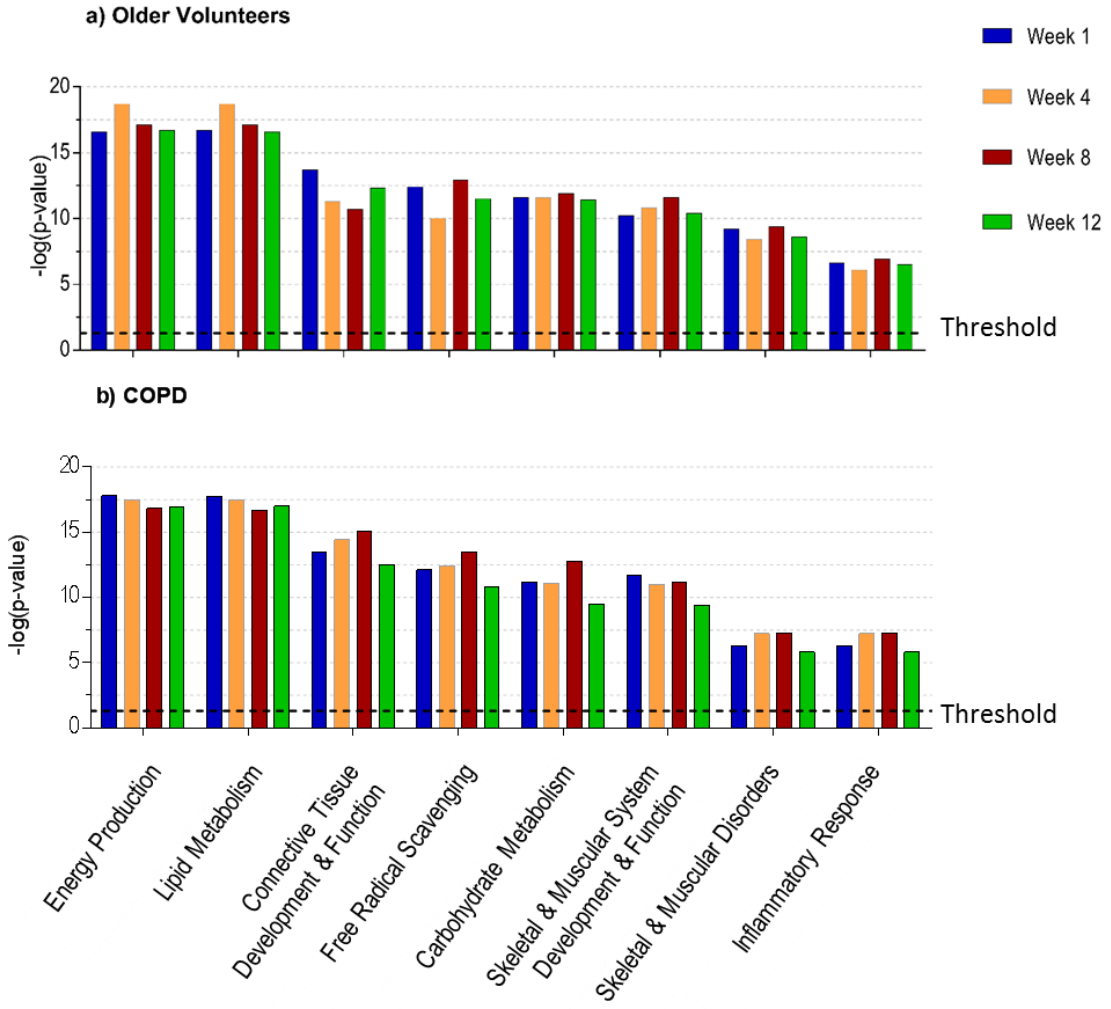


Figure 6-6. Muscle biological functions significantly altered from baseline by exercise over the course of an 8 week endurance cycling training intervention and subsequent 4 week detraining period (week 12) as identified by IPA in a) Older HC and b) COPD Patients. Y axis indicates the probability that a biological function is significantly altered from baseline. Dashed line denotes threshold of statistical significance (-log(p-value) of 1.3 is equivalent to p = 0.05).

6.3.2 Protein Expression

6.3.2.1 Baseline Protein Expression

Protein expression at baseline corrected for abundance of the housekeeping protein (actin or lamin) and reported as arbitrary units is presented in Table 6.2. Abundance of HIF1α and IL6 was significantly higher in the COPD compared to the Older HC group at

baseline (p = 0.007, and p = 0.003 respectively). MSTN protein expression was higher in the Older HC group (p = 0.003). There were no further between group differences.

Table 6-2. Baseline protein expression in resting, fasted vastus lateralis tissue in COPD & Older HC groups.

Protein	Older HC Group		COPD Group		*p*
	Mean	SD	Mean	SD	
HIF1 α	0.01	0.01	0.05	0.05	0.007
IGF1	0.05	0.04	0.16	0.38	NS
p53	0.05	0.04	0.08	0.08	NS
IGFBP4	1.59	1.17	1.68	1.79	NS
(p)IGF1	0.15	0.14	0.13	0.14	NS
(p)p53	0.10	0.11	0.14	0.12	NS
IL6	0.22	0.19	0.71	0.68	0.003
TLR4	0.09	0.11	0.06	0.05	NS
PPARγ	0.28	0.38	0.17	0.21	NS
PYGM	1.51	1.21	0.97	0.70	NS
MSTN *	2.28	1.23	0.95	1.04	0.003

Protein content normalised for actin (apart from *MSTN, normalised to lamin). Arbitrary units: Optical Density (OD) target protein / OD actin. HIF1 α, hypoxia inducible factor 1 α; IGF1, insulin-like growth factor 1; p53, (tumour) protein 53; (p)p53, phosphorylated p53; IGFBP4, insulin-like growth factor binding protein 4; (p)IGF1, phosphorylated IGF1 receptor; IL6, interleukin 6; TLR4, toll-like receptor 4; PPARγ, peroxisome proliferator activated receptor γ; PYGM, glycogen phosphorylase (muscle); MSTN, myostatin. Between group differences tested with Mann-Whitney U test.

6.3.2.2 Influence of Aerobic Training and Exercise Withdrawal on Protein Expression

Representative images of Western blots from two subjects are shown in Figure 6.7. There was a small but statistically significant (p < 0.05) decrease in the abundance of MSTN (myostatin) during the eight week training period in the Older HC group (Figure 6.7, k). There were no significant changes in expression during training or exercise withdrawal for any of the targets measured in the COPD group, nor when the groups were collapsed to increase statistical power (all p > 0.05). As in the Older HC group, the individual protein expression responses to aerobic training and exercise withdrawal in

the COPD group were highly variable (Figure 6.8). This variability was not influenced by the individual baseline starting value (see figures in Appendix 14).

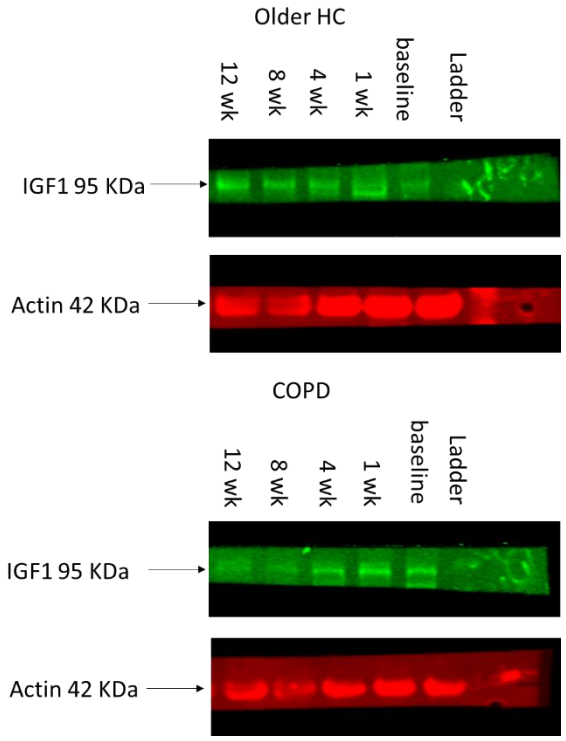


Figure 6-7. Representative Western blot images for one subject from the Older HC group and one subject from the COPD group for the target IGF1 (insulin-like growth factor) and actin loading control.

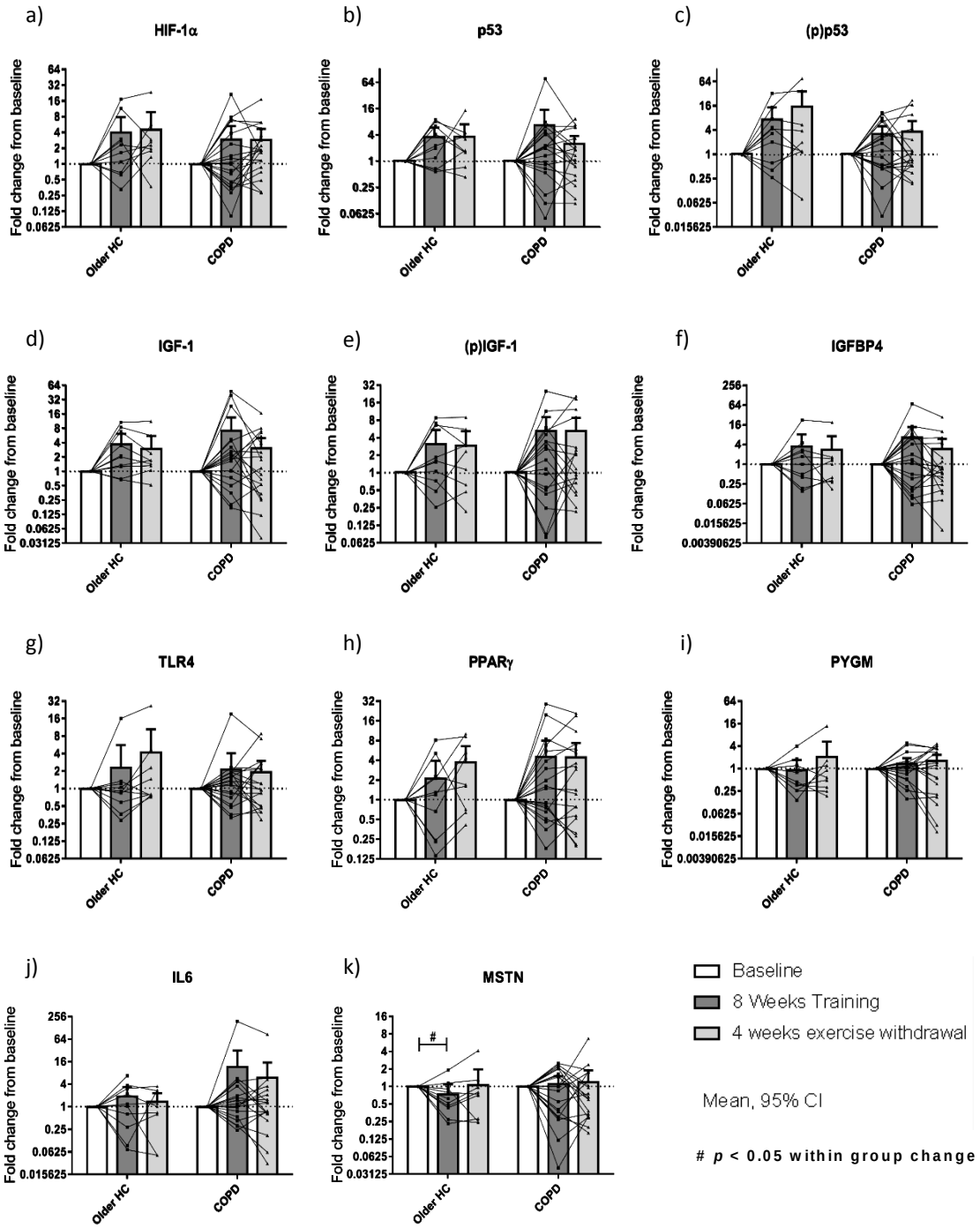


Figure 6-8. Change in muscle protein expression with exercise training and exercise withdrawal in COPD & Older HC groups. HIF-1α, hypoxia inducible factor 1 α; p53, (tumour) protein 53; (p)p53, phosphorylated p53; IGF1, insulin-like growth factor 1; (p)IGF1, phosphorylated IGF1 receptor; IGFBP4, insulin-like growth factor binding protein 4; TLR4, toll-like receptor 4; PPARγ, peroxisome proliferator activated receptor γ; PYGM, glycogen phosphorylase (muscle); IL6, interleukin 6; MSTN, myostatin. Bars represent group mean (95% CI), lines represent individual subject responses.

Due to the large inter-subject variability in protein abundance responses to exercise training and subsequent exercise withdrawal an attempt was made to identify defining characteristics of subjects that had experienced up- or down-regulation of individual proteins. The dichotomised groups were compared for age and BMI; groups with up- or down-regulation of proteins during exercise training were compared for baseline $\dot{V}O_2^{PEAK}$ and Peak Work; training intensity and metabolic stress during training (training intensity, W; baseline sub-maximal test RER; HR; and $\dot{V}E$ %MVV) as well as $\Delta\dot{V}O_2^{PEAK}$; groups defined by up- or down-regulation of proteins during exercise withdrawal were contrasted for $\Delta\dot{V}O_2^{PEAK}$ during that period.

Within the Older group, separation of individuals according to up- or down-regulation of the protein targets revealed no difference in the characteristics of individuals who had experienced an increase compared to a decrease in abundance of any of the proteins measured during the training or detraining period. Within the COPD group during the training period, patients who experienced reduced abundance of: IGF-1 were older (76.0 + 3.2 vs 68.1 $\pm$ 5.4 years, p = 0.014); p53 had lower W^{PEAK} at baseline (55.1 + 20.1 vs 82.4 $\pm$ 35.4 W, p = 0.045); and PPARγ had lower BMI (24.3 + 4.3 vs 32.3 $\pm$ 5.8 kg/m^2, p = 0.003) than those who experienced an increase in these proteins. When the COPD and Older HC groups were collapsed the only difference that reached statistical significance was that individuals in whom PPARγ was down regulated during the training period had significantly lower BMI at baseline (25.9 + 4.3 vs 30.8 $\pm$ 5.6 kg/m^2, p = 0.007).

During the exercise withdrawal period, individuals in the COPD group who experienced a reduction in abundance of PPARγ were significantly younger compared to those in whom the abundance of PPARγ increased (67.2 $\pm$ 5.6 vs 72.8 $\pm$ 5.4 years, p = 0.043). When the COPD and Older HC groups were collapsed, data for the detraining period showed that individuals with reduced IGFBP4 expression were significantly younger than those with increased IGFBP4 expression (68.1 $\pm$ 5.2 vs 73.5 $\pm$ 4.8 years, p = 0.009).

6.3.3 Gene – Protein Associations

In the Older HC group there was no correlation between change in mRNA abundance and change in protein abundance for any of the targets measured. In the COPD group there was a strong correlation between change in IL6 mRNA and protein expression from

week 0 – 1 (r_s = 0.736, p = 0.01) and a moderate correlation between mRNA and protein fold change for PYGM between week 8 – 12 (r_s = 0.456. p = 0.05).

When COPD and Older HC groups were combined moderate correlations emerged between mRNA and protein fold change between week 0- 1 for PYGM (r_s = 0.405, p = 0.04); and week 0 – 4 for IL6 (r_s = 0.503, p = 0.034). In the collapsed groups there was also a significant negative correlation between change in mRNA and protein abundance of IGFBP4 between week 0- 1 (r_s = -0.530, p = 0.005).

A large number of correlations were tested on this dataset without a control for type 1 error, thus the results should be interpreted cautiously and with reference to the scatter plots (Figure 6.9).

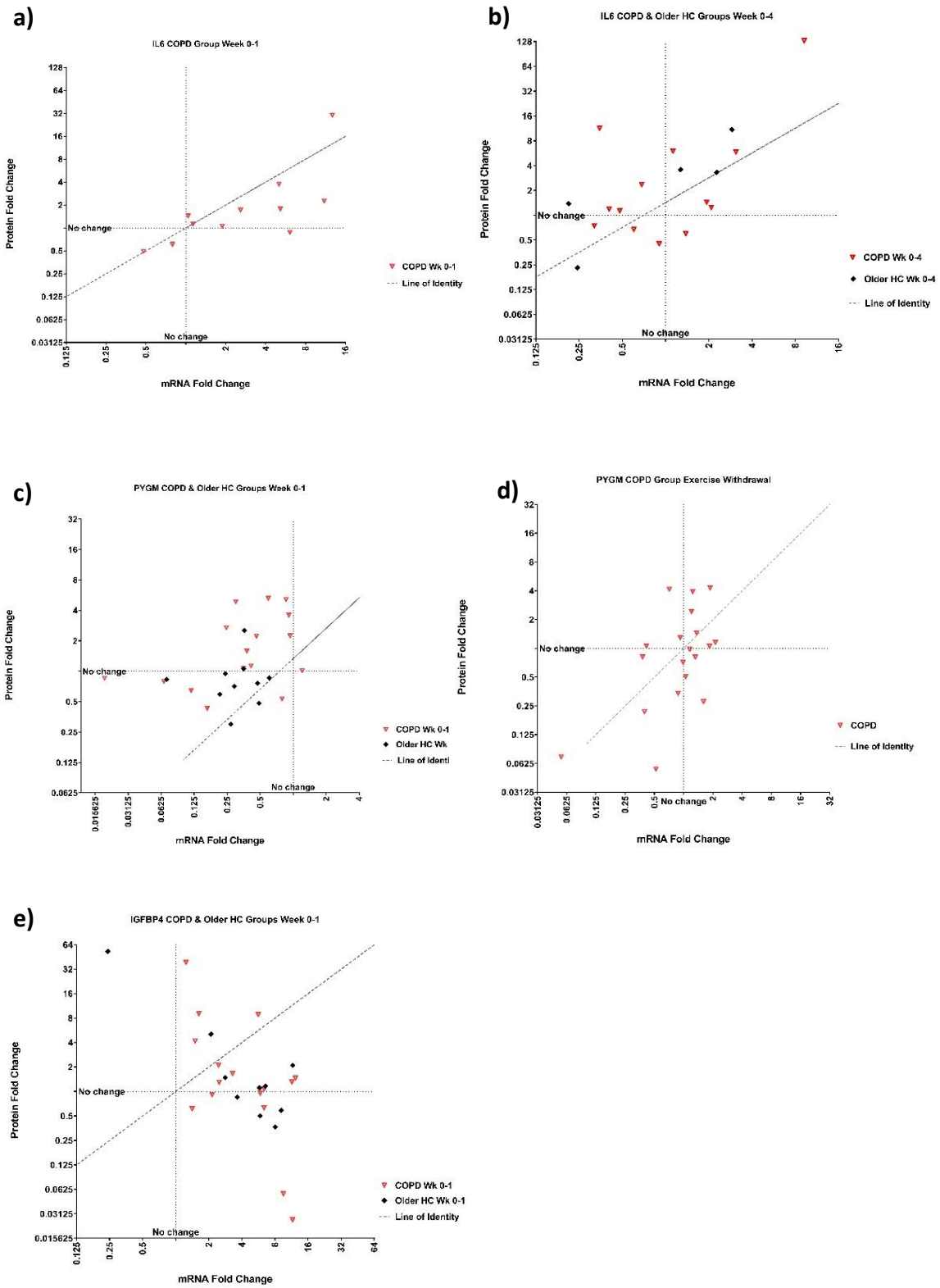


Figure 6-9. Significant muscle mRNA & protein product fold change correlations in COPD & Older HC groups. Line of identity demonstrates the trajectory of a perfect positive correlation.

6.4 Discussion

In patients with COPD and healthy age-matched controls, change in quadriceps mRNA abundance of targeted genes in response to aerobic training followed parallel time course and magnitude of change with the predicted influence on biological functions being almost indistinguishable between groups. The clear similarities in the response to aerobic training at a muscle mRNA level occurred despite significantly different whole-body cardiorespiratory responses in the two groups where $\dot{V}O_2^{PEAK}$ increased by 18% in the Older healthy group but did not change in the COPD group, suggesting a disassociation between muscle molecular responses and maximal aerobic capacity in COPD. Biological functions impacted by aerobic training (e.g. *Energy Production, Lipid Metabolism*) do have associations with cardiorespiratory adaptations observed in sub-maximal exercise testing where significant reductions in RER were observed at sub-maximal isowork in both COPD and healthy Older volunteers in the trained state indicating a shifting balance away from carbohydrate oxidation and towards lipid oxidation in contracting muscle. Changes in mRNA abundance prompted by training persisted throughout four subsequent weeks of exercise withdrawal in patients with COPD as they did in the healthy Older group despite a significant decline in peak oxygen uptake in the COPD group over the same period. None of the protein targets quantified changed in abundance in the COPD group during the exercise training or withdrawal period.

6.4.1 Baseline Comparisons

There were no statistically significant differences in the abundance of targeted mRNA transcripts in the *vastus lateralis* of COPD and Older heathy groups at baseline. There have been previous descriptions of differential gene expression in the quadriceps of patients with COPD compared to healthy controls: Debigaré *et al* (Debigare, Maltais et al. 2008) identified 57 transcripts that were differentially expressed in four COPD patients compared to four healthy controls using an Affymetrix microarray approach with subsequent verification using PCR highlighting no difference in IL6 expression (similar to the findings in this chapter) and differential expression of FOXO1 and IGF-1 (contrasting with the findings presented here). Tényi *et al* identified differences

between patients with COPD and healthy individuals in network modules identified in muscle mRNA microarray data which they described as showing abnormalities in skeletal muscle energy metabolism (Tenyi, Cano et al. 2018). An important factor to consider is that the work of Debigaré *et al* selected patients with COPD and muscle wasting, whereas the patients in this thesis did not have depleted muscle mass (Table 5.1 in Chapter 5). Tényi *et al* also found that COPD patients with low fat-free mass were less like the heathy controls than those with preserved muscle mass. Other work quantifying abundance of a small number of mRNA transcripts in quadriceps muscle (targeted by other authors during investigation of specific pathways and also measured in this study) has found that compared to healthy controls, patients with COPD had higher expression of HIF-1α (Remels, Gosker et al. 2015) and PFKM (van den Borst, Slot et al. 2013); and lower expression of PGC-1 α (van den Borst, Slot et al. 2013). Expression of HIF-1α mRNA trended towards being higher in patients with COPD in this study but failed to reach the threshold for statistical significance after correction for multiple testing (see Appendix 13). Additionally, the older group were selected to be physically inactive (average ~6000 steps per day) which fits with evidence that habitual physical activity levels or low muscle mass may be more influential that degree of airflow obstruction (Remels, Schrauwen et al. 2007; Rabinovich, Drost et al. 2015) in determining quadriceps gene expression.

6.4.2 mRNA responses to aerobic exercise training

Data presented in Chapter 3 show that Older and Young healthy volunteers made similar whole-body cardiorespiratory adaptations to eight weeks aerobic exercise (increased $\dot{V}O_2^{PEAK}$) and further to this, the response of mRNA transcripts in skeletal muscle selected due to their known responsiveness to exercise in young healthy populations and biological functions predicted to be influenced were the same (Chapter 4). Given that patients with COPD did not increase their peak oxygen uptake in response to the same moderate intensity aerobic training intervention in this study (Chapter 5) it is striking that the time course and magnitude of changes in *vastus lateralis* mRNA abundance closely matched those observed in Older Healthy volunteers (Figures 6.2 to 6.5). This finding differs to previous studies that have compared the mRNA responses to aerobic training in COPD and health which have concluded that there is a blunted or

dysfunctional response in patients with COPD. Previously published studies examining the impact of aerobic exercise training on mRNA abundance have concluded that mRNA responses in patients with COPD differ to those in healthy controls. One study reported blunted activation of pathways related to tissue remodelling and bioenergetics in COPD despite patients in that study outperforming healthy controls for improvement in $\dot{V}O_2^{PEAK}$ (Turan, Kalko et al. 2011). Another study reported cardiorespiratory adaptations to training in patients with COPD similar to those in the MATCH study reported in this thesis (no change in $\dot{V}O_2^{PEAK}$, but increased submaximal performance) but found a narrower response in muscle mRNA expression in patients with COPD (Radom-Aizik, Kaminski et al. 2007). Marín de Mas *et al* (Marín de Mas, Fanchon et al. 2016) employed a computational model-driven approach analysing data from three previous studies (Sala, Roca et al. 1999; Turan, Kalko et al. 2011; Rodriguez, Kalko et al. 2012) concluding that muscle mRNA responses are abnormal in COPD with possible effects on tissue remodelling and energy metabolism. Another report describes a blunted muscle mRNA response for genes associated with inflammation downstream of NFκB in a study of acute exercise (Mercken, Hageman et al. 2011). The findings from the MATCH Study contrast with this evidence from the literature. There are a number of possible explanations for these differences. In some studies the training workload performed by COPD and HC groups is not well described (Turan, Kalko et al. 2011; Marín de Mas, Fanchon et al. 2016) making it impossible to conclude whether differences in quadriceps gene expression result from the presence of COPD or the application of a different stimulus to the muscle. In the study reported here great care was taken to match relative workloads in the COPD and Older HC group and all training sessions were supervised. Whilst it is possible that a ventilatory limitation to peak exercise influenced training load at the muscle in this study (as discussed in Chapter 5) the intensity and duration of training was clearly sufficient to stimulate mRNA signalling in COPD patients to the same extent as in Older HCs indicating adaptive responses at the level of the muscle. Another analysis of mRNA microarray data from three previous studies concluded that network modules identified as influenced by aerobic training in COPD were distinct to those in health with COPD patients notably not experiencing an impact on oxidative phosphorylation or associated bioenergetics pathways (Tenyi, Cano et al. 2018), however the authors acknowledge that the response in COPD patients with normal fat-

free mass was more similar to healthy control subjects than that it was in patients with muscle wasting. The similarities observed in mRNA responses in COPD and Older HC groups in this chapter may in part be underpinned by the relatively well preserved lean mass of the COPD group. None of the previous training studies (Radom-Aizik, Kaminski et al. 2007; Turan, Kalko et al. 2011; Marín de Mas, Fanchon et al. 2016) report the time course of molecular adaptations to aerobic training (with one an acute study of only two hours duration (Mercken, Hageman et al. 2011), whereas the MATCH Study reports after one, four and eight weeks of training, with consistent results throughout. Another potential source of variation is that biopsies were performed in the fasted state for the MATCH Study whereas the feeding status of subjects in other studies is often not reported. Finally, and importantly, the selection of gene targets in the MATCH study differs to microarray analyses where a large portion of the genome is analysed. The MATCH Study can only inform us for the targets studied (that were selected due to known responsiveness in young healthy individuals), however the greater sensitivity and range of the PCR technique used allows detection of change in abundance that would be missed using a high-throughput microarray technique. Other studies that have employed a PCR technique selected different gene targets (e.g. (Mercken, Hageman et al. 2011)) and as such are not directly comparable with the results of this study.

The biological functions predicted by IPA to be influenced early (at 1 week) and throughout the training intervention (at 4 and 8 weeks) in Older healthy volunteers were found to relate closely to changes in cardiorespiratory measures (as discussed in Chapter 4): the *Lipid Metabolism, Energy Production* and *Carbohydrate Metabolism* functions all relate to altered fuel selection by mitochondria in the exercising muscle evidence of which was apparent with reduced RER at iso-work in the trained state and increased maximal mitochondrial ATP production rate (Figure 6.10, data courtesy of B Popat). Although patients with COPD did not increase $\dot{V}O_2^{PEAK}$ (likely due to a ventilatory limitation, as discussed in Chapter 5), they also demonstrated a significant reduction in RER at iso-work during submaximal testing in the trained state (Figure 5.5), suggesting that not only did they experience an effect of aerobic training but also a modification in mitochondrial fuel selection similar to that experienced by the Older healthy control group and in keeping with the predicted functional influence of changes in mRNA

abundance. In contrast to data from Older healthy controls however, maximal mitochondrial ATP production rate did not change in patients with COPD during the 8 week training period (Figure 6.10). The mitochondrial ATP production rate data relate to the maximal rate of ATP production in *ex vivo* isolated mitochondria stimulated with saturating concentrations of ADP and substrate. Training adaptations in patients with COPD were only apparent during sub-maximal constant work rate testing (reduced RER and HR). It is feasible that the measure of maximal mitochondrial function relate better to maximal exercise capacity (when ATP demand is greatest) and that training adaptations in fuel selection during sub-maximal exercise demonstrated by patients with COPD were underpinned by adaptations other than maximal mitochondrial ATP production rate, for example intramuscular lipid content, intramyocellular lipid droplet location and mobilisation of adipose and muscle lipid stores (Chee, Shannon et al. 2016). Adaptation in maximal ATP production rate of mitochondria may itself be dependent on the intensity of aerobic training with the absence of change in the COPD group relating to the absolute training intensity performed.

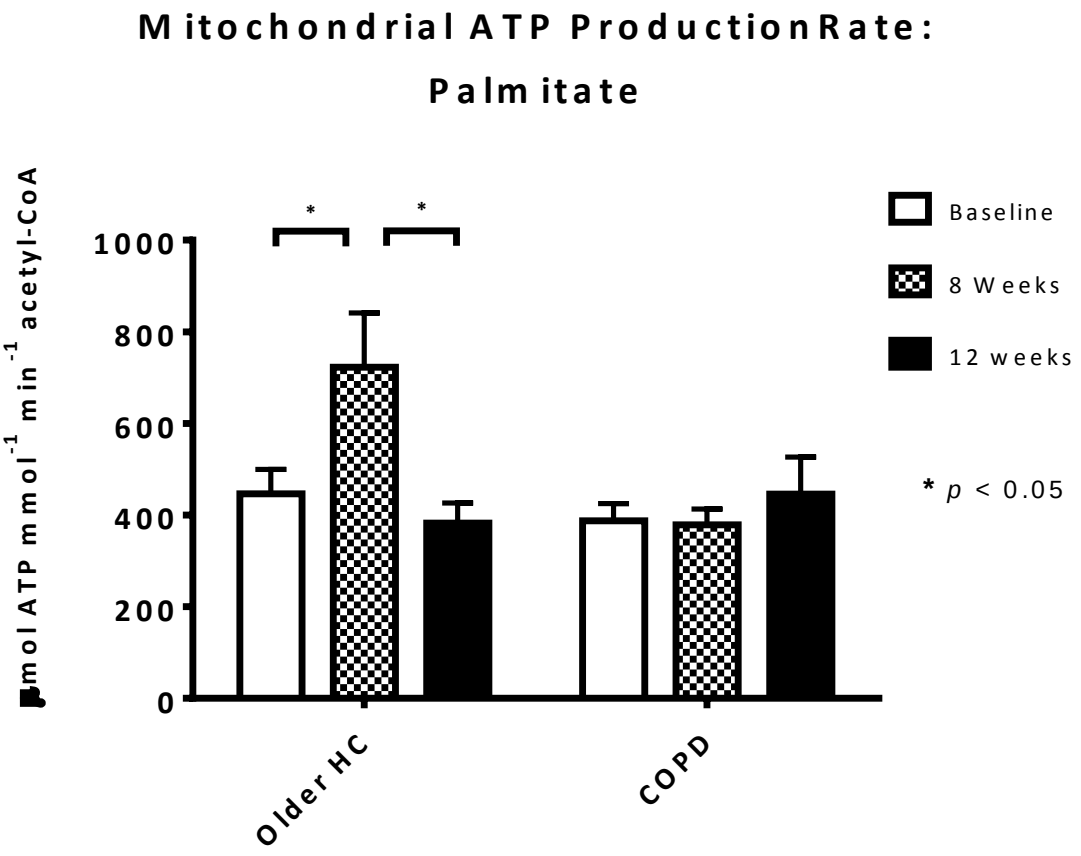


Figure 6-10. Maximal mitochondrial ATP production rate in COPD & Older HC groups (reproduced from unpublished work by B Popat)

The functions *Connective Tissue Development and Function*; *Skeletal and Muscular System Development and Function*; and *Skeletal and Muscular Disorders* are suggestive of muscle remodelling and structural adaptation. No detailed morphological studies of the skeletal muscle response to training were performed as part of this work and lean mass assessed by DEXA scan did not reveal any within-group changes in COPD or Older HC groups, thus it is not possible to relate impact on functional networks to morphological adaptations in this study.

In this study the *Free Radical Scavenging* function is modified in both COPD and Older HC groups. Previous investigations of free radical production and redox potential in COPD have produced contrasting results suggesting both increase (Rabinovich, Ardite et al. 2001) and decrease in oxidative stress with aerobic training in COPD (Rodriguez, Kalko

et al. 2012). Influence on the *Free Radical Scavenging* function in the current study suggests that at an mRNA level COPD and Older HC groups adapt similarly after aerobic training. This may be because participants in this study generally had well preserved muscle mass (Table 5.1) and evidence suggests that the COPD patients who experience a maladaptive oxidative stress response to aerobic training are those with low BMI (Rabinovich, Ardite et al. 2006). Similarly, as observed in healthy young and older volunteers (Chapter 4) the influence on the *Inflammatory Response* function is consistent with a normal intramuscular inflammatory response to exercise training.

6.5 mRNA responses to exercise withdrawal

This is the first study to report the influence of exercise withdrawal on muscle mRNA expression in patients with COPD. Figure 6.6 b shows that the influence of aerobic training on functional networks is not diminished by four weeks subsequent exercise withdrawal in patients with COPD. This observation closely parallels that in healthy people of a similar age (Figure 6.6 a). The influence of reduced muscular activity is of particular interest in the COPD population in regards to periods of acute inactivity resulting from acute exacerbation and systemic illness and in the context of the maintenance of benefits bought about by physical training in pulmonary rehabilitation programmes (Bolton, Bevan-Smith et al. 2013). The COPD group showed evidence of detraining after exercise withdrawal (reduced $\dot{V}O_2^{PEAK}$, Figure 5.2 a) however that does not appear to be related to mRNA signalling in the skeletal muscle.

6.6 Protein analysis

Baseline protein expression differed between groups for 3 targets. Myostatin is a known negative regulator of muscle mass and myostatin mRNA has previously been found to be elevated in COPD patients with reduced muscle mass compared to healthy controls (Plant, Brooks et al. 2010) however the data in this chapter show that myostatin protein was more abundant in the Older HC group than in the COPD group. Myostatin abundance did not appear to relate to muscle mass in the volunteers for this study in which measures of whole-body and thigh lean mass by DEXA were similar in patients with COPD and Older HC (Table 5.1). IL6 protein expression was higher in the COPD group which may indicate greater inflammation in the COPD muscle, however this contrasts with the findings of Barreiro *et al* (Barreiro, Schols et al. 2008) who found that

IL6 was not elevated in a group of COPD patients with similar characteristics to those in this chapter and concluded having assessed a range of cytokines that stable COPD patients did not demonstrate significant skeletal muscle inflammation. HIF1α protein was more abundant in COPD. HIF1α signalling (downstream of TNFα and NFκB) has been implicated in a shift towards glycolytic metabolism in COPD (Remels, Gosker et al. 2015) and in myotube models of extreme hypoxia HIF1α activation has been associated with the contrasting effects of inhibiting PPAR/PGC1α (with resultant influence on mitochondrial energy metabolism) while upregulating production of type I myosin contractile proteins (Slot, Schols et al. 2014), however patients described in this chapter did not show evidence of hypoxaemia at rest or after exhaustive exercise (peripheral capillary oxygen saturations pre- and post-exercise at baseline are found in Table 5.2).

In the COPD group, there were no changes in the abundance of proteins measured either with training or exercise withdrawal and there was only one change with training (decrease in myostatin discussed in Chapter 4) in the Older HC group. Although myostatin abundance is thought to have an inverse relationship with quadriceps strength, there were no detectable changes in muscle mass when assessed after 8 weeks training (data not shown).

With changes in protein abundance being highly variable between individuals (Figure 6.8) subjects who experienced either up- or down-regulation of protein targets were characterised according to demographic variables and their response to training and exercise withdrawal to look for common characteristics. There were significant differences in the age of patients with COPD (or COPD and Older HC groups collapsed) dependent on the direction of change in abundance of IGF1, PPARγ, and IGFBP4 during training or exercise withdrawal but although statistically significant, the magnitude of the difference in age was small (mean difference in age between groups ranged from 5.4 to 7.9 years) and is therefore unlikely to be a significant physiological factor. Individuals who experienced a decrease in abundance of PPARγ during the training period in the COPD group had lower BMI (24.3 + 4.3 vs 32.3 $\pm$ 5.8 kg/m^2) and this observation was also apparent when COPD and Older HC groups were collapsed. At an mRNA level, PPARγ is upregulated in skeletal muscle after resistance exercise and is thought to play a role in synthesis of fatty acids (Tsintzas, Stephens et al. 2017). PPARγ

gene expression is elevated in the muscle of obese compared to normal-weight individuals (Park, Ciaraldi et al. 1997). The abundance of PPARγ protein increased in individuals who were, on average obese in the current study and decreased in individuals who were normal weight raising the possibility that the change in abundance was part of an adaptation in lipid handling in skeletal muscle in response to exercise training that was influenced by obesity. COPD patients who experienced a reduction in p53 protein with training had significantly lower W^{PEAK} in maximal exercise testing at baseline (55.1 + 20.1 vs 82.4 $\pm$ 35.4 W). p53 is a transcription factor and as such influences multiple downstream processes which importantly include regulating mitochondrial biogenesis and influencing substrate metabolism in skeletal muscle (Bartlett, Close et al. 2014) and has been implicated in skeletal muscle atrophy prompted by immobilisation (Atherton, Greenhaff et al. 2016). Exercise induced activation of p53 may contribute to increases in mitochondrial content (Bartlett, Close et al. 2014). Individuals who increased p53 protein content with training had higher Peak Power at baseline and therefore trained at a higher absolute intensity compared to those with reduced p53 content so it is possible that a certain absolute threshold of metabolic stress must be placed on the exercising muscle in order to upregulate p53 abundance. An important note to consider is that change in abundance in the phosphorylated (activated) p53 was not associated with any particular defining characteristic among the subjects of this study nor did it significantly change in abundance in either group with exercise training or withdrawal.

6.7 Associations between gene and protein responses

There were no correlations between change in mRNA and change in protein abundance in the Older HC group, however in the COPD group some significant results emerged: the change in IL6 mRNA and protein abundance correlated during the first week of training; and the change in PYGM mRNA and protein abundance correlated during the exercise withdrawal period (Figure 6.9). It is possible that significant correlations were more likely to emerge in the COPD group due to the number of subjects compared to the Older HC group (19 vs 10 respectively). When the groups were collapsed it was again IL6 and PYGM that showed significant correlations in the first week and first four weeks of training respectively. An inverse correlation between change in abundance of IGFBP4

mRNA and protein was apparent for the first week of training when the groups were collapsed. The possible explanations for discrepancies between change in protein and mRNA abundance (both biological and methodological) are discussed in detail in Chapter 4 and would apply equally to the COPD population.

6.8 Limitations

Recruitment criteria were designed to match the COPD and Older healthy groups in all characteristics except lung function. Despite this, it is acknowledged that patients with COPD were less physically active that the Older HCs who took on average 50% more steps per day during pre-intervention monitoring (Figure 5.1). The difference in habitual physical activity may have impacted upon the expression of genes and proteins in baseline measures however the main focus of this work was to examine the relative response to a tightly supervised exercise training programme for which previous physical activity behaviour is less influential. Additionally, the smoking history of the two groups was significantly different (Table 5.1); all patients with COPD were ex-smokers while 60% of Older HCs had never smoked. In animal models, cigarette smoke exposure reduces endurance exercise tolerance, induces muscle weakness, influences muscle fibre signalling (including mRNA signalling) towards a pro-atrophy state (Caron, Morissette et al. 2013; Kamiide, Furuya et al. 2015; Kruger, Dischereit et al. 2015) and causes cell senescence in myotube cultures (Liu, Su et al. 2015), however there is evidence of reversal of signalling changes when smoke exposure ceases (Caron, Morissette et al. 2013). As all study volunteers reported no smoking within 12 months preceding study entry any effects of smoke exposure were minimised.

Previous reports have found that differences in mRNA expression between COPD and health after aerobic training are more pronounced when the patients with COPD also have low fat-free mass (Tenyi, Cano et al. 2018). The COPD patients described in this chapter had normal fat-free mass, therefore when interpreting these results consideration should be given to the possibility that patients with low fat-free mass may have responded differently.

As discussed in Chapter 5, patients with COPD had a ventilatory limitation which may have influenced exercise training intensity prescription and limited the physiological

load on the quadriceps muscle. Irrespective of this, data in this chapter show that the intensity of training undertaken by patients with COPD was sufficient to stimulate the same response in mRNA abundance seen in Older HCs. In this study there were no direct measures of cardiac output during exercise making it difficult to draw conclusions about the likely influence of cardiovascular adaptations in healthy individuals and those with COPD. Similarly substrate utilisation by the muscle can only be inferred from RER data. Further insights into the relative peripheral and cardiovascular adaptations could have been achieved with additional measures. Adaptive responses of skeletal muscle could have been obtained using tracer methods to directly measure lipid and glucose oxidation during exercise before and after the training programme and techniques such as cardiac MRI or echocardiogram might have elucidated the impact of training on cardiac output.

Finally, it is acknowledged that muscle morphology (specifically muscle fibre type proportion) may have differed between patients with COPD and Older HCs. It has previously been shown that there is a preferential loss of type I oxidative fibres in COPD leading to a relatively larger proportion of type II glycolytic fibres compared to healthy controls (Slot, van den Borst et al. 2014; Vogiatzis, Terzis et al. 2011). Distinct fibre phenotypes may react have different molecular reactions to exercise interventions. Due to limited tissue resource it was not possible to fibre-type the muscle samples from this study therefore it is not possible to speculate about any influence of fibre type proportion on the results presented here.

6.9 Implications

A reduced capacity for adaptation to training in terms of whole-body ($\dot{V}O_2^{PEAK}$; Chapter 5) and mitochondrial function (Figure 6.10) is apparent in COPD. Contrasting with this, skeletal muscle mRNA responses to aerobic training are not impaired in patients with COPD compared to healthy volunteers of a similar age. This implies that moderate intensity aerobic training is sufficient to initiate a normal skeletal muscle gene expression response for mRNA transcripts known to be responsive to aerobic exercise in young healthy individuals in COPD and that any defect in the adaptive process must lie downstream of mRNA transcription. This study was not able to definitively elucidate the contribution of protein expression to the diminished training response in COPD;

whilst protein measures did not correlate closely with mRNA in the COPD group, the same was true in healthy controls. Further work is warranted to investigate where in the pathway between mRNA expression and functional muscle adaptation (mitochondrial ATP production) COPD patients differ from healthy controls. If identified, this could potentially lead to a new therapeutic target for optimising skeletal muscle adaptation to training with a view to maximising the impact of rehabilitative exercise training. Alternatively, there may be no inherent defect in the skeletal muscle of patients with COPD, with different responses to training arising from the restricted relative and absolute training loads applied to the muscle in individuals limited by ventilatory constraints and dyspnoea. The impact of alternative training modes (for example interval training or single leg training) on the muscle molecular adaptive responses to exercise in COPD remains of interest as does their impact on cardiovascular training adaptations. No previous studies of COPD muscle molecular responses to aerobic training have examined time course data and the demonstration that mRNA responses to training are not only similar in COPD and health but occur early and are maintained throughout training and subsequent exercise withdrawal is novel. The maintenance of mRNA responses throughout the four week detraining period in both COPD and healthy controls suggests that the mRNA response to exercise withdrawal is not influenced by COPD and that differences in whole-body markers of detraining occur after transcription of mRNA.

6.10 Conclusions

Moderate intensity aerobic training for eight weeks prompted similar mRNA responses in the skeletal muscle of patients with COPD and healthy age-matched controls which were maintained throughout four subsequent weeks of exercise withdrawal. This contrasts with the different training and detraining responses at a muscle and whole-body level observed in the two groups where unlike healthy controls patients with COPD failed to increase peak oxygen uptake with training and experienced a reduction in peak oxygen uptake upon exercise withdrawal. Biological functions influenced at an mRNA level particularly those relating to fuel selection relate well to cardiorespiratory and skeletal muscle outcomes in health, but are not as clearly related in COPD. These results

suggest that any impairment in the capacity for patients with COPD to adapt to aerobic training occurs downstream of mRNA transcription and may indeed be due to constrained cardiovascular training adaptations due to low training intensities.

Chapter Seven

7 Acute impact of transcutaneous neuromuscular electrical stimulation or resistance exercise on skeletal muscle mRNA expression in COPD

7.1 Introduction

A programme of aerobic exercise training has been shown to influence mRNA expression and whole-body physiological responses to endurance exercise in patients with COPD (Chapters 5&6), however impaired aerobic capacity is not the only limit to exercise tolerance for patients with this condition. Muscle weakness is a common systemic feature of COPD and an important contributor to morbidity and mortality (Bernard, LeBlanc et al. 1998; Maltais, Decramer et al. 2014). There is substantial evidence demonstrating the benefits of resistance exercise (RE) training in COPD (Simpson, Killian et al. 1992; Clark, Cochrane et al. 2000; Casaburi, Bhasin et al. 2004) and it is currently recommended that this training modality training should be incorporated into pulmonary rehabilitation programmes (Spruit, Singh et al. 2013). However, not all patients with COPD are able to perform conventional voluntary resistance training of sufficient intensity to bring about meaningful gains in muscle mass and strength due to advanced deconditioning, acute exacerbation and associated dyspnoea or hospitalisation. Transcutaneous neuromuscular electrical stimulation (NMES) is a non-volitional means of evoking muscle contraction that places minimal demand on the cardiorespiratory system and does not induce significant dyspnoea (Sillen, Janssen et al. 2008; Sillen, Franssen et al. 2014). For these reasons, NMES may be an effective strategy for the maintenance or improvement of muscle function in settings as above, where voluntary RE training cannot be performed (Neder, Sword et al. 2002).

Clinical studies have suggested that NMES increases muscle mass and strength in COPD (Sillen, Franssen et al. 2013; Sillen, Franssen et al. 2014) despite typically generating contractile force of less than 15 – 20% of maximum voluntary isometric strength (Newham, Mills et al. 1983; Maddocks, Gao et al. 2013). To date however, understanding of the training adaptations to NMES at a muscle level, and how these

compare to those elicited by voluntary RE is limited. It is known that an unaccustomed bout of voluntary RE in COPD causes a change in expression of a wide range of mRNA transcripts (Constantin, Menon et al. 2013) but this information is lacking for NMES where it is likely that a smaller muscle mass will be recruited.

This chapter will test the hypotheses that in patients with COPD, a single bout of NMES or a single bout of RE will influence the abundance of targeted mRNA transcripts known to be responsive to RE in young healthy volunteers. A secondary research objective is to determine whether the magnitude of change in abundance of mRNA transcripts is similar in response to the two different muscle contraction modalities. In order to address these questions matched cohorts of COPD patients performed a single bout of either NMES or RE with quadriceps biopsies performed immediately before and 24 hours after intervention to allow quantification of mRNA abundance.

7.2 Methods

7.2.1 Participants

Patients with stable COPD (MRC Grade $\geq$ 3) matched for body composition and lung function were included in this study. Inclusion criteria are described in Chapter 2.4.2.

7.2.2 Study Design

The study protocol is described in Chapter 2.4.2. Briefly, subjects performed a single bout of either quadriceps NMES (30 min, 50 Hz stimulation frequency, pulse duration 300 µs, 15 s duty cycles with 5 s rest) or RE (5 sets of 30 maximal isokinetic knee extensions at 180°/s, 1 min rest between sets). Biopsies of the *vastus lateralis* of the exercised leg were performed immediately before and 24 hours after the exercise bout.

7.2.3 Measures

Tissue acquired from the muscle biopsies was analysed for abundance of 384 targeted transcripts using RT-PCR. Targets were selected due to their known responsiveness to resistance exercise in young healthy populations (Jones, Hill et al. 2004; Murton, Billeter et al. 2014) (see Chapter 2.10 for details).

7.2.4 Data Analysis

Gene expression data were analysed using the comparative C_T method ($\Delta\Delta C_T$) (Livak and Schmittgen 2001) using hydroxymethylbilane synthase (HMBS) which had stable C_T values across time points as the control gene. Significant change in abundance of the target gene relative to HMBS (ΔC_T) between baseline and 24 hrs was assessed by paired t-test with the False Discovery Rate (FDR) adjustment applied to control for multiple comparisons using the R Statistical Package (R Version 3.0.0, 2013-04-03, The R Foundation for Statistical Computing). Expression values are presented as fold change from baseline ($2^{-\Delta\Delta CT}$) and significant within-group change was defined by a FDR <5%. Between group differences in physiological variables were tested by t-test, Mann-Whitney U test (ordinal data), or Pearson X2 test (categorical data) and presented as mean ($\pm$ SD) or median (IQR).

7.3 Results

7.3.1 Patient Characteristics

Thirteen COPD patients received NMES and 13 patients performed voluntary RE. There were no significant differences between the groups for baseline measures of lung function, MRC grade or body composition (all $p > 0.05$)although there was a significant difference between the groups for smoking history with the RE group containing more current smokers (Table 7.1).

Table 7-1. Patient characteristics

	NMES	RE	*p*
Males / Females, n	7 / 6	8 / 5	NS
Age, years	63.6 (9.1)	64.2 (7.0)	NS
Height, m	1.61 (9)	1.65 (11)	NS
Body Mass, kg	66.4 (12.5)	65.4 (20.5)	NS
BMI, kg/m^2	25.6 (4.2)	23.7 (5.6)	NS
FFMI, kg/m^2	16.8 (1.7)	16.4 (2.0)	NS
FEV_1, l	1.02 (0.34)	1.02 (0.47)	NS
FEV_1, % Predicted	45.5 (19.3)	40.3 (16.7)	NS
FEV_1/ FVC	0.41 (0.10)	0.44 (0.13)	NS
Smoking Status (Current / Ex / Never)	1 / 11 / 1	7 / 6 / 0	.031
Pack Years Smoked	44.3 (23.5)	56.2 (35.8)	NS
MRC Grade $	4 (3-4)	4 (3-4)	NS
$, median (IQR).			

7.3.2 Exercise bout

The mean (SD) peak torque generated during RE was 38 ($\pm$13) Nm and the mean work done over 5 sets of 30 isometric knee extensions by the voluntary RE group was 2482 ($\pm$925) J. The electrical stimulation current during NMES was 39.3 ($\pm$12.7) mA.

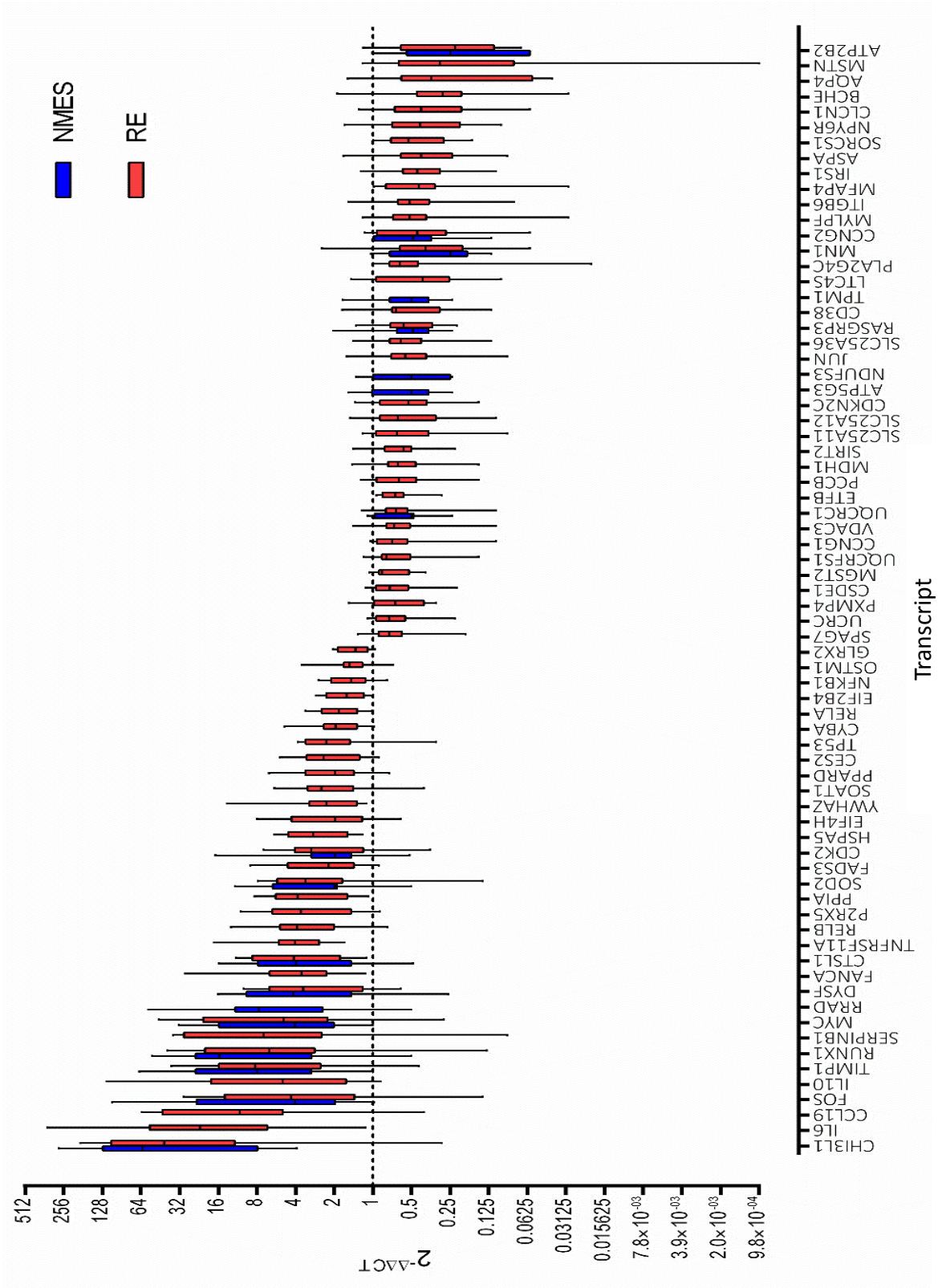


Figure 7-1. Genes significantly altered in expression 24 hr after NMES (18 transcripts) or RE (68 transcripts), FDR < 5%. Data expressed as fold change from baseline (baseline value = 1, dashed line). Y axis is a log2 scale. Abbreviated gene names are defined in Appendix 6

7.3.3 mRNA Expression

Twenty-four hr after NMES 18 mRNA transcripts were significantly changed in abundance with 68 mRNA transcripts significantly changed in abundance 24 hr after RE (both FDR <5% compared to baseline, Figure 7.1).Change in abundance of 14 gene transcripts were common to both NMES and RE with no significant difference in the magnitude of fold change between groups for these common genes ($p > 0.05$). The within group variation in the response to NMES and RE is depicted in Figure 7.2.

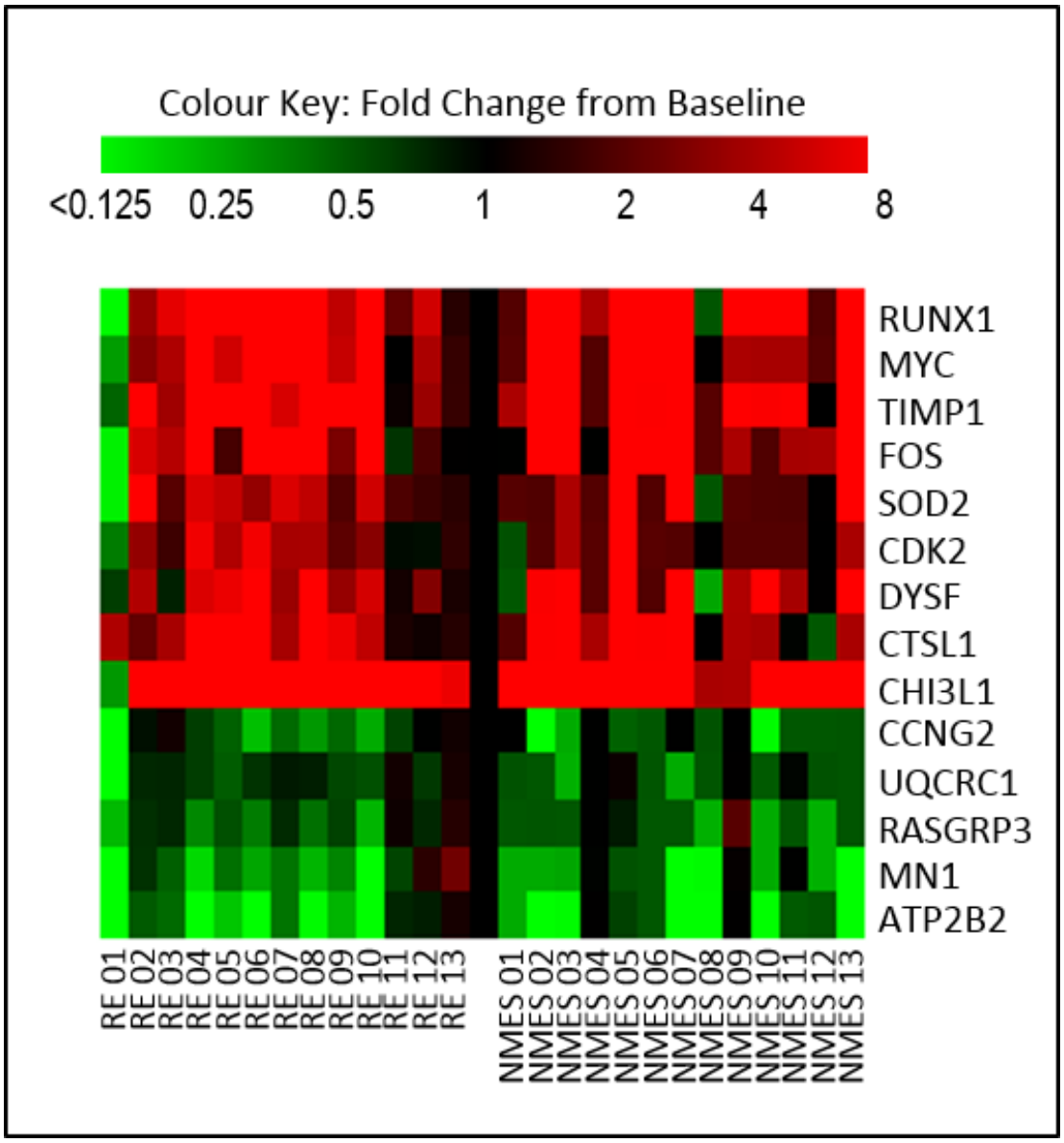


Figure 7-2. Heatmap demonstrating individual variation in response to NMES and RE for the 14 transcripts that were significantly influenced by both interventions. Scale capped at 8 fold change, baseline value = 1. Abbreviated gene names used in this figure are defined in Appendix 6.

The 14 transcripts significantly influenced by both NMES and RE perform a range of physiological roles which are detailed in Table 7.2, accompanied by fold change data. Genes with physiological roles associated with muscle hypertrophy, growth, repair, regeneration and anti-wasting respectively (RUNX1, MYC, TIMP1, FOS and DYSF) were

upregulated following both NMES and RE. Other upregulated transcripts CTSL1, CHI3L1, CDK2 and SOD2 have physiological roles relating to protein breakdown, anti-inflammatory action, cell cycle regulation and anti-oxidant action, respectively. Down-regulated transcripts CCNG2, ATP2B2 and RASGRP3 are influential in cell cycle / signalling regulation. RASGRP3 also has a physiological role in cancer, as does MN1, with both of these transcripts downregulated after both interventions. Another transcript downregulated following both NMES and RE, UQCRC1, codes for a mitochondrial subunit.

Table 7-2. Transcripts significantly altered in expression following a bout of NMES or RE.

Target Name	Description	Physiological Role	NMES		RE	
			Fold Change	FDR %	Fold Change	FDR %
Increased abundance after both NMES and RE						
CHI3L1	chitinase 3-like 1	Protects muscle against inflammation.	84.4	<0.001	59.3	0.003
RUNX1	runt-related transcription factor 1	Upregulated by exercise. May prevent muscle wasting.	15.7	0.005	12.6	0.009
MYC	v-myc avian myelocytomatosis viral oncogene homolog	Muscle growth via ribosomal biogenesis.	10.3	0.011	11.5	0.006
FOS	FBJ murine osteosarcoma viral oncogene homolog	Transcription factor for pro-hypertrophy genes.	17.6	0.011	8.7	0.029
TIMP1	TIMP metallopeptidase inhibitor 1	Promotes cell proliferation, inhibits apoptosis. May promote angiogenesis.	16.7	0.024	11.2	0.007
SOD2	superoxide dismutase 2, mitochondrial	Anti-oxidant.	3.6	0.029	3.6	0.036
DYSF	dysferlin	Muscle repair / regeneration.	6.2	0.033	4.2	0.013
CDK2	cyclin-dependent kinase 2	Cell cycle regulation.	3.2	0.033	3.1	0.025
CTSL	cathepsin L	Protein breakdown.	5.2	0.027	5.3	0.003
Decreased abundance after both NMES and RE						
MN1	meningioma (disrupted in balanced translocation) 1	Cancer.	0.4	0.024	0.6	0.01
CCNG2	cyclin G2	Cell cycle.	0.6	0.024	0.6	0.016
RASGRP3	RAS guanyl releasing protein 3	Cell signalling.	0.6	0.024	0.6	0.036
ATP2B2	ATPase, Ca++ transporting, plasma membrane 2	ATP Pump controlling intracellular calcium level.	0.4	0.024	0.4	0.003
UQCRC1	ubiquinol-cytochrome c reductase core protein I	Mitochondrial Subunit.	0.6	0.027	0.7	0.025

Fold change from baseline, $2^{-\Delta\Delta C_T}$; significant within group change, FDR < 5%; no between group differences in fold change (all $p > 0.05$).

7.4 Discussion

This study has shown that voluntary resistance exercise influences a substantially broader range of transcripts than transcutaneous electrically evoked muscle contraction (68 *vs*. 18 respectively). A small number of transcripts (representing 14 genes) responded similarly to the two interventions. It is therefore apparent that the NMES intervention employed in this study was not sufficient to stimulate the same breadth of transcriptional response generated by maximal voluntary RE in COPD, although there is some overlap in the mRNA response to the two interventions.

This is the first study to examine the influence of NMES on the expression of a broad range of mRNA transcripts and furthermore to compare this response to that following a bout of voluntary RE in well-matched groups of COPD patients. There is existing evidence of a transcriptional response to NMES in young non-weight trained individuals (Bickel, Slade et al. 2005), but this had not previously been reported for patients with COPD. NMES has previously been shown to influence phosphorylation of p70S6K, a regulator of muscle protein synthesis (Vivodtzev, Debigare et al. 2012), and influence muscle fibre size (Dal Corso, Napolis et al. 2007), suggesting that repeated bouts of NMES in COPD promote muscle fibre adaptation. More is known about the molecular changes in muscle following a bout of voluntary RE in COPD; expression of gene transcripts with functions relating to muscle protein synthesis and breakdown, myogenesis, transcription factors, and inflammation have previously been shown to respond to a bout of isokinetic resistance exercise in COPD patients (Constantin, Menon et al. 2013) in a manner comparable to that shown in our study.

NMES was performed at the highest tolerable intensity in order to maximise muscle fibre recruitment, however the narrower range of gene transcription responses following NMES is likely to be a function of less muscle recruitment during NMES compared to RE. This is supported by the observation that the changes in mRNA abundance of the 14 common transcripts were similar between NMES and RE. A limitation of this study is that muscle tension development during NMES was not measured, but is known to typically be below 15% of maximal voluntary isometric force generation (Newham, Mills et al. 1983) and might therefore be assumed to have been considerably lower than that produced during RE (Vivodtzev, Debigare et al. 2012).

The contraction provoked by NMES does not follow Henneman's size principle (Henneman, Somjen et al. 1965) of motor unit recruitment, rather NMES depolarises larger motor neurones first, thus preferentially activating fast-muscle fibres (which tend to be on the periphery of the muscle bundle (Marqueste, Hug et al. 2003)), or may activate fibres in a spatially determined manner dependent on proximity to the skin-surface electrode (Bickel, Gregory et al. 2011). The genes differentially regulated by NMES may therefore have been influenced by the type of muscle fibre recruited during electrical stimulation; unlike RE where all fibres types are likely to have been recruited.

NMES improves muscle strength and function across a range of stimulation frequencies (Bourjeily-Habr, Rochester et al. 2002; Neder, Sword et al. 2002; Zanotti, Felicetti et al. 2003; Abdellaoui, Prefaut et al. 2011; Maddocks, Gao et al. 2013; Sillen, Franssen et al. 2014; Maddocks, Nolan et al. 2016), with efficacy dependent on stimulation intensity (Vivodtzev, Debigare et al. 2012), which determines contraction force. Stimulation intensity is limited by the tolerance of the individual and in healthy individuals there have been reports of pain during stimulation (Mackey, Bojsen-Moller et al. 2008). In health there have also been reports of NMES causing muscle damage and delayed onset soreness (Mackey, Bojsen-Moller et al. 2008; Hansen, Trappe et al. 2009; Nosaka, Aldayel et al. 2011) comparable to that caused by eccentric RE (Newham, Mills et al. 1983; Lauritzen, Paulsen et al. 2009) despite generating lower force (Crameri, Aagaard et al. 2007). However, NMES protocols utilised in chronic disease rehabilitation, that generally elicit lower contraction forces, appear to be well tolerated (Maddocks, Gao et al. 2013) and significantly increase muscle strength and mass, even when generating a force as low as 13% of maximal voluntary contraction (Vivodtzev, Debigare et al. 2012).

The transcriptional response of muscle to both NMES and RE was similar for 14 gene transcripts (Figure 7.2 & Table 7.2), whether these changes reflect mutual type II muscle fibre recruitment is unknown. Functions of shared upregulated transcripts were associated with muscle growth, repair and regeneration; anti-inflammatory / anti-oxidant action, and protein breakdown. In addition, there was common down-regulation of gene transcripts associated with cell cycle, cancer and mitochondrial function. Following both interventions, the most markedly upregulated mRNAs were CHI3L1 and RUNX1. CHI3L1 (chitinase-3-like protein 1) gene expression is known to be

induced by contractile activity (Gorgens, Hjorth et al. 2015), and the protein is associated with myoblast proliferation (Gorgens, Hjorth et al. 2015) and inhibition of the inflammatory response (Gorgens, Eckardt et al. 2014). RUNX1 (runt-related transcription factor 1) may be protective against disuse atrophy (Wang, Blagden et al. 2005) and there is a pronounced increase in expression when muscle is exercised after a period of immobilisation (MacNeil, Glover et al. 2014). RUNX1 may also be a target of MYOD1, which regulates myogenesis and skeletal muscle differentiation (Blum and Dynlacht 2013). Whilst the influence of any individual gene on muscle function or architecture is likely to be small, the strong induction of these two genes after both NMES and RE supports the notion that both interventions are influencing muscle cells towards a pro-growth state.

7.4.1 Implications

The broader influence of RE on mRNA expression compared to NMES suggests that voluntary RE is the preferable mode of muscle contraction to elicit the largest muscle transcriptional response in stable COPD patients. However, NMES within the limits of comfort for patients does elicit a pro-growth transcriptional response. As such this study reinforces the evidence that NMES has potential as a therapeutic adjunct to rehabilitative exercise in COPD. Further work is warranted to investigate the effect of repeated bouts of NMES and RE on molecular responses and physiological adaptation to a chronic intervention.

7.4.2 Limitations

This analysis is limited to the 384 transcripts targeted for PCR. These targets are representative of the normal mRNA response to resistance exercise in healthy volunteers, however it is possible that NMES influences expression of genes that were not measured. The fully quantitative and highly sensitive nature of the RT-PCR technique employed in this study does however allow characterisation of a wide range of expression values for the genes targeted. The timing of the post-exercise biopsy 24 hours after NMES or RE does not provide time course data for mRNA abundance, however additional time points would have required patients to undergo additional biopsy procedures and previous work has shown expression of genes related to skeletal muscle mass regulation is altered 24 hr post-RE in COPD and health (Constantin, Menon

et al. 2013; Murton, Billeter et al. 2014). The data for this analysis were drawn from two separate study cohorts, however the groups were well matched for age, gender and body composition and adhered to a carefully planned study day protocol. Therefore we are confident that the differences in gene expression observed after the two interventions were as a result of the contraction mode, rather than a characteristic of the two groups.

7.4.3 Conclusion

This study demonstrates that a single bout of RE influences the abundance of a far wider selection of mRNA transcripts than a single bout of NMES in the quadriceps of patients with COPD. There is however a commonality of response for a small subset of mRNA transcripts with functions associated with muscle growth, repair and regeneration. Based on this evidence, voluntary RE would appear to be the preferable mode of exercise intervention to elicit the largest muscle transcriptional response in stable COPD patients. However, NMES within the limits of comfort for patients does elicit a pro-growth transcriptional response. Similar to the investigations described earlier in this thesis employing aerobic training, work to investigate the effect of repeated bouts of NMES and RE on molecular responses and functional adaptations to a chronic intervention are warranted.

Chapter Eight

Final conclusions

8.1 Introduction

The work described in this thesis aimed to examine the influence of advanced age and of COPD on the whole-body and skeletal muscle molecular time course responses to aerobic exercise training and subsequent exercise withdrawal, and to describe the influence of an acute bout of electrically stimulated muscle contraction on mRNA abundance in the quadriceps of patients with COPD compared to voluntary contraction. To this end, and with the help of colleagues, volunteers who were young (18 – 35 years), older (60 – 80 years) or who had COPD (aged 60 – 80 years) were recruited to complete an eight-week programme of supervised aerobic cycling exercise at an intensity corresponding to of 65% peak work (determined by incremental cycle ergometer exercise testing). Resting state *vastus lateralis* muscle biopsies and cardiopulmonary exercise testing were performed at baseline and repeated after one, four and eight weeks of training as well as after a four week period of exercise withdrawal subsequent to the training period. A separate cohort of patients with COPD volunteered to have *vastus lateralis* biopsies before and 24 hours after a single bout of resistance exercise or high-frequency neuromuscular electrical stimulation and the abundance of 384 mRNA transcripts known to be responsive to resistance exercise in health were quantified.

8.2 Summary of findings

At baseline, the peak rate of oxygen uptake in an incremental test was 34% lower in sedentary older adults compared to young sedentary individuals which is in keeping with the findings of previous studies. Lower aerobic capacity in the older group was unlikely to be a consequence of reduced habitual physical activity as step count measured by accelerometry was similar in the two groups thus raising the possibility that the difference may be a sign of biological ageing. The response to aerobic training at the same relative intensity was however very similar with young and older groups increasing $\dot{V}O_2^{PEAK}$ by 21% and 18% respectively, suggesting that the capacity to make training

adaptations is maintained in older volunteers. Patients with COPD displayed an 18% lower peak oxygen uptake (adjusted for lean mass) at baseline compared to healthy age-matched controls. This deficit is smaller than the 50% commonly cited in the literature, probably due to closer matching of habitual physical activity between patients with COPD and age-matched controls in this study. Unlike their healthy peers, patients with COPD did not increase $\dot{V}O_2^{PEAK}$ after eight weeks aerobic training at the same relative intensity. Patients with COPD did however show signs of physiological adaptation to exercise training in sub-maximal testing with RER and HR both being lower in isowork, sub-maximal exercise testing at week eight compared to baseline. That $\dot{V}O_2^{PEAK}$ did not increase in patients with COPD may be the result of a number of different factors. Patients with COPD on average reached 103% of their predicted maximal voluntary ventilation which combined with associated dyspnoea may have truncated the incremental exercise test prematurely. Resultant constraints on exercise training intensity (which was prescribed from the incremental test) may have limited the training impulse experienced by the muscles and cardiovascular system during continuous exercise and therefore constrained training adaptations. It is also possible that the patients with COPD experienced an unidentified barrier in the adaptive processes downstream of changes in abundance of mRNA (which were the same in young, older and COPD groups despite significantly different absolute training loads) resulting in the absence of a change in palmitate stimulated maximal mitochondrial ATP production rate in COPD. It is also possible that there is no muscle-specific defect in the adaptation to aerobic exercise training in patient with COPD and that failure to increase peak oxygen uptake is related to adaptations (or lack thereof) in cardiac output which was not measured in this study and likely related to training intensity.

The effect of four weeks exercise withdrawal at the end of the training programme was similar for young and older healthy volunteers; $\dot{V}O_2^{PEAK}$ did not decrease and peak power, which did decline significantly, was still significantly greater than it had been at baseline. The time course of these detraining adaptations is interesting and shows that the advanced age of the older group did not predispose them to more marked losses of aerobic fitness over a four week period compared with young volunteers, with none of the measures from incremental or sub-maximal testing having returned to pre-training

values. Curiously, patients with COPD achieved a lower $\dot{V}O_2^{PEAK}$ in testing after four weeks of exercise withdrawal despite having made no gains during the training period and objectively measures habitual physical activity levels not changing from baseline values. The reduction in $\dot{V}O_2^{PEAK}$ may be a sign of rapid deconditioning in this group, however RER and HR during submaximal testing at isowork was unaltered during the same period perhaps suggesting that the decline in $\dot{V}O_2^{PEAK}$ during incremental exercise was due to sub-optimal effort at the end of a lengthy and demanding study period.

The results of the mRNA bioinformatics analysis revealed that older healthy adults experience similar responses to young healthy individuals and to patients with COPD. Changes in mRNA abundance with exercise training predicted influence on functional networks relating to fuel selection, tissue remodelling and inflammation that were apparent in all three groups. The influence of exercise training on mRNA functional networks apparent early in the training programme (one week), was maintained throughout the course of the programme (four and eight weeks) and was unchanged after four weeks of exercise withdrawal in all three groups. Evidence from the literature had previously suggested that changes in mRNA abundance may differ between young and older volunteers after resistance exercise training, but given that the young and older healthy individuals described in this thesis made similar cardiorespiratory adaptations to aerobic exercise it is perhaps not surprising that there were similar responses at the level of mRNA in the exercising muscle. That the mRNA responses in the COPD group were so similar to those in the age-matched older healthy group was unexpected, but demonstrates that a robust muscle translational response is maintained in patients with COPD who have preserved muscle mass despite disease related deconditioning being evident at baseline. As discussed above, ventilatory limitations and associated dyspnoea may have limited the absolute (and indeed relative) training intensity at the level of the muscle, a factor which might be assumed to limit physiological adaptation. The mRNA targets in this study were selected as they were previously identified as being highly responsive to exercise training in young healthy volunteers. It would appear that the intensity of cycling exercise achieved by the COPD group was sufficient to stimulate the same influence on the same biological functions observed in health and that any deficits in skeletal muscle remodelling in response to

the training protocol lie outside of the range of targets examined in this study, downstream of mRNA transcription or at a more systemic level. It is also important to remember that patients with COPD did show additional evidence of exercise adaptation with reduced RER at submaximal isowork in the trained state suggesting a shift from carbohydrate towards lipid oxidation in the trained state. The documentation of the magnitude and time course of mRNA changes during exercise training and subsequent exercise withdrawal is a novel contribution to research understanding and it was apparent that not only did mRNA abundance changes occur early (apparent after only three sessions (one week) of training), but they were maintained throughout the exercise period and to the end of the exercise withdrawal period. The maintenance of changes in mRNA abundance relative to the baseline biopsy after a period of exercise withdrawal corresponds with evidence from cardiopulmonary exercise testing that showed none of the groups studied experienced full reversal of their recent training adaptations.

Analysis of a small selection of protein targets revealed that IGF1 abundance was lower in older compared to young healthy volunteers and that patients with COPD had higher IL6 and HIF1α, but lower myostatin protein abundance compared to age-matched older healthy controls at baseline. Change in abundance of protein targets with training and exercise withdrawal showed substantial inter-subject variability but some trends emerged including changes in abundance of p53, PPARγ, IGF1 and myostatin proteins in the young or older groups which may signpost towards alterations in fuel selection or muscle mass regulation. In the COPD group none of the changes in protein abundance with training or exercise withdrawal reached statistical significance. The absence of correlations between change in abundance of mRNA and its protein product highlights the complex nature of the biochemical responses to an intervention such as aerobic exercise training and suggests that protein translation, and not mRNA transcription was limiting to muscle level adaptation. The inter-individual variation in protein level responses was large and whilst novel in the context of mRNA abundance changes, remains unresolved.

The study of mRNA responses to acute muscle contraction revealed a far broader influence of voluntary resistance exercise compared to non-volitional NMES evoked

muscle contraction. That the voluntary exercise made the greatest impact may be explained by lower muscle fibre recruitment during NMES as it is known that this type of non-volitional contraction generally produces lower contractile force (a marker of motor unit recruitment) compared to volitional resistance exercise. It is interesting however that NMES did elicit an mRNA response and that the transcripts upregulated following NMES are associated with pro-growth functions.

8.3 Study limitations

Undertaking a prospective study of integrative physiology involving the recruitment of elderly volunteers, including those with a chronic disease is not without its challenges. Inevitably the demanding nature of the protocol led to the exclusion of frailer patients and in all groups there were dropouts from the study due to illness or social reasons. That the studies described in this thesis were completed is testimony to the motivation and generosity of all the study volunteers and a credit to the small research team of which I was a part. The aerobic training study described in this thesis was designed and implemented in a manner that yielded sufficient data to answer the research questions posed. Strengths of the design include the objective monitoring of habitual physical activity and the tight control of the training protocol. The research questions posed were novel, particularly in the areas of the time course of changes in mRNA abundance and the inclusion of an exercise withdrawal phase. The number of measures of physiological function and muscle biopsies included in the MATCH study was ambitious, but were all delivered allowing analyses of multiple mRNA and protein targets set against the context of carefully characterised cardiorespiratory measures. One point of consideration in the interpretation of responses to the training programme is the influence of training load prescription across three groups: young and older healthy sedentary volunteers and patients with COPD. As discussed earlier, peak exercise performance may have been limited by ventilatory constraints and associated dyspnoea whereas it is normally the case that healthy individuals experience a cardiovascular limitation to incremental exercise. Training at an intensity corresponding to 65% of the baseline peak power from the incremental exercise test was demanding but achievable for the healthy groups and elicited very similar training responses. Patients with COPD have previously been shown

to benefit from high intensity aerobic training and it is possible that the continuous cycle exercise at 65% of peak power was not optimised for them. The training intensity was chosen as it is an intensity that requires contribution from both lipid and carbohydrate oxidation to meet ATP requirements and therefore is likely to stimulate muscle mitochondrial adaptation as well as cardiovascular adaptation (all of which were evident in the healthy young and older groups). The incremental test may have been prematurely truncated in some patients with COPD as they reached the limit of their reduced ventilatory capacity such that they may have been prescribed exercise at 65% of a falsely low maximum. Whilst this means that the COPD group may have been able to exercise at a higher proportion of their peak power (pulmonary rehabilitation programmes use walking speeds 85% of maximum), the healthy groups would then have been unable to tolerate the intensity. Data from the COPD group should be interpreted in the context of moderate intensity training; the effects of high intensity training on the outcomes measured in this study remain unknown for this group of patients with COPD. Pooled data for the whole COPD group suggest the presence of a ventilatory limitation (the group reached a mean of 103% MVV in baseline incremental testing) however heterogeneity within the group means that some patients will have reached their ventilatory limit whilst some will not. The group size in this study (n = 19) was too small to permit a sub-group analysis of patients with and without a ventilatory limitation and a larger study may have shed light on the relative training responses of patients with different ventilatory constraints.

The choice of 96 mRNA targets in the MATCH study allowed quantification across a wide range of expression values using a highly sensitive RT-PCR technique for targets of known importance in adaptation to aerobic training. It is however acknowledged that other genes may be responsive to aerobic exercise training and withdrawal that were not measured in this study. Likewise the selection of 11 protein targets gives a narrow snapshot of influence on the proteome in response to exercise and selection of protein targets based on the response of mRNA at single time points may not have identified proteins most likely to make stable changes in abundance across time.

A criticism of the acute study of NMES vs resistance exercise is that the contractile force was not measured during NMES and therefore the work done could not be matched.

However, muscle contraction evoked by NMES is not smooth and coordinated, rather it causes “twitching” of individual and small groups of muscle fibres which can at times be temporally uncoordinated therefore reducing force generated at the tendon. Only if the muscle reaches full tetanus (which can cause intolerable discomfort for the patient) are all the fibres contracting at the same time. The contraction intensities were matched in a very pragmatic manner which was that resistance exercise was performed maximally and NMES was performed at the maximum tolerable intensity. In this respect the muscle contractions evoked represent what would occur in a clinical setting and the gene expression results relate to that. One consideration that would have improved the design of the study would have been for the same patient to undergo both NMES and resistance exercise on separate legs which would have minimised inter-subject variability, although this would have doubled the number of biopsies per subject.

8.4 Future directions

Having shown that young and older adults adapt similarly to eight weeks moderate intensity aerobic training and that adaptations are not fully reversed four weeks after training ceased, there are two questions that immediately stand out with relevance to healthy sedentary populations. Firstly, can the training intervention employed in this study be optimised to produce improvements in aerobic fitness of even larger magnitude? There is gathering evidence for the effectiveness of interval training of varying intensities and volumes and the use of different modes of exercise (e.g. walking, running, other whole-body exercise) should also be considered as should the duration of the intervention. Finding a balance between optimal physiological training load and an intervention that is tolerable and appealing to the individual is key to maximising gains in cardiorespiratory fitness. The second question is what is the minimum volume of exercise necessary to maintain newly gained aerobic fitness? No matter how highly optimised and personalised an aerobic training programme is, once the external motivation of exercise supervision ends it is unlikely that all previously sedentary individuals will persist with a rigorous training regime. The data in this thesis show that cardiorespiratory training adaptations are not lost in four weeks of total exercise training cessation and that the mRNA response is maintained, but a study examining the

effect of various reduced training loads for a much longer duration would provide evidence of what the minimum requirement is to maintain the benefits of an aerobic exercise training programme in the long term.

That maintaining training adaptations is desirable and indeed essential to maximise health benefits seems intuitive to health professionals and exercise scientists. As discussed in earlier chapters, low cardiorespiratory fitness is a known risk factor for morbidity and mortality in healthy populations of all ages. What is less clear is whether intervening to increase aerobic capacity reduces the risk of disease or early death. The biggest reduction in cardiovascular risk in observational studies is between the individuals with the very lowest aerobic capacity and those who have moderately better aerobic fitness (the reduction in risk between individuals who are fit and those who are very highly trained is much smaller) (Kaminsky, Arena et al. 2013), so it may be that it is more important to make modest improvements in the fitness of very sedentary individuals and to maintain those gains in the long-term than to attempt to make very large gains in aerobic fitness. There is however relatively little evidence on what effect an intervention to increase cardiorespiratory fitness has on long-term health outcomes. It is possible that individuals with a genetic predisposition for longevity also have naturally higher aerobic capacity independent of exercise training status. It would require huge resources to perform a trial of sufficient size and duration to test the long-term effects of changes in aerobic capacity, however data are emerging from large longitudinal cohort studies, particularly in the United States which include measures of cardiorespiratory fitness and may answer this question.

The standout finding from the study of aerobic training in patients with COPD presented in this thesis is that despite training at lower absolute intensities and the absence of an increase in $\dot{V}O_2^{PEAK}$, the mRNA responses in the *vastus lateralis* were strikingly similar to those in the healthy control group. Work currently underway within the research team is investigating the effects of the same aerobic training and exercise cessation study on the abundance and ATP production capacity of mitochondria in the skeletal muscle of patients with COPD and healthy controls. Early data suggest that maximal mitochondrial ATP production capacity does not adapt in patients with COPD as it does in healthy controls. If confirmed, this would open up an interesting area of investigation: where in

the pathway between transcription of mRNA and adaptation in mitochondrial function do the responses to exercise diverge between healthy volunteers and patients with COPD? Secondly, is there a means of intervening to maximise mitochondrial training adaptations in COPD, either through optimisation of training protocols (for example interval training, single-leg training, eccentric exercise or any other means of increasing the muscle-specific training intensity) or with pharmaceutical agents? Whilst the health objectives of improving aerobic fitness in healthy sedentary populations focus on disease and mortality risk reduction, the benefits of maximised aerobic capacity for patients with COPD are much more immediate. Very low aerobic capacity causing premature lactic acidosis and fatigue has significant negative impact on quality of life for patients with COPD; optimisation of skeletal muscle function has the potential to improve functional capacity and reduce symptom burden and should therefore be an area of focus, particularly as treatments for the primary lung pathology are currently limited.

Periods of inactivity are inevitable for patients with COPD due exacerbations and fluctuations in symptom severity. In the stable state, changes in mRNA abundance prompted by an aerobic training programme persist throughout four weeks of exercise withdrawal. It would be of interest to know whether this indicates that the skeletal muscle is still primed for training adaptations, and the time course of training adaptations at the skeletal muscle and whole-body level if training were to be resumed. Would recently trained individuals respond more quickly than untrained individuals? Similar to the question posed for healthy individuals, it is of great interest to know what minimum training load is necessary to maintain the benefits of an intensive short-term training programme in patient with COPD.

Muscle weakness as well as reduced aerobic capacity impairs quality of life for some patients with COPD. The study of a single bout of resistance exercise or NMES revealed differences in the acute mRNA responses to the different interventions. NMES is an appealing intervention for use with patients who are too breathless or deconditioned to perform traditional resistance exercise training. The logical progression from the study presented in this thesis is to examine the effects of a chronic NMES training intervention both on molecular markers and cellular adaptations (for example muscle fibre cross

sectional area and satellite cell activation) as well as functional outcomes. More evidence of functional benefit, supported by mechanistic evidence is required before NMES can be recommended as a clinical intervention as part of a pulmonary rehabilitation package but the potential of this intervention to benefit the weakest and most frail patients with COPD make it worthy of further investigation.

8.5 Concluding remarks

Evidence presented in this thesis suggests that for sedentary, but otherwise healthy individuals advanced age is not a barrier to improving cardiorespiratory fitness. Furthermore, older volunteers did not demonstrate signs of accelerated deconditioning compared to young individuals over a four week period after the cessation of training. Changes in mRNA abundance in quadriceps muscle occurred early in the intervention period in both young and older groups and were maintained throughout training and exercise withdrawal, apparently preceding and out lasting training adaptations that were measurable at a whole-body level. mRNA changes predicted influence over biological functions relating to fuel selection which agreed with data from sub-maximal exercise testing that showed reductions in RER in the trained state signifying a shift away from carbohydrate and towards lipid oxidation in muscle fibres suggesting that transcription of mRNA is not a limitation to training adaptations in COPD.

That patients with COPD did not experience the same cardiorespiratory adaptations to training employing matched relative moderate intensity exercise compared to age-matched controls is not unprecedented; it is known that patients must train at a high intensity to increase $\dot{V}O_2^{PEAK}$ and ventilatory constraints may limit incremental exercise performance. It was apparent however that patients with COPD did make physiological adaptations both during sub-maximal exercise (reduced RER and HR) and at an mRNA level where the influence of exercise on predicted biological functions was strikingly similar to healthy controls, suggesting that mRNA transcripts known to be responsive to aerobic training in health also respond in patients with COPD despite lower absolute training intensities.

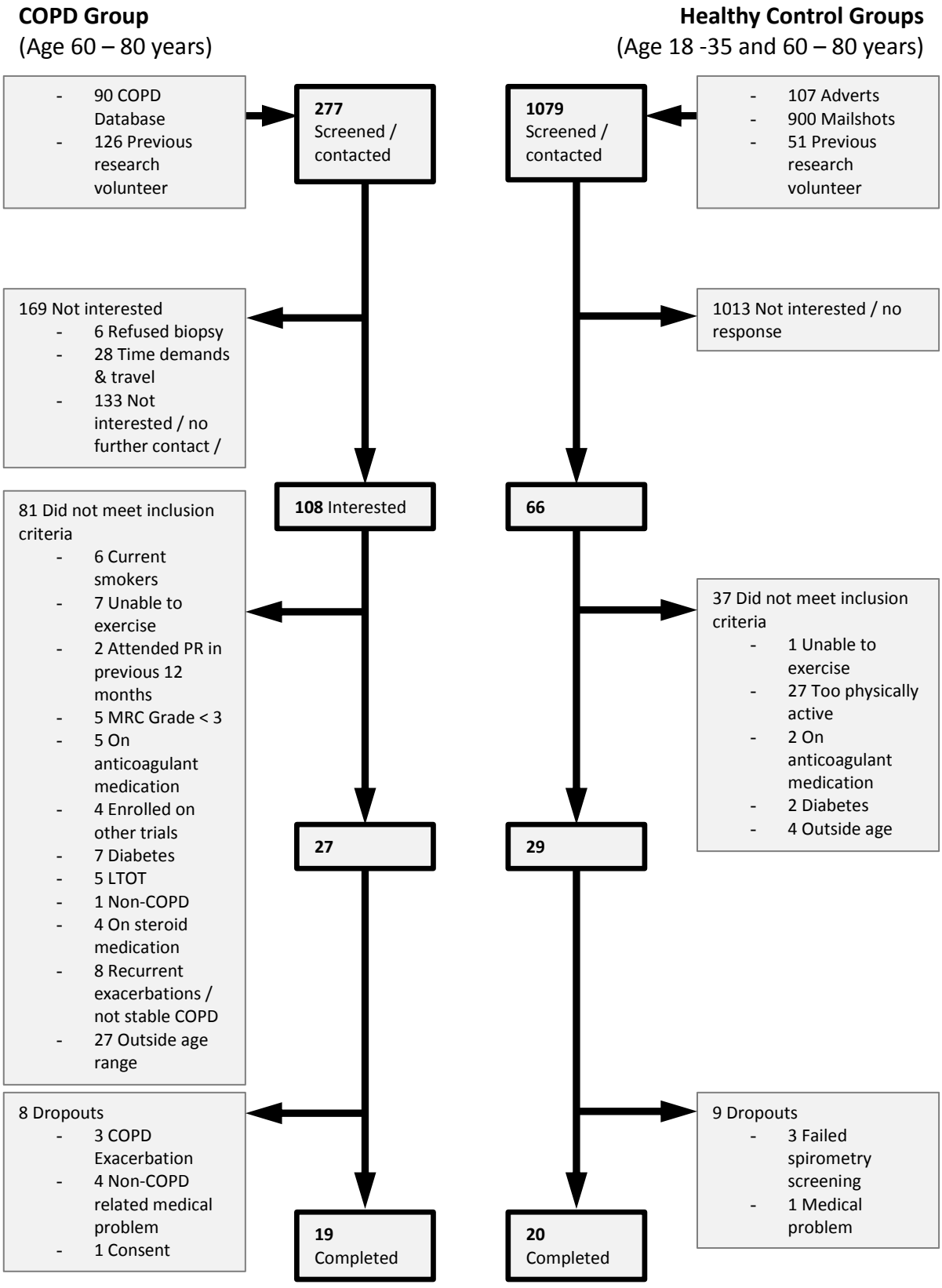
Whilst the mRNA response of patients with COPD to aerobic exercise was similar to that observed in healthy individuals, the acute study of two different modes of muscle

contraction in COPD showed clear differences. A bout of resistance exercise influenced the abundance of a far broader selection of mRNA transcripts than a bout of NMES. Although resistance exercise appeared to be superior to NMES for stimulating mRNA responses when both interventions were performed at the maximal tolerable intensity, non-volitional muscle contractions evoked by NMES did stimulate change in abundance of mRNA molecules associated with pro-growth signalling.

Appendices

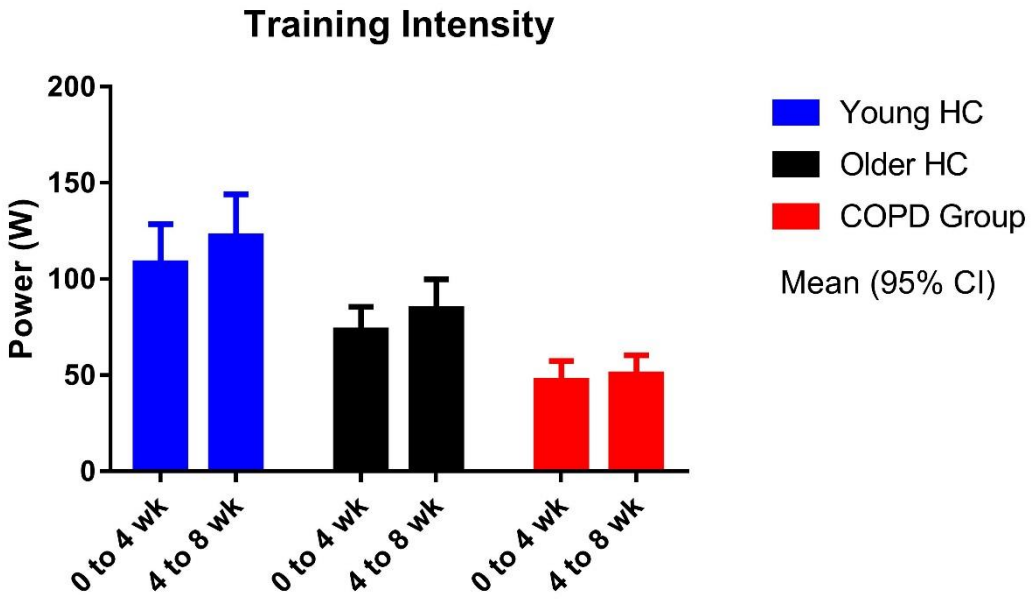
Appendix 1

Recruitment Flow: MATCH Aerobic Training Study



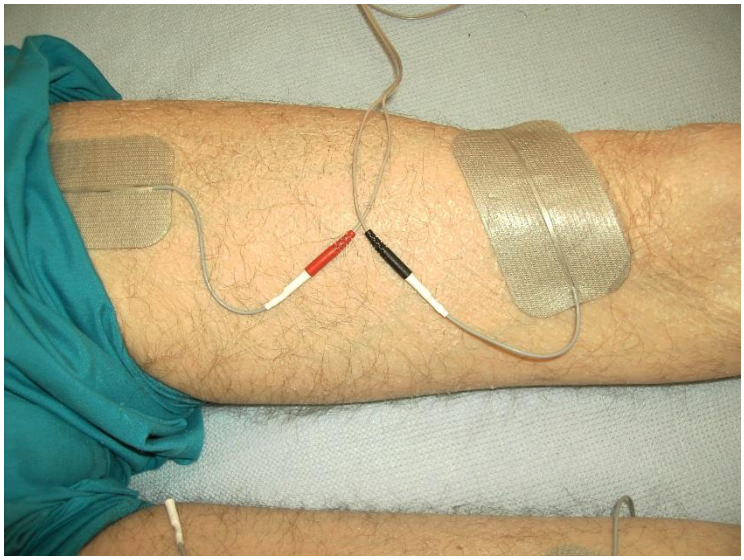
Appendix 2

Exercise training intensity performed by Young, Older and COPD groups during the MATCH Study.

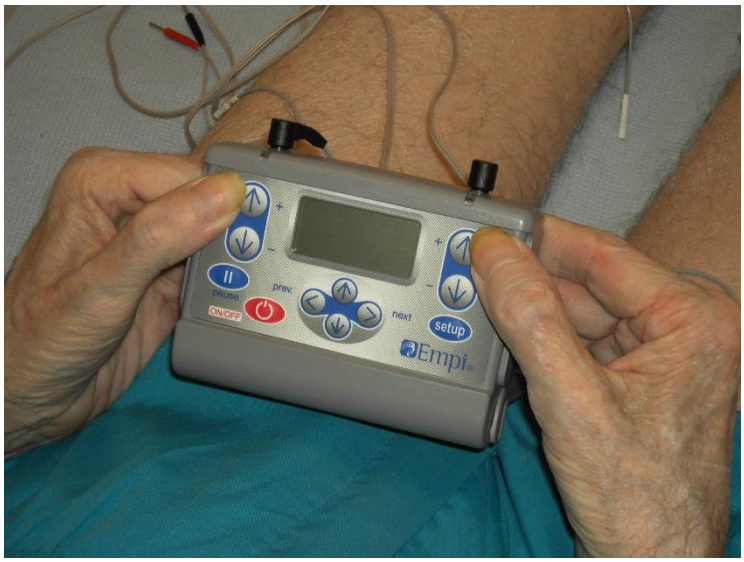


Appendix 3

Electrode Placement for neuromuscular electrical stimulation



Electrode placement on left thigh of participant.



Hand-held NMES device under the control of a study volunteer.

Appendix 4

Cardiopulmonary Exercise Testing Protocols: MATCH Aerobic Training Study

Maximal Aerobic Exercise Capacity: Ramp Protocol Overview	
Time (min)	
0	Recording starts. Subject sits at rest on cycle ergometer.
3	Subject starts unloaded pedalling (0 W) at 60 rpm
6	Ramp in workload starts. Subject continues to pedal at 60 rpm
End test	Workload continues to increase at the pre-determined rate until the subject is unable to continue, or maintain the required cadence of 60 rpm due to exhaustion or symptom burden, or until investigators stop the test due to safety concerns.

Sub-Maximal Protocol Overview	
Time (min)	
0	Recording starts. Subject sits at rest on cycle ergometer.
3	Subject starts unloaded pedalling (0 W) at 60 rpm
6	Workload starts to increase and will ramp from 0 W to the target load over a period of 60 s. Subject continues to pedal at 60 rpm
7	Target workload reached. Endurance time measured from this point to end of test.
37 **or** **End test**	Workload remains at the pre-determined wattage until the subject is unable to continue, or maintain the required cadence of 60 rpm due to exhaustion or symptom burden, or until investigators stop the test due to safety concerns. The upper limit for the endurance time test is 30 minutes, meaning that the test is terminated by the investigators in the event that a subject is still capable of continuing when the timer reaches 37 min.

Appendix 5

RNA Extraction Protocol

Reagents used for RNA Extraction

Product	**Company**	**Catalogue number**
Tri reagent	Life Technologies (Ambion)	AM9738
Glycogen (molecular grade)	Thermo Scientific	#R0561
Chloroform (molecular grade)	Sigma	
Isoamylic alcohol / Isopropanol (molecular grade)	Sigma	

Protocol for RNA Extraction using TRI Reagent (Ambion / Applied Biosystems)

1. Transfer tissue to a 4ml cryovial (silicon seal, sterile) keep on ice.
2. Add 1ml TRI Reagent solution (contains phenol and guanidine thiocyanate in monophasic solution to inhibit RNase activity) to the vial containing the muscle piece
3. Add 20µl of glycogen solution 10µg/µl (stock is 20µg/µl, dilute with DD H_2O)
4. Homogenise with Power Gen 1000 for 20-30 sec speed 2.5. Repeat if particles are still visible.
5. Incubate the homogenate at room temperature for 5 min
6. Transfer the muscle extract to sterilised Eppendorf tube
7. Add 200 µl chloroform (fume hood)
8. Manually shake the tubes for 15 sec. Keep finger on tube's cap at all times.
9. Incubate at room temperature for 2-3 min
10. Spin at 12,000g for 15 min, @ +4 ˚C
11. Transfer the water phase (clear) to a new labelled Eppendorf but avoid getting the organic phase (coloured) or interphase (white)
12. Add 500 µl iso-propanol and incubate for 10 min at room temperature

[Step 12 can also be left at -20 ˚C overnight]

1. Spin samples at 12,000g for 15 min at +4 ˚C
2. Remove supernatant (pour off, but take care if pellet is loose)
3. Wash pellet in 1 ml of 75% ethanol (EtOH, analysis grade; diluted with RNase free water) by vortexing for a few seconds. The pellet should float up.
4. Spin at 8000g for 10 min
5. Remove supernatant (pour off – care if pellet is loose!). Air dry over tissue paper for a few minutes.
6. If required, remove further supernatant using Gilson pipette (50 µl volume). Take care not to lose any of the pellet
7. Re-dissolve RNA pellet in 50 µl RNase free water. Put on ice.

Store at -80 ˚C until needed.

Reverse Transcription Protocol

Reagents used for Reverse Transcription

Product	**Company**	**Catalogue number**
Random Primers (dilute to 50 µM: 49.505 µl Random Primers + 200.496 µl DNase/RNase free H_2O)	Promega	C1181
dNTPs dATP, dCTP, dGTP, dTTP(40 µmol each). Dilute 1 in 10 (i.e. 10 µl of each dNTP + 60 µl DNase/RNase free H_2O)	Promega	U1240
Recombinant RNasin Ribonuclease Inhibitor	Promega	N2511
Superscript III Reverse Transcriptase	Life Technologies (Invitrogen)	18080-044
DTT	Supplied with RT enzyme	
First strand buffer	Supplied with RT Enzyme	

Protocol for Reverse Transcription

1. Calculate volumes for 1 sample:

Calculate volume of RNA solution to contain 1 µg	x µl
Random primers	3 µl
dNTPs (Promega U1240) dil 1:10	1 µl
Water (RNase free, molecular grade)	x µl
Final volume	13 µl

2. Add RNA and reagents to a labelled (on top, not side) 0.5 ml thin walled PCR tube
3. Quick spin
4. Set tubes in Mastercycler*
5. Run programme “DesRTISS” (5 min @ 65 ˚C)
6. Remove tubes from Mastercycler and place on ice. Quick spin to settle any vapour.
7. Make Master Mix (MM). Make enough for 1 more sample than you have (cannot pipette whole volume from MM tube once mixed).

For 1 sample:	
Enzyme buffer	4 µl
DTT	1 µl
RNase inhibitor	1 µl
Total volume per sample	6 µl

8. Add 6 µl MM to each sample
9. Add 1 µl Reverse Transcriptase Enzyme* (SuperScript III, Life Technologies / Invitrogen) to each tube. Final volume of each sample is 20 µl
   *Keep RT on ice at all times
10. Quick spin
11. Set tubes in Mastercycler, run “DesRT2SS” programme (5 min @ 25 ˚C, 60 min @ 50 ˚C, 15 min @ 70 ˚C)
12. Remove from Master Cycler
13. Quick spin
14. Store at -20 ˚C or -80 ˚C

Thermal cycler machine is “Mastercycler gradient” (Eppendorf, Hamburg, Germany).

PCR Protocol. MATCH Aerobic Training Study.

Equipment & Reagents for PCR

TaqMan 7900HT Fast Real-Time PCR System	Life Technologies / Applied Biosystems, Paisley, UK
384 Wells TaqMan Low Density Array Microfluidic Cards (custom design)	Life Technologies / Applied Biosystems, Paisley, UK
TaqMan Gene Expression Master Mix (Cat no. 30124537)	Life Technologies / Applied Biosystems, Paisley, UK
Molecular grade H_2O (DNase & RNase free)	

Preparation of microfluidic cards:

1. Remove card from refrigerator and leave at room temperature for at least 10 minutes before use
2. Defrost cDNA suspension on ice
3. Calculate volumes for one sample (2 channels). There are 4 samples per card.

Master Mix		100 µl
cDNA Suspension (volume to contain 8 x 100ng cDNA)		x µl
Molecular grade water	(to make up volume)	x µl
Total volume		200 µl

4. Load 100 µl of sample onto each channel
5. Spin 2x 1:00 min at 1200 rpm
6. Seal channels
7. Cut off loading wells
8. Place in TaqMan PCR Instrument
9. Select protocol: ΔΔCt (RQ) protocol for 384 Wells TaqMan Low Density Array
10. Import card design (SDS file), label wells on card with appropriate subject number and timepoint, save data file, start run.

Thermal cycler protocol:

Stage 1	50.0 ˚C, 2:00 minutes
Stage 2	94.5 ˚C, 10:00 minutes
Stage 3	(40 repeats) 97.0 ˚C, 0:30 seconds, then 59.7 ˚C, 1:00 minute

PCR Protocol. NMES vs RE Acute Study.

Equipment & Reagents for PCR

TaqMan 7900HT Fast Real-Time PCR System	Life Technologies / Applied Biosystems, Paisley, UK
384 Wells TaqMan Low Density Array Microfluidic Cards (custom design)	Life Technologies / Applied Biosystems, Paisley, UK
TaqMan Gene Expression Master Mix (Cat no. 30124537)	Life Technologies / Applied Biosystems, Paisley, UK
Molecular grade H_2O (DNase & RNase free)	

Preparation of microfluidic cards:

1. Remove card from refrigerator and leave at room temperature for at least 10 minutes before use
2. Defrost cDNA suspension on ice
3. Calculate volumes for one sample (2 channels). There are 4 samples per card.

Master Mix		400 µl
cDNA Suspension (volume to contain 8 x 100ng cDNA)		x µl
Molecular grade water	(to make up volume)	x µl
Total volume		800 µl

4. Load 100 µl of sample onto each channel
5. Spin 2x 1:00 min at 1200 rpm
6. Seal channels
7. Cut off loading wells
8. Place in TaqMan PCR Instrument
9. Select protocol: ΔΔCt (RQ) protocol for 384 Wells TaqMan Low Density Array
10. Import card design (SDS file), label wells on card with appropriate subject number and timepoint, save data file, start run.

Thermal cycler protocol:

Stage 1	50.0 ˚C, 2:00 minutes
Stage 2	94.5 ˚C, 10:00 minutes
Stage 3	(40 repeats) 97.0 ˚C, 0:30 seconds, then 59.7 ˚C, 1:00 minute

Appendix 6

mRNA targets quantified by RT-PCR in the MATCH aerobic training study and acute NMES vs RE study.

MATCH Study

Abbreviation	Gene name	ID
Mitochondria		
MCU(CCDC109a)	mitochondrial calcium uniporter	Hs00293548_m1
SDHA	succinate dehydrogenase complex A	Hs00188166_m1
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1	Hs00957930_m1
IDH1	Isocitrate dehydrogenase 1	Hs00271858_m1
MDH2	malate dehydrogenase	Hs00938918_m1
NDUFA9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex	Hs00245308_m1
ALDH2	Aldehyde dehydrogenase 2 family (mitochondrial)	Hs01007998_m1
POLRMT	mitochondrial DNA-directed RNA polymerase	Hs04187596_g1
EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1	Hs00607050_m1
MTND6	NADH dehydrogenase subunit 6	Hs02596879_g1
MICU1(CBARA1)	mitochondrial calcium uptake 1	Hs00246104_m1
SOD2	superoxide dismutase, mitochondrial	Hs00167309_m1
UCP1	uncoupling protein 1 in mitochondria	Hs00222453_m1
UCP2	uncoupling protein 2 in mitochondria	Hs01075227_m1
UCP3	uncoupling protein 3 in mitochondria	Hs01106052_m1
MCUR1	mitochondrial calcium uniporter regulator 1	Hs00254417_m1
COX1	cytochrome c oxidase subunit I	Hs00156989_m1
MDH1B	malate dehydrogenase 1B, NAD (soluble)	Hs00332405_m1
ATP5O	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	Hs00426889_m1
p53	tumor protein p53	Hs01034249_m1
MTIF2	mitochondrial translational initiation factor 2	Hs01091373_m1
MRPL40	mitochondrial ribosomal protein L40	Hs00186843_m1
MTFR1	negative regulator of mammalian mtDNA transcription	Hs00206110_m1
KL	klotho	Hs00183100_m1

TFAM	transcription factor A, mitochondrial	Hs00273372_s1
TUFM	Tu translation elongation factor, mitochondrial	Hs00607042_gH
MRPL3	mitochondrial ribosomal protein L3	Hs00246665_m1
ME2	malic enzyme 2, NAD(+)-dependent, mitochondrial	Hs00929809_g1
MTRF1L	mitochondrial translational release factor 1-like	Hs00759012_s1
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa	Hs00190004_m1
MRPL18	mitochondrial ribosomal protein L18	Hs00204310_m1
MPC1	mitochondrial pyruvate carrier 1	Hs00211484_m1
MUL1	mitochondrial E3 ubiquitin protein ligase 1	Hs00226069_m1
NDUFA3	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3, 9kDa	Hs01547166_g1
TFB2M	transcription factor B2, mitochondrial	Hs00915025_m1
ND4	NADH dehydrogenase, subunit 4 (complex I)	Hs02596876_g1
MNF1	mitochondrial nucleoid factor 1	Hs00276889_m1
MTG2	mitochondrial ribosome-associated GTPase 2	Hs00534998_m1
CS	citrate synthase	Hs02574374_s1
HADH	b-hydroxyacyl-CoA dehydrogenase	Hs00193428_m1
NRF2/GABPA	nuclear respiratory factor 2	Hs00975961_g1
BCL2L1	apoptosis regulator	Hs00236329_m1
Angiogenesis		
ALOX12	arachidonate 12-lipoxygenase	Hs00167524_m1
PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	Hs01016719_m1
PPARGC1B	peroxisome proliferator-activated receptor gamma, coactivator 1 beta	Hs00991677_m1
VEGFA	Vascular endothelial growth factor A	Hs00900055_m1
Carbohydrate metabolism		
PDP2	pyruvate dehyrogenase phosphatase catalytic subunit 2	Hs00380020_m1
PFKM	phosphofructokinase, muscle	Hs00175997_m1
GSK3B	Glycogen synthase kinase-3 beta	Hs01047719_m1
HK1	hexokinase 1	Hs00175976_m1
HK2	hexokinase 2	Hs00606086_m1
PDK2	pyruvate dehydrogenase kinase, isozyme 2	Hs00176865_m1
PGM1	phosphoglucomutase 1	Hs01071897_m1
PKM	pyruvate kinase, muscle	Hs00987255_m1

PYGM	phosphorylase, glycogen, muscle	Hs00989942_m1
GYS1	glycogen synthase 1 (muscle)	Hs00157863_m1
Glut4	glucose transporter	Hs00168966_m1
PDK4	pyruvate dehydrogenase kinase, isozyme 4	Hs01037712_m1
GLUD1	glutamate dehydrogenase 1	Hs03989560_s1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
G6PD	Glucose-6-phosphate dehydrogenase	Hs00166169_m1
MSTN	myostatin	Hs00976237_m1
Lipid metabolism		
PLD4	phospholipase D family, member 4	Hs00975488_m1
FAXDC2	fatty acid hydroxylase domain containing 2	Hs00260753_m1
LPPR2	lipid phosphate phosphatase-related protein type 2	Hs01106565_m1
LOXHD1	lipoxygenase homology domains 1	Hs00329848_m1
HSPA14	heat shock 70kDa protein 14	Hs00212495_m1
HSPD1	heat shock 60kDa protein 1 (chaperonin)	Hs01036747_m1
ACOT1	acyl-CoA thioesterase 1	Hs04195130_s1
ADIPOQ	adiponectin	Hs00605917_m1
APOC1	Apolipoprotein C	Hs00155790_m1
CPT1B	carnitine palmitoyltransferase, muscle isoform	Hs00189258_m1
CPT1A	carnitine palmitoyltransferase, liver isoform	Hs00912671_m1
APOA1	Apolipoprotein A-I	Hs00163641_m1
PLA2	Phospholipase A2	Hs00179898_m1
FOXO1	Forkhead box protein O1	Hs01054576_m1
Insulin resistance		
IGF1	insulin-like growth factor 1 (somatomedin C)	Hs01547656_m1
INSR	Insulin receptor	Hs00961554_m1
IRS1	Insulin receptor substrate 1	Hs00178563_m1
PPARA	peroxisome proliferator-activated receptor alpha	Hs00947536_m1
PPARD	peroxisome proliferator-activated receptor delta	Hs00987011_m1
PPARG	peroxisome proliferator-activated receptor gamma	Hs01115513_m1
IGF2BP1	insulin-like growth factor 2 mRNA binding protein 1	Hs00198023_m1

IGFBP7	insulin-like growth factor binding protein 7	Hs00266026_m1
IGFBP4	insulin-like growth factor binding protein 4	Hs01057900_m1
FASL	Fas ligand (TNF superfamily, member 6)	Hs00181225_m1
IL6	interleukin 6 (interferon, beta 2)	Hs00985639_m1
IL10	interleukin 10	Hs00961622_m1
CCL19	chemokine (C-C motif) ligand 19	Hs00171149_m1
CCL22	chemokine (C-C motif) ligand 22	Hs01574247_m1
TLR4	toll-like receptor 4	Hs00152939_m1
Hypoxia		
HIF1a	Hypoxia-inducible factor 1-alpha	Hs00153153_m1
HIGD1A	HIG1 hypoxia inducible domain family, member 1A, mitochondrial	Hs00854612_gH
NRF1	nuclear respiratory factor 1	Hs00192316_m1
Endogenous genes		
HMBS	hydroxymethylbilane synthase	Hs00609297_m1
18S	ribosomal 18S	Hs99999901_s1

Abbreviation	Gene name	ID
18S	ribosomal 18S	Hs99999901_s1
AARS	alanyl-tRNA synthetase	Hs00609836_m1
ABR	active BCR-related gene	Hs00254300_m1
ACAA1	Peroxisomal 3-oxoacyl-Coenzyme A thiolase (ACAA1)	Hs00155616_m1
ACACA	H.sapiens mRNA for acetyl-CoA carboxylase. (mRNA)	Hs00167385_m1
ACCN3	amiloride-sensitive cation channel 3	Hs00245097_m1
ACO2	aconitase 2, mitochondrial	Hs00426616_g1
ACOX2	acyl-Coenzyme A oxidase 2, branched chain	Hs00185873_m1
ACTA1	actin, alpha 1, skeletal muscle	Hs00559403_m1
ACTN1	actinin, alpha 1	Hs00241650_m1
ACTN3	actinin, alpha 3	Hs00153812_m1
ADARB1	Adenosine deaminase (adenosine aminohydrolase)	Hs00953724_m1
ADAT1	adenosine deaminase, tRNA-specific 1	Hs00201527_m1
ADK	Adenosine kinase	Hs00417073_m1
ADRM1	cell membrane glycoprotein, 110000M(r) (surface antigen), adhesion regulating molecule 1	Hs00199645_m1
AK1	Adenylate kinase 1 (AK1)	Hs00176119_m1
AKR1A1	aldo-keto reductase family 1, member A1 (aldehyde reductase)	Hs00195992_m1
AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	Hs00413886_m1
Akt1	v-akt murine thymoma viral oncogene homolog 1	Hs00178289_m1
ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)	Hs00355914_m1
ALDOA	aldolase A, fructose-bisphosphate	Hs00605108_g1
AMPD3	adenosine monophosphate deaminase (isoform E)	Hs00983043_m1
ANK1	ankyrin 1, erythrocytic	Hs00220867_m1
ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	Hs00173317_m1
ANP32B	acidic (leucine-rich) nuclear phosphoprotein 32 family, member B	Hs00742713_s1
ANXA6	annexin A6	Hs00241765_m1
ANXA7	annexin A7	Hs00559413_m1
APOA2	Apolipoprotein A-II	Hs00155788_m1
APOC1	apolipoprotein C-I	Hs00155790_m1

AQP4	Aquaporin 4 (AQP4)	Hs00242342_m1
ARPC4	actin related protein 2/3 complex, subunit 4 (20 kD)	Hs00194811_m1
ARS2	arsenate resistance protein ARS2	Hs00367552_m1
ARTS1	Adipocyte-derived leucine aminopeptidase (KIAA0525), type 1 tumor necrosis factor receptor shedding aminopeptidase regulator	Hs00429970_m1
ASPA	aspartoacylase (aminoacylase 2, Canavan disease)	Hs00163703_m1
ATF4	activating transcription factor 4 (tax-responsive enhancer element B67)	Hs00909569_g1
ATP2A1	Sarcoplasmic/endoplasmic reticulum calcium ATPase (ATP2A1) (fast-twitch skeletal muscle isoform), ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	Hs01092295_m1
ATP2B2	Plasma membrane calcium ATPase isoform 2 (ATP2B2), ATPase, Ca++ transporting, plasma membrane 2	Hs01090447_m1
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	Hs00969569_m1
ATP5D	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	Hs00184622_m1
ATP5G1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 1	Hs00829069_s1
ATP5G3	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9) isoform 3	Hs00266085_m1
ATP5I	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit e	Hs00273015_m1
ATP5O	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (oligomycin sensitivity conferring protein)	Hs00426889_m1
BAD	BCL2-antagonist of cell death	Hs00188930_m1
BCHE	Butyrylcholinesterase	Hs00992319_m1
BPGM	2,3-bisphosphoglycerate mutase	Hs00156139_m1
BRCA1	breast cancer 1, early onset	Hs00173233_m1
BRP44L	HSPC040 protein, brain protein 44-like	Hs00211484_m1
BZW2	HSPC028 protein (similar to elongation factor), basic leucine zipper and W2 domains 2	Hs00204063_m1
C22orf9	Homo sapiens mRNA for KIAA0930 protein, partial cds.	Hs00292272_m1
CA14	carbonic anhydrase XIV	Hs00201626_m1

CALCOCO2	nuclear domain 10 protein, calcium binding and coiled-coil domain 2	Hs00414663_m1
CAPN3	Calpain 3	Hs00544982_m1
CARS2	TRNASTNTHETASE:tRNA synthetase homologue 4574912CD1 (incyte), cysteinyl-tRNA synthetase 2, mitochondrial (putative)	Hs00226049_m1
CASP10	caspase 10, apoptosis-related cysteine peptidase	Hs01017902_m1
CAST	calpastatin	Hs00156280_m1
CBFB	core-binding factor, beta subunit, acute myeloid leukemia 1	Hs00242387_m1
CCL19	chemokine (C-C motif) ligand 19	Hs00171149_m1
CCL22	chemokine (C-C motif) ligand 22	Hs00171080_m1
CCND1	cyclin D1	Hs00277039_m1
CCNG1	Cyclin G1	Hs00171112_m1
CCNG2	Cyclin G2	Hs00171119_m1
CCPG1	cell cycle progression 8 protein	Hs00393715_m1
CCR10	chemokine (C-C motif) receptor 10	Hs00706455_s1
CCT3	chaperonin containing TCP1, subunit 3 (gamma)	Hs00195623_m1
CCT4	chaperonin containing TCP1, subunit 4 (delta)	Hs00272345_m1
CD38	CD38 antigen (p45)	Hs00277045_m1
CDC2L2	Galactosyltransferase associated protein kinase P58/GTA cell division cycle 2-like 1 (PITSLRE proteins)	Hs00414449_m1
CDC2L2	cell division cycle 2-like 2 (PITSLRE proteins)	Hs02341397_m1
CDC42BPA	Serine/threonine protein kinase 7483085CD1 (incyte), CDC42 binding protein kinase alpha (DMPK-like)	Hs00207976_m1
CDH13	cadherin 13, H-cadherin (heart)	Hs00169908_m1
CDK2	Cyclin dependent kinase 2	Hs00608082_m1
CDK2AP2	tumor suppressor deleted in oral cancer-related 1	Hs00739856_g1
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	Hs00176227_m1
CES2	carboxylesterase 2 (intestine, liver)	Hs00187279_m1
CFLAR	CASP8 and FADD-like apoptosis regulator	Hs00153439_m1
CHAF1B	chromatin assembly factor 1, subunit B (p60)	Hs00601414_m1
CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	Hs00609691_m1
CHRNB1	cholinergic receptor, nicotinic, beta 1 (muscle)	Hs00181255_m1
CHRND	cholinergic receptor, nicotinic, delta	Hs00181284_m1
CHRNE	cholinergic receptor, nicotinic, epsilon	Hs00181084_m1

CKMT2	Sarcomeric mitochondrial creatine kinase	Hs00176502_m1
CLCN1	Chloride channel protein 1 (CLC-2), chloride channel 1, skeletal muscle (Thomsen disease, autosomal dominant)	Hs00892505_m1
CLCN2	chloride channel 2	Hs00189078_m1
CLIP1	restin (Reed-Steinberg cell-expressed intermediate filament-associated protein), CAP-GLY domain containing linker protein 1	Hs00418962_m1
CNBP	zinc finger protein 9 (a cellular retroviral nucleic acid binding protein), CCHC-type zinc finger, nucleic acid binding protein	Hs00231535_m1
COL4A1	collagen type IV, alpha1	Hs01007469_m1
COX4I1	cytochrome c oxidase subunit IV	Hs00971639_m1
COX5B	cytochrome c oxidase subunit Vb	Hs00426948_m1
COX6A2	cytochrome c oxidase subunit VIa polypeptide 2	Hs00193226_g1
COX6B1	cytochrome c oxidase subunit Vib, cytochrome c oxidase subunit Vib polypeptide 1 (ubiquitous)	Hs00266375_m1
COX7B	cytochrome c oxidase subunit VIIb	Hs00371307_m
COX7C	cytochrome c oxidase subunit VIIc	Hs01595220_g1
CPT1B	Carnitine palmitoyltransferase I (mitochondrial muscle isoform)	Hs00189258_m1
CREB1	cAMP responsive element binding protein 1	Hs00231713_m1
CSDE1	NRAS-related gene, cold shock domain containing E1, RNA-binding	Hs00200261_m1
CTNNA3	alpha-catenin-like protein, cadherin associated protein	Hs00379052_m1
CTSB	Cathepsin B (APP secretase)	Hs00947439_m1
CTSH	Cathepsin H	Hs00189291_m1
CTSL1	Cathepsin L1	Hs00377632_m1
CTSL2	Cathepsin L2	Hs00426731_m1
CYBA	cytochrome b-245, alpha polypeptide	Hs00609145_m1
CYC1	cytochrome c-1	Hs00357717_m1
CYLD	cylindromatosis (turban tumor syndrome)	Hs00211000_m1
DAPK1	death-associated protein kinase 1	Hs00234480_m1
DAXX	death-associated protein 6	Hs00154692_m1
DBI	diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)	Hs01554584_m1
DCI	dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenzyme A isomerase)	Hs00157239_m1
DECR1	2,4-dienoyl CoA reductase 1, mitochondrial	Hs00154728_m1
DEXI	MYLE protein, dexamethasone-induced transcript	Hs00360234_m1

DGKD	Diacylglycerol kinase eta (DAG kinase eta)	Hs01114141_m1
DLAT	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogease complex, mitochondrial (E2)	Hs00898876_m1
DLG5	Discs large protein P-dlg, discs, large homolog 5 (Drosophila)	Hs00610416_m1
DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	Hs00266011_m1
DNAJB5	DnaJ (Hsp40) homolog, subfamily B, member 5	Hs00273373_m1
DPT	dermatopontin	Hs00170030_m1
DUSP3	Dual specificity protein phosphatase homologue AB027004	Hs00969203_m1
DYNC1H1	Human mRNA for KIAA0325 gene, partial cds., dynein, cytoplasmic 1, heavy chain 1	Hs00378891_m1
DYSF	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	Hs00243339_m1
E2F1	E2F transcription factor 1	Hs00153451_m1
EEF1G	eukaryotic translation elongation factor 1 gamma	Hs01922638_u1
EIF2B4	DKFZP586J0119 protein, eukaryotic translation initiation factor 2B, subunit 4 delta, 67kDa	Hs00248984_m1
EIF2S1	eukaryotic translation initiation factor 2, subunit 1 (alpha, 35kD)	Hs00187953_m1
EIF2S2	eukaryotic translation initiation factor 3, subunit 2 (beta, 36kD)	Hs00830013_s1
EIF3K	Homo sapiens PRO1474 mRNA, complete cds., eukaryotic translation initiation factor 3, subunit K	Hs00795983_s1
EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1	Hs00607050_m1
EIF4H	Williams-Beuren syndrome chromosome region 1, eukaryotic translation initiation factor 4H	Hs00254535_m1
ENDOG	endonuclease G	Hs00172770_m1
ETFB	electron-transfer-flavoprotein, beta polypeptide	Hs01085511_m1
FADD	Fas (TNFRSF6)-associated via death domain	Hs00538709_m1
FADS3	fatty acid desaturase 3	Hs00222230_m1
FANCA	Fanconi anemia, complementation group A	Hs01116668_m1
FARP1	|FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 (chondrocyte-derived)	Hs00195010_m1
FARS2	phenylalanine-tRNA synthetase	Hs00255212_m1
FASLG	Fas ligand (TNF superfamily, member 6)	Hs00181225_m1

FASTK	Fas-activated serine/threonine kinase (FAST)	Hs00179113_m1
FBP2	fructose-1,6-bisphosphatase 2	Hs00427791_m1
FBXO32	MAFbx	Hs00369714_m1
FHL3	four and a half LIM domains 3	Hs00189516_m1
FLII	flightless I (Drosophila) homolog	Hs00157582_m1
FLNC	filamin C, gamma (actin-binding protein-280)	Hs00155124_m1
FNTA	farnesyltransferase, CAAX box, alpha	Hs00357739_m1
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	Hs00170630_m1
FOXO1A	forkhead box O1A (rhabdomyosarcoma)	Hs01054576_m1
FOXO3A	forkhead box O3A	Hs00818121_m1
FRAP1	FK506 binding protein 12-rapamycin associated protein 1	Hs00234508_m1
GAMT	Guanidinoacetate N-methyltransferase (GAMT)	Hs00164681_m1
GBP2	guanylate binding protein 2, interferon-inducible	Hs00894837_m1
GLP-1 R	Glucagon-like peptide 1 receptor (GLP-1 receptor)	Hs00157705_m1
GLRX2	glutaredoxin (thioltransferase)	Hs00375015_m1
GMPR	guanosine monophosphate reductase	Hs00199328_m1
GNB1L	transcription elongation factor B (SIII), polypeptide 1-like	Hs00223722_m1
GNG7	guanine nucleotide binding protein (G protein), gamma 7	Hs00192999_m1
GOT	Cytosolic aspartate aminotransferase, glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	Hs00157798_m1
GPC1	glypican 1	Hs00892476_m1
GPI	glucose phosphate isomerase	Hs00976711_m1
GPSN2	glycoprotein, synaptic 2	Hs00604016_gH
GPX4	GPx-4 phospholipid hydroperoxide glutathione	Hs00157812_m1
GRB10	growth factor receptor-bound protein 10	Hs00193409_m1
GRHPR	glyoxylate reductase/hydroxypyruvate reductase	Hs00201903_m1
GSK3B	glycogen synthase kinase 3 beta	Hs00275656_m1
GSTK1	glutathione S-transferase subunit 13 homolog	Hs00210861_m1
HMBS		Hs00609297_m1
HP1BP3	HP1-BP74, heterochromatin protein binding protein 74 gene	Hs00212448_m1
HPGD	15-hydroxy prostaglandin dehydrogenase	Hs00168359_m1

HPRT1		Hs99999909_m1
HRASLS	HRAS-like suppressor	Hs01082527_m1
HSPA1B	heat shock 70kD protein 1B	Hs01040501_sH
HSPA5	Homo sapiens mRNA for glucose-regulated protein (HSPA5 gene)., heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	Hs99999174_m1
HSPB1	heat shock 27kDa protein 1	Hs00356629_g1
HSPB2	heat shock 27kD protein 2	Hs00155436_m1
ICMT	Prenylcysteine carboxyl methyltransferase (PCCMT)	Hs00202655_m1
IDH1	isocitrate dehydrogenase 2 (NADP+), mitochondrial	Hs00271858_m1
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	Hs00395088_m1
IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	Hs01063858_m1
IL10	interleukin 10	Hs00174086_m1
IL12B	interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	Hs00233688_m1
IL18	interleukin 18 (interferon-gamma-inducing factor)	Hs00155517_m1
IL1A	interleukin 1, alpha	Hs00174092_m1
IL6	interleukin 6 (interferon, beta 2)	Hs00174131_m1
INS	insulin	Hs00355773_m1
INSR	insulin receptor	Hs00169631_m1
IRF7	interferon regulatory factor 7	Hs00185375_m1
IRS1	insulin receptor substrate 1	Hs00178563_m1
ITGA7	Integrin alpha-7	Hs00174397_m1
ITGB6	Integrin beta-6	Hs00168458_m1
JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	Hs00277190_s1
KALRN	kalirin, RhoGEF kinase	Hs00610179_m1
KCNN2	Apamin-sensitive small-conductance Ca2+-activated potassium channel (KCNN2), potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	Hs01030641_m1
KCNN3	Calcium-activated potassium channel (KCNN3), potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3	Hs00158463_m1

KCNN4	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	Hs00158470_m1
KIAA0368	Human mRNA for KIAA0368 gene, partial cds.	Hs00378772_m1
LAMB1	laminin, beta 1	Hs00158620_m1
LARGE	Acetylglucosaminyltransferase-like protein (LARGE)	Hs00893935_m1
LDHA	lactate dehydrogenase A	Hs00855332_g1
LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1)	Hs00355202_m1
LIAS	Homo sapiens mRNA for putative lipoic acid synthetase, partial.	Hs00398048_m1
LMO1	LIM domain only 1 (rhombotin 1)	Hs00231133_m1
LRP4	Homo sapiens mRNA for MEGF7, partial cds.low density lipo protein receptor	Hs00391006_m1
LTC4S	Leukotriene C4 synthase	Hs00168529_m1
MAF	C-MAF (short form), v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	Hs00193519_m1
MAN2C1	mannosidase, alpha, class 2C, member 1	Hs00428900_m1
MAP2K3	mitogen-activated protein kinase kinase 3	Hs00177127_m1
MAP3K12	Mitogen activated protein kinase kinase kinase 12	Hs00178982_m1
MAPT	microtubule-associated protein 4	Hs00902193_m1
MARS2	methionine-tRNA synthetase	Hs00536599_s1
MDH1	malate dehydrogenase 1, NAD (soluble)	Hs00195506_m1
MDH2	malate dehydrogenase 2, NAD (mitochondrial)	Hs00938918_m1
MEF2C	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C), myocyte enhancer factor 2C	Hs00231149_m1
MFAP4	MFA4_HUMAN :: MICROFIBRIL-ASSOCIATED GLYCOPROTEIN 4.	Hs00412974_m1
MFAP5	Microfibril-associated glycoprotein-2	Hs00185803_m1
MGMT	6-O-methylguanine-DNA methyltransferase (MGMT)	Hs00172470_m1
MGST2	Microsomal glutathione S-transferase 2 (MGST2)	Hs00182064_m1
MKRN1	makorin, ring finger protein, 1 , acts as E3 ubiquitin ligase.	Hs00831972_s1
MLL	Homo sapiens clone 23645 mRNA sequence, myeloid/lymphoid or mixed-	Hs00610538_m1

	lineage leukemia (trithorax homolog, Drosophila)	
MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase),	Hs00234422_m1
MN1	meningioma (disrupted in balanced translocation) 1	Hs00159202_m1
MORF4L2	MORF-related gene X, mortality factor 4 like 2	Hs00202211_m1
MRAS	muscle RAS oncogene homolog	Hs00171926_m1
MRCL3	Human myosin regulatory light chain mRNA, complete cds.	Hs00272402_m1
MRPL12	mitochondrial ribosomal protein L12	Hs00243186_m1
MRPS15	mitochondrial ribosomal protein S15	Hs00229834_m1
MRPS33	mitochondrial ribosomal protein S33	Hs00211431_m1
MSTN	Myostatin	Hs00193363_m1
MYBPH	myosin binding protein H	Hs00192226_m1
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	Hs00153408_m1
MYH4	myosin, heavy polypeptide 4, skeletal muscle	Hs00757977_m1
MYL1	MLE3_HUMAN :: MYOSIN LIGHT CHAIN 3, SKELETAL MUSCLE ISOFORM (A2 CATALYTIC) (ALKALI) (MLC3F).	Hs00365258_m1
MYL3	myosin, light polypeptide 3, alkali; ventricular, skeletal, slow	Hs00264820_m1
MYLPF	(clone PWHLC2-24) myosin light chain 2, fast skeletal myosin light chain 2	Hs00203261_m1
MYOD1	Myogenic factor3	Hs00159528_m1
MYOG	Myogenin (myogenic factor4)	Hs00231167_m1
MYOM2	myomesin (M-protein) 2 (165kD)	Hs00187676_m1
NAP1L1	nucleosome assembly protein 1-like 1	Hs00748775_s1
NCF1	neutrophil cytosolic factor 1 (47kD, chronic granulomatous disease, autosomal 1)	Hs00165362_m1
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10 (42kD)	Hs00190004_m1
NDUFA9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9 (39kD)	Hs00245308_m1
NDUFB5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5 (16kD, SGDH)	Hs00159582_m1
NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8 (19kD, ASHI)	Hs00428204_m1
NDUFC1	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1 (6kD, KFYI)	Hs00159587_m1

NDUFS1	NADH dehydrogenase (ubiquinone) Fe-S protein 1 (75kD) (NADH-coenzyme Q reductase)	Hs00192297_m1
NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3 (30kD) (NADH-coenzyme Q reductase)	Hs00190028_m1
NDUFS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7 (20kD) (NADH-coenzyme Q reductase)	Hs00257018_m1
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8 (23kD) (NADH-coenzyme Q reductase)	Hs00159597_m1
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1 (51kD)	Hs00200073_m1
NDUFV2	NADH dehydrogenase (ubiquinone) flavoprotein 2 (24kD)	Hs00221478_m1
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	Hs00765730_m1
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	Hs00174517_m1
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Hs00153283_m1
NOL3	nucleolar protein 3 (apoptosis repressor with CARD domain)	Hs00358724_g1
NPDC1	neural proliferation, differentiation and control, 1	Hs00209870_m1
NPY6R	Neuropeptide Y receptor type 6 (NPY6-R)	Hs00246222_s1
NSFL1C	p47 (LOC51674), mRNA.	Hs00820401_m1
NT5M	5 nucleotidase, mitochondrial	Hs00220234_m1
OLFM1	neuroblastoma (nerve tissue) protein, olfactomedin 1	Hs00255159_m1
OSBP	oxysterol binding protein	Hs00387304_m1
OSTM1	osteopetrosis associated transmembrane protein 1	Hs00204014_m1
P2X5	P2X purinoceptor 5	Hs00531938_m1
PACSIN3	SH3 domain-containing protein 6511 (LOC51165), mRNA, protein kinase C and casein kinase substrate in neurons 3.	Hs00367625_m1
PCCB	propionyl Coenzyme A carboxylase, beta polypeptide	Hs00166909_m1
PDK2	Pyruvate dehydrogenase kinase 2 (PDK2)	Hs00176865_m1
PDK4	pyruvate dehydrogenase kinase, isozyme 4	Hs00176875_m1
PDLIM7	enigma (LIM domain protein)	Hs00193775_m1
PECI	peroxisomal D3,D2-enoyl-CoA isomerase	Hs00196146_m1
PGAM2	phosphoglycerate mutase 2 (muscle)	Hs00165474_m1

PGM1	phosphoglucomutase 1	Hs00160062_m1
PI4KA	phosphatidylinositol 4-kinase type II	Hs00176931_m1
PKD2L2	polycystic kidney disease 2-like 2	Hs00205170_m1
PKM2	Pyruvate kinase M1	Hs00761782_s1
PKP4	plakophilin 4	Hs00269305_m1
PLA2G12A	group XII secreted phospholipase A2	Hs00830106_s1
PLA2G4C	Cytosolic phospholipase A2 gamma	Hs00234345_m1
PMVK	Phosphomevalonate kinase (PMKASE)	Hs01014319_m1
POLR1C	RNA polymerase I subunit hRPA39	Hs00191646_m1
POLRMT	polymerase (RNA) mitochondrial (DNA directed)	Hs00863453_g1
PPARA	peroxisome proliferative activated receptor, alpha	Hs00231882_m1
PPARD	peroxisome proliferative activated receptor, delta	Hs00602622_m1
PPIA	T-cell cyclophilin	Hs99999904_m1
PPIF	peptidylprolyl isomerase F (cyclophilin F)	Hs00194847_m1
PPME1	protein phosphatase methylesterase-1	Hs00211693_m1
PPP2R4	Protein phosphatase PP2A regulatory subunit delta (Delta-PR55) (KIAA1541) (fragment)	Hs00603515_m1
PRDX1	peroxiredoxin 1	Hs00602020_mH
PREPL	Serine protease KIAA0436 (fragment), prolyl endopeptidase-like	Hs00384909_m1
PRKAR1A	cAMP-dependent protein kinase type I-alpha regulatory chain, protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	Hs00267597_m1
PRMT5	Protein methyltransferase (JBP1)	Hs00272020_m1
PRPS1	phosphoribosyl pyrophosphate synthetase 1	Hs00751338_s1
PSAP	prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)	Hs01551096_m1
PSMB1	Proteasome component C5	Hs00427357_m1
PSMB10	Proteasome component MECL-1	Hs00160620_m1
PSMD3	proteasome (prosome, macropain) 26S subunit, non-ATPase, 3	Hs00160646_m1
PSMD7	Proteasome regulatory subunit S12/p40/Mov34, proteasome (prosome, macropain) 26S subunit, non-ATPase, 7	Hs00427396_m1
PSME1	proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)	Hs00389209_m1
PSME2	20S Proteasome	Hs01923165_u1
PSTPIP2	proline-serine-threonine phosphatase interacting protein 2	Hs00225798_m1

PTPN3	Protein tyrosine phosphatase H1	Hs00160708_m1
PXMP4	24 kDa intrinsic membrane protein, peroxisomal membrane protein 4, 24kDa	Hs01566603_m1
PYGM	Muscle glycogen phosphorylase, phosphorylase, glycogen; muscle (McArdle syndrome, glycogen storage disease type V)	Hs00989942_m1
RABL2A	RAB, member of RAS oncogene family-like 2B	Hs00255244_m1
RASGRP3	KIAA0846 protein; RAS guanyl releasing protein 3 (calcium and DAG-regulated)	Hs00209819_m1
RBM8A	RNA binding motif protein 8A	Hs00254802_s1
REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)	Hs00968436_m1
RELA	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	Hs00153294_m1
RELB	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	Hs00232399_m1
RFX2	regulatory factor X, 5 (influences HLA class II expression)	Hs01100925_m1
RPL12	Human ribosomal protein L12 mRNA. /FEA=mRNA /DB_XREF=gi:10439364 /UG=Hs.182979 ribosomal protein L12	Hs01652254_gH
RPL38	ribosomal protein L38	Hs00605263_g1
RPL3L	ribosomal protein L3-like	Hs00192564_m1
RPP14	ribonuclease P (14kD)	Hs00199878_m1
RRAD	Ras-related associated with diabetes	Hs00188163_m1
RRAGD	Rag D protein, Ras-related GTP binding D	Hs00222001_m1
RTN3	reticulon 3	Hs00428645_m1
RUBVBL2	RuvB (E coli homolog)-like 2	Hs01090542_m1
RUNX1	runt-related transcription factor 1,	Hs00231079_m1
RUNX3	Human DNA sequence from clone RP3-467L1 on chromosome 1p36	Hs00231709_m1
RXRA	Retinoic acid receptor RXR-alpha	Hs01067640_m1
RYR1	ryanodine receptor 1 (skeletal)	Hs00166991_m1
S100A13	S100 calcium-binding protein A13	Hs00195583_m1
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	Hs00188166_m1
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	Hs00167155_m1

SFN	stratifin (keratinocyte)	Hs00356613_m1
SFRS2	Homo sapiens, Similar to splicing factor, arginine/serine-rich 2 (SC-35), clone MGC:2622 IMAGE:3501687, mRNA, complete cds.	Hs00427515_g1
SIGIRR	single Ig IL-1R-related molecule	Hs00222347_m1
SIRT2	sirtuin (silent mating type information regulation 2, S.cerevisiae, homolog) 2	Hs00247263_m1
SIVA1	CD27-binding (Siva) protein, SIVA1, apoptosis-inducing factor	Hs00276002_m1
SLC16A3	Monocarboxylate transporter 3 MCT3	Hs00358829_m1
SLC25A11	2-oxoglutarate/malate carrier protein	Hs00185940_m1
SLC25A12	Mitochondrial carrier protein ARALAR1	Hs00186535_m1
SLC25A36	Mitochondrial carrier protein FLJ10618, solute carrier family 25, member 36	Hs00216785_m1
SLC25A4	Heart/skeletal muscle adenine nucleotide translocator (ANT1), solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4	Hs00154037_m1
SLC37A4	Glucose-6-phosphate transporter (G6PT)	Hs00259865_m1
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	Hs00231324_m1
SMARCC1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1	Hs00268265_m1
SOAT1	sterol O-acyltransferase (acyl-Coenzyme A: cholesterol acyltransferase) 1	Hs00162077_m1
SOCS2	STAT induced STAT inhibitor-2, suppressor of cytokine signaling 2	Hs00919620_m1
SOD2	superoxide dismutase 2, mitochondrial	Hs00167309_m1
SORCS1	Homo sapiens, sortilin 1, neurotensin receptor, clone MGC:15673 IMAGE:3349731, mRNA, complete cds.	Hs00364666_m1
SPAG7	Homo sapiens sperm associated antigen 7 (SPAG7), mRNA. /FEA=mRNA /GEN=SPAG7 /PROD=sperm associated antigen 7 /DB_XREF=gi:4757715 /UG=Hs.90436 sperm associated antigen 7 /FL=gb:AF047437.1 gb:NM_004890.1	Hs00191669_m1
SQSTM1	sequestosome 1 (Ubiquitin-binding protein p62)	Hs00177654_m1
SRF	serum response factor (c-fos serum response element-binding transcription factor)	Hs00182371_m1
ST3GAL1	Sialyltransferase 4A (SIAT4A)	Hs00161688_m1

SVIL	supervillin	Hs00931028_m1
SYNGR1	synaptogyrin 1	Hs00377475_m1
SYNGR2	synaptogyrin 2	Hs00855143_g1
TBC1D22A	Novel human gene mapping to chomosome 22q13.3 similar to yeast ORF YOR070C, putative GTPase Activator (start missing).	Hs00378709_m1
TBCC	tubulin-specific chaperone c	Hs00268437_s1
TCAP	titin-cap (telethonin)	Hs00366220_m1
TFR2	Transferrin receptor 2 alpha	Hs00162690_m1
TGFB1	transforming growth factor, beta 1 (Camurati-Engelmann disease)	Hs99999918_m1
TIMP1	TIMP metallopeptidase inhibitor 1	Hs00171558_m1
TNF	tumor necrosis factor (TNF superfamily, member 2)	Hs00174128_m1
TNFRSF11A	tumor necrosis factor receptor superfamily, member 11a, NFKB activator	Hs00187192_m1
TNNI2	troponin I, skeletal, fast	Hs00268536_g1
TNXB	tenascin XB	Hs00219553_m1
TOR1B	torsin family 1, member B (torsin B)	Hs00248082_m1
TOX4	KIAA0737 gene product	Hs00706258_s1
TP53	TP53 target gene 1, tumor protein p53 (Li-Fraumeni syndrome)	Hs00153349_m1
TPI1	triosephosphate isomerase 1	Hs01593134_gH
TPM1	tropomyosin 1 (alpha)	Hs00165966_m1
TPRKB	CGI-121 protein	Hs00274981_m1
TRIB2	GS3955 kinase	Hs01120543_m1
TRIM63	MuRF1, tripartite motif-containing 63	Hs00261590_m1
TSPYL2	cutaneous T-cell lymphoma-associated tumor antigen se20-4; differentially expressed nucleolar TGF-beta1 target protein (DENTT)	Hs00223173_m1
TXN2	thioredoxin, mitochondrial	Hs00429399_g1
UBE2Z	hypothetical protein FLJ13855, ubiquitin-conjugating enzyme E2Z	Hs00225039_m1
UCP1	uncoupling protein 1 (mitochondrial, proton carrier)	Hs00222453_m1
UCRC	ubiquinol-cytochrome c reductase complex (7.2 kD)	Hs00203593_m1
UPK3A	uroplakin 3 (urothelial differentiation marker)	Hs00199590_m1
UQCRB	ubiquinol-cytochrome c reductase binding protein	Hs00559884_m1
UQCRC1	Ubiquinol-cytochrome C reductase complex core protein 1	Hs00163415_m1

UQCRC2	Ubiquinol-cytochrome C reductase complex core protein 2	Hs00996395_m1
UQCRFS1	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	Hs00705563_s1
VCP	Valosin- containing protein	Hs00997642_m1
VCPIP1	valosin-containing protein	Hs00228106_m1
VDAC-3	voltage-dependent anion channel 3	Hs00366592_m1
WBP11	WW domain binding protein 11	Hs00255461_m1
XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining; Ku autoantigen, 80kD)	Hs00221707_m1
YWHAZ		Hs00237047_m1
ZC3H15	uncharacterized hypothalamus protein HT010	Hs00218440_m1
ZFAND5	ZNF216	Hs00826311_m1
ZMAT5	hypothetical protein, zinc finger matrin type 5, small nuclearribonucleoprotein 20 kDa protein)	Hs00219538_m1
ZNF330	nucleolar cysteine-rich protein, zinc finger protein 330	Hs00205572_m1

Appendix 7

Western blotting protocol

Protocol supplied by Despina Constantin who performed the wet lab analyses:

Western Blotting

Each muscle sample was homogenised in Tris buffer (Tris 50 mM/EDTA 1 mM pH 7.5) supplemented with proteases and phosphatases inhibitors (Sigma). After homogenisation, each muscle extract was centrifuged for 15 min at 10,000 g. The supernatant was collected and stored at -80°C for further protein immunoblotting according to Constantin et al (Constantin, Constantin-Teodosiu et al. 2007).

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.

The protein transfer was checked with Ponceau S red staining, before blocking the membrane in BSA, TBS, and Tween for 1 hr at RT.

Membranes were probed with the primary antibodies overnight at 4°C, obtained from Santa Cruz Biotechnology (Dallas, Texas, USA).

Membranes were washed in TBS-T and 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).

Antibodies supplied by Santa Cruz Biotechnology Inc.

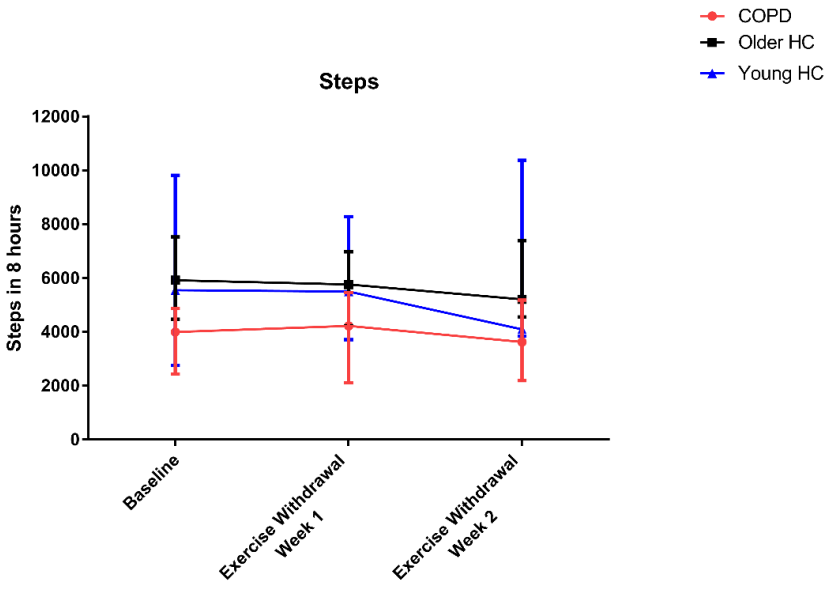
Protein Target	**Catalogue name / number**	**Epitope specificity**
HIF1 α	HIF-1α (3C144): sc-71247	mouse monoclonal
IGF1	IGF-I (W18): sc-74116	mouse monoclonal
p53	p53 (DO-1): sc-126	mouse monoclonal
IGFBP4	IGFBP4 (H-85): sc-13092	rabbit polyclonal
(p)IGF1	p-IGF-IR (2B9): sc-81499	mouse monoclonal
(p)p53	p-p53 (Ser 15): sc-101762	rabbit polyclonal
IL6	IL-6 (E-4): sc-28343	mouse monoclonal
TLR4	TLR4 (25): sc-293072	mouse monoclonal

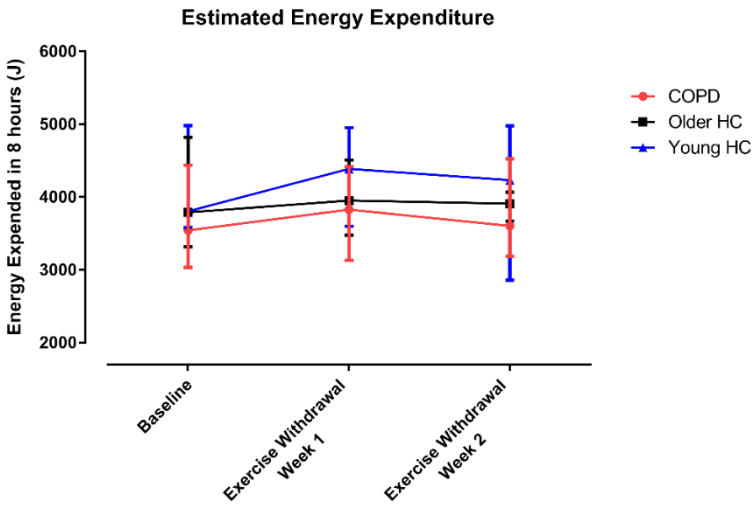
PPARγ	PPARγ (E-8): sc-7273	mouse monoclonal
PYGM	PYGM (U-23): sc-130860	rabbit polyclonal
MSTN *	GDF-8 (1L4): sc-134345	mouse monoclonal

Appendix 8

Physical activity data pre-intervention and during exercise withdrawal period.

Within group change in step count and estimated energy expenditure was assessed using Friedman's ANOVA. There was no significant difference in step count between time points for the COPD group (p = 0.411), Older Healthy group (p = 0.717) or Young Healthy group (p = 0.867). There was no significant difference between time points for energy expenditure in the COPD group (p = 0.846), Older Healthy Group (p = 0.895) or Young Healthy group (p = 0.651). Data on graphs are median and IQR.





Appendix 9

MATCH Study Baseline mRNA abundance in Young vs Older Group

Baseline gene expression (ΔC_T) for Young and Older groups with results of statistical comparison.

mRNA target	Older Group		Young Group		p	FDR
	Median	IQR	Median	IQR		
ACOT1	0.07	-0.40 - 0.66	-0.26	-0.62 - 0.28	0.67	0.89
ADIPOQ	0.21	-0.62 - 0.72	0.31	-0.68 - 0.70	0.91	0.96
ALDH2	-0.28	-0.48 - 0.03	0.06	-0.07 - 0.54	0.19	0.60
ALOX12	-0.01	-0.23 - 0.70	-0.63	-1.19 - 0.25	0.44	0.80
APOA1	-0.34	-0.72 - 0.65	0.18	-0.01 - 0.65	0.36	0.80
APOC1	-0.33	-0.60 - 1.02	0.06	-0.18 - 0.21	0.51	0.80
ATP5O	-0.15	-0.36 - 0.38	-0.48	-0.93 - 1.00	0.96	0.97
BCL2L1	-0.13	-0.88 - 0.46	0.47	-0.50 - 0.65	0.29	0.71
CCL19	0.30	0.03 - 0.76	0.30	-0.63 - 0.72	0.80	0.93
CCL22	-0.41	-1.16 - 0.50	0.34	-0.02 - 0.70	0.12	0.52
COX7A1	0.51	-0.14 - 0.85	-0.66	-1.16 - 0.35	0.15	0.53
CPT1A	-0.08	-1.38 - 0.61	0.32	-0.07 - 0.99	0.19	0.60
CPT1B.CHKB_CPT1B	-0.35	-0.85 - 0.19	0.63	-0.37 - 1.17	0.09	0.52
CS	-0.02	-0.38 - 0.85	-0.16	-0.83 - 0.27	0.52	0.80
EIF4EBP1	0.13	-0.40 - 1.07	-0.50	-0.76 - 0.26	0.08	0.52
18S	-0.61	-1.08 - 0.32	0.44	-0.41 - 0.87	0.13	0.52
FASLG	-0.38	-0.98 - 0.02	0.62	0.05 - 0.81	0.14	0.52
FAXDC2	-0.07	-0.70 - 0.26	0.02	-0.19 - 0.40	0.69	0.89

FOXO1	-0.56	-1.22 - 0.05	0.49	-0.15 - 1.17	0.02	0.43
G6PD	-0.24	-0.83 - 0.33	0.46	-0.01 - 0.86	0.06	0.49
GAPDH	0.13	-0.23 - 1.10	-0.46	-1.05 - 0.04	0.03	0.43
GLUD1	-0.60	-0.94 - 0.04	0.38	0.06 - 0.85	0.03	0.43
GSK3B	0.03	-0.49 - 0.23	0.30	0.05 - 0.85	0.33	0.77
GYS1	-0.37	-0.87 - 0.45	0.20	-0.29 - 0.62	0.29	0.71
HADH	-0.54	-0.87 - 0.39	-0.11	-0.39 - 0.36	0.37	0.80
HIF1A	-0.42	-1.02 - 0.24	-0.11	-0.30 - 0.69	0.13	0.52
HIGD1A	-0.35	-0.87 - 0.64	-0.13	-0.32 - 0.62	0.67	0.89
HK1	-0.58	-1.05 - 0.23	0.26	-0.53 - 0.82	0.40	0.80
HK2	-0.69	-0.96 - 0.11	0.04	-0.18 - 0.37	0.13	0.52
HNRNPM.MT_ND4.CCDC104	-0.37	-0.71 - 0.49	-0.09	-0.59 - 0.67	0.88	0.96
HSPA14	-0.42	-0.61 - 0.25	-0.05	-0.32 - 0.46	0.97	0.97
HSPD1	-0.38	-0.77 - 0.11	0.02	-0.25 - 0.80	0.46	0.80
IDH1	0.17	-0.38 - 0.57	0.07	-0.20 - 0.18	0.47	0.80
IGF1	-0.24	-0.31 - 0.96	-0.14	-0.60 - 0.03	0.44	0.80
IGFBP4	0.10	-0.84 - 0.58	0.34	-0.35 - 0.94	0.29	0.71
IGFBP7	0.21	-0.44 - 0.48	-0.21	-0.53 - 0.56	0.72	0.89
IL10	-0.67	-1.17 - 0.75	0.42	-0.16 - 0.98	0.10	0.52
IL6	-0.15	-0.50 - 0.45	-0.20	-0.36 - 0.31	0.97	0.97
INSR	-0.65	-1.17 - 0.01	0.18	-0.04 - 0.95	0.05	0.49
IRS1	0.35	0.14 - 0.75	-0.85	-1.14 - 0.23	0.03	0.43

KL	0.50	0.35 - 0.91	-0.25	-1.27 - 0.34	0.14	0.52
LOXHD1	-0.18	-0.55 - 0.05	0.30	-0.46 - 0.63	0.45	0.80
LPPR2	-0.18	-0.58 - 0.23	-0.36	-0.84 - 0.08	0.66	0.89
MCU	-0.73	-0.93 - 0.06	0.67	0.07 - 1.06	0.00	0.38
MCUR1	-0.29	-0.58 - 0.08	0.54	-0.65 - 0.84	0.38	0.80
MDH1B	-0.26	-0.97 - 0.06	0.31	0.23 - 0.89	0.09	0.52
MDH2	0.11	-0.69 - 0.82	-0.05	-0.48 - 0.33	0.94	0.97
ME2	-0.70	-0.81 - 0.05	0.67	-0.23 - 1.08	0.02	0.43
MICU1	-0.42	-0.95 - 0.13	0.20	-0.41 - 0.76	0.14	0.52
MNF1	-0.17	-0.69 - 0.79	0.11	-0.60 - 0.55	0.75	0.89
MPC1	0.42	-1.09 - 0.70	-0.33	-0.68 - 0.72	0.82	0.93
MRPL18	-0.26	-0.85 - 0.73	-0.10	-0.12 - 0.33	0.91	0.96
MRPL3	-0.28	-0.76 - 0.26	-0.12	-0.51 - 0.64	0.89	0.96
MRPL40	-0.61	-0.86 - 0.80	0.17	-0.47 - 0.93	0.40	0.80
MSTN	-0.09	-0.26 - 0.82	-0.44	-0.89 - 0.59	0.47	0.80
MT_ND6	-0.22	-0.47 - 0.44	0.08	-1.13 - 0.89	0.66	0.89
MTFR1	-0.46	-0.69 - 0.33	0.43	0.13 - 0.98	0.13	0.52
MTG2	-0.42	-0.72 - 0.01	0.26	-0.55 - 0.96	0.17	0.56
MTIF2	-0.29	-0.79 - 0.33	0.47	-0.54 - 0.78	0.22	0.63
MTRF1L	-0.21	-0.61 - 0.75	-0.21	-0.64 - 0.55	0.51	0.80
MUL1	-0.10	-0.95 - 0.31	0.33	-0.32 - 0.55	0.60	0.86
NDUFA10	-0.40	-0.82 - 0.44	0.35	-0.63 - 1.25	0.26	0.71
NDUFA3	0.34	-0.80 - 0.83	-0.24	-0.65 - 0.61	0.75	0.89

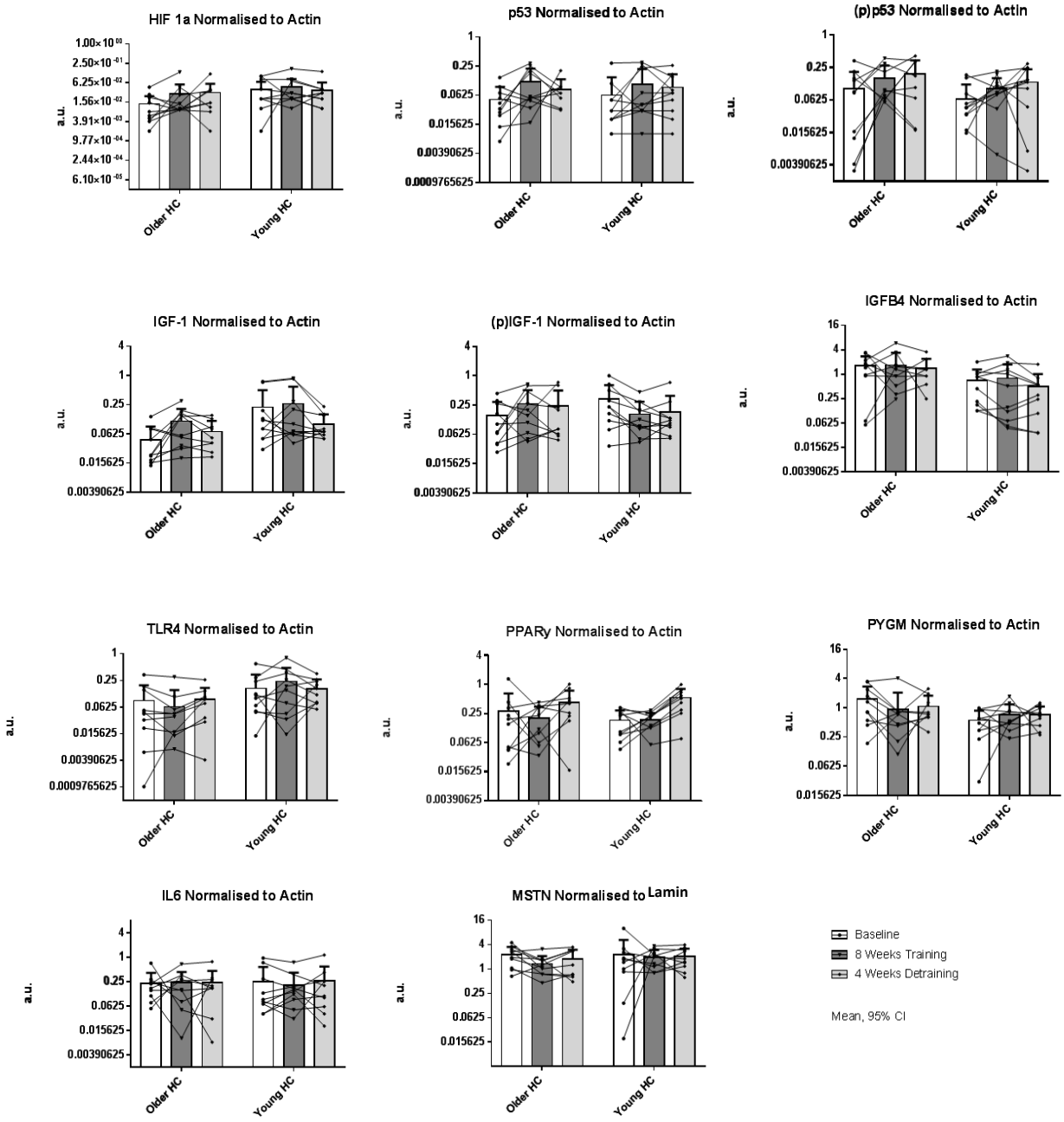
NDUFA9	0.45	0.03 - 0.70	-0.35	-0.91 - 0.40	0.06	0.49
NDUFV1	0.23	-0.34 - 0.42	-0.14	-0.35 - 0.13	0.76	0.89
NFE2L2	-0.73	-0.95 - 0.51	0.48	-0.13 - 0.76	0.09	0.52
NRF1	-0.54	-1.32 - 0.03	0.62	0.06 - 0.98	0.01	0.43
PDK2	0.03	-0.84 - 0.43	0.30	-0.90 - 0.83	0.51	0.80
PDK4	-0.02	-0.94 - 0.75	-0.04	-0.70 - 0.65	0.85	0.95
PDP2	-0.16	-0.49 - 0.02	0.21	-0.79 - 0.77	0.48	0.80
PFKM	-0.12	-0.66 - 1.01	-0.54	-0.81 - 0.74	0.45	0.80
PGM1	-0.07	-0.25 - 0.09	-0.05	-0.89 - 0.62	0.48	0.80
PKM	-0.15	-0.55 - 1.00	-0.42	-0.81 - 0.42	0.45	0.80
PLA2G2A	-0.04	-0.54 - 0.65	0.45	-0.80 - 1.02	0.76	0.89
POLRMT	-0.59	-1.02 - 0.16	0.58	0.08 - 0.70	0.04	0.43
PPARA	-0.47	-0.99 - 0.11	-0.04	-0.40 - 1.04	0.26	0.71
PPARD	-0.69	-1.08 - 0.03	0.51	-0.38 - 1.07	0.04	0.43
PPARG	0.36	-0.10 - 0.50	0.18	-0.51 - 0.47	0.67	0.89
PPARGC1A	0.13	-0.48 - 0.60	0.10	-0.51 - 0.46	0.56	0.84
PPARGC1B	-0.36	-1.08 - 0.76	0.17	-0.59 - 0.79	0.71	0.89
PYGM	-0.23	-0.46 - 0.39	-0.38	-0.99 - 0.80	0.73	0.89
SDHA	-0.02	-0.79 - 0.70	-0.11	-0.47 - 0.50	0.90	0.96
SLC2A4	-0.25	-1.02 - 0.61	0.44	-0.68 - 1.08	0.38	0.80
SOD2	-0.35	-1.05 - 0.45	0.19	-0.52 - 0.72	0.55	0.83
TFAM	-0.32	-0.69 - 0.63	0.06	-0.58 - 0.65	0.96	0.97
TFB2M	0.22	-0.40 - 0.40	-0.03	-0.31 - 0.45	0.74	0.89

TLR4	-0.33	-0.82 - 0.30	0.11	-0.47 - 0.83	0.30	0.71
TP53	-0.33	-0.94 - 0.50	0.27	-0.02 - 1.03	0.21	0.62
TUFM	-0.44	-1.09 - 0.65	0.29	-0.28 - 1.04	0.21	0.62
UCP2	-0.11	-0.78 - 0.65	0.08	-0.65 - 0.75	0.82	0.93
UCP3	0.51	-0.38 - 1.11	-0.31	-1.14 - 0.40	0.15	0.52
VEGFA	-0.04	-0.87 - 0.45	-0.05	-0.22 - 0.83	0.59	0.86

Baseline mRNA abundance (ΔCT) for target gene expressed relative to HMBS. Unadjusted p values are from 2-sided t-test. Adjustment for multiple comparisons was performed using the FDR method.

Appendix 10

MATCH Study change in protein abundance expressed in arbitrary units. Older vs Young Group

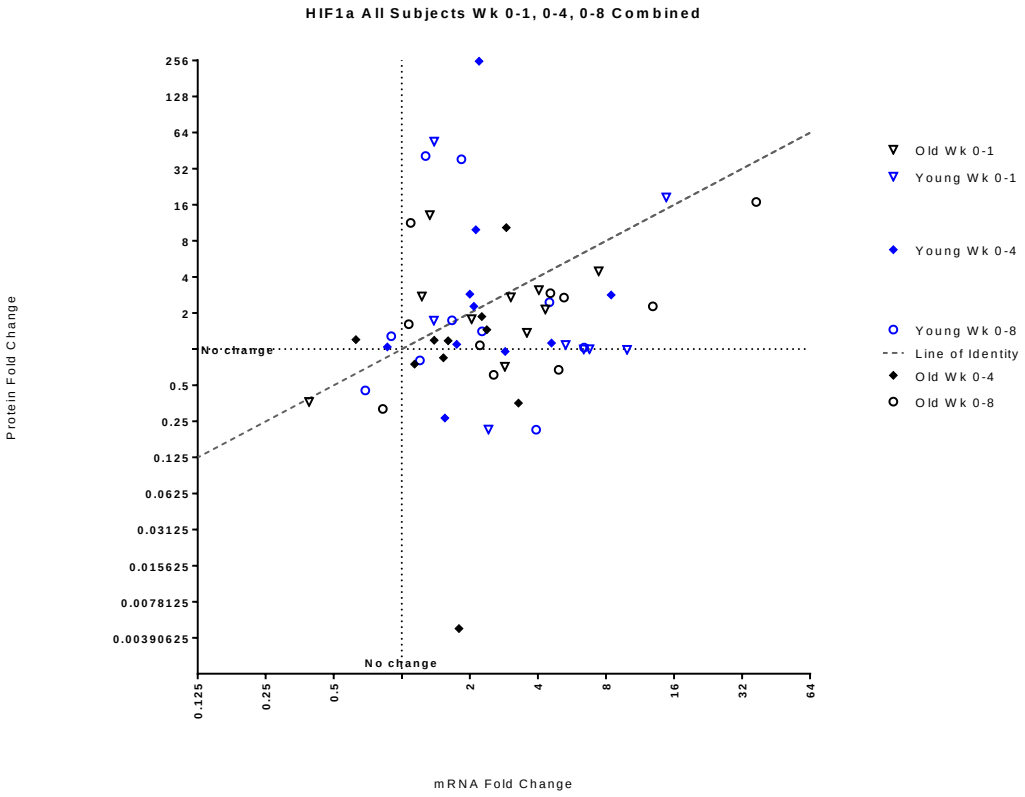


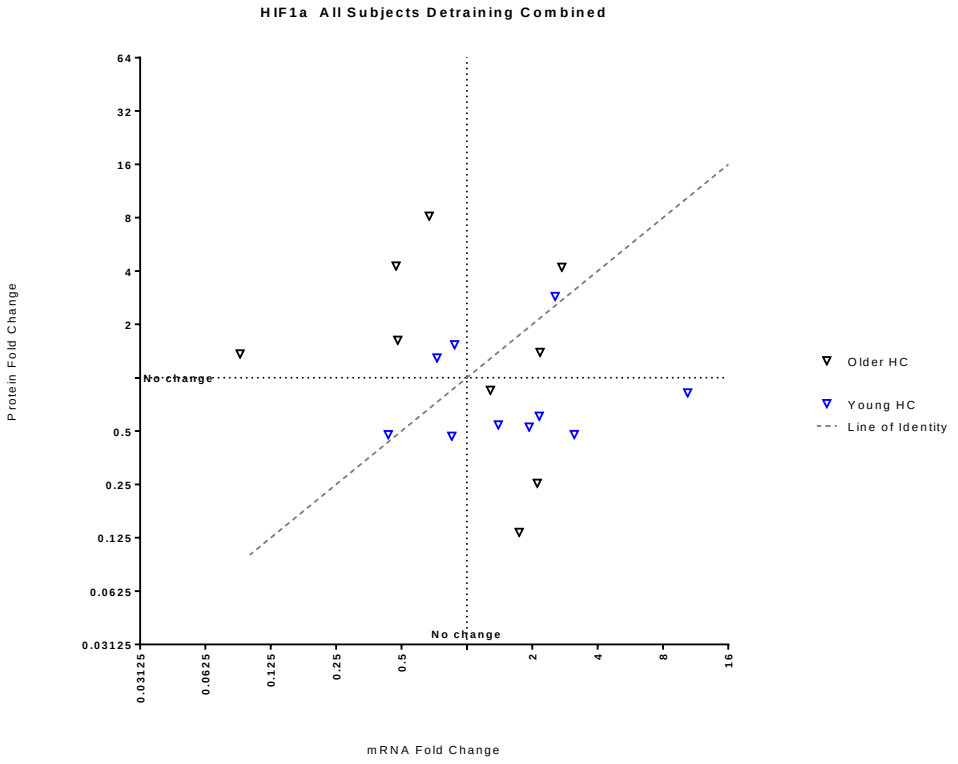
Change in protein abundance with exercise training and exercise withdrawal expressed relative to a housekeeping protein. HIF-1α, hypoxia inducible factor 1 α; p53, (tumour) protein 53; (p)p53, phosphorylated p53; IGF1, insulin-like growth factor 1; (p)IGF1, phosphorylated IGF1 receptor ; IGFBP4, insulin-like growth factor binding protein 4; TLR4, toll-like receptor 4; PPARγ, peroxisome proliferator activated receptor γ; PYGM, glycogen phosphorylase (muscle); IL6, interleukin 6; MSTN, myostatin.

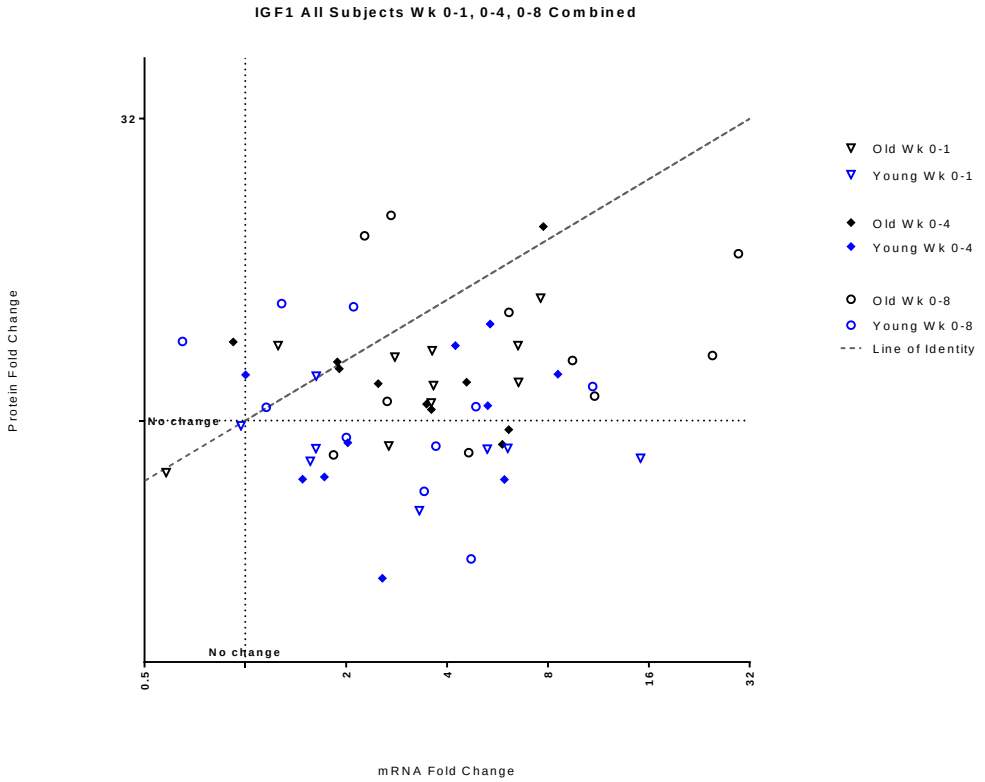
Appendix 11

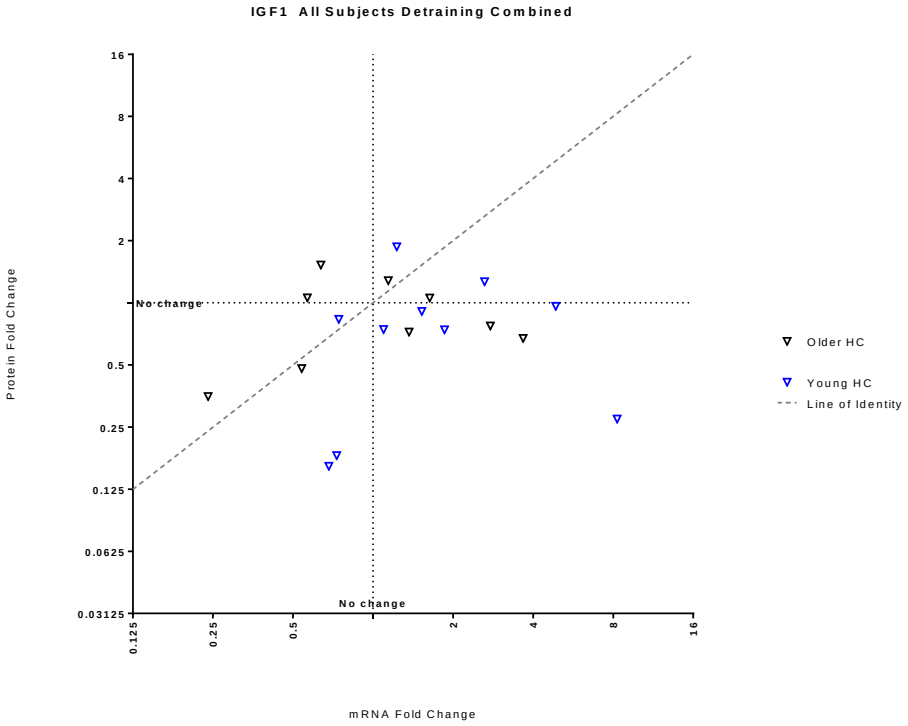
MATCH Study mRNA & protein fold-change correlations for Young and Older Groups.

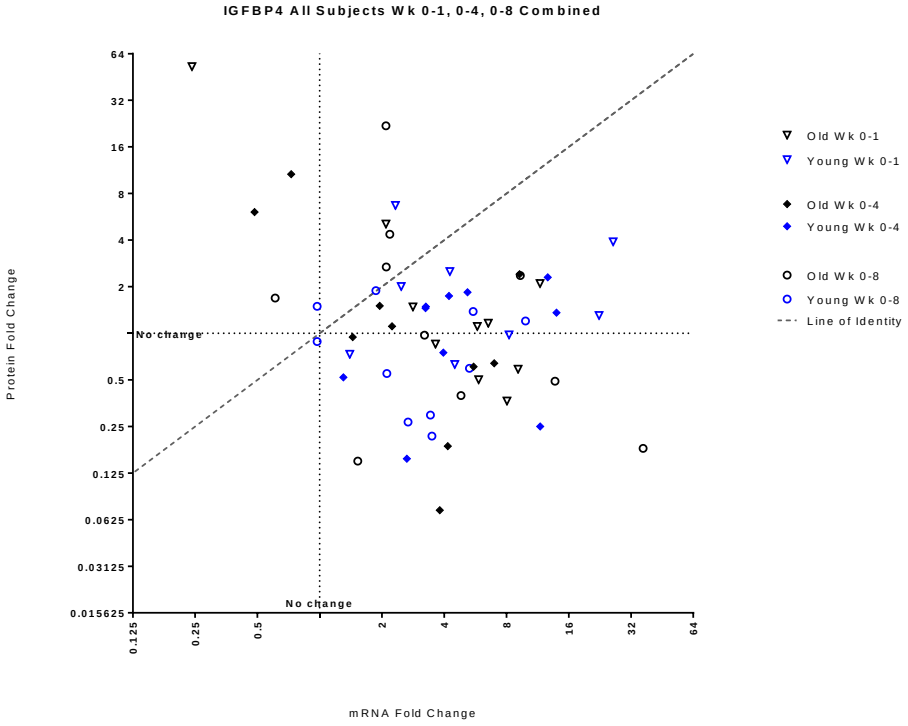
All correlations are non-significant ($p > 0.05$) unless stated.

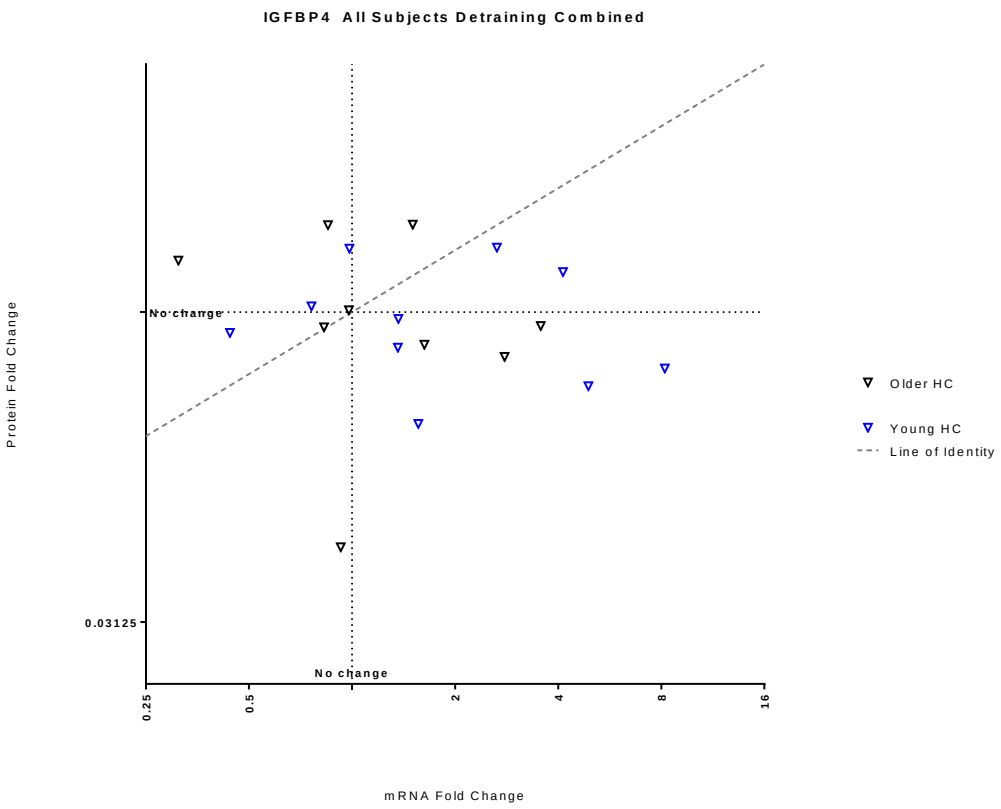


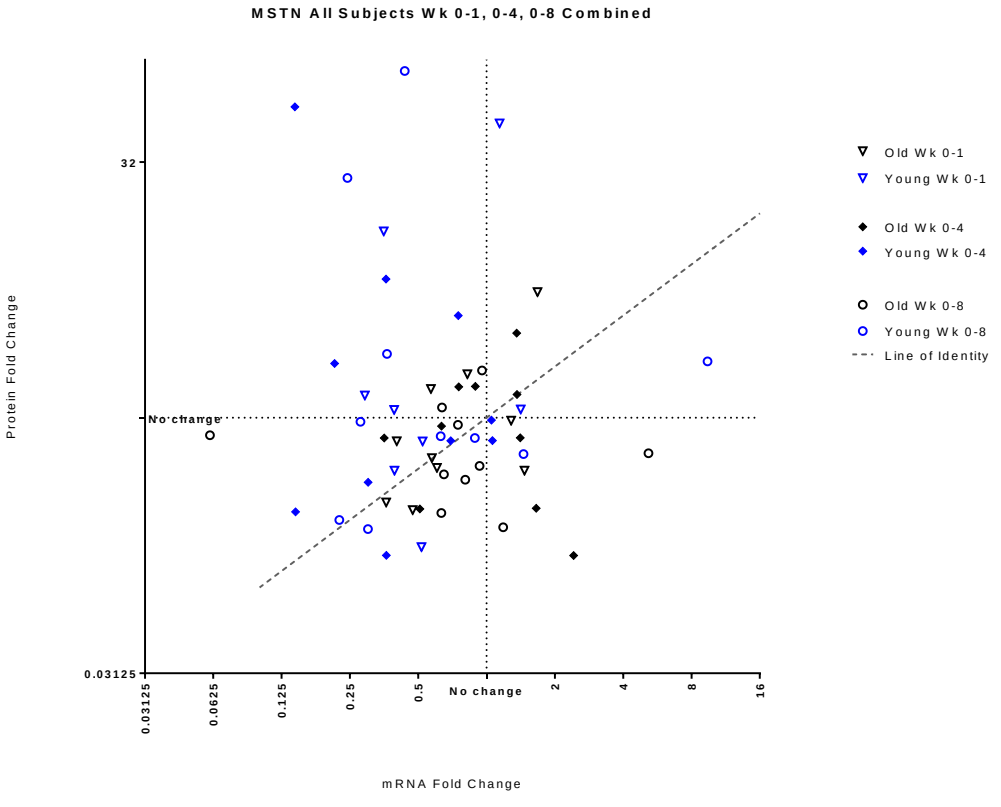

HIF1a All Subjects Detraining Combined
Protein Fold Change
64
32
16
8
4
2
No change
0.5
0.25
0.125
0.0625
0.03125
No change
0.03125
0.0625
0.125
0.25
0.5
2
4
8
16
mRNA Fold Change
Older HC
Young HC
Line of Identity

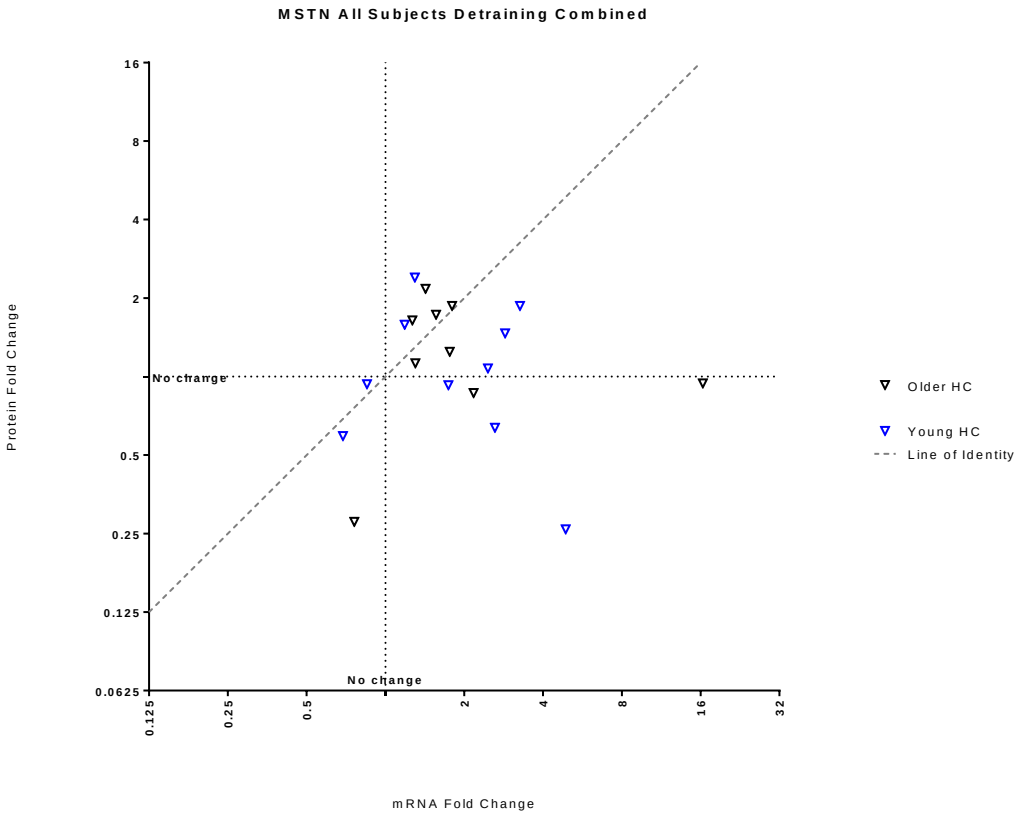

IGF1 All Subjects Wk 0-1, 0-4, 0-8 Combined
Protein Fold Change
32
No change
No change
0.5
2
4
8
16
32
mRNA Fold Change
Old Wk 0-1
Young Wk 0-1
Old Wk 0-4
Young Wk 0-4
Old Wk 0-8
Young Wk 0-8
Line of Identity

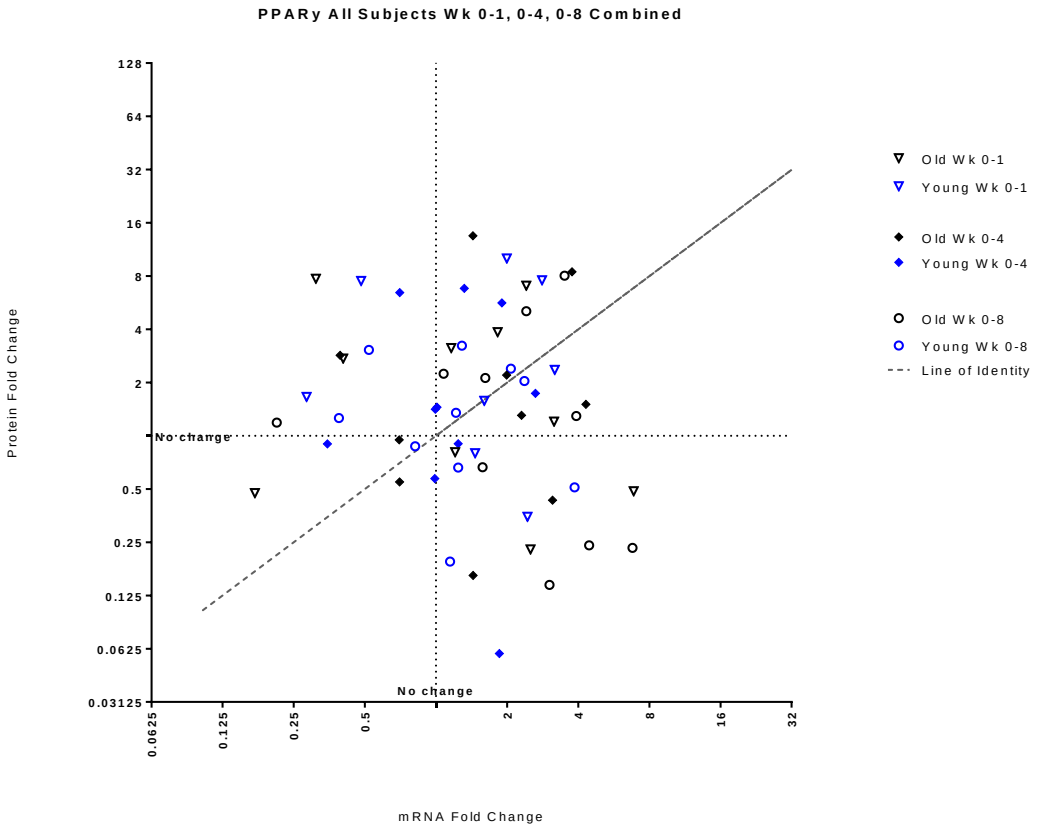

IGF1 All Subjects Detraining Combined
Protein Fold Change
mRNA Fold Change
No change
No change
16
8
4
2
0.5
0.25
0.125
0.0625
0.03125
0.125
0.25
0.5
2
4
8
16
Older HC
Young HC
Line of Identity

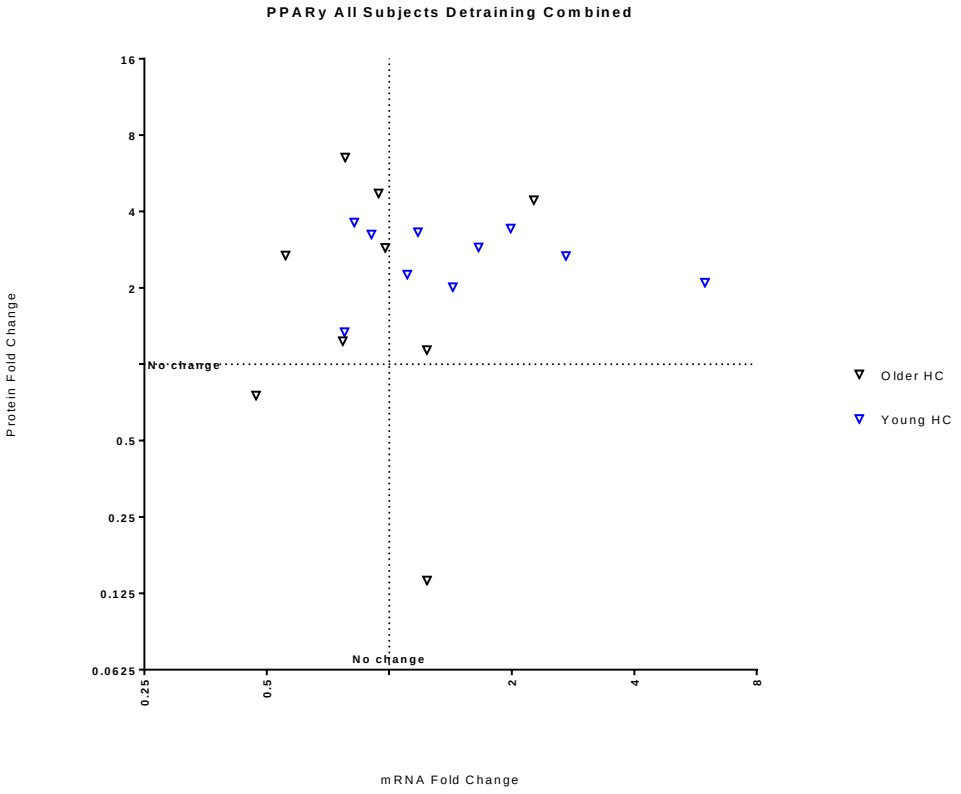

IGFBP4 All Subjects Wk 0-1, 0-4, 0-8 Combined
Protein Fold Change
mRNA Fold Change
No change
No change
64
32
16
8
4
2
0.5
0.25
0.125
0.0625
0.03125
0.015625
0.125
0.25
0.5
2
4
8
16
32
64
Old Wk 0-1
Young Wk 0-1
Old Wk 0-4
Young Wk 0-4
Old Wk 0-8
Young Wk 0-8
Line of Identity

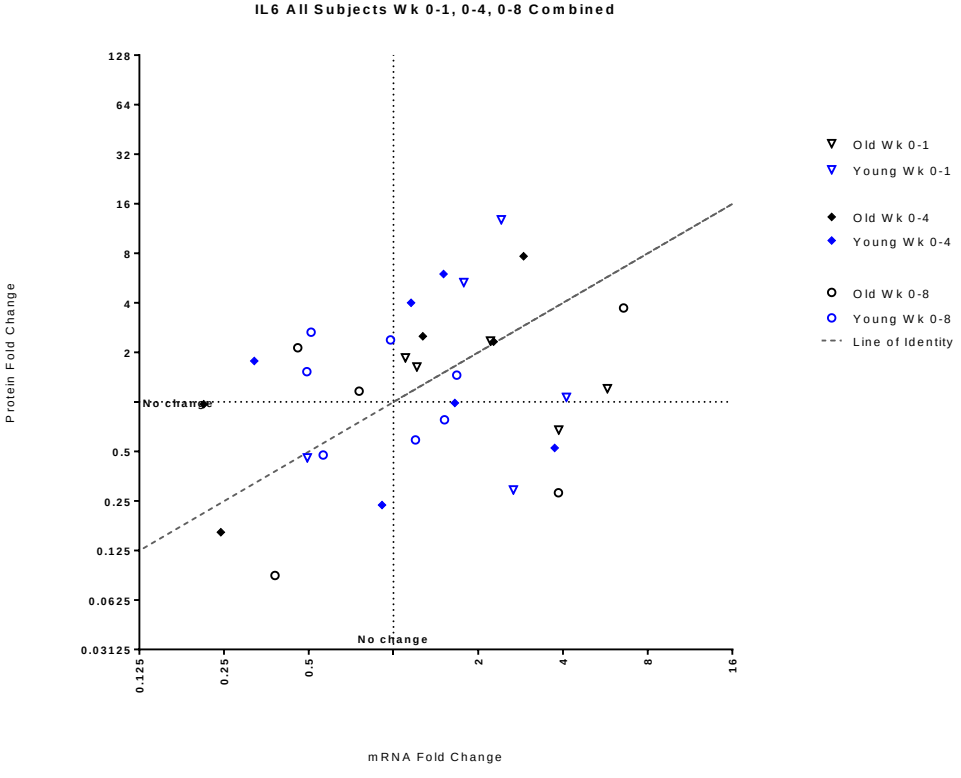

IGFBP4 All Subjects Detraining Combined
Protein Fold Change
No change
0.03125
No change
0.25
0.5
2
4
8
16
mRNA Fold Change
Older HC
Young HC
Line of Identity

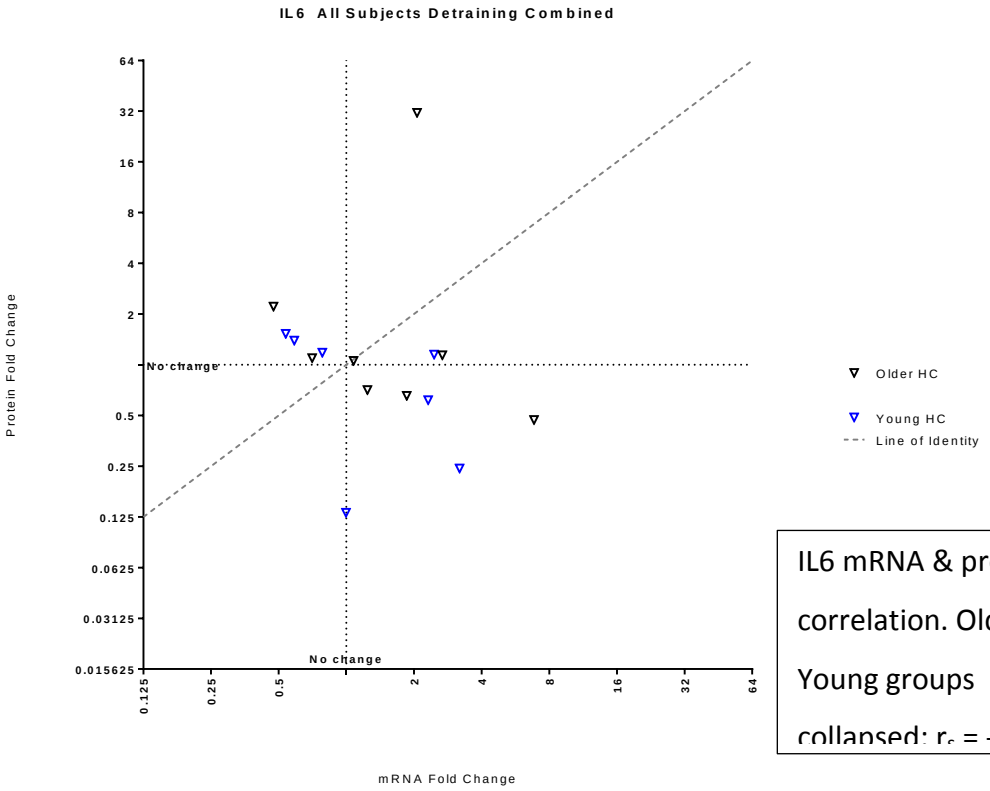

MSTN All Subjects Wk 0-1, 0-4, 0-8 Combined
Protein Fold Change
32
No change
0.03125
0.03125
0.0625
0.125
0.25
0.5
No change
2
4
8
16
mRNA Fold Change
Old Wk 0-1
Young Wk 0-1
Old Wk 0-4
Young Wk 0-4
Old Wk 0-8
Young Wk 0-8
Line of Identity


MSTN All Subjects Detraining Combined
Protein Fold Change
mRNA Fold Change
No change
No change
16
8
4
2
0.5
0.25
0.125
0.0625
0.125
0.25
0.5
2
4
8
16
32
Older HC
Young HC
Line of Identity

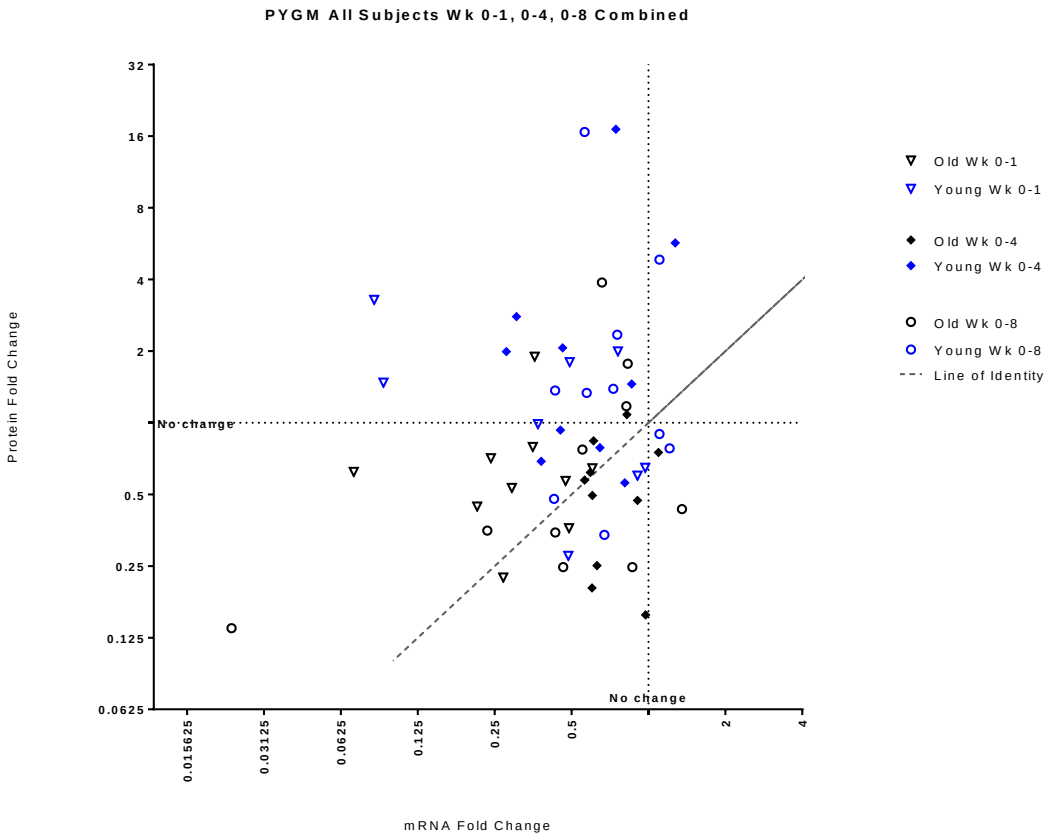

PPARγ All Subjects Wk 0-1, 0-4, 0-8 Combined
Protein Fold Change
mRNA Fold Change
No change
No change
128
64
32
16
8
4
2
0.5
0.25
0.125
0.0625
0.03125
0.0625
0.125
0.25
0.5
2
4
8
16
32
Old Wk 0-1
Young Wk 0-1
Old Wk 0-4
Young Wk 0-4
Old Wk 0-8
Young Wk 0-8
Line of Identity

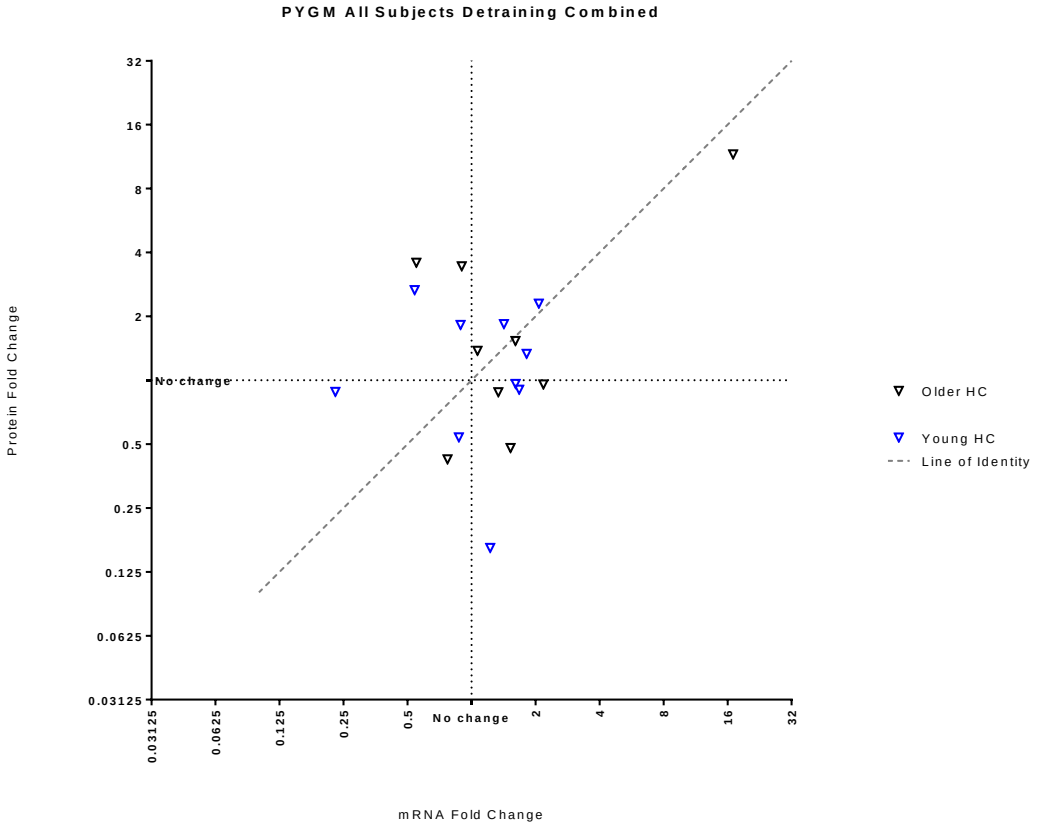

PPARy All Subjects Detraining Combined
Protein Fold Change
mRNA Fold Change
No change
No change
16
8
4
2
0.5
0.25
0.125
0.0625
0.25
0.5
2
4
8
Older HC
Young HC

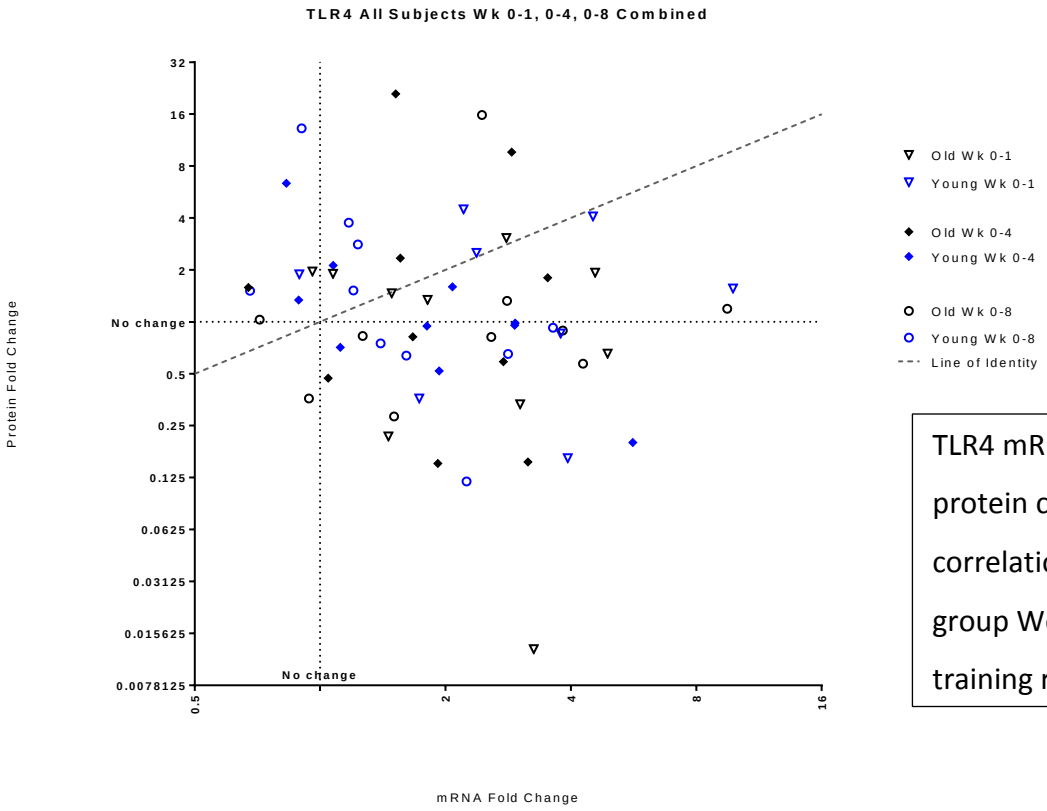

IL6 All Subjects Wk 0-1, 0-4, 0-8 Combined
Protein Fold Change
mRNA Fold Change
No change
No change
128
64
32
16
8
4
2
0.5
0.25
0.125
0.0625
0.03125
0.125
0.25
0.5
2
4
8
16
Old Wk 0-1
Young Wk 0-1
Old Wk 0-4
Young Wk 0-4
Old Wk 0-8
Young Wk 0-8
Line of Identity



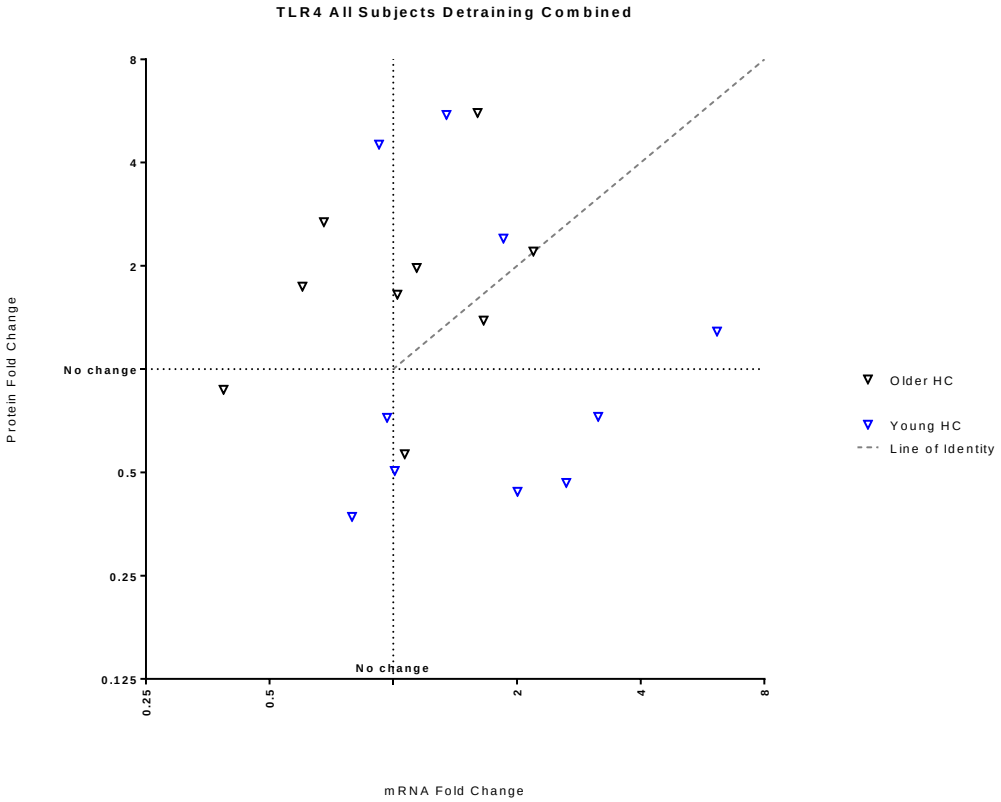
IL6 mRNA & protein correlation. Older & Young groups collapsed: r_s = -0.518, *p*

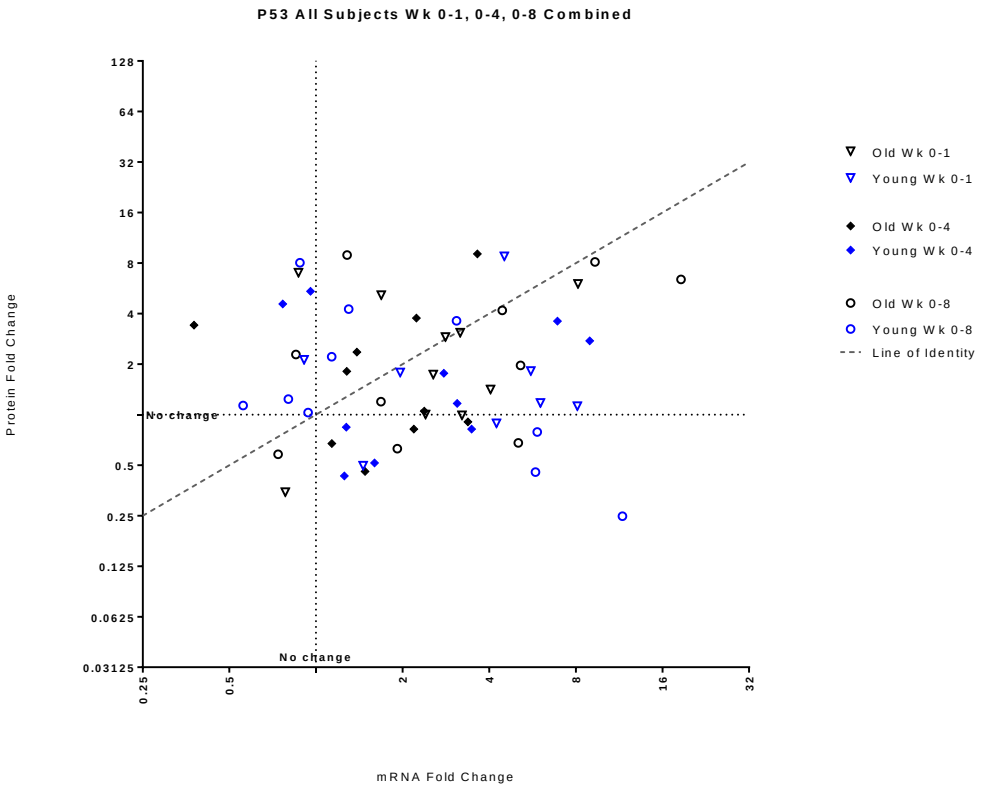

PYGM All Subjects Wk 0-1, 0-4, 0-8 Combined
Protein Fold Change
mRNA Fold Change
No change
No change
32
16
8
4
2
0.5
0.25
0.125
0.0625
0.015625
0.03125
0.0625
0.125
0.25
0.5
2
4
Old Wk 0-1
Young Wk 0-1
Old Wk 0-4
Young Wk 0-4
Old Wk 0-8
Young Wk 0-8
Line of Identity

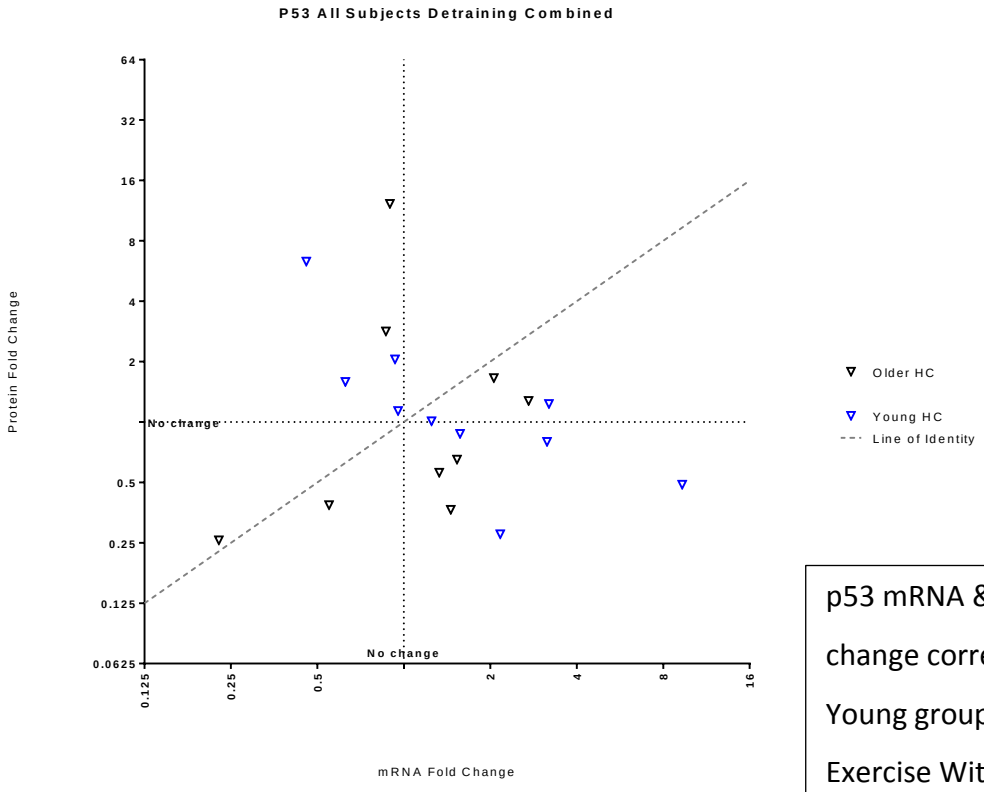

PYGM All Subjects Detraining Combined
Protein Fold Change
mRNA Fold Change
No change
No change
32
16
8
4
2
0.5
0.25
0.125
0.0625
0.03125
0.03125
0.0625
0.125
0.25
0.5
2
4
8
16
32
Older HC
Young HC
Line of Identity



TLR4 mRNA & protein change correlation. Young group Week 0-8 of training r_s = -.709, *p*


TLR4 All Subjects Detraining Combined
Protein Fold Change
mRNA Fold Change
No change
No change
8
4
2
0.5
0.25
0.125
0.25
0.5
2
4
8
Older HC
Young HC
Line of Identity

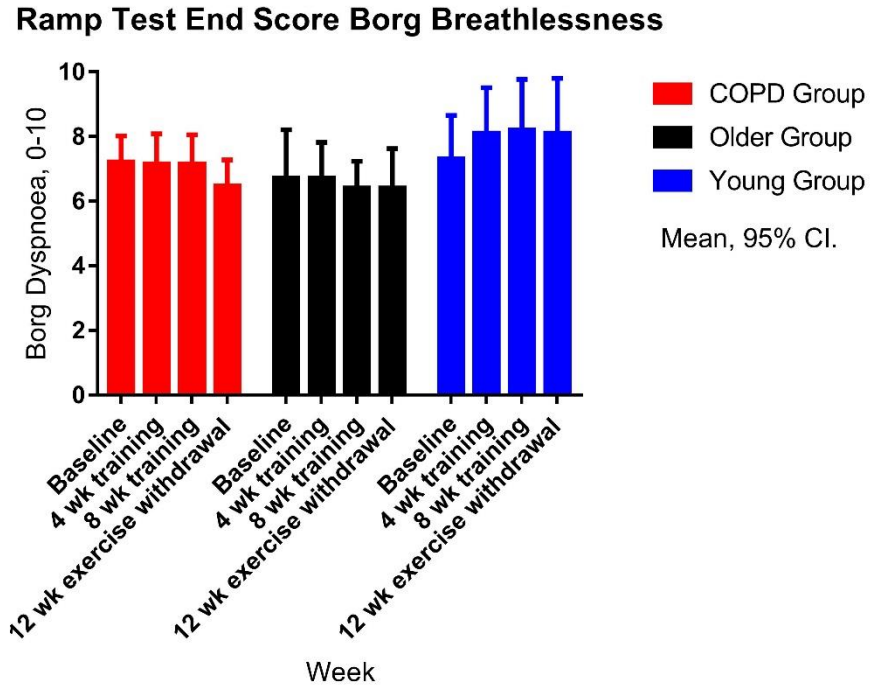

P53 All Subjects Wk 0-1, 0-4, 0-8 Combined
Protein Fold Change
mRNA Fold Change
No change
No change
128
64
32
16
8
4
2
0.5
0.25
0.125
0.0625
0.03125
0.25
0.5
2
4
8
16
32
Old Wk 0-1
Young Wk 0-1
Old Wk 0-4
Young Wk 0-4
Old Wk 0-8
Young Wk 0-8
Line of Identity

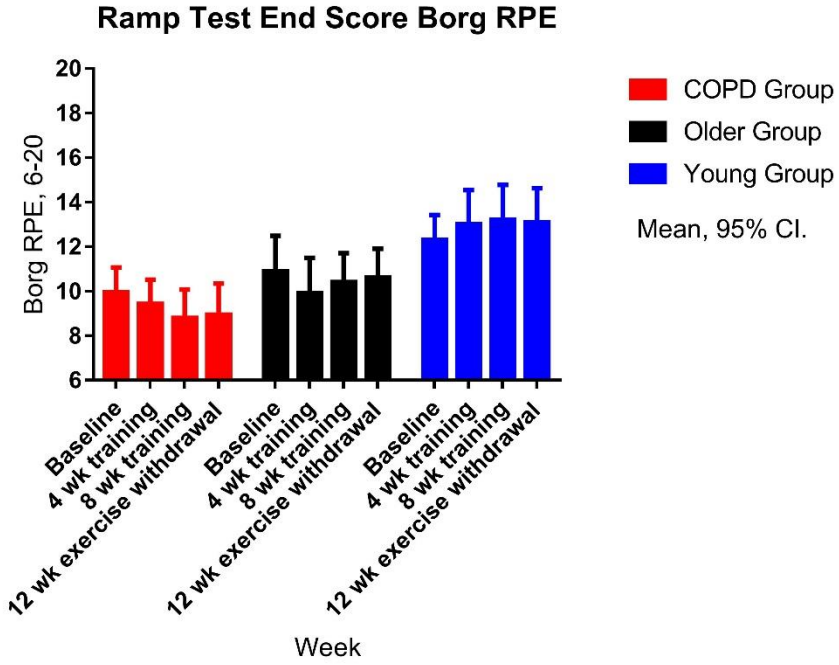


p53 mRNA & protein change correlation. Young group Exercise Withdrawal period r_s = -.758, *p* =

Appendix 12

Ratings of breathlessness and perceived exertion at termination of the incremental exercise test at all study time points





Appendix 13

Baseline gene expression (ΔC_T) for COPD and Older Healthy Control groups with results of statistical comparison.

mRNA Target	Older Group		COPD Group		*p*	FDR
	Median	IQR	Median	IQR		
ACOT1	1.88	1.53 - 2.33	2.03	1.48 - 2.43	0.45	0.62
ADIPOQ	1.385	-0.72 - 2.66	0.57	-0.22 - 1.04	0.73	0.86
ALDH2	-2.32	-2.44 - -2.14	-2.14	-2.62 - -1.92	0.57	0.74
ALOX12	6.16	5.99 - 6.70	6.78	6.05 - 7.40	0.31	0.57
APOA1	5.45	4.90 - 6.87	6.72	5.80 - 7.36	0.74	0.86
APOC1	1.935	1.64 - 3.39	2.5	2.23 - 3.43	0.32	0.57
ATP5O	-4.1	-4.19 - -3.89	-3.6	-3.82 - -3.44	0.00	0.09
BCL2L1	-1.12	-1.57 - 0.77	-0.93	-1.11 - 0.60	0.08	0.38
CCL19	6.35	5.82 - 7.25	6.665	6.21 - 7.28	0.24	0.50
CCL22	5.285	4.31 - 6.47	6.785	6.17 - 7.01	0.05	0.34
COX7A1	-6.155	-6.47 - -5.99	-5.98	-6.26 - -5.80	0.13	0.40
CPT1A	0.925	-0.06 - 1.44	1.05	0.74 - 1.47	0.23	0.50
CPT1B.CHKB_CPT1B	-2.56	-2.88 - -2.22	-2.53	-2.71 - -2.09	0.39	0.60
CS	-3.91	-4.10 - -3.45	-3.71	-3.95 - -3.31	0.41	0.60
EIF4EBP1	-0.965	-1.25 - -0.46	-0.58	-0.89 - -0.31	0.26	0.50
18S	-17.825	-18.03 - -17.42	-17.48	-17.82 - -17.24	0.19	0.46
FASLG	5.88	5.29 - 6.27	7.05	6.28 - 7.58	0.03	0.34
FAXDC2	0.105	-0.39 - 0.37	0.38	0.16 - 0.79	0.19	0.46

FOXO1	-1.06	-1.48	-	-0.73	-0.84	-1.24	-	-0.23	0.25	0.50
G6PD	1.23	0.80	-	1.65	1.67	1.22	-	1.94	0.04	0.34
GAPDH	-9.665	-9.88	-	-9.08	-9.71	-9.93	-	-9.32	0.41	0.60
GLUD1	1.58	1.33	-	2.00	2.38	2.07	-	2.72	0.01	0.23
GSK3B	-2.405	-2.82	-	-2.24	-2.24	-2.45	-	-1.96	0.06	0.37
GYS1	-4.645	-4.88	-	-4.27	-4.49	-4.88	-	-4.29	0.96	0.96
HADH	-3.34	-3.56	-	-2.74	-2.91	-3.08	-	-2.53	0.09	0.38
HIF1A	-0.765	-1.13	-	-0.36	0.02	-0.46	-	-0.19	0.00	0.19
HIGD1A	-2.345	-2.67	-	-1.73	-2.24	-2.63	-	-1.86	0.67	0.83
HK1	-2.68	-2.93	-	-2.25	-2.09	-2.44	-	-1.91	0.10	0.38
HK2	-1.04	-1.36	-	-0.10	0.03	-0.60	-	-0.60	0.15	0.43
HNRNPM.MT_ND4.CCDC104	-12.945	-13.12	-	-12.49	-12.81	-13.00	-	-12.46	0.77	0.88
HSPA14	1.31	1.20	-	1.70	1.48	1.22	-	1.57	0.64	0.82
HSPD1	3.405	3.12	-	3.76	3.52	3.17	-	3.77	0.80	0.89
IDH1	0.64	0.17	-	0.98	0.63	0.49	-	1.06	0.79	0.89
IGF1	-0.025	-0.07	-	0.67	0.52	0.23	-	0.92	0.12	0.40
IGFBP4	-0.01	-0.84	-	0.41	0.05	-0.13	-	0.53	0.17	0.45
IGFBP7	-1.51	-2.00	-	-1.31	-1.46	-1.61	-	-1.22	0.44	0.62
IL10	5.93	5.46	-	7.26	7.065	6.43	-	7.40	0.12	0.40
IL6	5.78	5.54	-	6.19	6.23	5.56	-	6.76	0.39	0.60
INSR	-1.335	-1.64	-	-0.95	-0.85	-1.46	-	-0.56	0.28	0.52
IRS1	0.265	0.10	-	0.61	0.7	-0.06	-	1.11	0.45	0.62

KL	5.29	5.16	-	5.65	4.76	4.56	-	5.44	0.35	0.59
LOXHD1	5.09	4.73	-	5.31	5.865	5.57	-	6.74	0.01	0.21
LPPR2	0.07	-0.26	-	0.41	0.39	0.24	-	0.65	0.42	0.60
MCU	-1.72	-1.84	-	-1.27	-1.4	-1.71	-	-1.01	0.08	0.38
MCUR1	2.045	1.85	-	2.30	2.5	2.11	-	2.86	0.15	0.43
MDH1B	5.75	5.17	-	6.01	5.76	5.12	-	6.17	0.95	0.96
MDH2	-6.39	-6.74	-	-6.08	-6.42	-6.60	-	-6.20	0.88	0.92
ME2	-1.315	-1.39	-	-0.85	-0.88	-1.21	-	-0.72	0.11	0.38
MICU1	-2.715	-2.99	-	-2.44	-2.51	-2.61	-	-2.18	0.16	0.44
MNF1	-2.115	-2.45	-	-1.50	-1.82	-2.09	-	-1.66	0.68	0.83
MPC1	-3.705	-4.73	-	-3.52	-3.77	-4.68	-	-3.52	0.82	0.90
MRPL18	-1.075	-1.43	-	-0.49	-0.81	-0.97	-	-0.59	0.57	0.74
MRPL3	-2.41	-2.74	-	-2.03	-2.26	-2.50	-	-1.94	0.95	0.96
MRPL40	-2.135	-2.27	-	-1.39	-1.98	-2.28	-	-1.62	0.93	0.96
MSTN	0.395	0.25	-	1.19	0.94	0.55	-	1.48	0.26	0.50
MT_ND6	-11.18	-11.36	-	-10.71	-10.77	-10.97	-	-10.38	0.35	0.59
MTFR1	-0.005	-0.15	-	0.51	0.51	0.31	-	0.79	0.08	0.38
MTG2	-0.59	-0.75	-	-0.37	-0.26	-0.49	-	-0.04	0.09	0.38
MTIF2	-0.99	-1.22	-	-0.71	-0.61	-0.76	-	-0.50	0.02	0.34
MTRF1L	4.27	4.05	-	4.79	4.75	4.30	-	5.49	0.19	0.46
MUL1	-0.515	-0.88	-	-0.34	-0.26	-0.43	-	-0.07	0.13	0.40
NDUFA10	-2.71	-2.87	-	-2.39	-2.29	-2.72	-	-2.14	0.08	0.38
NDUFA3	-5.71	-6.11	-	-5.54	-5.65	-5.82	-	-5.42	0.10	0.38

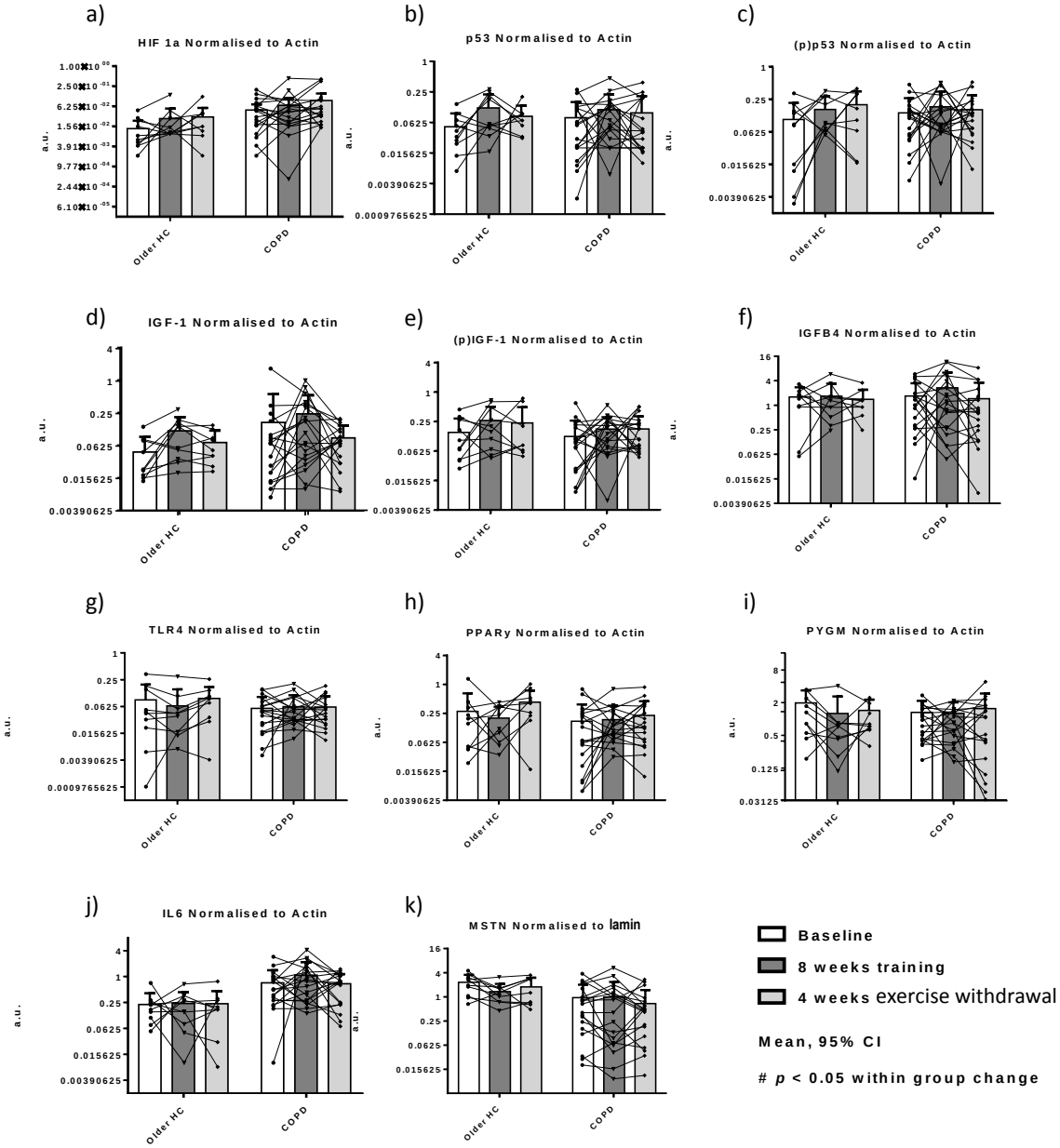
NDUFA9	-1.915	-2.15 - -1.78	-1.97	-2.16 - -1.63	0.85	0.92
NDUFV1	-3.975	-4.20 - -3.90	-3.93	-4.11 - -3.59	0.25	0.50
NFE2L2	-1.795	-1.96 - -0.84	-1.02	-1.31 - -0.89	0.13	0.40
NRF1	0.72	0.32 - 1.01	1.09	0.84 - 1.23	0.07	0.38
PDK2	-4.32	-4.69 - -4.15	-3.96	-4.14 - -3.87	0.20	0.46
PDK4	-3.195	-4.56 - -2.04	-4.05	-5.02 - -2.81	0.28	0.52
PDP2	3.2	3.02 - 3.28	3.45	3.15 - 3.79	0.24	0.50
PFKM	-6.605	-6.86 - -6.08	-6.56	-6.81 - -6.41	0.69	0.84
PGM1	-5.7	-5.81 - -5.60	-5.27	-5.51 - -5.08	0.18	0.46
PKM	-6.445	-6.71 - -5.70	-6.26	-6.61 - -5.59	0.64	0.82
PLA2G2A	-0.175	-1.16 - 1.19	-0.03	-0.81 - 1.03	0.37	0.60
POLRMT	-0.885	-1.15 - -0.63	-0.7	-0.99 - -0.36	0.42	0.60
PPARA	-2.025	-2.32 - -1.70	-1.59	-1.74 - -1.40	0.04	0.34
PPARD	-1.09	-1.32 - -0.67	-0.31	-0.76 - -0.23	0.01	0.21
PPARG	2.19	1.72 - 2.33	2.04	1.78 - 2.55	0.40	0.60
PPARGC1A	-0.99	-1.29 - -0.76	-0.94	-1.14 - -0.62	0.87	0.92
PPARGC1B	0.675	0.12 - 1.53	0.85	0.71 - 1.43	0.86	0.92
PYGM	-8.58	-8.69 - -8.30	-8.44	-8.59 - -8.08	0.71	0.85
SDHA	-3.55	-3.86 - -3.26	-3.28	-3.63 - -3.09	0.35	0.59
SLC2A4	-4.25	-4.63 - -3.83	-3.92	-4.14 - -3.57	0.04	0.34
SOD2	-4.24	-4.70 - -3.71	-4.07	-4.33 - -3.96	0.77	0.88
TFAM	-1.46	-1.69 - -0.88	-1.01	-1.28 - -0.96	0.41	0.60
TFB2M	2.2	1.74 - 2.33	2.2	1.97 - 2.50	0.21	0.48

TLR4	1.27	0.92 - 1.73	1.77	1.56 - 1.99	0.10	0.38
TP53	0.665	0.30 - 1.16	1.05	0.42 - 1.35	0.23	0.50
TUFM	-2.185	-2.42 - -1.80	-1.94	2.03 - 1.70	0.05	0.34
UCP2	-2.855	-3.38 - -2.26	-2.94	3.20 - 2.56	0.88	0.92
UCP3	-2.435	-3.12 - -1.98	-2.88	3.35 - 2.25	0.39	0.60
VEGFA	-3.35	-3.71 - -3.14	-2.97	3.40 - 2.63	0.05	0.34

Baseline mRNA abundance (ΔCT) for target gene expressed relative to HMBS. Unadjusted p values are from 2-sided t-test. Adjustment for multiple comparisons was performed using the FDR method.

Appendix 14

MATCH Study change in protein abundance expressed in arbitrary units. Older vs COPD Group



Change in protein abundance with exercise training and exercise withdrawal expressed relative to a housekeeping protein. HIF-1α, hypoxia inducible factor 1 α; p53, (tumour) protein 53; (p)p53, phosphorylated p53; IGF1, insulin-like growth factor 1; (p)IGF1, phosphorylated IGF1 receptor ; IGFBP4, insulin-like growth factor binding protein 4; TLR4, toll-like receptor 4; PPARγ, peroxisome proliferator activated receptor γ; PYGM, glycogen phosphorylase (muscle); IL6, interleukin 6; MSTN, myostatin.

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