

The manifestations of ageing in the pathophysiology of skeletal muscle dysfunction in COPD

A thesis submitted for the degree of Doctor of Philosophy

September 2014

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Abstract

The aim of this thesis was to address the impact of the skeletal muscle adaptations that occur in Chronic Obstructive Pulmonary Disease (COPD) and subsequently to identify tools that may be relevant in the management of patients with structural and functional muscle abnormalities.

To establish whether the type I to II fibre shift that occurs in COPD is associated with mortality, a retrospective multicentre analysis of 392 stable COPD outpatients was performed. Across the whole cohort, fibre shift was an independent predictor of mortality in a model also including age and FEV₁%predicted. In patients with GOLD stage III or IV disease, quadriceps strength, but not fibre shift had an independent association with an increased risk of mortality. Therapies targeting quadriceps fibre shift and weakness may be of therapeutic value; practical tools that identify relevant patients may have clinical utility. Since the pathophysiological adaptations that occur may be considered to be a manifestation of accelerated biological ageing, potentially relevant physical and biological markers of ageing were identified following a review of the literature.

The Short Physical Performance Battery (SPPB) is used in gerontology to assess lower limb function, but its determinants have not been previously evaluated in COPD. SPPB score was measured in 109 stable COPD outpatients; 31 also had a quadriceps biopsy. Quadriceps strength and exercise capacity were the only independent predictors of SPPB score. Stratification by SPPB score identified patients with locomotor muscle atrophy and impairment in strength, exercise capacity and daily physical activity. Patients with a reduced SPPB score had a higher proportion of type II fibres. Subsequently, the SPPB potentially has practical utility in the assessment of COPD patients.

Systemic Klotho and GDF-15 levels are associated with mortality in non-selected populations; these proteins were evaluated initially in the serum and then the skeletal muscle of healthy controls, smokers and COPD patients. Circulating Klotho levels were reduced in COPD and smokers, related to quadriceps strength and increased after successful smoking cessation. Serum GDF-15 levels were elevated in COPD, and related to a marker of systemic oxidative damage and locomotor muscle atrophy. Klotho and GDF-15 were expressed in skeletal muscle. Quadriceps GDF-15 expression was elevated in COPD patients as compared to controls and diaphragm expression. Klotho levels were reduced in the locomotor muscle of human smokers and smoke-exposed mice. Humans had relatively higher Klotho levels in respiratory muscle. Quadriceps Klotho levels positively correlated with local protein carbonylation and were also unexpectedly elevated in patients with established skeletal muscle dysfunction; immunohistochemistry confirmed that Klotho was associated with regenerating muscle fibres. Modulation of Klotho and GDF-15 signalling, and potentially other age-related molecules, may provide therapeutic options to COPD patients.

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Awards arising from this thesis

May 2014, American Thoracic Society Abstract Scholarship Award: Assembly on Pulmonary Rehabilitation.

May 2013, American Thoracic Society Abstract Scholarship Award: ATS Assembly on Clinical Problems Funded by The National Emphysema Foundation.

Acknowledgements

I would like to thank my PhD supervisors, Professor Michael Polkey, Dr. Paul Kemp, Dr. William Man and Dr. Amanda Natanek for their guidance over the past three years. I am also grateful to Dr. Nicholas Hopkinson for his valuable input and Dr. Mark Griffiths in his role as mentor. I am especially grateful to Professor Polkey for his boundless enthusiasm and support from the inception to completion of this thesis. Dr. Man and Dr. Hopkinson kindly provided support and assistance in the recruitment of patients and Dr. Man provided guidance in the formation of this thesis. I would like to thank Dr. Kemp for his guidance, support, patience and when necessary, practical assistance in the accrual of the molecular work presented in this thesis, and Dr. Natanek for her enthusiasm, unofficial mentorship and knowledge. I am grateful for the support provided by surgical colleagues, especially Mr. Simon Jordan, in the provision of surgical muscle biopsies and to Mr. Winston Banya at the Royal Brompton for his statistical advice. I also wish to thank the members of the Royal Brompton and Harefield Lung Function departments for testing the participants and the staff involved in the smoking cessation clinics at both the Royal Brompton and Harefield Hospitals.

I am grateful to Prof. Francois Maltais from Québec, Prof. Ioannis Vogiatzis from Athens and Prof. Joaquim Gea from Barcelona and their respective teams for sharing their data for the multicentre mortality study presented in Chapter 3. I am also grateful for the help of Dr. Divya Mohan, Dr. Manuel Baz, Dr. Samantha Kon and Dr. Jane Canavan for their assistance in recruitment and collection of some of the data for the SPPB study described in Chapter 4 and to Dr. Anna Donaldson for her assistance with collection of some of the data for Chapters 5 & 6. All of the molecular analyses were performed by myself, but I am extremely grateful to the assistance and guidance provided by Dr. Amy Lewis throughout my time in the molecular laboratory and to Dr. Jen Lee and Professor Nic Wells for providing the electroporated mouse tissue studied in Chapter 6.

During the formation of this thesis I have had the privilege of working with many other colleagues who I now consider friends. Many thanks for the support, knowledge and assistance that you have provided throughout. Specifically, I would like to say thank you to Dr. Gulam Haji, Dr. Dario Marolini, Dr. Dinesh Shrikrishna, Ms. Rebecca Tanner, Ms. Cayley Smith, Dr. Zaid Zoumot, Dr. Katrina Curtis, Dr. Yogini Raste, Ms Amy Clark, Ms. Dede Lori, Mr. Samuel Clark, Ms. Claire Davey and Ms. Julia Kelly.

Finally, I wish to express my deepest gratitude to the participants who agreed to take part in my studies, without their assistance, the work presented would simply not have been possible.

Dedication

My parents have provided a lifetime of support and encouragement, for this I will be eternally grateful. I would like to dedicate this thesis to my wife, Rinal, for her unwavering patience, understanding and support throughout and my baby boy, Shayan, for providing inspiration.

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Abbreviations List

1,25(OH)₂D - 1,25-dihydroxyvitamin D
p70S6K - 70-kD ribosomal S6 protein kinase
8-oxo-dG - 8-oxo-2'deoxyguanosine
ADO - Age, Dyspnoea, Obstruction
PaO₂ - Arterial oxygen content
BIA - Bioelectrical impedance
BMI - Body Mass Index
BODE - Body Mass Index, Airflow obstruction, Dyspnoea, Exercise capacity
CFi - Capillary to fibre ratio
CPET - Cardiopulmonary exercise testing
COPD - Chronic Obstructive Pulmonary Disease
CRQ - Chronic Respiratory Questionnaire
CCQ - Clinical COPD Questionnaire
cDNA - complementary DNA
CT - Computed tomography
CAT - COPD Assessment Test
C_T - Cycle threshold
DNA - Deoxyribonucleic acid
DEXA - Dual energy X-ray absorptiometry
ELISA - Enzyme linked immunosorbent assay
4E-BP1 - Eukaryotic initiation factor 4E binding protein-1
FFM - Fat free mass
FGFRs - FGF receptors
FGF - Fibroblast growth factor
FGF-23 - Fibroblast growth factor-23
FEV₁ - Forced expiratory volume in one second
FVC - Forced vital capacity
FoxO - Forkhead box O
FRC - Functional residual capacity
GOLD - Global Initiative for Chronic Obstructive Lung Disease
GDF-15 - Growth and differentiation factor-15
GDF-8 - Growth differentiation factor-8

ISW - Incremental shuttle walk

IC - Inspiratory capacity

IGF-1 - Insulin-like growth factor-1

KL - Klotho

MIC-1 - Macrophage inhibitory cytokine-1

MRI - Magnetic resonance imaging

mTOR - Mammalian target of rapamycin

MMPs - Matrix metalloproteinases

MRC - Medical Research Council

MTCSA - Mid-thigh cross-sectional area determined by CT scan

mMRC - modified MRC

MURF-1 - Muscle ring finger-1

CSA - myofibre cross-sectional area

MHC - Myosin heavy chain

NNMT - Nicotinamide N-methyltransferase

VO₂peak - Peak oxygen uptake

PAL - Physical activity level

PCR - Polymerase Chain Reaction

QMVC - Quadriceps maximum voluntary contraction

RF_{CSA} – *Rectus femoris* cross-sectional area

RT-qPCR - Real-time quantitative PCR

RV - Residual volume

SPPB - Short Physical Performance Battery

6MWT - Six minute walk test

SGRQ - St. George's Respiratory Questionnaire

Time ≥3METs - Time spent in at least moderate physical activity

TIMPs - Tissue Inhibitors of Metalloproteinases

TLC - Total lung capacity

TL_{CO} - Transfer of carbon monoxide

TGF-β - Transforming growth factor-β

TNF-α - Tumour necrosis factor-α

TwQ - Twitch quadriceps tension

VL - *Vastus lateralis*

Chapter 1 : Introduction

1.1 Background

1.1.1 Chronic Obstructive Pulmonary Disease (COPD); Overview

COPD is a debilitating disease that significantly impacts function and healthcare use. The inflammatory response of the lung to the inhalation of noxious particles or gases, most commonly from cigarette smoking, results at least in 20% of smokers, in a progressive disease. COPD is characterised by expiratory airflow obstruction with minimal reversibility and parenchymal destruction. In the United Kingdom, the prevalence of the disease in adults over 35 is more than 10% (Shahab, Jarvis et al. 2006) and it is the cause of 30 000 deaths per year in England and Wales alone (ONS 2004). Mean age of diagnosis is at 67 years. Up to half of all COPD patients remain undiagnosed so the recorded prevalence of COPD may only be 50% of the actual prevalence; those that are diagnosed often present relatively late having already developed significant airflow obstruction. Despite being preventable and treatable, at a global level, the burden of COPD is expected to rise because of the continued exposure to risk factors and an ageing population. In fact the latest data indicates that COPD is now the third leading cause of mortality worldwide (Lozano, Naghavi et al. 2012).

The chronic airflow obstruction that characterises COPD is a consequence of small airways disease (obliterative bronchiolitis) and parenchymal destruction (emphysema). Structural adaptations including narrowing of the small airways and destruction of the lung parenchyma occur in the setting of chronic inflammation. The impaired elastic recoil of the lung that occurs with parenchymal

destruction predisposes the airway to collapse during expiration. Furthermore, obstruction of peripheral airways results in the progressive trapping of air which results in hyperinflation.

Although cigarette smoking is the most recognised risk factor for COPD, non-smokers may also develop COPD (Eisner, Anthonisen et al. 2010). Additionally, even in the context of the same smoking history, not all smokers develop COPD. Consistent with this, twin studies demonstrate that around 60% of an individual's susceptibility to developing COPD may be explained by genetic factors (Ingebrigtsen, Thomsen et al. 2010). COPD is a disease that is more prevalent in older populations and usually requires a chronic exposure to relevant risk factors; however, those with genetic susceptibility may develop the disease earlier. Alpha-1 antitrypsin deficiency is the most widely recognised genetic risk factor; whilst only directly relevant to a relative minority, it does illustrate that the interaction between environmental and host factors may be relevant in the development of COPD (Tomashefski, Crystal et al. 2004). Further genomic and proteomic studies may identify other host factors that are relevant to the development of COPD in other populations. In this regard, an interaction between several relevant risk factors such as the inhalation of noxious particles, ageing and host defence mechanisms may complicate the development of COPD.

In addition to cigarette smoking, other relevant inhaled agents including organic and inorganic dusts, chemicals and fumes may initiate an abnormal inflammatory response in the lung. Not only does this response result in the destruction of tissue, but it also disrupts the normal repair mechanisms and results in progressive fibrosis of the small airways which further adds to the airflow limitation and air trapping.

The extent of inflammation and fibrosis relates to the level of airflow obstruction and probably the accelerated decline in lung function, monitored as the forced expiratory volume in one second (FEV₁) that is observed in COPD (Hogg, Chu et al. 2004). Mucus hypersecretion is a feature of chronic

bronchitis which clinically manifests as a chronic productive cough. A proportion of COPD patients develop this aspect of the disease which is a consequence of goblet cell hyperplasia in response to the stimulation of growth factors in the setting of chronic airway irritation. Exacerbations of COPD may be triggered by infection with viruses, bacteria, environmental pollutants or other unknown factors. Exacerbations and the systemic aspects of COPD significantly impact the severity of the disease.

1.1.2 The pathogenesis of the pulmonary aspects of COPD

The pathological changes that occur in COPD patients as a consequence of chronic inflammation and impaired repair mechanisms extend from the airways and parenchyma to the pulmonary vasculature. The mechanisms for the amplified inflammatory response are yet to be established, but as the inflammation of the lung persists even after smoking cessation, factors other than inhaled exposures, including genetic factors are also likely to be relevant (Cosio, Saetta et al. 2009). A range of inflammatory agents have been shown to be elevated in COPD. Chemotactic factors such as IL-8 attract circulating inflammatory cells whereas proinflammatory cytokines including IL-6 and TNF- α amplify the inflammatory process. Elevated levels of cytotoxic (CD8) Tc1 lymphocytes are present in smokers that develop the disease and contribute to the local inflammatory mediators that are also released by other inflammatory cells (Barnes, Shapiro et al. 2003).

There is evidence of an imbalance in proteases and anti-proteases in the lungs of COPD patients. Whilst proteases derived from inflammatory and epithelial cells are responsible for the breakdown of connective tissue, anti-proteases counteract this process, for example, deficiency of the anti-protease α 1-antitrypsin is recognised to incur proteolytic damage. Specifically, the destruction of elastin by proteases may be an important feature of emphysema and is likely to be irreversible.

Oxidative stress is a consequence of an increased production of reactive oxygen species (ROS) or as a result of reduced antioxidant capacity. Oxidative stress may have an important role in the tissue damage that occurs in COPD (Rahman 2005) by directly inflicting injury at a cellular level as well as by activating local inflammation. Oxidants are released by activated inflammatory cells including neutrophils and macrophages in response to cigarette smoke and other inhaled particulate matter. Antioxidants may be enzymatic or non-enzymatic. An example of the former is the superoxide dismutase family, whilst ascorbate and alpha-tocopherol are examples of the latter (Lin and Thomas 2010). In COPD, markers of oxidative stress have been demonstrated to be raised in the sputum, exhaled breath condensate, systemic circulation and organs distant to the inhalation of noxious particles (Lin and Thomas 2010; Kirkham and Barnes 2013).

1.1.3 Systemic pathophysiological adaptations in COPD

Whilst milder forms of the disease may go unrecognised, more advanced disease is characterised by progressive dyspnoea and impaired exercise tolerance. Although COPD has traditionally been considered a disease of the respiratory system, it is now recognised that systemic manifestations of the disease are an important source of morbidity and mortality (Sabit, Bolton et al. 2007; Swallow, Reyes et al. 2007; Barnes and Celli 2009). In fact it has recently been established that the systemic facets of the disease may occur early on in the course of the disease (Shrikrishna, Patel et al. 2012). This is of importance as pharmacological amelioration of lung function impairment translates to relatively small improvements in exercise capacity (Grove, Lipworth et al. 1996). Furthermore, even when lung function is restored following lung transplantation, exercise performance does not usually return to normal (Levy, Ernst et al. 1993). Systemic manifestations potentiate the morbidity and

mortality conferred by the pulmonary aspects of the disease and therefore require consideration in their own right.

The development of systemic manifestations may be a consequence of common susceptibility to shared pathological factors with subsequent development of multiple organ effects, or alternatively, a consequence of the spillage of respiratory burdens and events into the systemic circulation and other compartments (see Figure 1.1). In this regard, ageing may be taken to be an example of the former and inflammation could be considered an example of the latter. Although the pulmonary adaptations that occur in COPD are essentially irreversible, systemic manifestations may be amenable to intervention (classically by pulmonary rehabilitation). Therefore, in the pathophysiological evaluation of COPD, on the one hand, systemic manifestations may be considered as co-morbid conditions with management focussing on systemic burdens, on the other, as a complication with management focussing on the lungs in the first instance. Further work is necessary to elucidate the mechanisms involved in the development of systemic manifestations of the disease, this will likely guide future therapeutic approaches. Subsequently, at present, it is important to consider each aspect of the disease independently and this is the focus of the remainder of section 1.1.3.

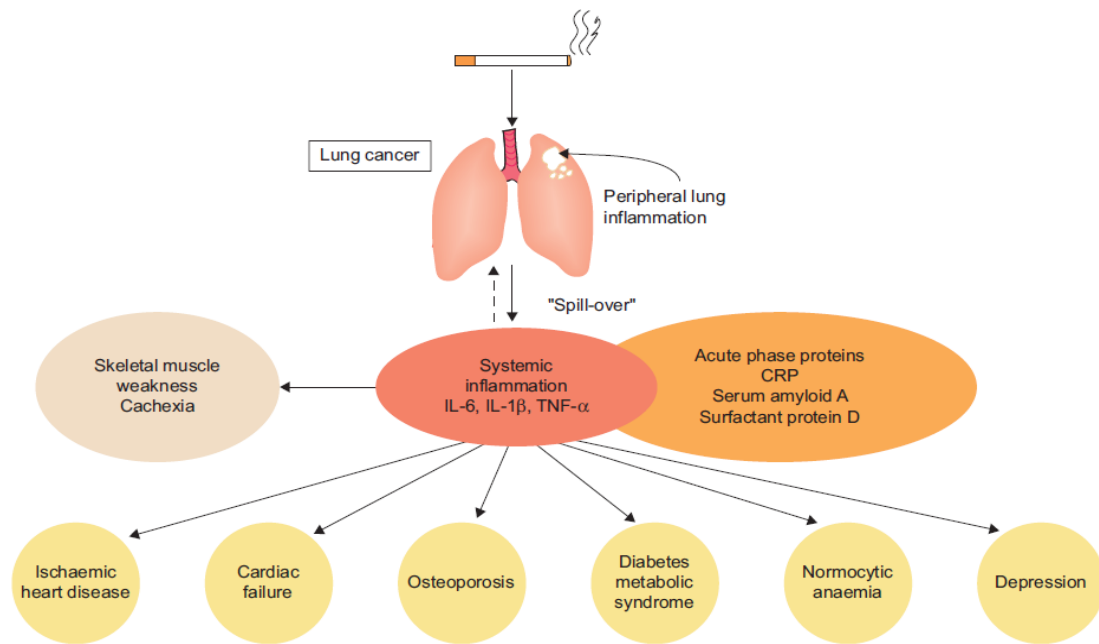


Figure 1.1: The potential systemic manifestations in a “spill-over” model of COPD.
(Barnes and Celli 2009)

The musculoskeletal system

Skeletal muscle dysfunction is a prevalent finding in COPD and is an important source of morbidity and mortality. Many chronic disease processes are associated with cachexia, although this is often considered to be a late-stage phenomenon. In COPD, it has recently been established that loss of muscle strength occurs early on in the disease process and this may precede the development of cachexia assessed by fat free mass (Hopkinson, Tennant et al. 2007). Most studies have focussed on the quadriceps muscles which are the main locomotor muscles and display a loss of mass and function in COPD (Bernard, LeBlanc et al. 1998; Shrikrishna, Patel et al. 2012). Furthermore, these muscles are predisposed to structural and molecular adaptations that are associated with a reduced oxidative capacity (Nataneek, Gosker et al. 2013). Whilst inactivity is considered the main driver for many of these adaptations, as evidenced by predominant locomotor muscle dysfunction, various other factors have been implicated including oxidative stress, systemic inflammation, hypoxia,

pharmacological agents, nutritional status and genetic factors. A detailed description of the functional, structural and molecular adaptations is provided in section 1.1.9. Many of these adaptations are accentuated during COPD exacerbations, which at a clinical level manifest as a loss of muscle strength (Sprit, Gosselink et al. 2003).

Patients with COPD are exposed to many of the risk factors relevant to the development of osteoporosis. Therefore, it is unsurprising that COPD patients have a high prevalence of osteoporosis (Graat-Verboom, Wouters et al. 2009; Duckers, Evans et al. 2011). Smoking cessation and increasing physical activity are important aspects of managing COPD and are translatable to the management of osteoporosis. Vitamin D deficiency in COPD patients is a likely biological consequence of reduced sun exposure and impaired nutritional status (Janssens, Bouillon et al. 2010). Vitamin D deficiency is not only a well-established risk factor for osteoporosis, but also impacts other aspects of the disease including inflammation (Janssens, Lehouck et al. 2009). Several inflammatory mediators, including TNF- α and IL-6, stimulate osteoclasts which may result in bone resorption (Boyle, Simonet et al. 2003). There is a significant relationship between the degree of emphysema on CT and the bone mineral density (Ohara, Hirai et al. 2008). The relationship between osteoporosis and physical function in COPD and osteoporosis is yet to be fully established, although they are expected to relate given that bone density relates to fat-free mass (Bolton, Ionescu et al. 2004).

The cardiovascular system

In the setting of shared risk factors, COPD is a common finding in patients with cardiovascular disease; pertinently patients with milder COPD are more likely to die from a cardiac event than respiratory failure (Calverley, Anderson et al. 2007). Systemic inflammation and oxidative stress are involved in the development of atherosclerosis; other relevant considerations include activation of the sympathetic nervous system, hyperinflation and chronic hypoxia.

In the setting of acute COPD exacerbations, a rise in troponin T reflecting cardiac damage frequently occurs and is associated with increased mortality (Høiseth, Neukamm et al. 2011). It is recognised that raised intrathoracic pressures in the setting of hyperinflation reduces cardiac filling and subsequently cardiac output (Stark-Leyva, Beck et al. 2004). In patients being evaluated for lung volume reduction surgery, the prevalence of mild pulmonary hypertension is around 50% (Thabut, Dauriat et al. 2005). However, a relatively small proportion of patients have significant pulmonary hypertension, indicating that airflow limitation is not the most important driver of elevated pulmonary pressure. Patients exposed to chronic hypoxia are pre-disposed to destruction of the pulmonary vasculature with subsequent right ventricular hypertrophy. When the right heart is no longer able to sustain adequate output to meet demand, right heart failure may ensue.

The neurological system

Patients with COPD may develop central and peripheral nervous system adaptations. The cardiovascular adaptations that occur in COPD have already been discussed; stroke may be considered a neurological expression of this and is more prevalent in COPD, especially following exacerbation (Donaldson, Hurst et al. 2010). Patients with hypoxia may develop cognitive impairment which particularly affects the processing of information and verbal memory, and may consequently impact their ability to action management plans or their awareness of deterioration. Psychological manifestations including depression and anxiety may be detected by tools such as The Hospital Anxiety and Depression Scale. There are no disease specific features of depression in COPD, which is considered to occur in response to the physical aspects of the disease. In addition to mainstream treatment strategies, anxiety in COPD may be addressed by patient education and learning techniques for breathing control.

The metabolic system

The prevalence of diabetes mellitus is increased in patients with COPD, with an odds ratio of up to 1.5 in severe disease (Mannino, Thorn et al. 2008), the mechanisms behind this increase are not fully established. Pertinently, inflammatory cytokines including TNF- α and IL-6 may induce insulin resistance (Spranger, Kroke et al. 2003). Hyperglycaemia is common during exacerbations of COPD, even in those without previous evidence of diabetes; furthermore, it is associated with a history of current smoking (Koskela, Salonen et al. 2013).

1.1.4 The role of ageing in the development of COPD

Epidemiological considerations

Ageing is the age-related decline in physiological processes and functions that are necessary for survival. Many of the pathophysiological features that are observed in COPD also occur in the ageing lung independent to any exposure to smoke (see Figure 1.2). Furthermore, most patients with COPD are above the age of 40, despite smoking normally having commenced decades earlier, raising the hypothesis that chronic smoke exposure accelerates the ageing process or that an increased susceptibility to ageing itself may be important to the pathogenesis of COPD. In individuals aged 65 or over the prevalence of COPD is 14.2%, whereas in those aged 40 and over prevalence is 9.9% (Halbert, Natoli et al. 2006). The association between COPD and ageing may be expected to result in a significant increase in the morbidity, mortality and socioeconomic burden of disease. Contextually, from 1970 to 2010, global male life expectancy increased from 56 to 68 years, female life expectancy similarly increased to an improved life expectancy of 73 years (Wang, Dwyer-Lindgren et al. 2013).

Pathophysiological similarities

There is a growing body of evidence to suggest that smokers and patients with COPD are prone to early ageing; certainly even those who do not smoke are at risk of developing airflow obstruction and reduced elastic lung recoil that progresses with age (Fletcher and Peto 1977). Ageing is recognised to be a proinflammatory state and there is an overlap between the dysregulation of the immune system that is observed in COPD and senescence. IL-6 and TNF- α increase with age and an age-dependent oligoclonal expansion of CD-8 cells accompanies a fall in naive T cells (Vallejo 2006). This dysregulation of the adaptive immune system with age results in the upregulation of the innate

immune system, the recruitment of inflammatory cells in the lungs of smokers is likely to trigger and perpetuate an inflammatory cascade in susceptible individuals (Cosio, Saetta et al. 2009).

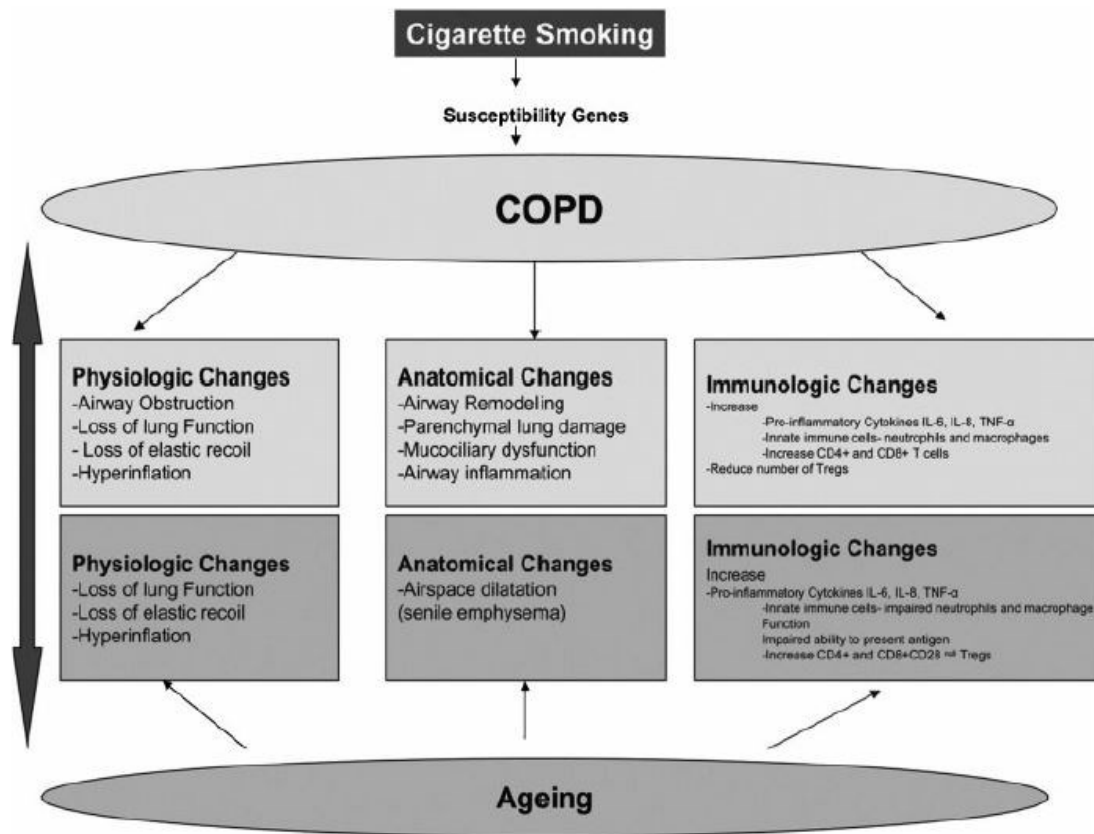


Figure 1.2: Physiological, anatomical and immunological changes with smoking and ageing.

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Ageing is associated with the development of frailty, a phenotype that results in the increased vulnerability to stresses consequent to the decline of various physiological systems. The frailty phenotype is typified by several recurring pathophysiological entities that are also common in COPD, including sarcopenia, anorexia, osteoporosis, fatigue and poor physical health. The fact that skin wrinkling, a clinical marker of ageing, is accelerated in smokers supports the notion that smoking may initiate effects outside of the pulmonary system (Aizen and Gilhar 2001). The degree of

wrinkling correlates with quantitative measurements of emphysema, which supports the notion that smoking may influence systemic agents which either protect against or confer these adaptations (Patel, Loo et al. 2006). Systemic agents that mediate ageing may mediate some of the pathophysiological adaptations, including the development of skeletal muscle dysfunction, that are observed in COPD.

Pathophysiological mechanisms

As with virtually every other disease, chronological age is a predictor of mortality in COPD (Puhan, Garcia-Aymerich et al. 2009), although not when nutritional status or exercise capacity are included as predictors (Celli, Cote et al. 2004). Consistent with this, in COPD, biological processes and markers of ageing may be influenced by other relevant risk factors such as smoking. Telomeres are DNA caps located at the end of chromosomes. Telomeres protect against DNA degradation and alteration, consequently they, specifically their length, are a marker of biological ageing. Telomere length is reduced in the circulating leukocytes of COPD patients, furthermore, it relates to mortality in those with disease (Lee, Sandford et al. 2012). However, the effect may be modest: Lee *et al.* only just demonstrated statistical significance ($p=0.04$) with a hazard ratio of 1.29 when evaluating the risk of total mortality in a study of over 4000 COPD patients, furthermore current smoking status did not influence telomere length. It may be that smoking has an influence at an earlier stage of life; alternatively, other markers of ageing may be more relevant in COPD. Interestingly, chronological age was not different amongst patients divided into quartiles according to telomere length, indicating that other processes are more relevant than chronological age in determining telomere length in the context of COPD. The mechanisms of telomere shortening in COPD are unknown, although oxidative stress and genetic predisposition may be relevant. Study of other potential age-related markers may identify pathophysiological mechanisms that underlie the disease process, potential biomarkers and possibly even therapeutic targets.

It may be that clinical measures that are established as having discriminatory value in general older populations may be relevant in the assessment of COPD patients. Despite this, there are important pathophysiological differences, detailed later, that differentiate COPD and normal ageing and these considerations may complicate the extension of these tests to the COPD population.

Oxidative stress is increased in both COPD and ageing and may be a process that interlinks the two. Glutathione is an antioxidant that is obtained either through the diet or by production within the body. It is normally concentrated in fluid lining the epithelium. Mouse models indicate that exposure to cigarette smoke not only induces glutathione production in the lung, but also that this response is blunted with increasing age (Gould, Min et al. 2013). Furthermore, reactive nitrogen species found in cigarette smoke and produced endogenously, have also been implicated in the pathogenesis of COPD. Contextually, alveolar nitric oxide production may be particularly important as levels relate to the degree of airflow obstruction (Brindicci, Kharitonov et al. 2009).

There is evidence that the oxidative damage caused by cigarette smoke is not isolated to the lungs, possibly explaining some of the systemic manifestations observed in COPD. In this regard, Barreiro *et al.* demonstrated that cigarette smoke exerts direct oxidative adaptations to muscle proteins that may contribute to muscle dysfunction and depletion in both smokers and patients with COPD (Barreiro, Peinado et al. 2010). Furthermore, Kim *et al.* demonstrated whilst investigating nicotinamide N-methyltransferase (NNMT) expression, that improving resistance to oxidative stress may ameliorate these effects (Kim, Mofarrahi et al. 2010). NNMT expression was up-regulated in the diaphragm and quadriceps muscles of patients with COPD, relatively more so in the quadriceps, and negatively correlated with COPD severity and limb muscle wasting. Overexpression of NNMT promoted myoblast proliferation and migration whilst reducing protein oxidation and hydrogen

peroxide-induced cell death, implicating an adaptive response that might enhance myogenesis and defence against oxidative damage.

1.1.5 The functional evaluation of the COPD patient

Patients thought to be at risk of COPD should be assessed by spirometry in order to detect airflow obstruction, an FEV_1/FVC ratio of below 70% allows a diagnosis of to be established (Rabe, Hurd et al. 2007) and appropriate interventions to be administered. Although international guidelines continue to classify COPD severity on the degree of airflow obstruction (Table 1) (Rabe, Hurd et al. 2007), FEV_1 poorly predicts dyspnoea, health status, functional impairment and mortality. For these reasons, the latest iteration of the GOLD guidelines includes symptom scoring and exacerbation frequency in addition to FEV_1 in the routine assessment of COPD patients (detailed further below).

Stage 1: mild	$FEV_1/FVC < 0.70$ $FEV_1 \geq 80\%$ predicted
Stage II: moderate	$FEV_1/FVC < 0.70$ $50\% \leq FEV_1 < 80\%$ predicted
Stage III: severe	$FEV_1/FVC < 0.70$ $30\% \leq FEV_1 < 50\%$ predicted
Stage IV: very severe	$FEV_1/FVC < 0.70$ $FEV_1 < 30\%$ predicted <i>or</i> $FEV_1 < 50\%$ predicted plus chronic respiratory failure

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

Patients with COPD frequently complain of dyspnoea that is exacerbated by exercise. Impaired exercise capacity frequently results in a reduced ability to perform routine activities such as shopping and socialising, and in more advanced cases this may even extend to limiting more basic activities such as dressing, bathing, walking up the stairs and performing household chores. The evaluation of the functional status of patients with COPD is pertinent and provides an indication of

the effects of the disease on each individual and subsequently guides management. Various tools may be used to provide an assessment of functional status in COPD, each has their own limitations.

Pulmonary function testing

Pulmonary function measurements include spirometry (comprising the forced expiratory volume in 1 second (FEV₁) and forced vital capacity (FVC)), lung volumes and gas diffusion capacity.

The decline in FEV₁ over time has been traditionally used to indicate disease progression in patients with COPD. The diagnosis and staging of COPD is based on spirometric data as already discussed and the methodology for measuring spirometry has been standardised and is regarded to be highly reproducible when performed properly (Miller, Hankinson et al. 2005). Spirometry allows the detection of COPD and subsequently intervention in early stages of the disease before the appearance of symptoms. Furthermore, a reduction in FEV₁ is a risk factor for all-cause mortality (Young, Hopkins et al. 2007). However, given that spirometry only evaluates the pulmonary aspects of the disease, it is unsurprising that patients with a similar FEV₁ may have very different underlying phenotypes. Even when focussing on the respiratory aspects of the disease, a patient may have significant emphysema without significant airflow limitation being evident on spirometry. Subsequently, whilst crucial in the evaluation of the COPD patient, the FEV₁ alone does not provide sufficient information to adequately characterise a patient.

Progressive hyperinflation due not only to airflow limitation but also loss of elastic recoil of the lung parenchyma increases the static inspiratory burden and significantly impinges upon the respiratory reserve. Symptomatically, these changes manifest as an increased sense of effort and dyspnoea. The assessment of lung volume is more technically demanding than spirometry and requires specific training. Lung volume, measured as the total lung capacity (TLC), functional residual capacity (FRC)

and the residual volume (RV), may be measured at rest to detect static hyperinflation or on exercise to detect dynamic hyperinflation. A decrease in the inspiratory capacity (IC) indicates an impact on the respiratory reserve. The sniff inspiratory nasal pressure (SnIP) primarily reflects the severity of hyperinflation in COPD and Moore *et al.* have previously demonstrated that the SnIP has at least as much power in predicting mortality as the IC/TLC ratio (Moore, Soler *et al.* 2010). Markers of dynamic hyperinflation relate better with exertional dyspnoea than FEV₁ (O'Donnell and Laveneziana 2006). Despite being of prognostic relevance (Martinez, Foster *et al.* 2006), there is no standardised classification, nor are there established minimally important differences for the various markers of hyperinflation. In this context, the course of hyperinflation in COPD patients is unclear.

Diffusion studies estimate oxygen transfer across the alveolus into the pulmonary vasculature and therefore detect the functional consequences of parenchymal destruction. Gas exchange abnormalities with resultant hypoxaemia and hypercapnia tend to progress during the course of the disease process. Impaired ventilatory drive may also manifest as carbon dioxide retention in the setting of the demands incurred by severe obstruction and hyperinflation. Furthermore, abnormalities in the alveolar bed and pulmonary vasculature may also manifest as gas exchange abnormalities. Contextually, the transfer of carbon monoxide (TL_{CO}) expressed as a percentage of that predicted, was the only lung function parameter to independently predict mortality in stable COPD outpatients, the only other independent predictors in this model were age and arterial oxygen content (PaO₂) (Boutou, Shrikrishna *et al.* 2013).

Dyspnoea and health status

Breathlessness is the most prevalent symptom to be reported by COPD patients. Dyspnoea is a major source of morbidity and limits functional capacity in patients with COPD. Alleviating dyspnoea is an important goal in the management of the COPD patient. Numerous methods of assessing

dyspnoea exist; in COPD, dyspnoea scales are most commonly used. The Borg scale is often used to determine an individual's perception of dyspnoea during exercise testing. Similarly, the Medical Research Council (MRC) dyspnoea score provides a summary of the patient's subjective assessment of their own functional limitation on account of dyspnoea.

Improving health status is another important consideration in the management of COPD. Given that health status is a multidimensional concept, the evaluation of health status has classically necessitated the use of multidomain instruments; numerous instruments that have been designed for this purpose. The St. George's Respiratory Questionnaire (SGRQ) and the Chronic Respiratory Questionnaire (CRQ) are the most widely established measures of health status in COPD. The SGRQ is a 76 item disease-specific questionnaire that evaluates the effect of the disease across three domains - symptoms, impacts and activities. Scores correlate with measures of disease severity and functional ability (Jones, Quirk et al. 1991; Jones, Quirk et al. 1992). It has been shown to be reproducible and valid, with ability to detect changes over time and response to intervention (Jones, Quirk et al. 1992; Welte, Miravittles et al. 2009). The CRQ is also a validated and reliable questionnaire; it may be self or interviewer-administered. The CRQ evaluates 20 aspects across four domains, specifically dyspnoea, fatigue, emotional function and mastery. Although it detects changes at an individual level, it is not suited for comparisons between populations (Jones, Miravittles et al. 2012). Despite their established value, the SGRQ and CRQ are not widely practised due to their complexity and time pressures in routine clinical practice.

Simple questionnaires have now been designed to assist in the clinical evaluation of health status, an example is the COPD Assessment Test (CAT) which was developed in 2009 with a view to obtaining valuable information in a short period of time (Jones, Harding et al. 2009). Despite only having 8 items applicable to a wide range of impacts in COPD patients, it has been validated and found to be reliable in different populations and correlates well with longer more established measures including

the SGRQ (Jones, Brusselle et al. 2011). Furthermore, it detects improvement following intervention (Dodd, Hogg et al. 2011) and recovery following exacerbation (Agusti, Soler et al. 2012). The Clinical COPD Questionnaire (CCQ) is another brief, easy to use tool that has been developed in the past decade. It has demonstrated good reliability, validity and responsiveness both at an individual and group level (Jones, Miravittles et al. 2012).

Exercise capacity

Exercise performance provides an integrated assessment of the respiratory and systemic aspects of the disease including skeletal muscle function, cardiovascular impairment, chronological age, co-morbidity and cognitive function. Despite the successful introduction of simple tools to assess health status in clinical practice, the assessment of exercise capacity in COPD is currently mostly confined to research studies and for the evaluation of the response to pulmonary rehabilitation. Established measures of exercise capacity include the gold standard measure of evaluating performance on a cycle ergometer or treadmill with cardiopulmonary monitoring, cardiopulmonary exercise testing (CPET), and field walking tests which are simpler, cheaper and more relevant to the daily functioning of patients. Impaired exercise capacity identified by peak oxygen uptake (VO_{2peak}) or by simple field tests predicts mortality better than FEV_1 (Oga, Nishimura et al. 2007). The six-minute walk test (6MWT), a self-paced test, is the most recognised field walking test (Redelmeier, Bayoumi et al. 1997) and provides an accurate assessment of functional exercise capacity and performance, correlating well with CPET results. However, the 6MWT requires a significant amount of space (in particular the minimum corridor length is 30m) and is prone to a learning effect (American Thoracic Society 2002). Other timed walking tests include the 12-minute walk test and the 2-minute walk test, which only differ in their duration when compared to the 6MWT and in fact convey very similar information to the 6MWT (Butland, Pang et al. 1982). The incremental shuttle walk test (ISWT) is an externally paced test assessed over a 10 metre course that is less affected by motivation and relates

strongly to VO_2 peak (Singh, Morgan et al. 1994). However, the ISWT is less well validated than the 6MWT, furthermore at a patient level, incremental external pacing may not reflect regular daily practice. Similarly, whilst progressive, incremental treadmill and cycle tests allow the collection of detailed physiological and metabolic data, the rigors of the testing protocols are not suited to all patients, especially those who are limited in performing simple tasks of daily living. In this context the introduction of simple functional performance tools that may be used to screen for impaired performance may be advantageous in the routine evaluation of the COPD patient.

Physical activity

Whilst the measurement of performance is important in the evaluation of the COPD patient, it does not necessarily relate to what patients actually do in their own environment. Whole body physical activity is significantly reduced in COPD (Pitta, Troosters et al. 2005), where it is predictive of hospitalisation (Garcia-Aymerich, Farrero et al. 2003), lung function decline (Garcia-Aymerich, Lange et al. 2007) and mortality (Waschki, Kirsten et al. 2011). Physical activity may be assessed by questionnaires or measured objectively by pedometers or accelerometers, although neither are regularly performed in clinical practice, the available evidence suggests that self-reported data correlates poorly with direct measurement (Garfield, Canavan et al. 2011). Questionnaires are limited by their inherent lack of objectivity and potential for recall bias. Objective assessment of physical activity requires the patient to wear an activity monitor for an extended period of time and then return for the device outputs to be measured, this may be expensive and poorly tolerated. Furthermore, whilst advanced accelerometers allow the detection of poor compliance, the risk of observational bias with a greater level of activity occurring during the assessment period is difficult to detect (Casaburi 2007).

Multimodal scoring indices

Composite scoring indices that recognise the importance of systemic disturbance have been developed to predict mortality in COPD (Celli, Cote et al. 2004). The BODE index comprises an assessment of Body Mass Index (BMI), airflow obstruction, dyspnoea and exercise capacity; it is likely to be loss of skeletal muscle mass that is represented as the BMI component conferring a poor prognosis (Marquis, Debigare et al. 2002; Celli, Cote et al. 2004). The BODE index provides better power than its individual components and predicts exacerbations in addition to mortality (Marin, Carrizo et al. 2009), although its predictive value in less severe disease is unclear. The relevance of age as a prognostic factor in COPD is recognised as one of three components predictive of mortality in the Age, Dyspnoea, Obstruction (ADO) index (Puhan, Garcia-Aymerich et al. 2009). The fact that the modified MRC (mMRC) dyspnoea score, which has the same identifiers as the MRC dyspnoea score but is scored from 0-4 to allow the BODE index to total 10, has utility in both of these prognostic scoring indices is of interest. A detailed inspection of the components of the mMRC indicates that it may be taken to be a patient's subjective assessment of their own performance level. Contextually, it is unsurprising that the latest iteration of the GOLD guidelines incorporates the addition of either the mMRC or CAT score to the spirometric evaluation of the COPD patient (see Figure 1.3). The fact that simple tools such as the mMRC and CAT have been included in the symptomatic evaluation of the disease at a clinical level demonstrates the attraction of simple, validated tools that are easily practicable. Whilst both the mMRC dyspnoea and CAT score relate to systemic features such as skeletal muscle strength, they also relate to lung function parameters; tools that do not relate to lung function impairment but detect systemic impairment may be of significant practical value.

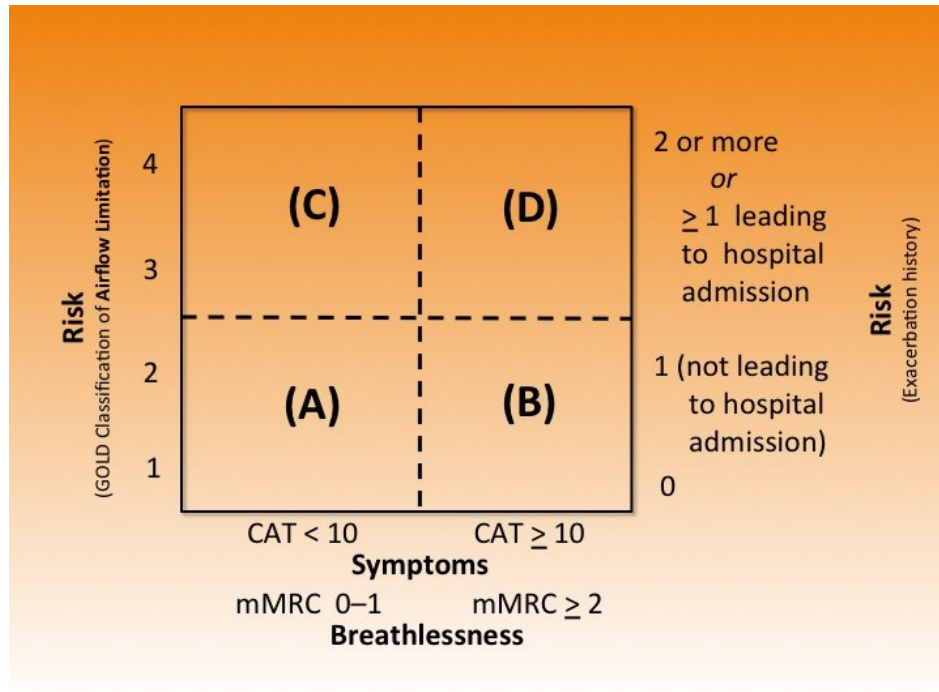


Figure 1.3: Assessment including dyspnoea, symptom scoring and FEV_1

From the Global Strategy for Diagnosis, Management and Prevention of COPD 2014, © Global Initiative for Chronic Obstructive Lung Disease (GOLD), all rights reserved. Available from <http://www.goldcopd.org>.

Skeletal muscle function

The relevance of skeletal muscle function in COPD has already been briefly discussed and is detailed further below, as are the validated assessment methods currently practiced (Section 1.1.8); however, the direct evaluation of skeletal muscle function in COPD is not commonly performed in clinical practice. Tests that may be used to evaluate relevant parameters such as strength and muscle bulk are only used in research practice due to prohibitive costs, time constraints and the need for expertise.

Tools that identify patients at risk of adverse events are particularly attractive, especially if the deficits detected may respond to intervention. In this context, since skeletal muscle defects are

treatable (Troosters, Probst et al. 2010), simple practical tools that detect muscle dysfunction may impact clinical practice and screening for interventional studies.

1.1.6 The relevance of skeletal muscle dysfunction in COPD

Skeletal muscle dysfunction in COPD predicts functional capacity (Saey, Debigare et al. 2003), quality of life (Montes de Oca, Torres et al. 2006), healthcare use (Decramer, Gosselink et al. 1997) and mortality (Swallow, Reyes et al. 2007), independent of lung function impairment, at least in patients seen in hospital practice. The quadriceps and the diaphragm are the most commonly studied skeletal muscles; however, regional differences in phenotype and predisposition to pathophysiological processes highlight the inherent complexity of the skeletal muscle dysfunction that occurs in COPD.

The importance of skeletal muscle dysfunction in COPD is highlighted by the fact that pulmonary rehabilitation is by far the most effective treatment in COPD, improving exercise capacity (Troosters, Gosselink et al. 2000), dyspnoea (Reardon, Awad et al. 1994), health status (Troosters, Gosselink et al. 2000; Bourbeau, Julien et al. 2003), health care utilisation (Bourbeau, Julien et al. 2003) and mortality indices (Cote and Celli 2005). The improvements observed after pulmonary rehabilitation are mediated largely through skeletal muscle adaptations to physical training (Bernard, Whittom et al. 1999; Troosters, Gosselink et al. 2000; Vogiatzis, Terzis et al. 2011); in fact classically successful pulmonary rehabilitation programs do not change FEV₁ (Griffiths, Burr et al. 2000). Thus, in contrast to the largely irreversible pulmonary adaptations, pharmacological management of skeletal muscle dysfunction represents a potential tool to improve function and quality of life in COPD.

1.1.7 The structure of skeletal muscle

The classification of skeletal muscle

Three types of muscle tissues exist, namely skeletal, smooth and cardiac muscle. These muscle tissues may be classified according to morphology or function. In the former they are differentiated into striated or non-striated muscle. Functionally, they are differentiated into voluntary or involuntary depending on whether conscious control of contraction occurs. Skeletal and cardiac muscle are forms of striated muscle on account of an appearance of alternating light and dark bands on light microscopy. However, cardiac muscle differs from skeletal muscle as activation of cardiac muscle is involuntary. The diaphragm and quadriceps are both striated skeletal muscle, which at least in health, have very similar properties.

Human skeletal muscle fibres

Muscle fibres are elongated and tubular structures that contain multiple superficial nuclei in addition to mitochondria and other organelles. Three skeletal muscle fibres with different phenotypic properties exist in humans, type I, type IIa and type IIx, each expressing a predominating myosin heavy chain (MHC) isoform. Type I fibres are slow-twitch, aerobic fibres that have oxidative properties with elevated oxidative enzyme content, mitochondrial numbers and capillarity. Type IIx fibres are fast-twitch anaerobic fibres with high glycolytic enzyme content. Type IIa fibres have mixed properties, they are fast-twitch fibres but have an oxidative profile similar to type I fibres.

The structural basis of muscle contraction

Skeletal muscles are able to perform voluntary contractions to allow movement under the control of the somatic nervous system. The human body contains several hundred skeletal muscles which function to generate force for breathing and locomotion, postural support and maintaining body temperature. Each skeletal muscle is comprised of bundles of muscle fibres (Figure 1.4), or fascicles, enclosed by the epimysium. The bundles are separated by the connective tissue fibres of the perimysium and within the bundles, muscle fibres are surrounded by the endomysium. Nerve fibres penetrate the epimysium, branch through the perimysium and enter the endomysium to innervate individual muscle fibres. The sarcolemma is the cell membrane of a muscle fibre and encloses the sarcoplasm or the cytoplasm of the muscle fibre. The sarcolemma is electrically polarised, it is the sudden change in transmembrane potential of a muscle fibre that initiates muscle contraction. Therefore the sheath provided by the endomysium plays an important role in electrically insulating individual muscle fibres from each other. Given that skeletal muscle fibres are very large and all regions of a fibre need to contract simultaneously, electrical action potential are rapidly transmitted to the interior of the cell by transverse tubules that extend from the sarcolemma to the sarcoplasm. Within individual muscle fibres, branches of transverse tubules surround myofibrils, which are essentially 'muscle threads' that are aligned in parallel across the length of the muscle. The myofibrils are composed of bundles of myofilaments, which are protein filaments consisting mostly of actin and myosin. It is the myofibrils that are able to shorten and are responsible for muscle contraction. These myofilaments are organized into a series of sarcomeres, which are the smallest functional units of contraction. In developed muscle, sarcomeres are able to respond to stimuli, subsequently allowing the functional adaptation of muscle.

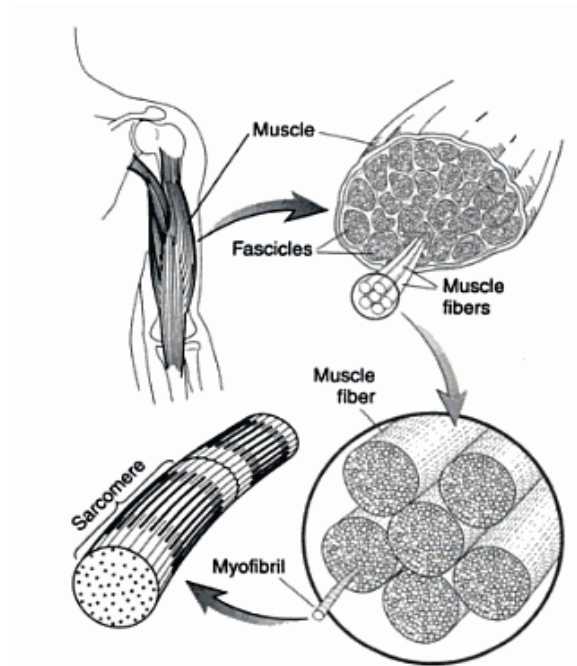


Figure 1.4: The structural hierarchy of skeletal muscle.
 Lieber (2002) *Skeletal Muscle Structure, Function, and Plasticity* (2nd ed).
 USA: Lippincott Williams & Wilkins

The functional units of muscle contraction

Sarcomeres are comprised of thick and thin filaments and it is the interaction between the two that enables muscle contraction to occur. Thick filaments are mostly composed of the protein myosin; whilst actin is the predominating protein in thin filaments, they also contain tropomyosin and troponin. There are characteristic dark bands (anisotropic, A bands) and light bands (isotropic, I bands) that appear when viewed under polarized light. The thick filaments are located in the centre of a sarcomere within the A band, which also includes portions of thin filaments, the M line, H zone and the zone of overlap. The central portion of each thick filament is connected to its neighbours by the dark-staining proteins of the M line which help stabilize the structure. In a resting sarcomere, the H zone is a lighter region on either side of the M line; it contains thick filaments but no thin filaments. In the zone of overlap, thin filaments are situated between the thick filaments. The I band contains thin filaments but not thick filaments and extends from the A band of one sarcomere to the

A band of the next (Figure 1.5). Adjacent sarcomeres are delineated by the Z disc which consists of proteins called connectins which interconnect thin filaments of adjacent sarcomeres. Thin filaments extend from the Z disc at either end of the sarcomere toward the M line and into the zone of overlap. Cytoskeletal crosslinking proteins such as titin contribute to the elasticity of the structure.

When a skeletal muscle fibre contracts, the H bands and I bands get smaller, the zones of overlap increase, the Z discs move closer together, and the width of the A bands remains constant (Figure 1.5). The contraction ends once the fibre has shortened by about a third, this coincides with the elimination of the I bands. These observations make sense only if the thin filaments are sliding toward the centre of the sarcomere, alongside the thick filaments. This explanation for the physical changes that occur during contraction is the 'sliding filament' theory.

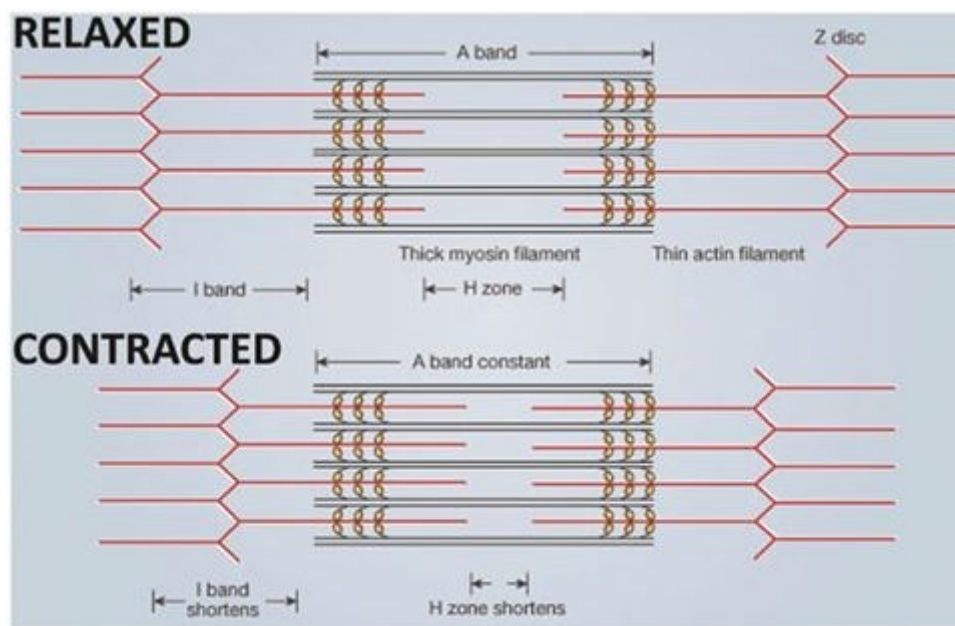


Figure 1.5: The structure and function of a sarcomere.
Adapted from (Krans 2010).

1.1.8 The assessment of skeletal muscle mass, structure and function

Muscle dysfunction is defined as loss of at least one of the two major physiological properties, strength and endurance. Strength is a term given for the capacity to develop a short maximal contraction; it is mostly determined by muscle mass, resting length, the number of motor units recruited and contractile velocity. Endurance is the capacity of muscle to maintain a submaximal force over time; it provides an integrated assessment of the oxygen delivery and utilisation of the muscle, including fibre proportion, capillarity, oxidative enzymes and mitochondrial function. Fatigue is another term used in the evaluation of muscle function, it describes the fall in strength that occurs over a set period of time.

The direct assessment of skeletal muscle function in COPD is currently confined to research facilities because of the need for specialist equipment, expertise and time. The assessment of muscle performance in the context of exercise capacity is more commonly undertaken, although once again outside of specialist centres this is not routinely performed. Furthermore, in COPD, pulmonary limitation and possible concomitant cardiovascular aspects of the disease may have significant impact on performance. Therefore, simple assessment tools that have been validated in other populations may not be applicable to patients with COPD. The methods used to assess skeletal muscle mass, structure and function are detailed here, as are their applicability to COPD populations.

Muscle mass

Various methods may be used to measure muscle mass, each has their own advantages, contextually there is no widely accepted gold standard. It is recognised that BMI is an independent prognostic

factor in COPD (Schols, Slangen et al. 1998), however, it does not include an assessment of body composition or account for regional (i.e. upper vs lower limb) differences.

Fat-free mass (FFM) evaluates functional muscle tissue in addition to other skeletal components, providing more detailed information than BMI. FFM may be measured for the whole body or regionally. In COPD, the relative ease and validity of measuring FFM extends its value in evaluating the response to pulmonary rehabilitation. Skinfold anthropometry estimates body composition by using callipers to determine the thickness of the skin and underlying fat at various body sites. An estimate of fat mass based on 4 calliper measurements allows FFM to be estimated from the subtraction of fat mass from the body mass estimate. Whilst cheap and relatively simple, this method does not allow discriminatory evaluation of different muscle groups and is limited by inaccuracies in the measurement of fat mass that may not be uniformly distributed. On account of the low cost, ease of use and portability, bioelectrical impedance (BIA) is the method usually used for assessing FFM in clinical practice. A differential current is passed through the body and FFM conducts electricity better than fat on account of having a high fluid electrolyte content. Whole body impedance is converted to a volume estimate and as with other populations, to improve accuracy, disease specific regression equations have been validated in COPD (Steiner, Barton et al. 2002).

Dual energy X-ray absorptiometry (DEXA) determines FFM accurately via the differential attenuation of two x-ray beams passing through the body, subsequently reconstructed by computer, to provide quantitative measures of fat mass, bone mass and bone-free lean mass. Although expensive and requiring specialist expertise, DEXA has been validated against deuterium dilution in COPD and can be performed quickly, with the additional ability to provide regional measurements and bone mass data (Engelen, Schols et al. 1998). All techniques that measure FFM depend upon the need for stable hydration. The different methods do not relate well, BIA more accurately reflecting DEXA measurements than skinfold anthropometry. Despite this, BIA is predisposed to inaccuracy in the

setting of fluid shift and may lack sensitivity to detect interventional change (Nelson, Fiatarone et al. 1996).

Computed tomography (CT) and magnetic resonance imaging (MRI) are well-established radiological techniques that may be used to determine regional muscle mass, their ability to isolate and measure the size and cross-sectional area of different muscles or groups makes them the most accurate representation of local muscle mass. Although CT uses ionising radiation in order to differentiate tissues according to the attenuation of x-rays, MRI does not use ionising radiation, but generates an image by use of a strong magnetic field; this method provides greater contrast between the soft tissues than produced by CT. Both CT and MRI have been used in the evaluation of COPD patients (Marquis, Debigare et al. 2002; Mathur, Takai et al. 2008); however, routine use of both is limited by time, expense, and the need for specialist equipment and technical expertise. Ultrasound provides images through the reflection of high-frequency sound waves from tissue interfaces. It allows the quick, portable and relatively cost-effective determination of the cross-sectional area and thickness of superficial muscles without requiring exposure to ionising radiation. Despite the potential for operator variance, our group has found it to be a reliable tool. We have published on the use of ultrasound in determining *rectus femoris* cross-sectional area in COPD patients. In this context, we have found it to be a valid tool with measurements relating well with other measures of muscle bulk and strength (Seymour, Ward et al. 2009; Shrikrishna, Patel et al. 2012).

Muscle structure and composition

Although radiological techniques allow the size of different muscle groups to be evaluated and even the evaluation of other relevant tissues including fat, they do not provide important data on the content of the muscle. Although a muscle biopsy will not directly provide functional details, it can provide information on the histological structure of the muscle including the fibre type content, the

cross-sectional area of the myofibres, the oxidative enzyme content, capillarity and mitochondrial content and function. Furthermore, it is possible to measure nucleic acid and protein content of relevant molecules or processes that may have a role in regulating muscle phenotype from the tissue obtained. Muscle biopsies may be obtained surgically, by using a specialist Bergstrom needle that mechanically slices tissue through an aperture when suction pressure is applied (Bergstrom 1962), or by a micro-biopsy needle technique that operates on the release of a trigger. Despite the valuable information provided from a muscle biopsy, it not only requires that the patient undergoes an invasive procedure, but also specialist expertise. Consistent with this, whilst the technique is now commonly used in the evaluation of skeletal muscle dysfunction in COPD, data is often limited to relatively small cohorts; given the heterogeneity of the disease this may result in contradictory findings.

Muscle function

Strength may be evaluated by volitional methods which require maximal effort or non-volitional methods which are independent of patient or operator motivation. Volitional methods may test static (i.e. isometric) or dynamic contraction; the former is simpler although dynamic contractions may more closely mirror the demands of daily life. Cable tensiometers and the maximum weight that can be lifted during a single weight-lifting exercise (1 repetition maximum, 1RM) are more simple approaches; however, the values obtained depend upon the equipment used. Furthermore, the latter test is not suited to frail individuals. Dynamometers evaluate the application of an external force against a spring or electrical force transducer, requiring the assessor to be able to generate a greater force than the muscle group being tested. All of these methods have been employed in the evaluation of COPD patients (Wilson, Rogers et al. 1986; Simpson, Killian et al. 1992). Isokinetic dynamometry allows the measurement of strength over a range of positions and velocities with the use of computerised equipment. Whilst providing detailed information, widespread use of isokinetic

dynamometry is limited by the expense, relative complexity of the technique and its poor relationship with functional ability. Non-volitional methods are more technically demanding and require the use of more expensive equipment, and remain limited to the domain of research. Supramaximal magnetic nerve stimulation works by the discharge of a magnetic field over a peripheral nerve resulting in a muscle action potential in the muscle it supplies, the resultant tension generated has a constant relationship with the maximal tetanic tension (Polkey, Kyroussis et al. 1996). The technique has been developed by our group and quadriceps strength has been shown to be lower in COPD patients than controls when using this technique (Man, Soliman et al. 2003).

Muscle fatigue may be evaluated by monitoring strength using volitional and non-volitional techniques after loaded exercise; the use of non-volitional techniques is particularly advantageous in this setting (Man, Soliman et al. 2003). Swallow *et al.* have reported on the use of a novel technique using repetitive electrical stimulation to evaluate muscle endurance in COPD patients (Swallow, Gosker et al. 2007). Whilst of potential value in the assessment of novel therapeutic approaches, the requirements of time, expensive equipment and expertise limit clinical applicability.

Physical performance

The role of exercise tests in the context of COPD have already been discussed; both laboratory and field based tests are well established in COPD. However, performance is often limited by the pulmonary or cardiovascular aspects of the disease, furthermore whether CPET performance on a bike or a treadmill relates to the daily life of the COPD patient is questionable. Field walking tests such as the six-minute walk test (6MWT) and the incremental shuttle walk (ISW) are more widely practiced as they do not require the use of expensive laboratory equipment. They are predictive of important adverse outcomes and relate to quadriceps strength, however, they do still require the

use of equipment or considerable space. Furthermore, in the case of the 6MWT, a practise walk is advised with a 1 hour wait between repeat testing (American Thoracic Society 2002).

Simple functional assessment tools, have been validated in general geriatric populations, however, the applicability of these tools to COPD populations has not been evaluated until recently. If validated, these tests may be of particular value in the efficient screening of patients in routine clinical practice or in stratifying patients prior to entering research studies.

The Short Physical Performance Battery

The Short Physical Performance Battery (SPPB) has been recommended by consensus working groups as a primary functional outcome measure in frail older persons (Bhasin, Cress et al. 2008) and also as a screening tool for sarcopenia (Cruz-Jentoft, Baeyens et al. 2010). As the SPPB was initially designed to evaluate community populations, it does not require specialist equipment or personnel and may be tested in a short period of time. It summates the ability to perform three functionally relevant tasks, assessing standing balance, habitual gait speed and standing ability. It has been validated in community-dwelling older populations where it detects those at risk of mortality, nursing home admission, hospitalization and disability (Guralnik, Simonsick et al. 1994; Guralnik, Ferrucci et al. 1995). Furthermore, in general populations it relates to both functional exercise capacity (Latham, Mehta et al. 2008) and skeletal muscle function (Puthoff and Nielsen 2007).

Thus far, most studies evaluating the SPPB have been in community-dwelling older adults, the majority of whom do not have respiratory disease. Our group has recently validated two components of the SPPB, namely habitual gait speed and the sit-to-stand in COPD (Kon, Patel et al. 2012; Jones, Kon et al. 2013), however, the SPPB has not been specifically validated in COPD. The respiratory and systemic manifestations of the disease may impact the validity of the SPPB in this

population. Available data in COPD populations is from community dwelling individuals with milder disease (Eisner, Blanc et al. 2007); the applicability and relevance to those with more severe impairment is unknown. Furthermore, the relationship between SPPB score and the structural constitution of the lower limb muscles has not been established. This is particularly important as opposing patterns are observed in COPD patients and the healthy elderly (Natanek, Gosker et al. 2013) (detailed in 1.1.9), in this context, the rapid assessment of several relevant functions as provided by the SPPB may be advantageous in the routine screening of COPD patients.

1.1.9 Skeletal muscle adaptations in COPD

Functional adaptations

Skeletal muscle dysfunction in COPD manifests clinically as reduced strength and endurance. Quadriceps strength as assessed by volitional and non-volitional techniques is reduced in patients with COPD as compared to age-matched controls (Bernard, LeBlanc et al. 1998; Man, Soliman et al. 2003). Seymour *et al.* have shown that quadriceps weakness is present in all stages of COPD, with a prevalence of 31% in GOLD stage I/II and 38% in GOLD stage IV disease (Seymour, Spruit et al. 2010), the difference between severe and mild disease not being statistically different. Furthermore, patients with COPD are susceptible to fatigue as manifest by a rapid decline in performance during either repeated exercise (Mador, Deniz et al. 2003) or during continuous exercise (Man, Soliman et al. 2003). It has been demonstrated that the quadriceps exhibit reduced endurance and increased fatigability in COPD (Mador, Deniz et al. 2003; Allaire, Maltais et al. 2004; Swallow, Gosker et al. 2007). Significant reductions in the cross-sectional area of the mid-thigh and *rectus femoris* demonstrate loss of muscle bulk in the legs of COPD patients (Bernard, LeBlanc et al. 1998; Seymour, Ward et al. 2009). Muscle atrophy in the setting of a normal BMI is more prevalent than muscle

atrophy occurring in nutritionally depleted individuals (Vermeeren, Creutzberg et al. 2006). Additionally, quadriceps weakness is twice as prevalent as fat-free mass depletion, indicating that it precedes cachexia (Franssen, Broekhuizen et al. 2005). These factors indicate that quadriceps weakness is not merely an epiphenomenon. In COPD patients, quadriceps strength normalises when mid-thigh cross sectional area is accounted for, suggesting that the restoration of muscle mass may normalize strength (Bernard, LeBlanc et al. 1998).

The functional changes and loss of mass that are demonstrated in the quadriceps are not universally seen in other muscle groups. The *abductor pollicis*, *rectus abdominii*, *latissimus dorsi*, *pectoralis major* and diaphragm muscles have been reported to demonstrate preserved strength (Polkey, Kyroussis et al. 1996; Bernard, LeBlanc et al. 1998; Man, Soliman et al. 2003; Man, Hopkinson et al. 2005). While studies of the lower limbs have tended to focus on the quadriceps, which are well characterised, data regarding the upper limbs is conflicting. The interpretation of data reporting both preserved upper limb function (Bernard, LeBlanc et al. 1998; Gosselink, Troosters et al. 2000; Man, Soliman et al. 2003) and reduced function (Gosselink, Troosters et al. 2000; Franssen, Broekhuizen et al. 2005) is difficult. Methodological differences including the muscle groups isolated, measurement techniques and patient demographics probably explain these diverse observations. Importantly, the upper limbs may not be subjected to the same level of disuse as the lower limbs in COPD, furthermore, proximal upper limb muscles are recruited as accessory muscles when there is increased ventilatory demand. Given histological and metabolic differences (as discussed below), it is conceivable that the upper limbs and lower limbs may not be subjected to the same pathological consequences.

The diaphragm, is not only relatively resistant to fatigue (Polkey, Kyroussis et al. 1995; Polkey, Kyroussis et al. 1997), but also exhibits improved metabolic efficiency in COPD (Stubbings, Moore et al. 2008). The different clinical phenotype expressed by the diaphragm and the quadriceps indicates

that local factors play an important role in skeletal muscle dysfunction. The diaphragm is the main inspiratory muscle; in COPD it faces an increased burden due to airflow obstruction and hyperinflation. The quadriceps, however, as the main locomotor muscles, tend to be predisposed to disuse. Despite different levels of activation, both are exposed to systemic disturbance in COPD, such as that provided by oxidative stress. Comparative studies of diaphragm and quadriceps muscle samples obtained from the same individuals may delineate the role of systemic and local factors in the skeletal muscle dysfunction observed in COPD.

Structural adaptations

The functional changes seen in COPD are accompanied by changes at a cellular level involving fibre atrophy, fibre type shift, oxidative capacity, oxidative stress and capillarisation. As a consequence of exhibiting the most relevant clinical changes and aided by the relative ease of *vastus lateralis* biopsy, the quadriceps are the most structurally characterised skeletal muscles. The regional muscle mass observed in COPD patients is further explained by the evaluation of single muscle fibre cross-sectional areas. In the quadriceps, atrophy of type IIx fibres is most prominent. (Gosker, Engelen et al. 2002).

The disparity in clinical phenotype that occurs in skeletal muscle groups in COPD is mirrored at a cellular level. Although healthy ageing is characterised by an increase in type I (slow-twitch, oxidative) fibres (Larsson 1983), in COPD the quadriceps exhibit a decreased proportion of type I fibres and an increased proportion of type IIx (fast-twitch, glycolytic) fibres (see Figure 1.6) (Whittom, Jobin et al. 1998; Gosker, Zeegers et al. 2007). However, studies have tended to focus on patients with spirometrically advanced disease. Increased fatigability and reduced exercise capacity in COPD is likely to be related to the reduced proportion of oxidative type I fibres (Natanek, Gosker et al. 2013). Consistent with this fibre type shift, the *vastus lateralis* exhibits reduced oxidative

capacity (Jakobsson, Jorfeldt et al. 1995; Maltais, Simard et al. 1996; Gosker, Engelen et al. 2002), mitochondrial density (Gosker, Hesselink et al. 2007) and in cachectic patients, mitochondrial function (Rabinovich, Bastos et al. 2007). Whether *vastus lateralis* glycolytic activity is increased remains debated (Jakobsson, Jorfeldt et al. 1995; Maltais, Simard et al. 1996). Furthermore, our group recently published data from a large cohort demonstrating that quadriceps morphology is heterogeneous in COPD, with isolated fibre shift occurring in 31%, isolated fibre atrophy in 20% and a combination of the two in 25% (Natanek, Gosker et al. 2013). Fibre type shift is not observed in the upper limbs and oxidative enzyme capacity of the deltoids remains preserved (Gea, Pasto et al. 2001).

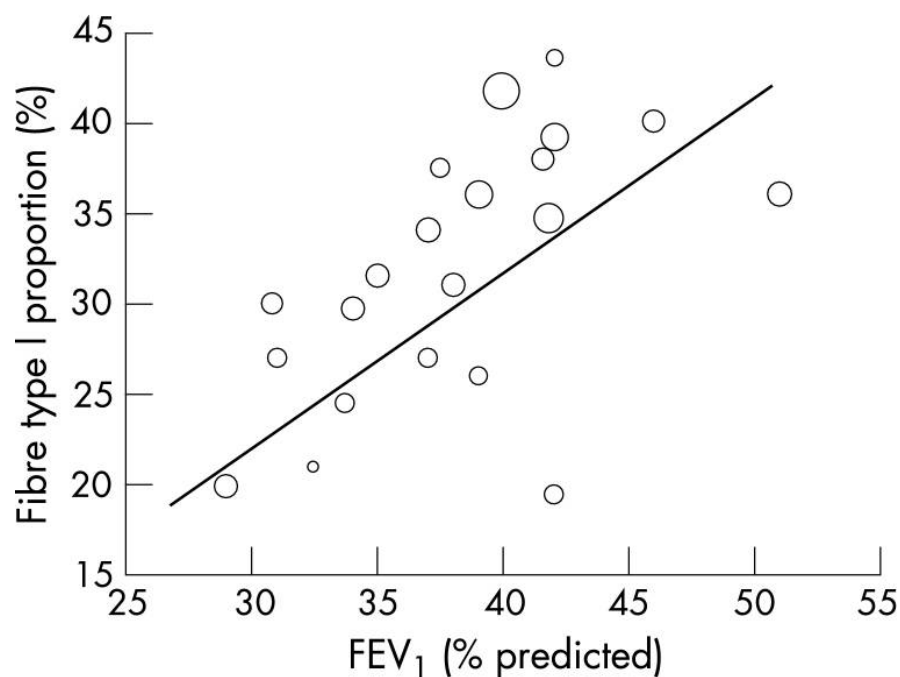


Figure 1.6: The relationship between *vastus lateralis* type I fibre proportion and FEV₁ (Gosker, Zeegers et al. 2007). Circle size represents cohort size in this meta-analysis.

As seen in long-term endurance training, the diaphragm in COPD exhibits an increased type I fibre proportion (Howald, Hoppeler et al. 1985). Additionally, mitochondrial function (Wijnhoven, Janssen et al. 2006), oxidative capacity (Levine, Kaiser et al. 1997; Levine, Gregory et al. 2002; Wijnhoven,

Janssen et al. 2006) and capillarisation (Doucet, Debigare et al. 2004) are improved in the diaphragm. The parasternal intercostal and external intercostal muscles also display features of adaptation to the chronically elevated mechanical load (Ribera, N'Guessan et al. 2003; Levine, Nguyen et al. 2006). However, concurrent electron transport chain blockade and excessive production of reactive oxygen species in both the *vastus lateralis* and external intercostals in mild-moderate COPD may allude to systemic effects also mediating pathophysiological adaptations (Puente-Maestu, Perez-Parra et al. 2009).

Molecular adaptations

Skeletal muscle mass is determined by the balance between anabolic and catabolic pathways (see Figure 1.7) and is additionally influenced by the homeostatic balance provided by apoptosis of myofibre nuclei and the recruitment of myonuclei from muscle progenitor cells, such as satellite cells. Current knowledge of the molecular biology and the factors which influence skeletal muscle is mostly based on animal studies and other disease models such as diabetes mellitus; the pathways that determine hypertrophy or atrophy in COPD patients are yet to be fully defined, although evidence based on muscle biopsies obtained from human COPD populations is growing. It remains unclear whether reduced protein synthesis or increased degradation is the major cause of muscle loss in COPD, as is whether molecular pathways are disease specific.

Akt has a central role in protein synthesis by not only stimulating the hypertrophy pathways, but also through the inhibition of atrophy pathways (Glass 2005); it is stimulated by insulin-like growth factor-1 (IGF-1), exercise and insulin and acts on downstream targets (Rommel, Bodine et al. 2001). Although circulating IGF-1 levels are not different in COPD patients stratified according to lung function impairment (Piehl-Aulin, Jones et al. 2009) or quadriceps bulk (Debigare, Marquis et al. 2003), locally produced IGF-1 is likely to be more relevant in activating Akt signalling in locomotor

muscle (Crul, Spruit et al. 2007; Vogiatzis, Simoes et al. 2010). On phosphorylation, Akt activates the mammalian target of rapamycin (mTOR) which upregulates 70-kD ribosomal S6 protein kinase (p70S6K) (Rommel, Bodine et al. 2001) and inhibits eukaryotic initiation factor 4E binding protein-1 (4E-BP1) (Hara, Yonezawa et al. 1997). These actions promote protein synthesis. Consistent with this, IGF-1 mRNA levels have been demonstrated to be reduced in the quadriceps of hospitalised and stable COPD patients as compared to healthy controls (Crul, Spruit et al. 2007), additionally, increased exercise capacity and fibre size following pulmonary rehabilitation relate to the upregulation of IGF-1 (Vogiatzis, Stratakos et al. 2007). However, contrary to this, our group has found IGF-1 to be elevated in a cohort of stable patients with more severe lung function impairment (Lewis, Riddoch-Contreras et al. 2012), which considered in isolation may be consistent with an attempt at regeneration failing in the absence of other necessary stimuli. These inconsistent findings may indicate the relevance of subtle differences in cohorts and the complexity of the molecular mechanisms underlying muscle adaptations in COPD. These considerations may also explain unexpected findings observed in other cohorts.

Several proteolytic pathways seem to regulate skeletal muscle degradation; the ubiquitin-proteasome pathway is thought to be the rate-limiting system involved in this process. Muscle inactivity, glucocorticoids and pro-inflammatory cytokines (TNF- α , IL-1 and IL-6) have been shown to induce the ubiquitin-proteasome pathway via the ubiquitin-ligases atrogin-1 and Muscle ring finger-1 (MuRF-1) (Jagoe, Lecker et al. 2002). Molecules of ubiquitin attach to proteins that are marked to be degraded by the ubiquitin-ligases. Proteasomes are large multiunit complexes that selectively degrade intracellular proteins via enzymatic action. In addition to stimulating the hypertrophy pathways, Akt inhibits muscle atrophy by inhibiting forkhead box O (FoxO) mediated atrogin-1 and MuRF-1 expression. MuRF-1 and atrogin-1 are expressed at significantly greater levels in animal models of skeletal muscle atrophy (Bodine, Latres et al. 2001; Gomes, Lecker et al. 2001). Despite confirmation of elevated atrogin-1 and MuRF-1 mRNA and elevated FoxO-1 protein content in a

small cohort of COPD patients, there was no difference in expression between those with clinical atrophy and those without (Doucet, Russell et al. 2007). Furthermore, our group did not find elevated MuRF-1 or atrogen expression in the quadriceps of COPD patients (Natanek, Riddoch-Contreras et al. 2013). Additionally, Doucet *et al.* surprisingly found raised levels of Akt. Elevated Akt in this setting may once again be another indication of an attempted corrective mechanism aimed at restoring muscle mass. This observation may alternatively be considered to provide evidence that mechanisms independent of Akt regulate the transcription of atrogen-1 and MuRF-1 in the context of COPD associated cachexia.

In a study comparing 12 COPD patients with 7 controls, mRNA expression of atrogen-1, MuRF-1 and FoxO-1 was elevated in the quadriceps as compared to the diaphragm in COPD patients (Doucet, Dube et al. 2010). While p70S6K was down-regulated in the quadriceps, there was no difference in Akt itself or other downstream targets. There was no difference in the expression of either the hypertrophy pathway or ubiquitin-proteasome pathway constituents in controls. This suggests that the changes observed are part of the adaptations seen in COPD and that the ubiquitin-proteasome is fundamental to this process. An important confounding factor in this study was that over half of the patients had active malignancy, raising the possibility of cancer induced cachexia and other influences on molecular adaptations observed. Nonetheless, the evaluation of novel biological markers in the respiratory and locomotor muscles of COPD patients in the absence of other systemic diseases is of interest.

One mechanism that atrogen-1 and MuRF-1 may be regulated independent to Akt may be via peroxisome proliferator activated receptor (PPAR) expression (see Figure 1.7). PPARs are a nuclear receptor family of transcription factors, 3 isoforms exist, namely α , β/δ and γ . The PPAR- δ and PPAR- α isoforms are highly expressed in skeletal muscle. PPARs and PPAR- γ co-activator 1 α (PGC-1 α) regulate mitochondrial biogenesis, oxidative capacity and fibre type shift towards oxidative type 1

fibres (Luquet, Lopez-Soriano et al. 2003; Koves, Li et al. 2005); PGC-1 α also seems to play an important role in muscle adaptations to exercise training. Remels *et al* demonstrated PPAR- δ protein levels and PGC 1- α to be reduced in patients with COPD, and that cachectic patients have reduced PPAR- α expression (Remels, Schrauwen et al. 2007). However, the study by Remels *et al* was performed in a small cohort of patients and was not correlated to measures of muscle function. Nonetheless, given that at least in cell models, PGC-1 α influences a fibre shift towards type I fibres (Lin, Wu et al. 2002), this pathway remains an area of interest, although the relevance of fibre shift in the quadriceps of patients with COPD is yet to be established.

Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF- κ B) has also been reported to mediate muscle atrophy via actions on MuRF-1 (Cai, Frantz et al. 2004) and there is evidence to indicate that NF- κ B is activated in COPD patients with cachexia (Vogiatzis, Simoes et al. 2010). The role of mitogen activated protein kinases (MAPK) in the skeletal muscle of patients with COPD remains unclear. In COPD patients with muscle wasting, atrogin-1 protein expression was elevated and related to the activity and expression of the MAPKs p38, JNK and ERK 1/2, which inversely related to quadriceps bulk (Lemire, Debigare et al. 2013). However, Riddoch-Contreras *et al* did not observe differences in either MAPK activation or expression in a much larger cohort of COPD patients, nor did they demonstrate any relationship with either muscle mass or function (Riddoch-Contreras, George et al. 2013).

Growth differentiation factor-8 (GDF-8), also known as myostatin, is a member of the transforming growth factor- β family (TGF- β) super-family and is recognised to have potent inhibitory effects on muscle mass (McPherron, Lawler et al. 1997). GDF-8 has been demonstrated to be elevated at a transcript level in weak COPD patients (Plant, Brooks et al. 2010), furthermore, it has been shown that quadriceps levels inversely relate to strength and functional exercise capacity (Man, Natanek et al. 2010). GDF-8 inhibits the differentiation of myoblasts through the downregulation of myogenic

differentiation factors including MyoD and myogenin (Thomas, Langley et al. 2000) and satellite cell proliferation by the upregulation of the cyclin-dependent kinase inhibitor (p21) (Langley, Thomas et al. 2002). Additionally, GDF-8 may influence muscle breakdown via effects on the ubiquitin-proteasome pathway (McFarlane, Plummer et al. 2006).

Further work is necessary to delineate the molecular adaptations that occur in the skeletal muscle of COPD patients. Sample size, patient demographics and clinical phenotype are likely to be relevant as are potential confounding factors that may be involved in the aetiology of these adaptations such as smoking and physical activity. The evaluation of muscle biopsies obtained from large cohorts of well-characterised patients, interventional studies and different muscle groups within the same patients may contribute significantly to the understanding of the mechanisms underlying skeletal muscle dysfunction and guide the development of therapeutic targets.

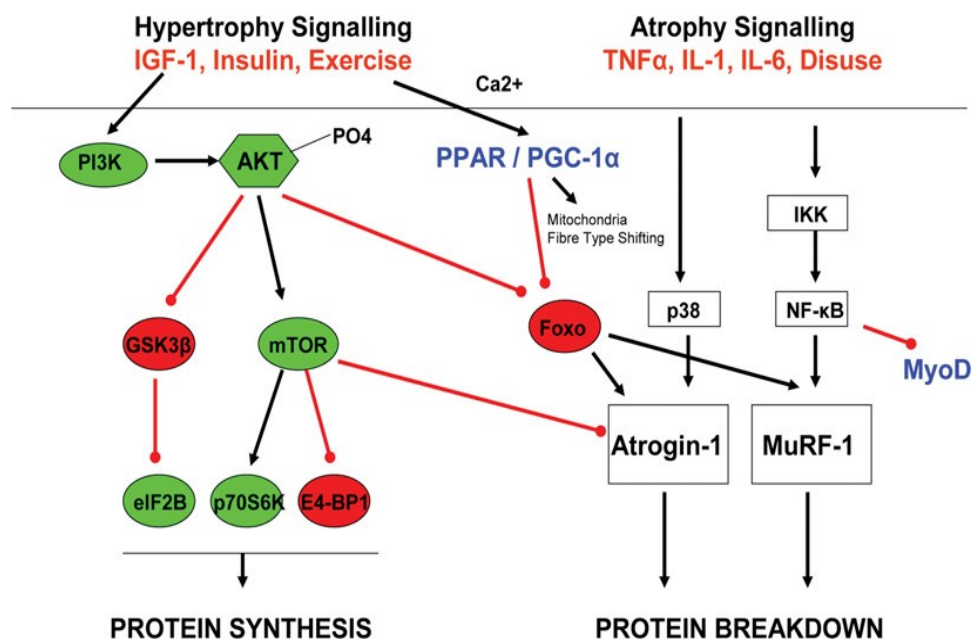


Figure 1.7: Interacting catabolic and anabolic pathways in skeletal muscle. Reproduced with permission, from Man WD, Kemp P, Moxham J, Polkey MI (2009). Skeletal muscle dysfunction in COPD: clinical and laboratory observations. *Clin. Sci.* 117(7):251-64. © the Biochemical Society.

1.1.10 The aetiology of skeletal muscle dysfunction in COPD

A combination of interplaying aetiological factors may underlie skeletal muscle dysfunction in COPD including smoking, disuse, systemic inflammation, ageing, oxidative stress, nitrosative stress, inadequate regeneration and malnutrition.

Cigarette Smoking

Tobacco smoking is the most important risk factor for COPD, with up to 50% of smokers developing the disease (Chapman, Mannino et al. 2006). The deleterious effects of cigarette smoking and the general health benefits to be achieved from smoking cessation are well established (Levy 1981; Peto, Darby et al. 2000; Taylor, Hasselblad et al. 2002). With regards to lung function, susceptible smokers who stop smoking will not revert to having a normal FEV₁, but the average loss of FEV₁ will revert back to that seen in non-smokers (Fletcher and Peto 1977).

Several studies have confirmed the presence of structural adaptations to skeletal muscle following smoke exposure. Rats exposed to smoke have reduced muscle fibre cross-sectional area and changes in muscle fibre type proportions (Nakatani 2002; Nakatani, Nakashima et al. 2003). In smokers, Orlander *et al* found fibre type changes and reduced muscle enzyme activity, similar characteristics to that seen in COPD (Orlander, Kiessling et al. 1979). However, without pulmonary function testing, discrimination between a direct smoking effect and COPD is difficult. Further clarification has been provided by Montes de Oca *et al* who showed that the quadriceps of non-COPD smokers have type 1 fibre atrophy, a higher proportion of fibres with a lower oxidative capacity and higher glycolytic capacity as compared to healthy subjects (Montes de Oca, Loeb et al. 2008). In this study, there was no evidence of local inflammation and markers of oxidative and nitrosative stress were normal. The absence of local inflammation may be a consequence of this not being pertinent to smoking and

skeletal muscle dysfunction, or alternatively, that inflammation had occurred prior to muscle sampling. Consistent with these findings, smokers have been shown to have greater muscle fatigability in comparison to non-smokers (Wust, Morse et al. 2008). These findings are pertinent as unlike the damage conferred by smoking to the lungs, deleterious skeletal muscle adaptations may respond to intervention (Whittom, Jobin et al. 1998; Bernard, Whittom et al. 1999; Lacasse, Goldstein et al. 2006).

Following on from the work of Montes de Oca *et al.*, the mechanism through which skeletal muscle is affected whilst the lungs remain preserved is of interest; furthermore, it remains to be determined whether skeletal muscle adaptations respond to smoking cessation. Increased fatigability may be a consequence of fibre type shift towards more fatigable fibres, oxidative stress, inflammation or reduced oxidative capacity of the muscle.

Alterations in either the IGF-1/Akt hypertrophy or the ubiquitin-proteasome pathways may mediate skeletal muscle atrophy in smokers (Schiaffino and Mammucari 2011). Contrary to the work of Montes de Oca *et al* in muscle, others have demonstrated a heightened systemic inflammatory response in smokers (Bermudez, Rifai et al. 2002). Additionally, in smoke exposed mice, downregulation of PGC-1 α is seen in response to raised levels of the inflammatory cytokine TNF- α and this is associated with increased MuRF-1 and atrogin-1 expression, a reduced oxidative phenotype and muscle atrophy (Tang, Wagner et al. 2010). Given that PGC-1 α expression also induces type 1 fibre shift, changes in PGC-1 α expression may mediate smoking related skeletal muscle changes (Lin, Wu et al. 2002).

Our group has previously demonstrated that in a COPD cohort, in comparison to the other patients, those who stopped smoking had a significant increase in fat free mass (Hopkinson, Tennant et al. 2007). Furthermore, smoking cessation in postmenopausal women results in increased muscle mass,

without significant changes in physical activity or calorific intake (Kleppinger, Litt et al. 2010). The relationship of smoking cessation to clinical phenotype and relevant molecular pathways are yet to be established. Of particular importance, is the relationship of smoking to physical activity and exercise capacity. Evidence of molecular adaptations occurring in 'healthy smokers' raises the possibility of the identification of specific targets that have an aetiological role in the pathogenesis of skeletal muscle dysfunction in COPD. Furthermore, reversal of skeletal muscle adaptations, possibly even in those with COPD, who are successful in quitting may suggest that manipulation of these molecules may improve skeletal muscle phenotype (Montes de Oca, Loeb et al. 2008). A detailed understanding of the mechanisms involved could lead to the development of targeted therapeutic interventions.

Physical inactivity

Exertional dyspnoea is a common complaint amongst COPD patients and translates to a reduction in physical activity (Pitta, Troosters et al. 2005). Physical activity is particularly relevant as many of the changes seen in smoking related skeletal muscle dysfunction are seen in prolonged physical inactivity such as the fibre type shift toward type II fibres, reduced oxidative capacity and greater oxidative stress (Jakobsson, Jorfeldt et al. 1995; Maltais, Simard et al. 1996; Whittom, Jobin et al. 1998; Gosker, Zeegers et al. 2007). Following acute exacerbations, COPD patients may have reduced physical activity for several weeks (Pitta, Troosters et al. 2006), an important consideration given that quadriceps weakness occurs within a week of hospital admission (Spruit, Gosselink et al. 2003). Despite this, functional capacity 3 months after an admission for an acute exacerbation may be significantly improved with the administration of early pulmonary rehabilitation (Man, Polkey et al. 2004). Furthermore, as higher levels of physical activity moderate lung function decline and COPD risk in smokers (Garcia-Aymerich, Lange et al. 2007), physical activity may also be pertinent to the development of skeletal muscle adaptations in smokers. In COPD, the diaphragm is trained in the

setting of the burdens of hyperinflation and airflow obstruction (Levine, Kaiser et al. 1997), whereas the locomotor muscles are relatively underactive in the setting of symptomatic dyspnoea. Therefore, it seems likely that the compartmental disparity in the phenotype observed between the respiratory and locomotor muscles is due to different levels of physical activation.

Systemic inflammation

It is well recognised that patients with COPD not only have an inflammatory response in their lungs, but have systemic inflammation, especially when the disease is more severe and during exacerbations (Hurst, Donaldson et al. 2006). This may be detected in the form of circulating cytokines such as TNF- α , acute phase reactant proteins including CRP, or inflammatory cells (Gan, Man et al. 2004). Whilst smoking is recognised to cause systemic inflammation, inflammation persists even after smoking cessation (Willemse, ten Hacken et al. 2005; Garcia-Rio, Miravittles et al. 2010; Simpson, McDonald et al. 2013). Regular physical activity suppresses systemic inflammatory markers including the inflammatory cytokine tumour necrosis factor- α (TNF- α) (Das 2004) (Clarkson and Thompson 2000). Systemic inflammation is associated with an accelerated decline in lung function (Donaldson, Seemungal et al. 2005). Consistent with this, it is probably through the modulation of systemic inflammation that higher levels of physical activity moderate lung function decline and COPD risk in smokers (Garcia-Aymerich, Lange et al. 2007) and as already discussed, physical activity may be pertinent to skeletal muscle changes in smokers.

Peripheral muscle wasting and reduced strength have been shown to be associated with circulating acute phase reactants and cytokines (Schols, Buurman et al. 1996; Eid, Ionescu et al. 2001). However, the role of specific inflammatory markers within muscle remains debated. For example, initial data suggested that systemic cytokines such as TNF- α are increased in COPD patients with muscle wasting (Di Francia, Barbier et al. 1994); this was supported by patients with high muscle

TNF- α transcript levels having a lower BMI and a tendency toward a lower FFMI compared to control subjects (Remels, Gosker et al. 2010). The latter study extended these findings to demonstrate that in cultured muscle cells TNF- α stimulation impaired markers relevant to an oxidative phenotype in an NF- κ B dependent manner. Supporting a role for NF- κ B in the skeletal muscle dysfunction of COPD, NF- κ B activation is more prevalent in patients with a low BMI (Agusti, Morla et al. 2004). Despite these findings, in another study, Barreiro and colleagues found reduced muscle TNF- α protein levels in the quadriceps of patients with severe COPD as compared to controls, they also reported TNF- α levels to be positively correlated with muscle strength (Barreiro, Schols et al. 2008). Barreiro *et al.* observed no difference in IL-6, interferon- γ or TGF- β protein levels within the muscle of severe COPD patients as compared to controls, refuting inflammation within the muscle having a significant role in the pathogenesis of skeletal muscle dysfunction in COPD (Barreiro, Schols et al. 2008).

Ageing

Despite the fact that the quadriceps of COPD patients exhibit the opposite fibre shift pattern to that observed in normal healthy ageing, there are similarities between healthy ageing muscle and the skeletal muscle of COPD. The limb muscles of older individuals are reduced in size and have more fat and connective tissue than younger individuals (Lexell 1995). Furthermore, muscle strength is markedly reduced with age; quadriceps strength is 39% lower in the eighth decade as compared to the third decade of life (Young, Stokes et al. 1985). The onset of physiological decline in muscle function observed in healthy populations varies at an individual level on account of variance in genetic factors, baseline status, physical activity, nutrition and stressors. Decline in strength relates to the degree of atrophy of type II fibres, a process that is also observed in COPD. Furthermore, normal ageing predisposes to other factors thought to be relevant to muscle dysfunction in COPD, including systemic inflammation and physical inactivity. In fact in trained individuals the effects of

ageing on muscle are mitigated (Klitgaard, Mantoni et al. 1990). It is plausible that the molecular adaptations observed in the muscle of COPD and older healthy populations may be similar.

Satellite cells are the major source of myogenic precursor cells that contribute to the maintenance of muscle mass, hypertrophy and repair. Therefore, satellite cell senescence may be important in age-related muscle atrophy; progressive shortening of satellite cell telomeres plays a significant role in their senescence. In the *tibialis anterior* of healthy older aged individuals, satellite cells are reduced in number (Kadi, Charifi et al. 2004) as compared to younger individuals with a similar physical activity pattern, however, telomere length is not reduced (Elodie, Jan et al. 2008). Therefore in the setting of maintained physical activity, despite reduced numbers, the replicative function of the remaining satellite cells may not be impaired by age. Mechanisms that maintain telomere length may be upregulated in this population. Recently formed and regenerating myofibres display a central nucleus, myogenic regulatory factors subsequently aid the migration of the nucleus toward the periphery to form a mature fibre; centralised nuclei may be considered markers of muscle regeneration. In COPD, GOLD stage III and IV patients with preserved quadriceps muscle mass have increased numbers of centralised nuclei as compared to controls and patients with atrophy (Theriault, Pare et al. 2012). Furthermore, telomere length was reduced in the COPD patients, particularly those with muscle atrophy. Telomere shortening in this context may indicate an increased number of senescent satellite cells in those with muscle atrophy and an impaired ability to regenerate. More recently, the same group have extended their findings to demonstrate that myogenesis signalling was also altered in patients with COPD. In cultured satellite cells, there was a reduced capacity for differentiation, consistent with impaired skeletal muscle regeneration in COPD (Theriault, Pare et al. 2014).

Oxidative and nitrosative stress

Increased oxidative or nitrosative stress may result in cellular damage and have functional consequences. Pertinently, hypoxia and inflammation may initiate oxidative stress (Koechlin, Couillard et al. 2004). In COPD, peroxidation products have been shown to be elevated in the systemic circulation at rest, following submaximal exercise and during exacerbation (Supinski and Callahan 2007). There is data that indicates the presence of increased antioxidants in muscle; likely in response to the increased oxidative burden (Gosker, Bast et al. 2005; Barreiro, Rabinovich et al. 2009). Consistent with this scenario, Barreiro and colleagues demonstrated increased muscle oxidation, manifest as protein carbonylation, in the quadriceps of patients with COPD and that levels inversely related to quadriceps strength (Barreiro, Schols et al. 2008). Barreiro and colleagues extended these findings to also demonstrate increased protein oxidation in the quadriceps of smokers and in the respiratory and limb muscles of guinea pigs chronically exposed to cigarette smoke in the absence of increased local inflammation, implicating a direct role for cigarette smoke inducing muscle protein oxidative damage (Barreiro, Peinado et al. 2010).

However, in another cohort of severe COPD patients, although muscle oxidative stress was also found to be increased, irrespective of body composition, protein ubiquitination was only increased in patients exhibiting muscle atrophy. The inference, at least from this latter cohort of 29 COPD patients, is that oxidative stress may not directly modulate muscle protein loss (Fermoselle, Rabinovich et al. 2012). The conflicting role for oxidative stress within the skeletal muscle of COPD patients is further confused by studies that show that unlike in controls, quadriceps antioxidant enzymes do not rise after acute exercise in patients (Couillard, Maltais et al. 2003). Given that oxidative stress is increased by exercise in COPD patients (Couillard, Maltais et al. 2003) and the widely accepted benefits that may be achieved from exercise, the role for oxidative stress in the skeletal of COPD patients is clearly complex.

In addition to evidence of oxidative stress, there is evidence of up-regulation of inducible nitric oxide synthetase and nitrotyrosine formation in the skeletal muscle of COPD patients (Agusti, Morla et al. 2004). This is supported by the work of Barreiro *et al* who also demonstrated a significant rise in protein nitration in the respiratory and limb muscles of cigarette smoke exposed rodents, although they did not replicate these findings in human smokers (Barreiro, Peinado et al. 2010). However, the finding of reduced expression of the constitutive nitric oxide synthases in smokers does indicate that smoking impairs the normal process of nitric oxide generation in humans.

Further work is necessary to find agents that ameliorate oxidative and nitrosative burdens, at a local or a systemic level, and to establish whether they are of benefit in COPD.

1.1.11 Age related biomarkers of potential pathophysiological relevance in COPD

Accelerated biological ageing may be important in the pathophysiology of COPD and pertinent to the pathogenesis of the skeletal muscle adaptations that are observed, however, further work is required to corroborate or refute this. Studies evaluating skeletal muscle dysfunction in COPD have thus far mostly focused on moderate and severe disease, rather than milder disease. Characterisation of patients with milder disease is important as it allows assessment before significant ventilatory limitation. Given that smoking may have a direct influence on the expression of systemic agents, levels of relevant circulating factors in smokers without COPD may provide useful information, where relevant the response to smoking cessation may detail whether the influence of smoking is reversible. The influence of physical activity is an important consideration given that in COPD, pathophysiological adaptations mostly occur in the locomotor muscles and that physical activity can mitigate the effects of ageing on skeletal muscle. Concurrent determination of molecular activity in the respiratory muscles and quadriceps of COPD patients may provide further evidence to

the mechanisms behind the response to chronically divergent levels of activity and systemic disturbance. Furthermore, there is a significant body of evidence supporting systemic inflammation and oxidative stress as having consequences outside of the pulmonary system, processes that are also relevant to the ageing process. Potential ageing related biomarkers of interest are discussed further here.

Klotho and fibroblast growth factor-23

Klotho is a signalling protein that is expressed as a circulating 'anti-ageing' hormone and as a transmembrane protein (Matsumura, Aizawa et al. 1998). The role of both forms of Klotho and their relevant mechanisms of action are yet to be clearly elucidated. *Klotho* knockout mice have a shortened life span and develop emphysema, sarcopenia, thin skin, osteoporosis and vascular calcification (Kuro-o, Matsumura et al. 1997; Iida, Kanki et al. 2011). Klotho has a humoral role in mineral metabolism and growth factor signalling, it is a co-receptor for fibroblast growth factor-23 (FGF-23) (Kurosaki, Ogawa et al. 2006) and influences vitamin D expression (Tsujioka, Kurotani et al. 2003). Our group has recently demonstrated that circulating 1,25-dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$) relates to quadriceps strength and $25(\text{OH})\text{D}$ to quadriceps MHCIIa expression in those without COPD (Jackson, Shrikrishna et al. 2013). Vitamin D levels were reduced in smokers and patients with COPD in this study, however, vitamin D levels did not relate to any muscle specific measures in either the cohort as a whole or in COPD patients.

In general populations, circulating Klotho levels predict grip strength (Semba, Cappola et al. 2012), physical performance (Crasto, Semba et al. 2012) and mortality (Semba, Cappola et al. 2011). Furthermore, levels may be altered in disease, including diabetes mellitus and chronic renal disease (Devaraj, Syed et al. 2012). The consequences of low expression may be reversed (Wang and Sun 2009) resulting in extended rodent lifespan (Kurosaki, Yamamoto et al. 2005). Despite a very recent

study demonstrating circulating Klotho to be reduced in gestational female smokers (Lam-Rachlin, Romero et al. 2013), limited data is available in COPD. In fact, published papers are confined to two studies focusing on genotyping, neither showing genetic polymorphisms to be associated with disease status or severity (Sotiriou, Kukuivitis et al. 2010; Kim, Oh et al. 2011). Furthermore, there is no data available on transmembrane Klotho expression within the skeletal muscle of humans.

FGF-23 is a bone derived hormone that belongs to the fibroblast growth factor (FGF) subfamily of endocrine factors and mediates phosphate regulating actions in the kidney through FGF receptors (FGFRs), FGFR1 is the most prominent of these. The transmembrane form of Klotho is required for the functioning of FGF-23, significantly enhancing the binding affinity of FGF-23 to the FGFRs (Kurosu, Ogawa et al. 2006). In mice, the ablation of Klotho leads to accelerated ageing in the setting of elevated levels of $1,25(\text{OH})_2\text{D}$, hypercalcemia and severe hyperphosphatemia (Kuro-o, Matsumura et al. 1997; Kurosu, Ogawa et al. 2006; Wang and Sun 2009). Mice lacking a functional FGF-23 allele have an almost identical phenotype to that observed in Klotho deficient mice (Kurosu, Ogawa et al. 2006). Evaluating potential roles for Klotho and FGF-23 in the skeletal muscle dysfunction of COPD may be of potential therapeutic interest.

Matrix metalloproteinase-9

Matrix metalloproteinases (MMPs) have a central role in lung remodelling in COPD (Vernooy, Lindeman et al. 2004; Vignola, Paganin et al. 2004). They are a family of zinc and calcium dependent enzymes capable of degrading all components of the extracellular matrix. Elevated cellular (Segura-Valdez, Pardo et al. 2000), pulmonary (Russell, Culpitt et al. 2002) and systemic (Brajer, Batura-Gabryel et al. 2008) MMP-9 activity is associated with airflow obstruction (Vignola, Riccobono et al. 1998) and emphysematous changes in COPD (Vignola, Paganin et al. 2004). Whilst Tissue Inhibitors of Metalloproteinases (TIMPs) are endogenous inhibitors, doxycycline and statins also inhibit MMP-9

activity. MMP-9 levels in the sputum increase with age (Simpson, McDonald et al. 2013). MMP-9 activity is related to arterial stiffness and skin elasticity (Maclay, McAllister et al. 2012); furthermore, it is produced by inflammatory cells and satellite muscle cells. MMP-9 activity in the tendons of rats increases with age and may contribute to tendinopathy (Yu, Pang et al. 2013). Interestingly, the Klotho mouse model has been shown to have increased MMP-9 and reduced TIMP-1 inhibitor levels in the lung only after development of emphysema (Funada, Nishimura et al. 2004).

In muscle, MMP-9 levels are increased in patients with Duchenne's muscular dystrophy and the mdx-mouse model; furthermore, inhibition of MMP-9 improves structure and contractile function (Li, Mittal et al. 2009). Inhibition also improves soleus muscle regeneration (Zimowska, Olszynski et al. 2012) and *vastus lateralis* MMP-9 mRNA levels increase in response to exercise training, possibly having a role in extracellular remodelling (Hoier, Nordsborg et al. 2012). Muscle data on MMP-9 expression in human cohorts is limited and to my knowledge there is no data in COPD cohorts.

Growth differentiation factor-15

TGF- β signalling is believed to have an important role in determining muscle mass and has already been discussed in the context of GDF-8 (section 1.9.3). Our group has established that quadriceps GDF-8 levels inversely relate to function in COPD patients (Man, Natanek et al. 2010). Our group also recently demonstrated that plasma growth and differentiation factor-15 (GDF-15, also known as Macrophage inhibitory cytokine-1, MIC-1), another member of TGF- β signalling family, acutely increases following cardiothoracic surgery and that levels stay elevated for up to 1 week in those who develop muscle wasting, unlike those who did not display wasting (Bloch, Lee et al. 2013). Evidence of GDF-15 having a negative influence on muscle mass was corroborated by *in-vitro* work demonstrating that it causes myotube atrophy.

GDF-15 protein levels are increased in the airway epithelium of human COPD smokers as compared to healthy non-smokers, an effect of smoking was confirmed by cigarette smoke exposure up-regulating GDF-15 expression in airway epithelial cells (Wu, Jiang et al. 2011). Whether GDF-15 is elevated in the systemic circulation of patients with COPD, in the setting of the chronic stressors described in section 1.10 is unknown. Furthermore, it remains to be determined if GDF-15 levels in COPD relate to relevant clinical parameters. Despite this, in prostate cancer, GDF-15 levels relate to BMI indicating that GDF-15 may be relevant in the cachexia observed in chronic disease (Johnen, Lin et al. 2007). Johnen *et al.* extended these findings by xenografting mice with prostate malignancies, elevated GDF-15 levels were associated with marked weight, fat and lean tissue loss mediated by reduced food intake and reversed by an antibody to GDF-15. Circulating GDF-15 levels have been shown to relate to chronological age (Vila, Riedl et al. 2011; Berberoglu, Aktas et al. 2014) and cognitive decline (Fuchs, Trollor et al. 2013). Consistent with these findings, serum GDF-15 is recognised as a predictor of all-cause mortality, with an adjusted odds ratio of 3.38 identified (Wiklund, Bennet et al. 2010).

1.2 Aims and structure of this thesis

The overall aim of this thesis was to assess at a physiological, biological and structural level the role of age-related markers in the skeletal muscle dysfunction of COPD. The general methods used for all studies in this thesis are described in Chapter 2.

Given that the predominant quadriceps fibre type in COPD opposes that seen in healthy older adults, yet it remains unclear how this relates to relevant patient related outcomes, the relationship between fibre type and mortality was assessed. This study is presented in Chapter 3.

The functional assessment of patients with COPD is highly relevant, but not often implemented in clinical practice. Chapter 4 describes a study that sought to evaluate a simple functional assessment tool, the SPPB, in COPD and to establish whether the pulmonary aspects of the disease prevent this tool from identifying patients with physiological and structural evidence of skeletal muscle abnormality.

A study that aimed to evaluate the expression of age-related circulating biomarkers, specifically Klotho, FGF-23, MMP-9 and GDF-15 in COPD and establish whether they relate to skeletal muscle function in COPD is described in Chapter 5. Subsequently, an extension of this work was to determine whether Klotho expression is influenced by smoking. Given the paucity of data available in muscle, a study evaluating the expression of GDF-15 and transmembrane Klotho within skeletal muscle is reported in Chapter 6.

The overall findings of these studies are discussed in Chapter 7, with a further description of potential areas of further work.

1.3 Hypotheses

1. Quadriceps fibre type proportion relates to mortality in COPD.
2. The SPPB relates to important muscle parameters, including quadriceps strength and fibre type, and will subsequently identify a phenotype with skeletal muscle dysfunction in COPD.
3. Circulating age-related proteins will be altered in COPD, and relate to quadriceps strength and exercise capacity.
4. Klotho is expressed in skeletal muscle and levels will be influenced by smoking, the presence of COPD, and muscle contractile activity.
5. GDF-15 is expressed in skeletal muscle and levels will be altered in disease and relate to relevant physiological parameters such as muscle bulk.

Chapter 2 : Methods

2.1 Regulatory Procedures

The studies received research ethical committee approval (West London REC 3: 10/H0706/9; North West London REC: 11/LO/1636; North London REC: 11/H0717/3; NRES Committee London – Chelsea: 12/LO/0523) and all patients provided written informed consent for their involvement. The research was conducted in accordance with Good Clinical Practice guidelines.

2.2 Subject selection

COPD patients

Patients with GOLD stage I to IV disease according the Global Initiative in Obstructive Lung Disease guidelines (see Table 1) (Rabe, Hurd et al. 2007) were recruited either via the muscle laboratory database, or respiratory outpatients at the Royal Brompton and Harefield Hospitals. Patients undergoing thoracic surgery were identified by the surgical theatre lists or from thoracic surgery outpatients.

Controls

Control subjects were recruited from a database of subjects previously studied at the Royal Brompton Hospital or smoking cessation clinics at either the Royal Brompton or Harefield Hospital.

2.3 Subject entry criteria

Inclusion criteria

All participants:

Male or female aged 40 - 90 years inclusive.

COPD patients:

Current or ex-smokers with a smoking history ≥ 15 pack years.

FEV₁/FVC ratio < 70% on entry to the study.

Controls:

Current smokers (at least 5 cigarettes or equivalent per day) or never smoker (<1 pack year), with an FEV₁ of >80% and FEV/FVC of >70%.

Exclusion criteria

All participants:

Significant systemic co-morbidity including renal, hepatic and cardiac disease.

Subjects who are scheduled or awaiting in-patient surgery during the study.

Contra-indication to biopsy for research purpose (e.g bleeding disorder).

Joint or neurological disease which would preclude exercise.

COPD patients:

Significant respiratory disease other than COPD.

History of or currently taking steroids or other drugs known to affect muscle function.

Controls:

Significant respiratory disease.

Taking steroids or other drugs known to affect muscle function.

2.4 Anthropometric measurements

Height (in metres, m) without shoes was measured using a wall-mounted measure and weight (in kilograms, kg) by standardised scales. The body mass index (BMI) was calculated using these measurements.

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

2.5 Carbon monoxide measurement

Exhaled carbon monoxide (CO) was measured by subjects exhaling through a handheld CO meter, Smokecheck (Micromedical Ltd, Kent, UK) whilst wearing a nose-clip.

2.6 Pulmonary function testing

Pulmonary function tests were performed by physiologists within the Lung Function Departments at the Royal Brompton and Harefield Hospitals.

Spirometry

Post-bronchodilator spirometry was performed with the Vitalograph 2120 (Vitalograph Ltd, Buckinghamshire) according to standard criteria using a pneumotachograph with the integration of flow to derive volume (Miller, Hankinson et al. 2005). The device was calibrated daily with a 1L syringe with a view to ensuring an accuracy of within 3% of the calibration volume. Subjects were asked to take a deep inspiration, completely filling the lungs and were then asked to perform a maximal forced exhalation through a disposable mouthpiece attached to a filter. This was repeated until 3 attempts within 5% of each other were obtained, the best forced expiratory volume in 1 second (FEV₁) and forced vital capacity (FVC) achieved were recorded.

Lung volume and gas transfer

Lung volumes were measured using body plethysmography (Wanger, Clausen et al. 2005). Gas transfer was determined by inhaling a known concentration of carbon monoxide (CO) and measuring the end expiratory CO concentration after a single breath hold and adjusting for alveolar volume (Compact Master lab system, Jaeger, Germany) (Macintyre, Crapo et al. 2005). All tests were performed and interpreted in accordance with ATS/ERS guidelines (Macintyre, Crapo et al. 2005; Wanger, Clausen et al. 2005). Calibrations on all equipment were undertaken regularly. Predictive values were expressed in accordance with the British Thoracic Society guidelines (British Thoracic Society 1997).

Capillarised blood sampling

Arterialized capillary earlobe blood samples were taken at rest with the subject breathing room air.

2.7 Dyspnoea

The MRC dyspnoea score was introduced over 50 years ago, initially in the assessment of patients with chronic bronchitis (Mahler and Wells 1988). It has prognostic validity and correlates well with relevant other clinical and physiological measures. Patients simply select the most relevant of 5 statements describing levels of breathlessness during routine physical activities.

2.8 The assessment of health status

Subjects completed the St. George's Respiratory Questionnaire for COPD patients (SGRQ-C). This is a self-administered questionnaire validated for the assessment of health status in COPD patients. The SGRQ-C is a 40 item tool specifically assessing the impact of COPD on health-related quality of life, it is scored out of 100 (higher scores denoting poorer health status), with sub-scores for symptoms, activity and impact on daily life (Meguro, Barley et al. 2007).

2.9 Six minute walk test

A six minute walk test (6MWT) was performed in accordance with the American Thoracic Society guidelines, except that the practice walk was omitted (American Thoracic Society 2002) over a 30m track. Subjects were seated for 10 minutes before starting the route and pre-test arterial oxygen

saturation (SaO₂) and heart rate were recorded with an N-200E Nellcor oximeter (Nellcor, Covidien-Nellcor™ Boulder, USA). Standardised instructions were given to each subject. Subjects were asked to walk as far as they could without running or jogging. Each 30m lap covered was manually recorded and the duration of the test was timed using a stopwatch. Standard prompts were provided at minute intervals and the test was stopped at the end of 6 minutes at which point the heart rate and SaO₂ were measured and the total distance walked to the nearest metre calculated. Extreme dyspnoea, chest pain or leg pain prompted early termination of the test.

2.10 Body composition

Fat free mass (FFM) was measured by bioelectrical impedance analysis using a Bodystat 4000 device (Bodystat, Isle of Man, UK). Subjects lay supine for 10 minutes before measurement. Silver chloride electrodes (Maersk Medical, Stonehouse, UK) were placed in the midline position of the dominant hand and foot. For the hand, one electrode was placed at the dorsal aspect of the hand 2cm proximal to the metacarpophalangeal joints and one at the level of the ulnar head of the wrist. For the foot, one electrode was placed 2cm proximal to the metatarsophalangeal joints, the other at the level of the malleoli. As equations derived from healthy populations tend to overestimate FFM in COPD patients, the FFM was corrected using disease specific regression equations for COPD patients (Steiner, Barton et al. 2002). The device's internal algorithms were used for healthy subjects.

COPD males:

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

COPD females:

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

Fat free mass index (FFMI) was calculated by dividing FFM by the height (in metres) squared. Individuals with a FFMI below 16 kg/m² for males and 15 kg/m² for females were considered to have fat free mass depletion (Schols, Soeters et al. 1993; Steiner, Barton et al. 2002).

2.11 Quadriceps strength

The leg ipsilateral to the dominant hand was tested. Subjects were studied supine with the knee flexed at 90° over the end of the chair. An inextensible strap was placed around the ankle and connected to a strain gauge (Straininstall, Isle of Wight, UK; range 0-100kg) mounted to the back of the chair.

Isometric maximal voluntary contraction (QMVC)

Quadriceps maximum voluntary contraction was measured as described by Edwards *et al.* (Edwards, Young et al. 1977), see Figure 2.1. Signals from the strain gauge were processed as a digital output by a Powerlab recording unit (ADInstruments, Oxford, UK) connected to a PC running LabChart software (ADInstruments) sampling at 10 kHz. Calibration with a known weight was performed before QMVC measurement. Subjects were asked to extend their knee with maximal effort against the strap for 5 seconds, standardised encouragement was provided until there was not any further increase in QMVC visualised. The QMVC reported in kilograms (kg), was the highest value achieved in 5 efforts, with a 30 second resting period between each effort. The QMVC was normalised according to the body-mass-index and patients with a QMVC/BMI below 1.2 were identified as being weak (Swallow, Reyes et al. 2007). The % predicted QMVC was determined as according to regression equations based on age, gender and FFM (Edwards, Young et al. 1977; Seymour, Spruit et al. 2010).

Predicted QMVC force in kg =

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

The residual standard deviation from the analysis of Seymour *et al* was 8.58 kg; therefore, patients with an ((observed-predicted QMVC)/ 8.58) <-1.645, were considered weak (Seymour, Spruit *et al.* 2010).



Figure 2.1: Strain gauge set-up for measurement of QMVC.

Twitch quadriceps tension (TwQ)

TwQ muscle tension was measured using the technique described by Polkey and colleagues (Polkey, Kyroussis *et al.* 1996). A 70 mm figure of eight coil head powered by a double Magstim 200 stimulator (Magstim Co, Whitland, UK) was positioned high in the femoral triangle just lateral to the femoral artery, overlying the path of the femoral nerve (see Figure 2.2). Minor positional

adjustments during observed stimulations allowed the optimal position to be determined and marked. The generated force was amplified with the signals passed via a Powerlab recording unit (ADInstruments) to a PC running LabChart software (ADInstruments) sampling at 10 kHz. At least a 20 second interval between each twitch was observed to avoid twitch on twitch potentiation. Supramaximality of femoral twitch (TwQ) response was confirmed by measuring the force generated over a range of magnetic power outputs in order to generate a stimulus response curve. To assess the magnitude of twitch potentiation, a stimulation at 100% stimulator output was performed 5 seconds after an MVC was performed. The value recorded should be the largest TwQ obtained. The mean tension generated by 5 stimulations at 100% magnetic power output was calculated to provide TwQ.



Figure 2.2: Measurement of TwQ.

2.12 Ultrasound of the *rectus femoris*

Cross-sectional area of the *rectus femoris* (RF_{CSA}) was measured by B-mode ultrasonography using an 8 MHz 5.6 cm linear transducer (PLM805, Toshiba Medical Systems, Crawley, UK), according to the methods of Seymour *et al.* (Seymour, Ward et al. 2009). Subjects were positioned supine with the leg supported in passive extension. A marking was made on the superior aspect of the dominant thigh at 3/5 of the distance from the anterior superior iliac spine to the superior patellar border. The transducer was placed on this marking perpendicular to the longitudinal axis of the *rectus femoris* muscle, with generous quantities of gel placed on the skin to enhance image acquisition. Subjects were asked to contract and relax their quadriceps in order to delineate the muscle septa prior to the US image being frozen. RF_{CSA} was calculated from a frozen image by using a cursor to outline the inner echogenic line of the *rectus femoris*. RF_{CSA} was taken as the mean of three consecutive measurements that were within 10% of each other.

2.13 Physical activity

Physical activity was measured by a multisensory armband that incorporates a biaxial accelerometer and energy expenditure measurements (SenseWear Pro Armband, Bodymedia, Pittsburgh, USA) worn on the upper aspect of the right arm over the triceps muscle (see Figure 2.3). To improve reliability, the armband was worn throughout a 7 day period, with the subject only removing it when washing such that at least 22.5 hours of data was collected over a 24 hour period (Watz, Waschki et al. 2009). Data was analysed over a period of 4 working days and 2 weekend days. The outputs recorded were the mean daily step count, the time spent in at least moderate physical activity (time ≥ 3 METs) and the physical activity level (PAL), defined as total energy expenditure per minute divided by sleep energy expenditure per minute (Watz, Waschki et al. 2009).



Figure 2.3: Sensewear armband.

2.14 Venous blood sampling and serum processing

Up to 20mls of blood was venesected in the morning before other tests were performed and where applicable, before muscle biopsies were taken. Blood was collected into serum gel clotting activator tubes to allow subsequent storage as serum. After mixing, the tubes were stored upright at ambient temperature for 30 minutes to allow the serum to separate and then centrifuged for 10 minutes at 1000g at room temperature. Care was taken to avoid agitation or contamination of the sample with cellular debris from the pellet and the supernatant pipetted into labelled 2mL polypropylene tubes and stored at -80°C until further analysis.

2.15 Quadriceps muscle sampling and processing

Using the technique described by Bergstrom (Bergstrom 1962) percutaneous needle biopsy of the *vastus lateralis* of the quadriceps was taken on the same side as the dominant hand. This was done under sterile conditions. The patient lay supine for 15 minutes before the procedure was performed in order to minimise the effects of contraction on the biopsy findings. With the patient supine, the skin was cleaned with 2% chlorhexidine/ 70% isopropyl alcohol. 5mls of 2% lignocaine was injected into the skin, subcutaneous tissue and *vastus lateralis* muscle. A 1cm incision was made and then the Bergstrom needle inserted through the subcutaneous tissues and through the fascia of the muscle. A 3-way tap was used to connect a 50ml syringe to the Bergstrom needle, in order to apply suction. The aperture of the needle was opened whilst an assistant applied concurrent suction. The needle was then rotated and then the procedure repeated with the needle cutting further pieces of muscle tissue at the same pass. The muscle tissue was divided into 2 parts. One portion was placed into cryovials and immediately frozen in liquid nitrogen and stored for protein and messenger RNA (mRNA) analysis. The other muscle samples were prepared for histochemical analysis. Initially, these samples were placed on saline-soaked filter paper within a petri-dish. These samples were then placed on a piece of cork and surrounded with Tissue-tek (OCT compound). The OCT embedded samples were then frozen in melting isopentane. All samples were then stored at -80°C.

2.16 Muscle sectioning

Muscle sectioning was performed by trained histopathology technicians. Frozen muscle samples (6µm thick) were cut at -20°C using a microtome with fibres predominantly orientated in a transverse manner. The sections were then stored at -80°C until further analysis.

2.17 Immunohistochemical detection of type I and II fibres

The immunohistochemical detection of muscle fibre type was performed by collaborators from AstraZeneca (Molndal, Sweden). Sections were fixed in 4% formalin for 30 minutes and were then transferred to phosphate buffered saline (PBS). Fibre type detection was carried out by dual-immunostaining using a Ventana Discovery XT Staining Module. All reagents, with the exception of detergent, were purchased from Ventana medical systems, Inc. Res IHC UltraMap AP XT and Res IHC Omni-UltraMap HRP XT procedures were selected for the anti-fast myosin and anti-slow myosin protocols respectively. The following user determined parameters were selected Res IHC UltraMap AP XT procedure; primary antibody MY-32; mouse (IgG1) anti-human fast myosin (Abcam #ab7784) [diluted 1:100], 60 minute incubation time; secondary antibody (UMap anti-Ms AP (#760-4313)) incubation time 4 minutes; Chromomap Blue kit (#760-161), substrate incubation time 16 minutes. Slides were then washed in 2% detergent and placed in Ventana reaction buffer before running the Res IHC Omni-UltraMap HRP XT procedure to detect slow myosin. A blocking agent was applied by hand to prevent binding to the MY-32 (AffiniPure Fab fragment goat anti-mouse, IgG (H+L) Jackson Immuno research laboratories #115-007-003 [diluted 1:13]), 60 minute incubation time); primary antibody NOQ7.5.4D mouse (IgG1) anti-human slow myosin (Abcam #ab11083) [diluted 1:6400], incubation time 60 minutes; UMap anti-Ms HRP (#760-4313) incubation time 4 minutes; ChromoMap DAB kit (#760-159), substrate incubation time 8 minutes. Slides were then washed in 2% detergent before dehydrating and mounted using Pertex mounting media.

Primary antibodies and blocking fragments were diluted in Ab Diluent (Ventana #760-108) and controls were run for both the fast and slow myosin using antibody isotypes in place of primary antibody.

Fibres were classified as being either type I/slow (brown) or type II/fast (blue) by brightfield microscopy (see Appendix image). All of the transverse fibres within a biopsy section were analysed, resulting in a minimum of 100 fibres being analysed for each biopsy specimen. Dual stained fibres were excluded from the analysis.

2.18 Myofibre cross-sectional area measurement

The myofibre cross-sectional area (CSA) of a minimum of 50 of each fibre type was determined by drawing around fibres identified as being either type I/slow (brown) or type II/fast (blue) following immunohistochemical staining using ImageScope digital software (Aperio Technologies, CA, USA). The mean area for both fibre types for each biopsy was calculated. Dr. Manuel Baz performed these analyses.

2.19 Statistical analysis

Statistical analyses and graphical presentations were performed using a commercial computer programs, GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA) and SPSS V.16.0 (IBM, Illinois, USA). Details of the statistical methods used are provided in each of the relevant chapters.

Chapter 3 : The relationship between quadriceps fibre type and survival in COPD

3.1 Introduction

3.1.1 Background

COPD is now the third leading cause of mortality worldwide (Lozano, Naghavi et al. 2012). The forced expiratory volume in the first second (FEV₁) considered alone is a modest predictor of important outcomes including mortality in COPD (Boutou, Shrikrishna et al. 2013). Subsequently, multimodal scoring indices that recognise the importance of systemic disturbance have been developed to predict mortality in COPD (Celli, Cote et al. 2004), and these confer additional prognostic power (Celli, Cote et al. 2008). Skeletal muscle dysfunction in COPD, measured as weakness or whole muscle atrophy, has been shown to be associated with worse quality of life (Montes de Oca, Torres et al. 2006), reduced functional capacity (Saey, Debigare et al. 2003), increased healthcare use (Decramer, Gosselink et al. 1997) and increased mortality (Marquis, Debigare et al. 2002; Swallow, Reyes et al. 2007), independent of lung function impairment. It is likely therefore that loss of skeletal muscle mass is captured by the body mass index (BMI) component of the Body Mass Index, Airflow obstruction, Dyspnoea, Exercise capacity (BODE) index (Celli, Cote et al. 2004).

The skeletal muscle adaptations that occur in COPD have several clinical manifestations, including muscle weakness and muscle atrophy, although not atrophy of the two most common fibre types

(Natanek, Gosker et al. 2013). Most studies have focussed on the quadriceps, since these are the main locomotor muscles. Approximately 30% of COPD patients exhibit quadriceps weakness and atrophy (Seymour, Ward et al. 2009; Seymour, Spruit et al. 2010), which are both associated with increased mortality (Marquis, Debigare et al. 2002; Swallow, Reyes et al. 2007). The quadriceps also display adaptations at a structural level, of which the best characterised is a change in fibre composition such that there are fewer type I (oxidative) fibres and more type II (glycolytic) fibres. Gender specific cut-offs to identify the presence of fibre shift have recently been published (Natanek, Gosker et al. 2013). Pulmonary rehabilitation only partially affects fibre distribution (Vogiatzis, Simoes et al. 2010), and given that at least in animal models, drugs are now available which can switch fibre types (Narkar, Downes et al. 2008) it seems pertinent to establish whether fibre shift is associated with a poor prognosis in COPD, and if so, whether this is independent to other important parameters such as age.

3.1.2 Aims and hypotheses

The aim of the present study was to establish whether fibre type proportion or the occurrence of fibre shift (considered as a dichotomous variable) is associated with increased mortality in COPD, and if so, whether these predictive properties are independent to other relevant muscle parameters including quadriceps strength and bulk. Since it is currently unclear which muscle parameters confer greater risk in populations with different levels of airflow obstruction, we additionally sought to identify the most relevant predictors of mortality separately in those with spirometrically mild (GOLD stage I and II) and severe disease (GOLD stage III and IV). We hypothesised that quadriceps fibre type preponderance and especially the presence of fibre shift would be independent predictors of mortality in both groups.

3.2 Methods

3.2.1 Study design

Data were retrospectively collated from the largest international groups that have been responsible for investigating skeletal muscle dysfunction in COPD over the past 20 years. 7 other institutions with a history of publication in the field were approached; overall 4 institutions were able to contribute data (Tables 3.1-3.4). The methodology and the inclusion and exclusion criteria were set out after a review of the study protocols within the different centres, but prior to the collation of the data. Each relevant institution was required to provide a core dataset (see 3.2.2). Type I and II fibre preponderance, as established by immunohistochemistry of *vastus lateralis* muscle biopsy sections taken from stable COPD outpatients was recorded, the minimal requirement was for the evaluation of 100 fibres for each patient. Inclusion criteria included a formal diagnosis of COPD having been made on the basis of a smoking history of greater than 15 pack years with spirometry confirming an FEV₁/FVC ratio < 70% and an age above 40 at the time of initial data collection. Patients were excluded by each institution if there was a diagnosis of any respiratory disease other than COPD, if any other significant systemic co-morbidity had been diagnosed (including renal, hepatic and cardiac disease) and if patients were on drugs known to affect muscle function at the time of study entry. Mortality status, and subsequently where relevant, date of death, was checked at each institution by local and national database enquiry up until November 2013. Cause of death was not included as this data was not readily available in the majority of centres. Each patient had provided written consent for their involvement in research studies that had received local ethics committee approval. A database was provided by each institution as according to the parameters set out prior to the collation of the final database; all analyses were performed by the author.

3.2.2 Study parameters

Fibre preponderance, reported as the percentage of type II fibres (type II fibre %), was established by immunohistochemistry. Fibre shift was considered to have occurred when the proportion of type II fibres was >68% in men or >65% in females (Natanek, Gosker et al. 2013). Because the samples had been collected by different groups for different reasons a minimum core dataset was collated, the additional data required for inclusion in the analysis were age, gender and FEV₁ expressed as % predicted (FEV₁%pred).

Other parameters included in sub-analyses, where available, were body mass index (BMI), fat free mass index (FFMI) (Steiner, Barton et al. 2002), dominant leg isometric quadriceps maximum voluntary contraction expressed in kg (QMVC) and when normalised to Body mass index (QMVC/BMI) (Edwards, Young et al. 1977), mid-thigh cross-sectional area determined by CT scan (MTCSA), plethysmographic lung volumes (expressed as a percentage of the residual volume normalised to total lung capacity, RV/TLC) and gas transfer measurements (expressed as percent predicted value for the carbon monoxide transfer factor corrected for haemoglobin, TLCOC) (Macintyre, Crapo et al. 2005; Wanger, Clausen et al. 2005). Due to the methodological approach, whilst these data were available in the majority, they were not available in all patients (see Appendix, 8.17).

3.2.3 Statistical Analysis

Data were analysed for the whole dataset and also after splitting the group into those with an FEV₁<50% and those with an FEV₁≥50%. Statistical analyses and graphical presentations were performed using GraphPad Prism 5 (GraphPad Software, San Diego, USA) or SPSS version 18 (IBM,

USA). Significance was set at a p-value of ≤ 0.05 . Differences between groups were assessed by Student t-test or Fisher's exact test; predictors of mortality were identified by Cox Proportional Hazards. Data are presented as mean (SD) or median (IQR).

3.3 Results

3.3.1 Characteristics of the cohort

Data was collated on 392 stable COPD outpatients from 4 sites (London (n=162), Québec (n=102), Barcelona (n=68) and Athens (n=60)) who had undergone a *vastus lateralis* biopsy between 1995 and 2013. The characteristics of the different cohorts are presented in the Tables 3.1-3.4; the characteristics of the overall dataset including those with additional data are presented in Table 3.5 (and Appendix, 8.17).

Patients were followed up for a median of 1699 days (range 127-6601 days) up until the data analysis began in November 2013; 102 of 392 (26.7%) patients died during the follow up period (Table 3.5). Overall survival was 98.2% at 1 year. As might be expected, those that died had been followed up for a shorter time period than those that survived (1370 (816, 2396) days vs 1736 (1206, 2180) days; $p=0.03$).

151 patients had GOLD stage I/II disease and 241 had GOLD stage III/IV disease. The patients that died were older, had a lower FEV₁%pred and a greater male preponderance (Table 3.5A). 177 (45.1%) of the patients had fibre shift.

The patients that died had a higher percentage of type II fibres (69.5 (62.2, 76.3) % vs 66.0 (54.0, 74.2) %; $p=0.002$) and a higher proportion of patients with established fibre shift (58% vs 41%; $p=0.004$) using the Natanek criteria (Natanek, Gosker et al. 2013). In those with additional data available (Table 3.5B), BMI, FFMI, QMVC, MTCSA and TLCOc were all lower in those that died, however, QMVC/BMI was not. Those that died also had a higher RV/TLC.

Parameter	Number with available data	Number/ Mean (SD)/ Median (IQR)
Dead (%)	162	23
Time between biopsy and survival status check (days)	162	1645 (817, 1962)
Age (years)	162	66.3 (8)
Gender (%male)	162	64
FEV ₁ (%predicted)	162	42.7 (27.1, 59.3)
% type II fibres	162	65.9 (13.2)
Fibre shift (%)	162	42
FEV ₁ (L)	162	1.01 (0.75, 1.54)
BMI (kg/m ²)	162	24.6 (21.8, 28.9)
FFMI (kg/m ²)	162	16.5 (14.6, 18.2)
QMVC (kg)	162	29.8 (10)
MTCSA (cm ²)	42	100.6 (26)
TLCOc (%pred)	158	45.2 (19)
RV/TLC (%)	158	55.7 (11)

Table 3.1: Characteristics of the London cohort (n=162).

Parameter	Number with available data	Number/ Mean (SD)/ Median (IQR)
Dead (%)	102	26
Time between biopsy and survival status check (days)	102	1575 (1310, 2582)
Age (years)	102	65.5 (7)
Gender (%male)	102	78
FEV ₁ (%predicted)	102	50.5 (34.0, 88.3)
% type II fibres	102	58.5 (18)
Fibre shift (%)	102	32
FEV ₁ (L)	102	1.44 (0.89, 2.50)
BMI (kg/m ²)	102	25.8 (5)
FFMI (kg/m ²)	0	-
QMVC (kg)	0	-
MTCSA (cm ²)	102	79.2 (24)
TLCOc (%pred)	0	-
RV/TLC (%)	0	-

Table 3.2: Characteristics of the Québec cohort (n=102).

Parameter	Number with available data	Number/ Mean (SD)/ Median (IQR)
Dead (%)	68	24
Time between biopsy and survival status check (days)	68	1468 (1021, 1920)
Age (years)	68	65.5 (59.0, 73.0)
Gender (%male)	68	74
FEV ₁ (%predicted)	68	36.5 (15)
% type II fibres	68	71.5 (68.0, 77.0)
Fibre shift (%)	68	76
FEV ₁ (L)	0	-
BMI (kg/m ²)	0	-
FFMI (kg/m ²)	0	-
QMVC (kg)	59	29.4 (5)
MTCSA (cm ²)	0	-
TLCOc (%pred)	0	-
RV/TLC (%)	0	-

Table 3.3: Characteristics of the Barcelona cohort (n=68).

Parameter	Number with available data	Number/ Mean (SD)/ Median (IQR)
Dead (%)	60	35
Time between biopsy and survival status check (days)	60	2463 (812)
Age (years)	60	65.8 (9)
Gender (%male)	60	90
FEV ₁ (%predicted)	60	44.1 (18)
% type II fibres	60	63.2 (15)
Fibre shift (%)	60	40
FEV ₁ (L)	60	1.20 (0.5)
BMI (kg/m ²)	56	25.3 (23.5, 28.2)
FFMI (kg/m ²)	42	18.0 (2)
QMVC (kg)	60	26.6 (6)
MTCSA (cm ²)	0	-
TLCOc (%pred)	51	59.3 (34)
RV/TLC (%)	47	54.8 (15)

Table 3.4: Characteristics of the Athens cohort (n=60).

A)

Parameter	Alive (n=290)	Dead (n=102)	p-value
Age (years)	65 (9)	68 (8)	0.01
Gender (m:f)	203:87	84:18	0.02
FEV ₁ (%predicted)	47 (32, 68)	31 (25, 44)	<0.0001
% type II fibres	66.0 (54.0, 74.2)	69.5 (62.2, 76.3)	0.002
Fibre shift (yes:no)	118:172	59:43	0.004
Time between biopsy and survival status check (days)	1736 (1206, 2180)	1370 (816, 2396)	0.03

B)

Parameter	Number (alive:dead)	Alive	Dead	p-value
FEV ₁ (L)	324 (238:86)	1.27 (0.86, 1.87)	0.91 (0.69, 1.19)	<0.0001
BMI (kg/m ²)	320 (236:84)	26.2 (5)	24.3 (4)	0.01
FFMI (kg/m ²)	204 (153:51)	17.1 (2.6)	16.3 (2.0)	0.049
QMVC (kg)	281 (206:75)	29.0 (23.4, 35.0)	25.4 (21.0, 31.4)	0.01
QMVC/BMI	218 (160:58)	1.16 (0.4)	1.08 (0.4)	0.12
MTCSA (cm ²)	144 (102:42)	87.6 (69.0, 107.2)	71.4 (57.8, 91.9)	0.004
TLCOc (%pred)	209 (154:55)	50.9 (35.9, 63.9)	32.3 (24.3, 44.9)	<0.0001
RV/TLC (%)	205 (155:50)	58.2 (48.0, 72.5)	66.2 (57.3, 85.2)	0.009

Table 3.5: Characteristic data of those surviving and those that died over the follow-up period (n=392) in **A)** the cohort with core data and **B)** additional relevant parameters including the numbers in whom data was available. Data are presented as mean (SD) and median (IQR).

3.3.2 The predictors of mortality across the range of airflow obstruction

The univariate and multivariate predictors of mortality are shown in Table 3.6. Both type II fibre % (HR 1.02, 95% CI 1.01, 1.04; $p=0.002$) and the presence of fiber shift (HR 2.07, 95% CI 1.39, 3.09; $p=0.001$), Figure 3.1, were univariate predictors of mortality. Furthermore, age and FEV₁%predicted were also univariate predictors of mortality, however, gender was not. When age, FEV₁%predicted and %type II fibres were entered into a multivariate analysis, age (HR 1.07, 95%CI 1.04, 1.10; $p<0.0001$) and FEV₁%predicted (HR 0.97, 95%CI 0.95, 0.98; $p<0.0001$) were retained as independent predictors, while fibre type as a continuous variable just failed to be retained (HR 1.014, 95%CI 0.998, 1.030; $p=0.09$), Table 3.6A. However, in a multivariate analysis including fibre shift as a dichotomous variable in the analysis rather than fibre type as a continuous variable, fibre shift was retained (HR 1.60, 95%CI 1.06, 2.42; $p=0.03$) in addition to age (HR 1.07, 95%CI 1.04, 1.10; $p<0.001$) and FEV₁%predicted (HR 0.97, 95%CI 0.95, 0.98; $p<0.0001$), Table 3.6B and Figure 3.1.

Given that type II fibre % was not retained as an independent predictor of mortality when age and other lung function parameters were also considered, the relationship between these parameters and fibre type proportion was assessed by Spearman's rank correlation (Figure 3.2). There was a very weak relationship with age ($r=-0.12$, $p=0.01$), whilst moderate relationships with FEV₁ % predicted ($r=-0.44$, $p<0.0001$) and TLCOc % ($r=-0.33$, $p<0.0001$) were demonstrated. There was a greater degree of variance in the relationship in those with more preserved lung function.

A)

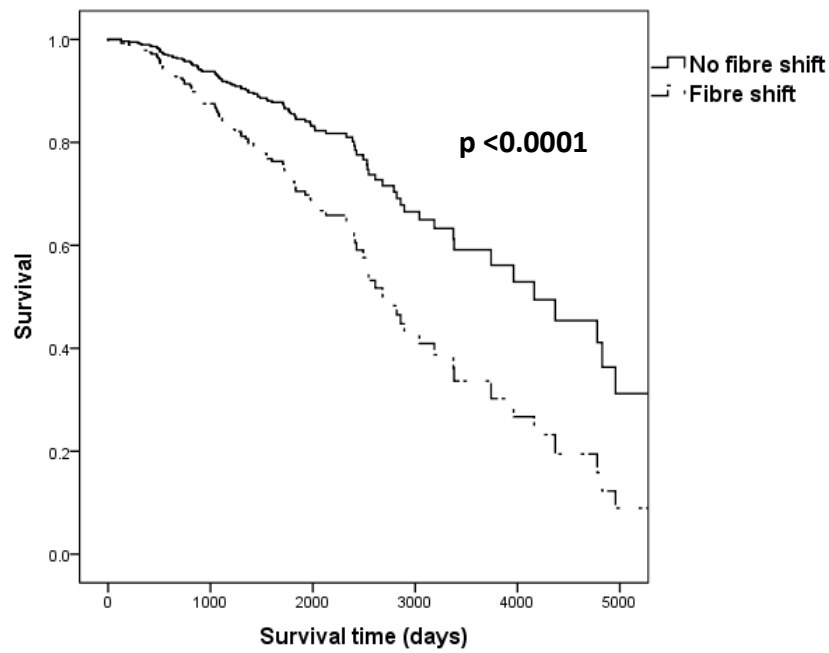
Parameter	% / Mean (SD)/ Median (IQR)	Univariate HR (95%CI)	p-value	Multivariate HR (95%CI)	p-value
Age (years)	65.9 (8)	1.05 (1.02, 1.07)	0.001	1.07 (1.04, 1.10)	<0.0001
Male gender (%)	73	1.35 (0.81, 2.25)	0.257	-	-
FEV₁ %pred	41.6 (28.0, 61.0)	0.97 (0.96, 0.98)	<0.0001	0.97 (0.95, 0.98)	<0.0001
% type II fibers	66.6 (56.0, 75.0)	1.02 (1.01, 1.04)	0.002	1.014 (0.998, 1.030)	0.09

B)

Parameter	% / Mean (SD)/ Median (IQR)	Univariate HR (95%CI)	p-value	Multivariate HR (95%CI)	p-value
Age (years)	65.9 (8)	1.05 (1.02, 1.07)	0.001	1.07 (1.04, 1.10)	<0.0001
Male gender (%)	73	1.35 (0.81, 2.25)	0.26	-	-
FEV₁ %pred	41.6 (28.0, 61.0)	0.97 (0.96, 0.98)	<0.0001	0.97 (0.95, 0.98)	<0.0001
Fibre shift present	45	2.07 (1.39, 3.09)	0.001	1.60 (1.06, 2.42)	0.027

Table 3.6: The univariate and multivariate analyses (across the whole cohort, n=392) including fibre preponderance as **A)** a continuous measure, and **B)** dichotomized into the occurrence of fibre shift.

A)



B)

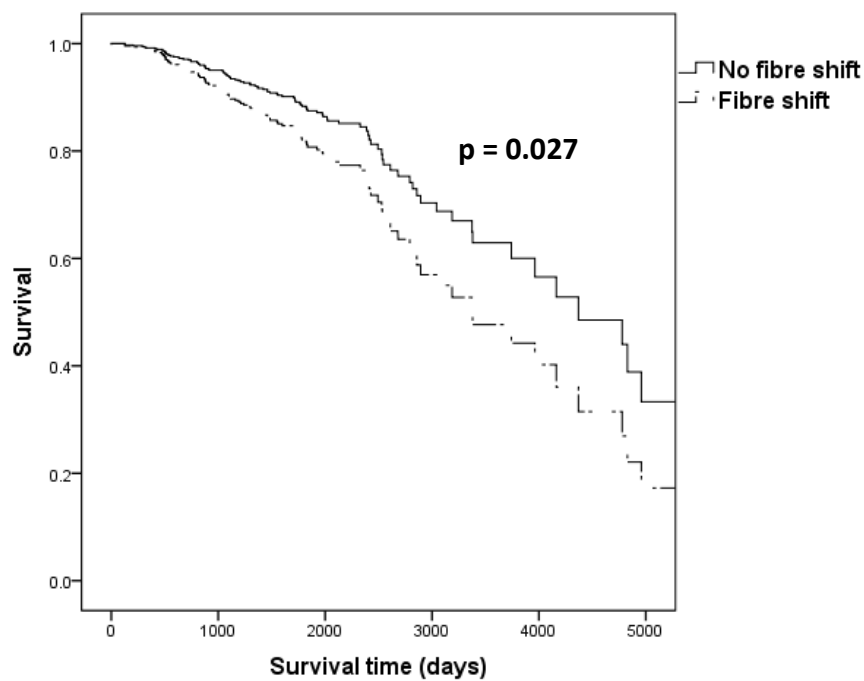
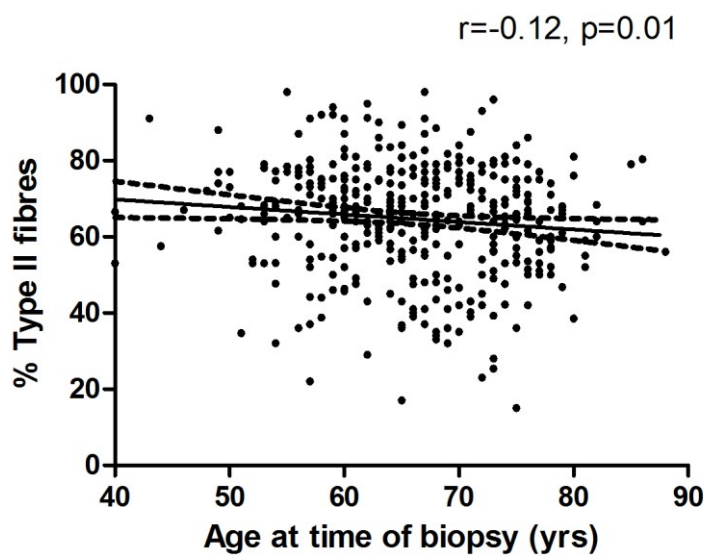


Figure 3.1: Survival curves for **A)** those with fibre shift (n=177) and those without fibre shift (n=215) and **B)** after adjusting for age and FEV₁ % predicted as co-variates.

A)



B)

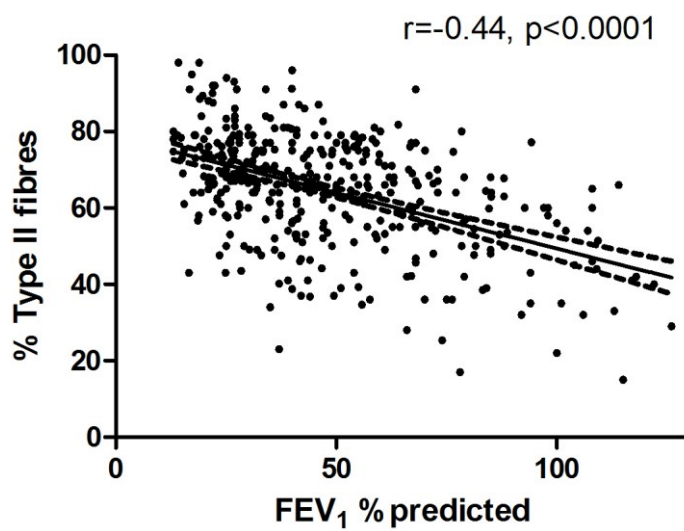


Figure 3.2: The relationship between type II fibre percentage with **A)** age and **B)** FEV₁ % predicted. The interrupted lines demonstrate the 95% CI.

3.3.3 Additional lung function parameters

FEV₁ expressed as an absolute value was available in 324 patients and was a univariate predictor of mortality (HR 0.32, 95%CI 0.19, 0.54; $p<0.0001$), when multivariate analyses were performed including FEV₁ expressed in litres (Table 3.7), FEV₁ (L) in addition to both % type II fibres as a continuous variable and the presence of fibre shift were retained as independent predictors of mortality.

TLCOC and RV/TLC data were available in 209 and 205 patients respectively. TLCOC (HR 0.98, 95% CI 0.966, 0.99; $p=0.003$), but not RV/TLC (HR 0.997, 95%CI 0.991, 1.003; $p=0.31$) was a univariate predictor of mortality. In multivariate analyses including TLCOCc, TLCOCc was retained as an independent predictor of mortality. After including TLCOC in the multivariate analysis, % type II fibres as a continuous variable ($p=0.16$) was not retained, however, the presence of fibre shift was ($p=0.03$), see Table 3.8.

3.3.4 Additional muscle parameters

BMI, FFMI, QMVC, QMVC/BMI and MTCSA were not univariate predictors of mortality across the cohort as a whole (see Table 3.9). Stratifying patients on the basis of validated cut-offs that have predictive properties in COPD (Table 3.10, Figures 3.3 and 3.4), did not identify those with an increased risk of mortality.

A)

	HR (95% CI)	p-value
FEV₁ (L)	0.32 (0.19, 0.54)	<0.0001

B)

	Multivariate HR (95% CI)	p-value
Age	1.06 (1.03, 1.09)	<0.0001
% type II fibres	1.018 (1.002, 1.035)	0.026
FEV₁ (L)	0.34 (0.19, 0.59)	<0.0001

C)

	Multivariate HR (95% CI)	p-value
Age	1.05 (1.02, 1.09)	<0.0001
Fibre shift present	1.66 (1.06, 2.60)	0.027
FEV₁ (L)	0.34 (0.19, 0.59)	<0.0001

Table 3.7: FEV₁ expressed as an absolute value (n=324) in **A)** a univariate analysis and in multivariate analyses including **B)** % type II fibres and **C)** fibre shift.

A)

	HR (95% CI)	p-value
TLCOc (%)	0.98 (0.966, 0.99)	0.003
RV/TLC (%)	0.997 (0.991, 1.003)	0.31

B)

	Multivariate HR (95% CI)	p-value
Age	1.08 (1.04, 1.12)	<0.0001
% type II fibres	1.02 (0.99, 1.04)	0.16
FEV₁%pred	0.979 (0.961, 0.997)	0.03
TLCOc (%)	0.985 (0.970, 1.000)	0.04

C)

	Multivariate HR (95% CI)	p-value
Age	1.08 (1.04, 1.13)	<0.0001
Fibre shift present	1.88 (1.06, 3.35)	0.03
FEV₁%pred	0.979 (0.961, 0.998)	0.03
TLCOc (%)	0.985 (0.971, 0.999)	0.03

Table 3.8: Univariate analyses evaluating **A)** TLCOc (n=209) and RV/TLC (n=205), with subsequent multivariate analyses including TLCOc in addition to other variables in **B)** and **C)**.

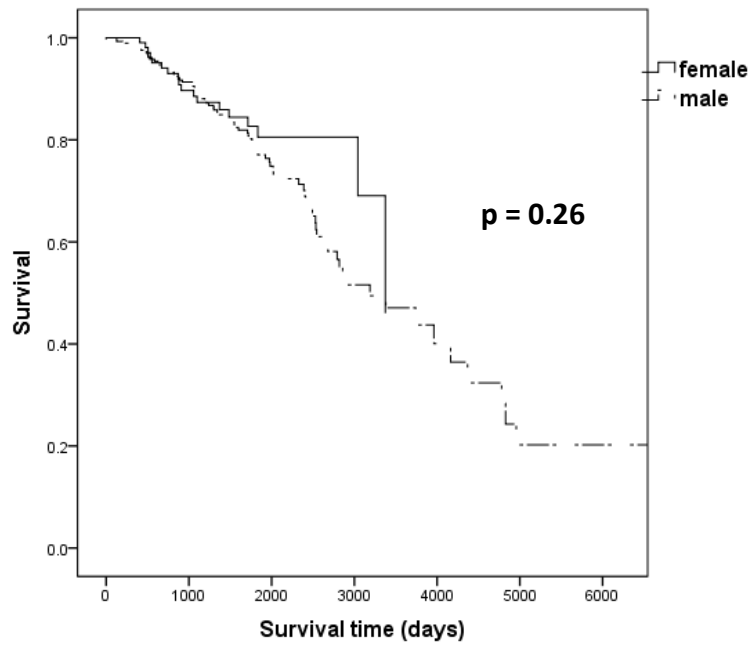
	Number	HR (95% CI)	p-value
BMI	320	0.956 (0.910, 1.006)	0.08
FFMI	204	0.90 (0.79, 1.02)	0.09
QMVC	281	0.974 (0.946, 1.003)	0.08
QMVC/BMI	281	0.67 (0.30, 1.53)	0.34
MTCSA	144	0.99 (0.98, 1.01)	0.41

Table 3.9: Univariate analyses evaluating other relevant muscle parameters including the number in whom data were available.

Parameter	Alive	Dead	p-value
FFMI<15or16 : FFMI >15or16	43:110	21:30	0.09
QMVC/BMI<120% : QMVC/BMI >120%	90:70	41:17	0.06
MTCSA<70 : MTCSA>70	26:76	20:22	0.02

Table 3.10: Variables dichotomized according to validated cut-offs.

A)



B)

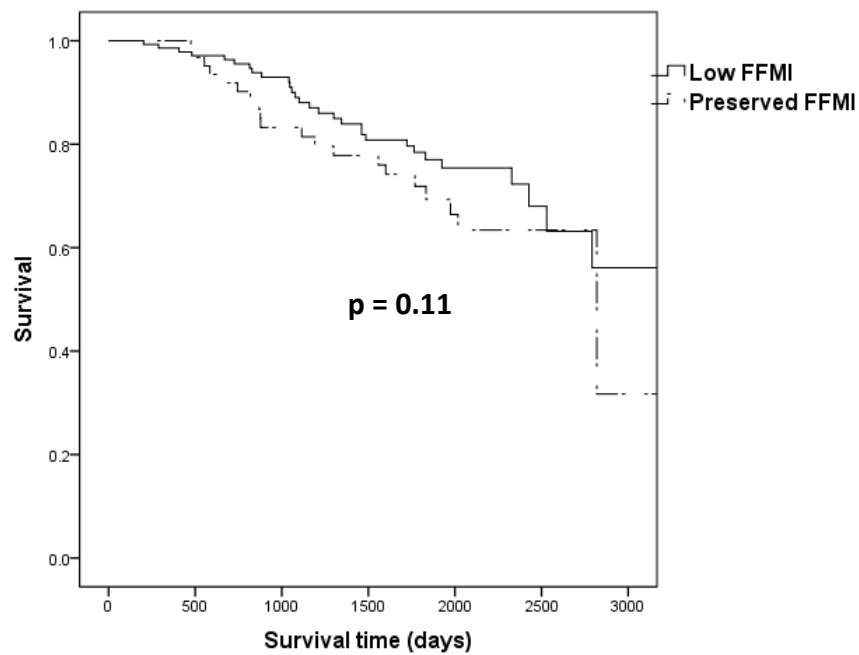
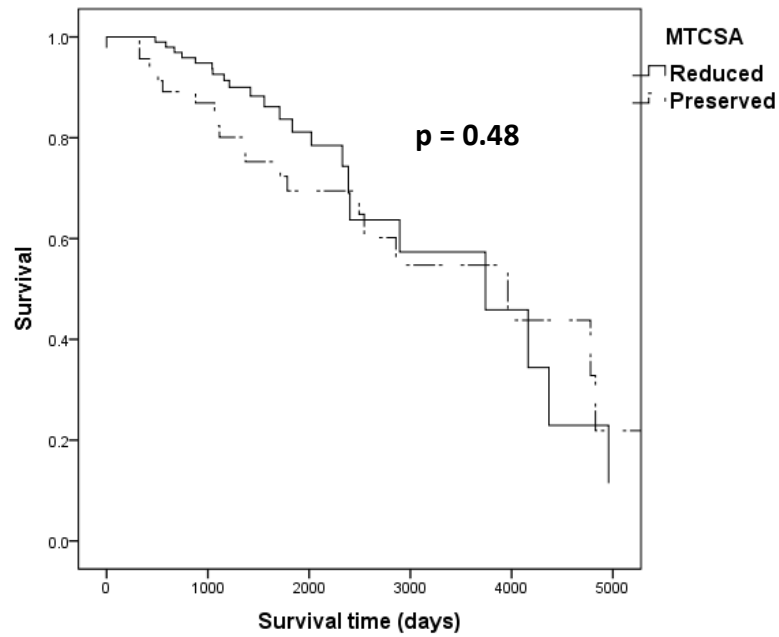


Figure 3.3: Survival curves for **A)** males (n=287) and females (n=105) and **B)** patients with a low FFMI (males <16, females <15 kg/m²; n=64) and patients with a preserved FFMI (n=140).

A)



B)

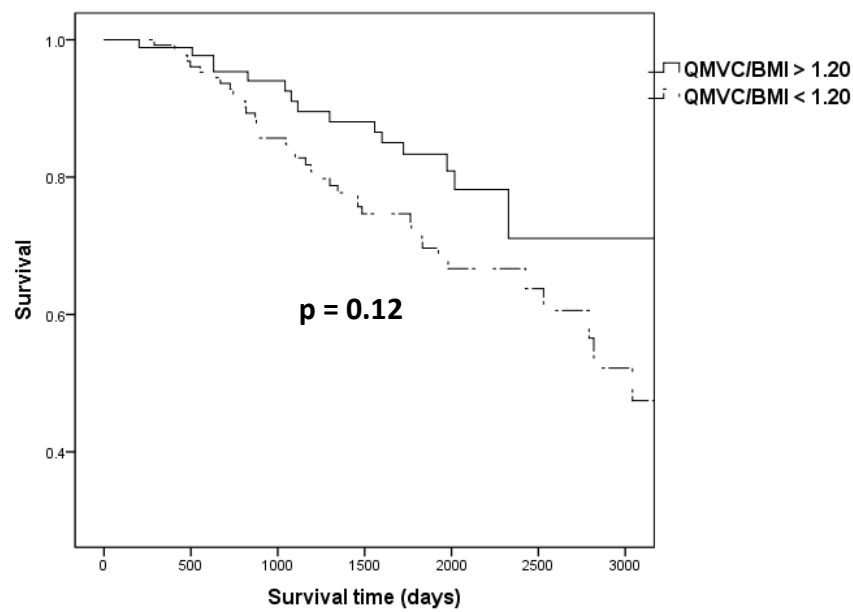


Figure 3.4: Survival curves for **A)** patients with a MTCSA of $<70\text{cm}^2$ ($n=46$) and patients with a MTCSA $\geq 70\text{cm}^2$ ($n=98$) and **B)** patients with a QMVC/BMI of <1.20 ($n=130$) and patients with a QMVC/BMI >1.20 ($n=88$).

3.3.5 The predictors of mortality after stratifying by FEV₁

The comparative characteristics of the groups after stratifying by FEV₁

The characteristics of patients with an FEV₁<50% as compared to those with an FEV₁≥50%, are presented in Table 3.11. The group with more severe airflow obstruction were younger, had a greater type II fibre %, a greater proportion of patients with established fibre shift and a greater proportion that had died (Table 3.11A). In those with additional parameters available, BMI, FFMI, MTCSA and TLCOc were all lower in those that died whilst RV/TLC was higher (Table 3.11B). The group with an FEV₁<50% had a greater proportion of patients with a reduced FFMI, although they did not have a greater proportion of patients with a reduced QMVC/BMI or MTCSA (Table 3.12).

Patients with an FEV₁≥50%

When limiting the analysis to those with an FEV₁≥50%, age was the only predictor of mortality (HR 1.16, 95%CI 1.07, 1.25, p<0.0001); Table 3.13.

A)

Parameter	FEV ₁ <50 % (n=241)	FEV ₁ ≥ 50% (n=151)	p-value
Age	65.1 (8)	67.1 (8)	0.02
Gender (m:f)	181:60	106:45	0.29
FEV ₁ %pred	31.0 (24.1, 40.0)	67.9 (57.0, 85.0)	-
Alive:dead	156:85	134:17	<0.0001
% type II fibers	69.0 (61.8, 77.0)	61.0 (49.0, 68.5)	<0.0001
Fiber shift (yes:no)	132:109	44:107	<0.0001
Time between biopsy and survival status check (days)	1769 (1101, 2225)	1481 (1081, 2038)	0.07

B)

Parameter	Number (FEV ₁ <50% : FEV ₁ ≥50%)	FEV ₁ <50 %	FEV ₁ ≥ 50%	p-value
BMI (kg/m ²)	320 (188:132)	24.0 (21.2, 27.0)	27.4 (5)	<0.0001
FFMI (kg/m ²)	204 (131:73)	16.4 (2)	17.9 (3)	<0.0001
QMVC (kg)	281 (186:95)	28.0 (23.0, 33.1)	30.0 (10)	0.30
QMVC/BMI	218 (138:80)	1.17 (0.3)	1.10 (0.84, 1.31)	0.17
MTCSA (cm ²)	144 (82:62)	80.6 (26)	91.8 (25)	0.01
TLCOc (%pred)	209 (127:82)	37.0 (25.9, 50.1)	57.5 (50.0, 69.3)	<0.0001
RV/TLC (%)	205 (126:79)	66.1 (57.5, 74.9)	48.5 (43.0, 62.4)	<0.0001

Table 3.11: Characteristics of those with an FEV₁<50 % as compared to those with FEV₁≥50% in **A)** the cohort with complete data and **B)** additional relevant parameters including the numbers in whom data was available. Data are presented as mean (SD) and median (IQR) or proportion.

Parameter	FEV ₁ <50 %	FEV ₁ ≥ 50%	p-value
FFMI<15or16:FFMI >15or16	49:82	15:58	0.02
QMVC/BMI<120%:QMVC/BMI >120%	81:57	49:31	0.78
MTCSA<70:MTCSA>70	31:51	15:47	0.10

Table 3.12: Variables dichotomized according to validated cut-offs.

A)

Parameter	Univariate HR (95%CI)	p-value
Age	1.16 (1.07, 1.25)	<0.0001
Male gender	4.75 (0.62, 36.66)	0.14
FEV ₁ %pred	0.98 (0.94, 1.10)	0.21
% type II fibres	1.01 (0.98, 1.05)	0.54
Fibre shift present	1.16 (0.40, 3.35)	0.78

B)

Parameter	Number	Univariate HR (95%CI)	p-value
FEV ₁ (L)	134	0.51 (0.21, 1.27)	0.148
BMI (kg/m ²)	132	1.04 (0.95, 1.15)	0.42
FFMI (kg/m ²)	55	1.109 (0.845, 1.457)	0.46
QMVC (kg)	92	1.03 (0.97, 1.09)	0.36
QMVC/BMI	77	1.74 (0.32, 9.41)	0.52
MTCSA (cm ²)	62	0.97 (0.92, 1.02)	0.22
TLCOc (%pred)	79	0.97 (0.94, 1.01)	0.12
RV/TLC (%)	76	0.995 (0.978, 1.012)	0.59

Table 3.13: The univariate predictors of mortality in patients with an FEV₁≥50% (n=151) in **A)** those with the core dataset, and **B)** those with additional parameters.

Patients with an FEV₁<50%

In this group, age, FEV₁%pred, type II fibre % and the presence of fibre shift were all univariate predictors of mortality (Table 3.14). In a multivariate analysis, age (HR 1.06, 95%CI 1.03, 1.09; $p<0.0001$) and FEV₁%predicted (HR 0.96, 95%CI 0.94, 0.99; $p<0.001$) were retained as independent predictors, whereas type II fibre % was not (HR 1.014, 95%CI 0.996, 1.032; $p=0.13$) (Table 3.14A). In a separate analysis including fibre shift as a dichotomous variable rather than fibre type as a continuous variable, fibre shift was retained as an independent predictor (HR 1.71, 95% CI 1.08, 2.71; $p=0.02$) in a model that also included age and FEV₁%predicted (Table 3.14B; Figure 3.5).

Considering the additional parameters in those with an FEV₁<50%, aside from FEV₁ expressed as an absolute value, QMVC (HR 0.96, 95%CI 0.93, 0.99; $p=0.02$) was the only other parameter that predicted mortality (Table 3.15). In multivariate analyses which also included QMVC as a parameter, $n=186$, type II fibre % and the presence of fibre shift ($p=0.25$ and 0.13 respectively) were not retained as independent predictors whilst QMVC was in each respective analysis (HR 0.962, 95%CI 0.930, 0.995; $p=0.03$ and HR 0.964, 95%CI 0.931, 0.997; $p=0.03$); Table 3.16. In this cohort, whilst stratifying patients according to FFMI and MTCSA did not identify those at risk of increased mortality, stratifying by QMVC/BMI did ($n=138$, $p=0.046$); Figure 3.6, although in a multivariate analysis including age, FEV₁%pred and fibre shift, none of these parameters were independent predictors of mortality.

A)

Parameter	Univariate HR (95%CI)	p-value	Multivariate HR (95%CI)	p-value
Age	1.04 (1.01, 1.07)	0.007	1.06 (1.03, 1.09)	<0.0001
Male gender	1.14 (0.67, 1.95)	0.63	-	-
FEV₁ %pred	0.97 (0.94, 0.99)	0.005	0.96 (0.94, 0.99)	<0.0001
% type II fibres	1.02 (1.00, 1.04)	0.03	1.014 (0.996, 1.032)	0.13

B)

Parameter	Univariate HR (95%CI)	p-value	Multivariate HR (95%CI)	p-value
Age	1.04 (1.01, 1.07)	0.007	1.06 (1.03, 1.09)	<0.0001
Male gender	1.14 (0.67, 1.95)	0.63	-	-
FEV₁ %pred	0.97 (0.94, 0.99)	0.005	0.96 (0.94, 0.99)	0.002
Fibre shift present	1.92 (1.22, 3.00)	0.004	1.71 (1.08, 2.71)	0.02

Table 3.14: The univariate predictors of mortality in patients with an FEV₁<50% (n=241) and multivariate analyses when including **A)** type II fibre %, and **B)** fibre shift in the model.

Parameter	Number	Univariate HR (95%CI)	p-value
FEV ₁ (L)	190	0.20 (0.08, 0.56)	0.002
BMI (kg/m ²)	188	0.95 (0.90, 1.01)	0.11
FFMI (kg/m ²)	131	0.90 (0.80, 1.04)	0.14
QMVC (kg)	186	0.96 (0.93, 0.99)	0.02
QMVC/BMI	138	0.45 (0.18, 1.12)	0.09
MTCSA (cm ²)	82	1.01 (0.99, 1.02)	0.436
TLCOc (%pred)	127	0.986 (0.970, 1.002)	0.08
RV/TLC (%)	126	0.99 (0.99, 1.00)	0.10

Table 3.15: The other univariate predictors of mortality in patients with an FEV₁<50%.

A)

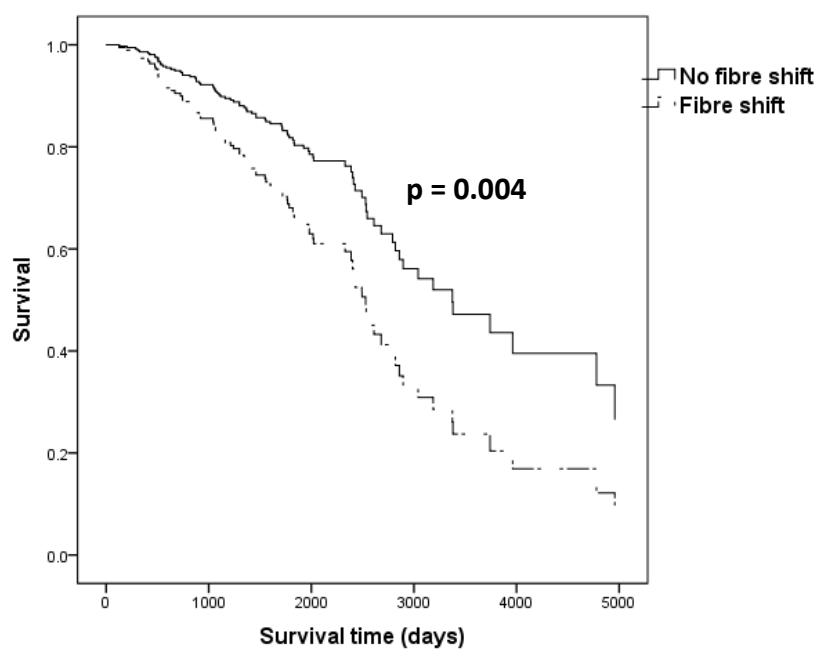
Parameter	Multivariate HR (95%CI)	p-value
Age	1.04 (1.01, 1.08)	0.01
FEV ₁ %pred	0.96 (0.94, 0.99)	0.02
% type II fibres	1.012 (0.992, 1.033)	0.25
QMVC	0.962 (0.930, 0.995)	0.03

B)

Parameter	Multivariate HR (95%CI)	p-value
Age	1.04 (1.01, 1.08)	0.02
FEV ₁ %pred	0.96 (0.94, 0.99)	0.01
Fibre shift present	1.52 (0.88, 2.63)	0.13
QMVC	0.964 (0.931, 0.997)	0.03

Table 3.16: Multivariate analyses including QMVC in patients with an FEV₁ <50% (n=186).

A)



B)

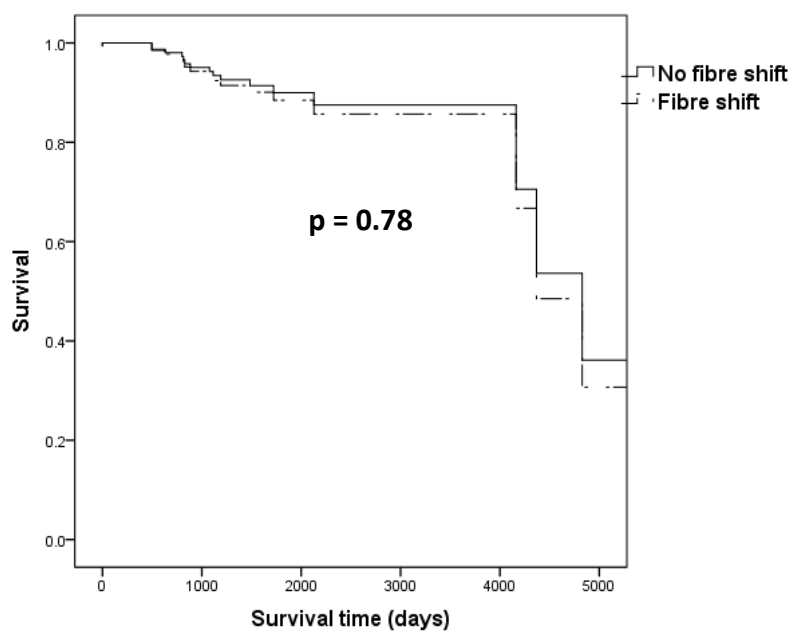
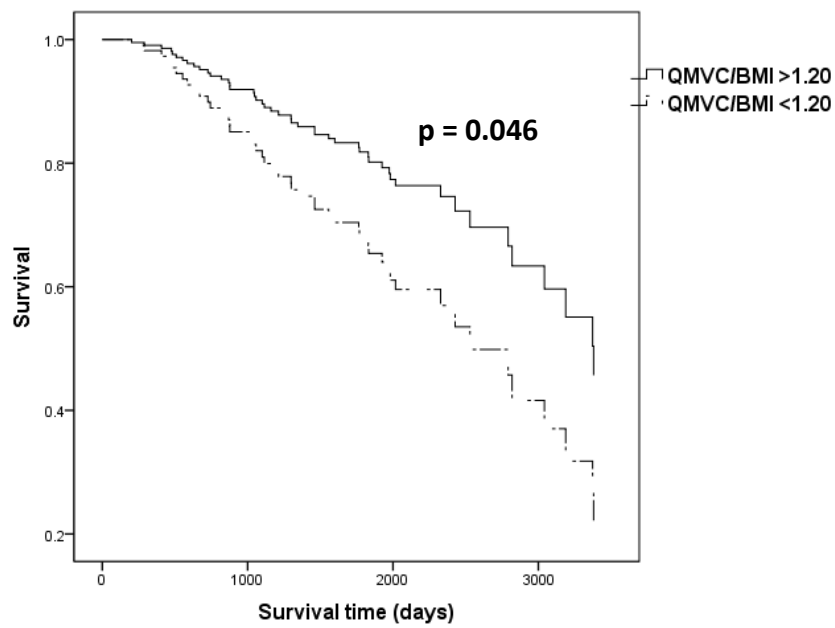


Figure 3.5: Survival curves for patients with and without fibre shift in **A)** those with an FEV₁ < 50% and **B)** those with an FEV₁ ≥ 50%.

A)



B)

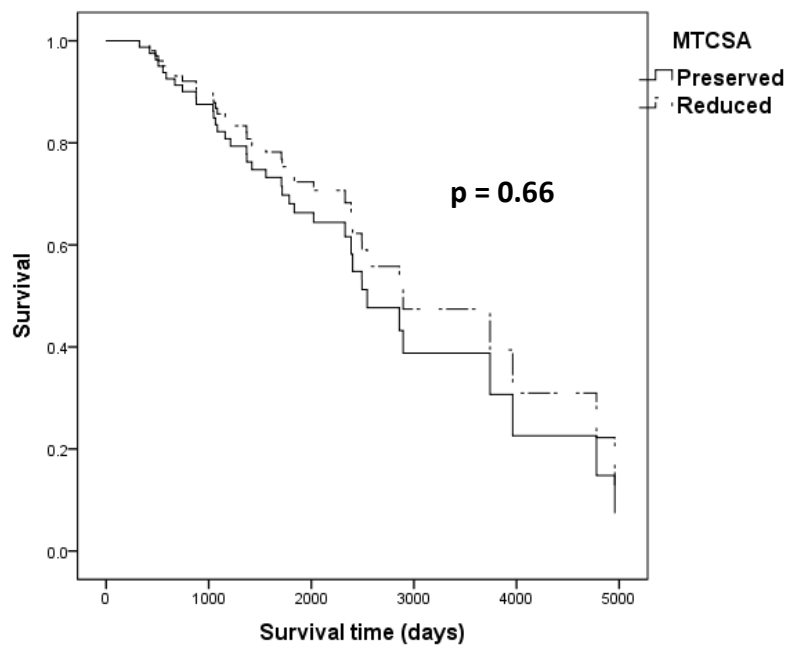


Figure 3.6: Survival curves for patients with an $FEV_1 < 50\%$, and **A)** with a QMVC/BMI of $< 120\%$ ($n=81$) and a QMVC/BMI $> 120\%$ ($n=57$) and **B)** MTCSA of $< 70\text{cm}^2$ ($n=8$) and a MTCSA $\geq 70\text{cm}^2$ ($n=74$) in patients with an $FEV_1 < 50\%$.

3.4 Discussion

3.4.1 Summary of the findings

The main finding of this study is that fibre shift in the *vastus lateralis* of COPD patients is associated with increased mortality and that this effect is independent of the recognised associations with FEV₁ and age. This finding was especially evident in patients in GOLD stage III/IV and was not detectable in GOLD I/II disease considered alone.

3.4.2 Significance of the findings

Lung function parameters and age appear to be the most important predictors of mortality; indeed age was the only parameter that was predictive of mortality irrespective of the level of airflow obstruction. This is unsurprising as ageing is characterised by physiological decline and increased age predisposes to a risk of adverse events in most populations including COPD patients (Martinez, Foster et al. 2006; Boutou, Shrikrishna et al. 2013). Moreover, given that longevity is finite, age is always a strong statistical predictor of death. For this reason while it is included in some composite indices, for example the ADO (Puhan, Garcia-Aymerich et al. 2009), it is not included in others even though addition of age strengthens prognostic power (Marin, Alfageme et al. 2013).

Measures of the severity of lung damage including those assessing airflow limitation, namely FEV₁ and the degree of emphysema, specifically TLC₀, were also retained in multivariate models (Boutou, Shrikrishna et al. 2013). FEV₁ was retained in all models either when expressed as a percentage of the predicted value or as an absolute value. Although it was not the remit of this

analysis to compare the predictive properties of different lung function indices, given that the cohort studied included COPD patients with a full range of spirometric severity, it is unsurprising that lung function indices were predictive of mortality. This observation has been previously made in connection with skeletal muscle dysfunction and COPD by Marquis *et al.* who demonstrated that the increased risk of mortality associated with a reduced mid-thigh cross sectional area was substantially less in those with an FEV₁ of greater than 50% predicted.

Several studies have shown that there is a shift towards a more glycolytic phenotype with a preponderance of type II fibres in COPD (Jakobsson, Jorfeldt *et al.* 1990; Whittom, Jobin *et al.* 1998; Gosker, Zeegers *et al.* 2007; Natanek, Gosker *et al.* 2013), and Natanek *et al.* have recently demonstrated that fibre shift, but not fibre atrophy, relates to both functional and peak exercise capacity in COPD (Natanek, Gosker *et al.* 2013). The findings of the present study validate the importance of the occurrence of fibre shift in a diverse cohort of stable COPD patients under outpatient specialist care and by confirming a relationship with mortality validate the thresholds reported by Natanek *et al.* (Natanek, Gosker *et al.* 2013). When only considering patients with GOLD stage III or IV disease, fibre type preponderance assessed either as a continuous variable or by the presence of established fibre shift were both univariate predictors, however, only the presence of fibre shift evaluated as a dichotomous variable was retained after incorporating age and the FEV₁ into the model. In this regard, whilst gender was not in itself predictive of mortality, the different fibre preponderances that are observed in males and females may be relevant and this is encompassed by the gender specific cut-offs used in this study (Natanek, Gosker *et al.* 2013).

Pertinently, the predictive properties of quadriceps strength and bulk have previously either been restricted to, or demonstrated a greater effect on survival, in those with more severe airflow obstruction (Marquis, Debigare *et al.* 2002; Swallow, Reyes *et al.* 2007). When limiting the current analysis to patients with GOLD stage III and IV disease, QMVC but not fibre shift was retained as an

independent predictor when including both variables in multivariate analysis. It should be recognised that the period of follow up and the characteristics of the cohorts in whom sub-analyses were performed were different from that of the cohort as a whole. Nonetheless, unlike the irreversible nature of chronological age and lung function impairment, the other parameters predictive of mortality in this cohort, quadriceps weakness and fibre shift respond to intervention (Bernard, Whittom et al. 1999; Vogiatzis, Terzis et al. 2011). The occurrence of fibre shift may be indicative of a reduced physiological reserve accrued over a prolonged period of time, but this is subsequently superseded by the functional relevance of reduced quadriceps strength in those with spirometrically advanced disease.

Given that quadriceps strength was not retained in the analysis of the whole cohort, fibre shift would appear to be of greater prognostic relevance than quadriceps strength across the full range of airflow obstruction. Natanek *et al.* demonstrated quadriceps morphology to be of such heterogeneity in COPD that phenotype is difficult to predict without a muscle biopsy (Natanek, Gosker et al. 2013), other recent data confirm that even patients with mild to moderate COPD may exhibit a loss of oxidative phenotype, including fibre type shift, in the absence of muscle wasting (van den Borst, Slot et al. 2013). In this regard, it may be hypothesised that fibre shift might precede loss of muscle mass; clearly a prospective study would be required to demonstrate this.

3.4.3 Critique of the Method

A population under outpatient specialist care was studied, who through referral bias are likely to have more significant disease than that observed in primary care; however, this risk was attenuated by performing sub-analyses in those with severe and milder levels of airflow obstruction. Although skeletal muscle dysfunction is present in preclinical COPD patients (Van Remoortel, Hornikx et al.

2014) and indeed muscle weakness is associated with dyspnoea in patients managed in primary care (Kelly, Elkin et al. 2013), the findings of this study are likely to be most applicable to secondary care patients.

The relevance of other structural properties of the quadriceps such as fibre size and capillarity could not be assessed as this data was not widely available; however, the relevance of these characteristics remain unclear in COPD. For example, in a large observational study, Natanek *et al.* did not identify fibre cross-sectional area to be reduced in COPD patients when compared to controls except in the marginal case of type IIx fibres which comprise below 5% of the fibre milieu (Natanek, Gosker et al. 2013), similarly, it remains to be established whether quadriceps capillarity is impaired in COPD (Whittom, Jobin et al. 1998). Nonetheless, given that performing a quadriceps muscle biopsy seems relevant in the assessment of the COPD patient, at least with regards to establishing the presence of fibre shift, comparative assessment of the structural findings identifiable from a muscle biopsy including fibre shift, fibre size and capillarity and their relationship to relevant clinical parameters warrants further study; the association of these parameters with physical performance is evaluated in Chapter 4.

It could be argued that the identification of fibre shift is impractical due to the requirement of a biopsy, in this regard, muscle biopsy is currently only undertaken in specialist research facilities. However, muscle biopsy and analysis is commonly undertaken in musculoskeletal and neurological practice and the procedure could be adopted into respiratory practice if the information obtained were to actively guide management. Furthermore, other tools may identify a likelihood of fibre shift and given that exercise capacity relates to fibre preponderance (Natanek, Gosker et al. 2013); simple functional assessment tools may obviate the need for a biopsy, in this regard the potential use of the SPPB is evaluated in Chapter 4. Furthermore, the identification of systemic biological markers of fibre shift and indeed also quadriceps strength may become possible in the future, for example,

Donaldson *et al.* recently identified that circulating levels of the muscle-specific microRNA, miR 499, may have a role in the identification of patients with fibre shift (Donaldson, Natanek et al. 2013). Nonetheless, the findings of the present study indicate that different skeletal muscle adaptations, including fibre shift and loss of strength, may not be uniformly relevant in different COPD populations. In this regard, functional and biological markers that identify both fibre shift and loss of muscle mass may be of value. Given the overall prognostic relevance of chronological age and the fact that the prognostic relevance of pathological skeletal muscle adaptations appear to be independent to this, and that the relationship between age and fibre type preponderance is very weak, altered biological ageing may be relevant to the pathogenesis of the skeletal muscle adaptations observed. A study of circulating biological markers of ageing that were identified as being potentially relevant following a review of the literature is presented in Chapter 5.

It has previously been shown that fibre shift is associated with impaired exercise performance (Natanek, Gosker et al. 2013) and that this is also associated with poor prognosis in COPD (Oga, Nishimura et al. 2003), it remains unclear whether fibre shift causes poor exercise tolerance or is a manifestation of reduced exercise capacity and physical activity, which is also of prognostic relevance (Waschki, Kirsten et al. 2011). Consequent to the retrospective nature of this analysis, measures of exercise capacity and physical activity were not available for analysis in this study. A prospective study would clearly have been preferable, and could also have encompassed other factors of relevance including relevant co-morbidities, the role of interventions such as pulmonary rehabilitation over the intervening period, exacerbation frequency, and the cause of death. Nonetheless, given the participant numbers, multicentre involvement and median follow up of more than 4 years such a prospective study of comparable size is unlikely to ever be done.

3.4.4 Conclusion

In a large outpatient COPD population, the presence of type II fibre shift was an independent predictor of survival. In patients with GOLD stage III and IV disease, quadriceps strength was a superior predictor of mortality than the presence of fibre shift. The identification of practical tools that detect quadriceps fibre shift and weakness in COPD may be clinically applicable; furthermore, identification of the molecular mechanisms pertaining to the development of these adaptations and targeted therapy is likely to be beneficial and these considerations are the focus of Chapters 4-6. In summary, these findings support the concept that fibre shift and skeletal muscle dysfunction contribute to mortality in COPD and suggests value in stratifying patients prior to entry into clinical trials, especially those where the proposed mode of action is to reverse fibre shift.

Chapter 4 : The Short Physical Performance

Battery in COPD

4.1 Introduction

4.1.1 Background

The SPPB has been recommended by consensus working groups as a screening tool for sarcopenia (Cruz-Jentoft, Baeyens et al. 2010) and also as a functional outcome measure in those who are frail (Bhasin, Cress et al. 2008). However, despite being well-characterised in older general populations, where it has been shown to identify individuals at risk of adverse events (Guralnik, Simonsick et al. 1994; Guralnik, Ferrucci et al. 1995), limited data is available in COPD.

Our group has recently validated the 4 metre gait speed and the sit-to-stand in COPD (Kon, Patel et al. 2012; Jones, Kon et al. 2013), two of the three components of the SPPB, and found that both provide useful information in the assessment of the COPD patient. Furthermore, balance, the third component of the SPPB, is impaired in COPD and deficits are associated with skeletal muscle weakness and reduced physical activity levels (Beauchamp, Sibley et al. 2012). Therefore, the SPPB provides a simple, integrated assessment of several relevant functions. Each of these tasks are relevant to daily life, those identified with having impairment may respond to intervention; in this regard our group has recently demonstrated that the sit-to-stand responds to pulmonary rehabilitation in COPD (Jones, Kon et al. 2013).

The determinants of the SPPB have not been systematically evaluated in patients with COPD, where respiratory and systemic manifestations including skeletal muscle dysfunction may affect performance. Therefore, whilst the SPPB provides valuable information in other populations, whether it has utility and adds any information beyond that detected by FEV₁ is presently unknown in COPD. The SPPB has validated cut-offs in general populations, with scores of 10-12 indicating minimal limitation, scores of 7-9 indicating limitation and scores of <7 indicating major limitation (Guralnik, Ferrucci et al. 1995). Determining the distribution of SPPB scores in a COPD population with a wide-range of airflow limitation may provide context as regards to the proportion of patients that have limitation as detected by the SPPB, or indeed whether the SPPB adequately discriminates patients with or without evidence of skeletal muscle dysfunction.

4.1.2 Aims and hypotheses

The aim of this study was to assess the characteristics of COPD patients that determine SPPB score. We hypothesized that the SPPB would detect impairment that was not detected by FEV₁ in COPD patients. Furthermore, we hypothesized that stratifying patients according to SPPB score would identify a phenotype with impaired function and abnormal features in a quadriceps biopsy. If confirmed, the SPPB could be a useful biomarker in identifying individuals who may benefit from intervention, such as pulmonary rehabilitation, and also entry into stratified medicine studies (Steiner, Roubenoff et al. 2012).

4.2 Methods

4.2.1 Study design and recruitment

This was a cross-sectional study where the data were collected prospectively from 4 studies investigating the pathophysiological determinants and associations of skeletal muscle dysfunction in COPD. Each study was approved by an ethical committee (West London REC 3: 10/H0706/9; North West London REC: 11/LO/1636; North London REC: 11/H0717/3; NRES Committee London – Chelsea: 12/LO/0523) and all patients provided written informed consent.

By design we sought to study a range of COPD severity, so patients with any degree of lung function impairment were included. 109 stable COPD patients that had been diagnosed according to GOLD guidelines (Rabe, Hurd et al. 2007) were recruited from respiratory clinics held within the Royal Brompton & Harefield NHS Foundation Trust and an ethically approved database. Patients within one month of an exacerbation or significant co-morbidity including unstable cardiac disease and predominant neurological or musculoskeletal limitation to mobilising were excluded.

4.2.2 Study measurements

Clinical features associated with SPPB

Patients performed the SPPB as according to the National Institute on Aging protocol (www.grc.nia.nih.gov/branches/ledb/sppb/); the scoring of the SPPB is shown in Figure 4.1. The SPPB consists of three components - standing balance, 4 metre gait speed and a 5 repetition sit to

stand test, each scored from 0-4, giving a total score out of 12 with higher scores indicating better performance. Phenotypic assessments were spirometry from which the FEV₁%pred (FEV₁ expressed as a percentage of that predicted) is reported, MRC, 6MWT distance, SGRQ, FFM and FFMI, QMVC - expressed as QMVC/BMI and as QMVC%pred, RF_{CSA} and daily step count (Sensewear; Bodymedia, Pittsburgh, Pennsylvania, USA). BODE and ADO scores were calculated as composite indices of mortality (Celli, Cote et al. 2004; Puhan, Garcia-Aymerich et al. 2009).

Within the same visit, inter-observer reproducibility of the SPPB was measured in 33 patients first by myself and then by another trained assessor (Damaris Ardelean, a clinician on secondment to the Harefield Pulmonary Rehabilitation Department). In a further 10 patients, inter-occasion reproducibility was assessed by measuring the SPPB on two occasions 7 days apart.

Biopsy features associated with SPPB

A *vastus lateralis* needle biopsy was performed in a convenience cohort of 31 COPD patients who in addition to the above assessments also had plethysmographic lung volumes (RV/TLC%) and gas transfer (KCOc%pred) measured. Immunohistochemistry was performed on the biopsy samples to determine fibre proportions, myofibre cross-sectional area (CSA) and the mean capillary to fibre ratio (C:Fi).

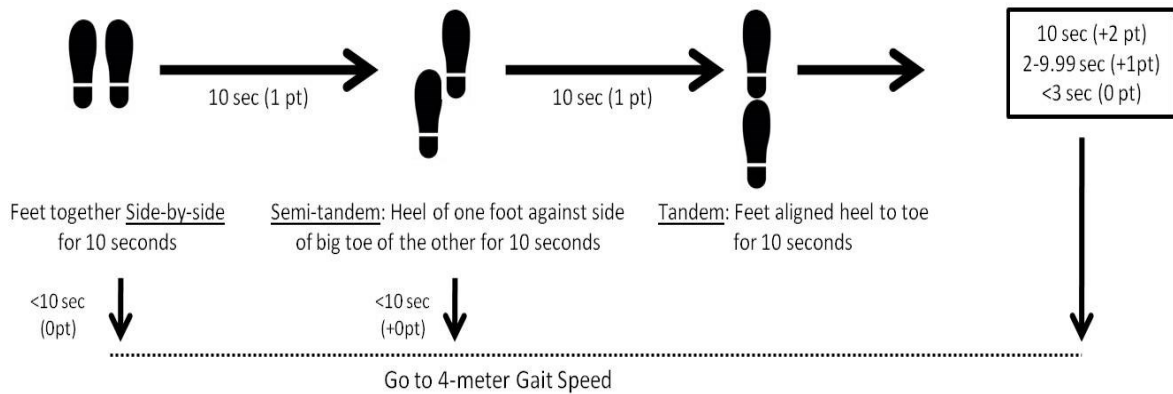
4.2.3 Statistical Analysis

Statistical analyses and graphical presentations were performed using GraphPad Prism 5 (GraphPad Software, San Diego, USA) or SPSS version 18 (IBM, USA). Significance was set at a 2-tailed p-value of ≤0.05. Spearman's rank correlation was used to assess the relationship between outcome measures.

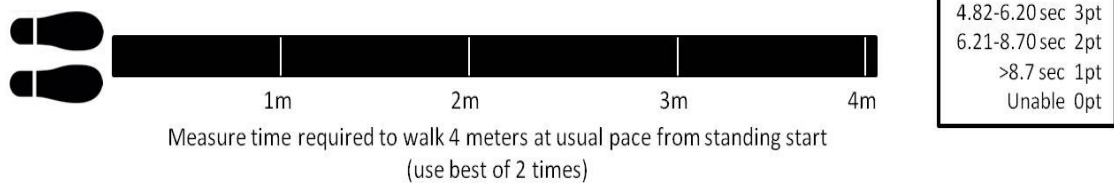
Logistic regression was performed to identify the predictors of SPPB impairment (SPPB <10). Patients were stratified according to SPPB score into 3 groups: minimal limitation (SPPB 10-12), limitation (SPPB 7-9) or major limitation (SPPB <7). Unpaired t-tests, Mann-Whitney tests or Chi-Squared test were used to compare between two groups and ANOVA or Kruskal-Wallis with post-hoc correction were used to compare 3 groups.

Short Physical Performance Battery

1. Standing Balance



2. Four-metre Gait Speed



3. Sit to Stand Test

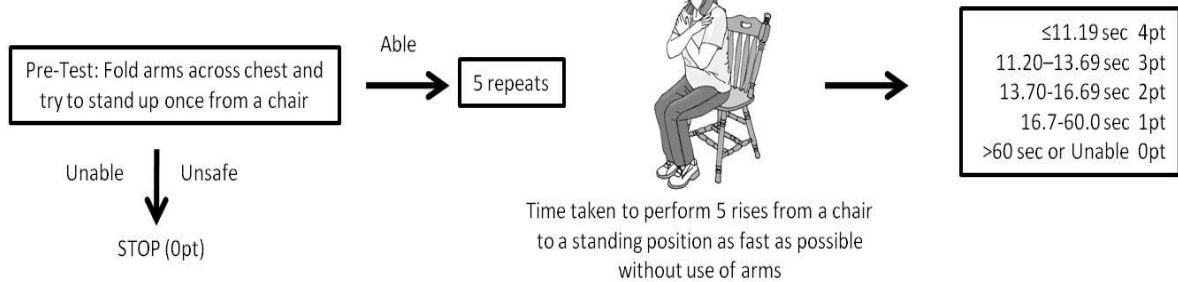


Figure 4.1: Scoring the SPPB, National Institute of Aging protocol. (www.grc.nia.nih.gov/branches/ledb/sppb/).

4.3 Results

4.3.1 Demographics and SPPB scores

109 patients were recruited; the demographic data and clinical characteristics are shown in Table 4.1. There were no differences in the demographic data or the clinical characteristics that were measured in the two cohorts. The SPPB scores achieved across the whole cohort are shown in Figure 4.2; 40% of the patients achieved a maximal score of 12.

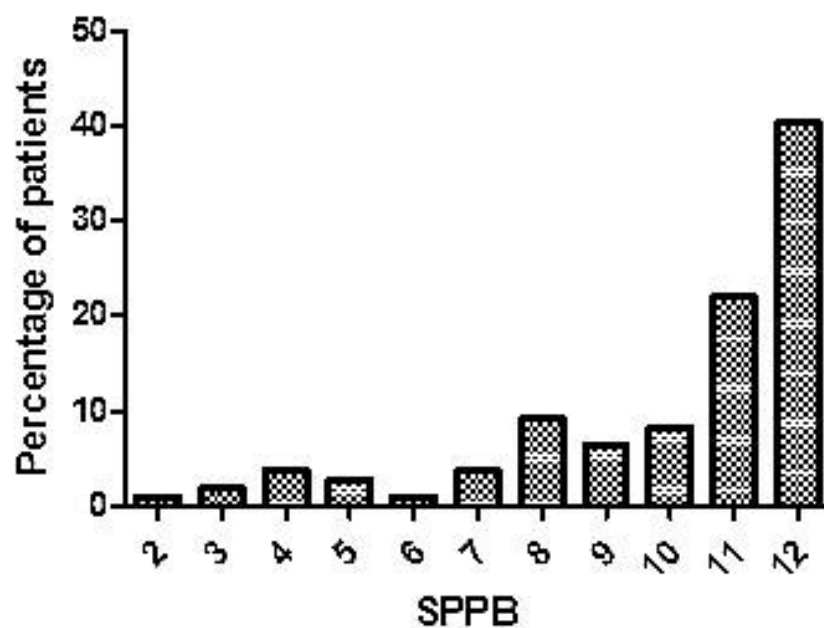


Figure 4.2: SPPB scores achieved across the cohort.

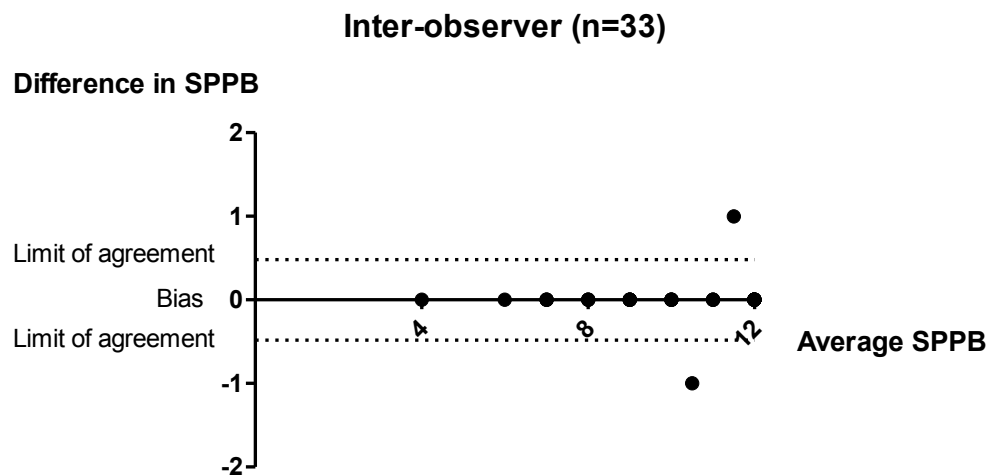
In the biopsy cohort (n=31), the muscle tissue obtained was not of sufficient size or quality to make fibre type and CSA analyses in 1 patient and capillarity measurements in 4 patients.

	Full cohort (n=109)	Biopsy cohort (n=31)	p-value
Age (years)	65 (9)	64 (10)	0.51
Gender (m:f)	45:64	16:15	0.13
Height (cm)	167 (9)	165 (10)	0.36
FEV₁%pred	48 (23)	52 (24)	0.39
RV/TLC (%)	-	50 (13)	-
KCOc%pred	-	71 (23)	-
BMI (kg/m²)	28.6 (18)	27.3 (7)	0.63
FFM (kg)	47.4 (10)	47.8 (12)	0.91
FFMI (kg/m²)	16.7 (3)	17.3 (3)	0.25
QMVC (kg)	30.0 (10)	29.5 (11)	0.80
QMVC%pred	71 (19)	67 (17)	0.24
QMVC/BMI	1.19 (0.4)	1.12 (0.4)	0.36
MRC dyspnoea	3.2 (1.2)	2.2 (1.2)	0.74
SGRQ	50 (21)	46 (22)	0.42
Steps/ day	5037 (3677)	5219 (3950)	0.84
6MWT (m)	381 (129)	393 (115)	0.62
RF_{CSA} (mm²)	529 (172)	470 (147)	0.12
BODE	3.7 (2.6)	3.4 (2.8)	0.47
ADO	4.3 (1.9)	4.3 (2.1)	0.88
SPPB score	10.1 (2.5)	10.2 (2.2)	0.85
% Type II fibres	-	62 (15)	-
Type I fibre CSA	-	4339 (993)	-
Type II fibre CSA	-	3323 (1001)	-
C:Fi	-	1.55 (0.3)	-

Table 4.1: Descriptive characteristics of the full cohort and of the sub-cohort in whom *vastus lateralis* biopsies were performed. Data presented as mean (standard deviation).

4.3.2 Reproducibility

A)

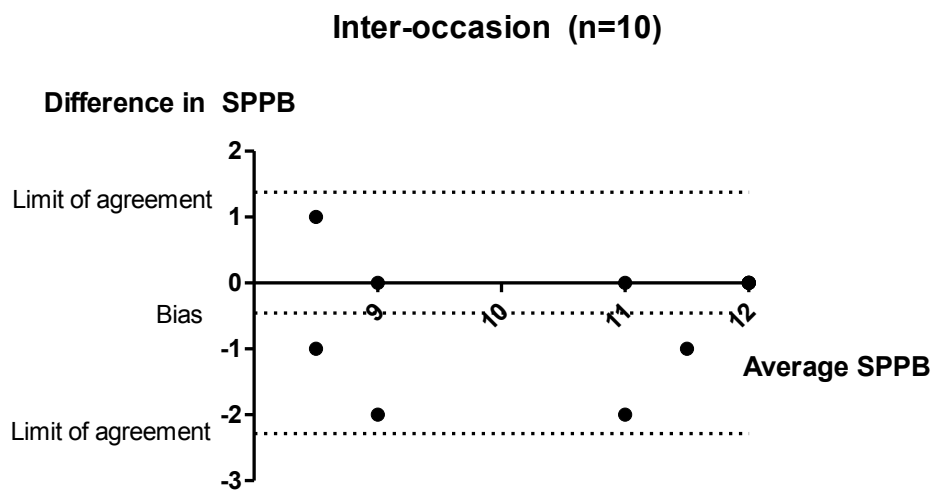


ICC 0.99 (95% CI 0.97-0.99)

Bias 0.00 (0.25)

95% limits of agreement (-0.48, 0.48)

B)



ICC 0.99 (95% CI 0.97-0.99)

Bias -0.45 (0.93)

95% limits of agreement (-2.28, 1.37)

Figure 4.3: Bland-Altman plots to demonstrate **A)** the inter-observer reproducibility and **B)** the inter-occasion reproducibility of the SPPB in COPD.

4.3.3 The relationship between the SPPB and other parameters

The relationship between the SPPB and other parameters is shown in Table 4.2.

In the cohort taken as a whole (n=109), the SPPB was unrelated to FEV₁%pred. However, the SPPB correlated significantly with the 6MWT distance and quadriceps strength, expressed as QMVC%pred and QMVC/BMI, and with RF_{CSA}, daily step count and FFM, but not FFMI. There were negative correlations with SGRQ, MRC dyspnoea and ADO and BODE indices.

In the biopsy cohort, there was no relationship between the SPPB and the % type II fibres, type I or II fibre CSA, C:Fi, nor with KCOc%pred, there was however, a weak negative relationship with RV/TLC.

	Full cohort (n=109)		Biopsy cohort (n=31)	
	r	p-value	r	p-value
Age	-0.19	0.05	0.03	0.87
Height	0.26	0.65	0.43	0.02
FEV ₁ %pred	0.05	0.60	0.39	0.03
RV/TLC	-	-	-0.43	0.02
KCOc%pred	-	-	0.21	0.29
BMI	-0.12	0.22	0.27	0.15
FFM	0.23	0.02	0.54	0.002
FFMI	0.16	0.10	0.40	0.03
QMVC	0.45	<0.0001	0.68	<0.0001
QMVC%pred	0.39	<0.0001	0.57	0.001
QMVC/BMI	0.39	<0.0001	0.48	0.006
MRC dyspnoea	-0.33	0.0003	-0.55	0.001
SGRQ	-0.33	0.002	-0.48	0.007
Steps/ day	0.22	0.047	0.28	0.14
6MWT	0.52	<0.0001	0.55	0.002
RF _{CSA}	0.46	<0.0001	0.62	<0.0001
BODE	-0.32	0.0006	-0.51	0.003
ADO	-0.28	0.003	-0.47	0.008
% Type II fibres	-	-	-0.30	0.11
Type I fibre CSA (μm^2)	-	-	0.13	0.51
Type II fibre CSA (μm^2)	-	-	0.11	0.51
C:Fi	-	-	0.27	0.17

Table 4.2: Spearman's rank correlations to assess the relationship between the SPPB and other relevant parameters.

4.3.4 Univariate and multivariate predictors of the SPPB

The clinical characteristics after stratifying into those with limitation (SPPB<10) and those with minimal limitation (SPPB 10-12) and the univariate and multivariate predictors of the SPPB are shown in Tables 4.3 and 4.4; data from the biopsy cohort are shown in Table 4.5. Daily step count could not be entered into the regression analyses as the data were not normally distributed.

In the cohort taken as a whole (n=109), age, MRC dyspnoea, SGRQ, QMVC, RF_{CSA} and 6MWT distance were univariate predictors of SPPB score. Gender, height, FEV₁%pred, BMI and FFMI were not predictors. RF_{CSA} showed a strong co-linearity with QMVC, in a multivariate analysis excluding RF_{CSA}, only QMVC (OR 0.89 (0.82, 0.96; p=0.0004)) and 6MWT (OR 0.99 (0.983, 0.996; p=0.003)) were retained as independent predictors. Even when RF_{CSA} was included in the multivariate analysis (Table 4.4), QMVC and 6MW remained the only independent predictors.

Regarding the biopsy characteristics, fibre type proportion, type I or II fibre CSA and C:Fi were not predictors of SPPB score (Table 4.5). Whilst RV/TLC was not a univariate predictor (p=0.06), KCOc%pred was (OR 0.969 (0.941, 0.999; p=0.04)), as were QMVC, RF_{CSA} and 6MWT distance. In a multivariate analysis, none of the univariate predictors were retained in the model (Table 4.5) and this did not change when RF_{CSA} was excluded from the analysis.

	Variable	SPPB ≥ 10 (n=77)	SPPB < 10 (n=32)	Univariate Analysis Odds ratio (95% CI)	p	Multivariate Analysis Odd Ratio (95% CI)	p
Demographics & spirometry	Age	64 (10)	68 (7)	1.05 (1.002, 1.11)	0.04	1.03 (0.96, 1.10)	0.45
	Gender (Female)	32 (42%)	13 (41%)	0.96 (0.42, 2.23)	0.93		
	Height (cm)	168 (10)	165 (9)	0.96 (0.92, 1.00)	0.06		
	FEV ₁ %pred	49 (24)	46 (24)	1.00 (0.98, 1.01)	0.62		
Dyspnoea & health status	MRC dyspnoea	2.9 (1.2)	3.9 (1.1)	2.23 (1.48, 3.36)	< 0.0001	1.06 (0.51, 2.19)	0.89
	SGRQ	45.6 (21)	59.2 (18.5)	1.04 (1.01, 1.06)	0.003	1.01 (0.96, 1.05)	0.76
Nutritional status	BMI (kg/m ²)	26.1 (6)	26.6 (7)	1.01 (0.95, 1.08)	0.68		
	FFMI (kg/m ²)	16.9 (2.8)	16.2 (2.5)	0.90 (0.76, 1.07)	0.25		
Quadriceps strength & bulk	QMVC (kg)	33 (9)	24 (7)	0.88 (0.82, 0.94)	<0.0001	0.89 (0.82, 0.96)	0.004
	RF _{CSA} (mm ²)	570 (161)	429 (157)	0.994 (0.990, 0.997)	0.001		
Physical activity & exercise capacity	Steps/day	5088 (2626, 7163)	2539 (1927, 5103)	-	-		
	6MWT (m)	427 (106)	269 (114)	0.987 (0.982, 0.992)	< 0.0001	0.990 (0.983, 0.996)	0.003

Table 4.3: Descriptive characteristics and logistic regression analyses of the predictors of SPPB score in the full cohort. Data presented as mean (standard deviation) or median (interquartile range).

	Variable	Univariate Analysis Odds ratio (95% CI)	p	Multivariable Analysis Odd Ratio (95% CI)	p
Demographics & spirometry	Age	1.05 (1.002, 1.11)	0.04	1.04 (0.96, 1.12)	0.33
	Gender (Female)	0.96 (0.42, 2.23)	0.93		
	Height	0.96 (0.92, 1.00)	0.06		
	FEV ₁ %pred	1.00 (0.98, 1.01)	0.62		
Dyspnoea & health status	MRC dyspnoea	2.23 (1.48, 3.36)	< 0.0001	0.73 (0.30, 1.81)	0.50
	SGRQ	1.04 (1.01, 1.06)	0.003	1.02 (0.97, 1.07)	0.38
Nutritional status	BMI	1.01 (0.95, 1.08)	0.68		
	FFMI	0.90 (0.76, 1.07)	0.25		
Quadriceps strength & bulk	QMVC	0.88 (0.82, 0.94)	<0.0001	0.88 (0.80, 0.98)	0.01
	RF _{CSA}	0.994 (0.990, 0.997)	0.001	0.997 (0.993, 1.002)	0.25
Physical activity & exercise capacity	Steps/day	-	-		
	6MWT	0.987 (0.982, 0.992)	< 0.0001	0.990 (0.983, 0.998)	0.01

Table 4.4: A multivariate regression analysis of the predictors of the SPPB when including *rectus femoris* cross-sectional area in the multivariate analysis.

	Variable	SPPB ≥ 10 (n=22)	SPPB < 10 (n=9)	Univariate Analysis Odds ratio (95% CI)	p	Multivariate Analysis Odd Ratio (95% CI)	p
Demographics	Age	63 (11)	65 (9)	1.03 (0.95, 1.11)	0.54		
	Gender (Female)	12 (55)	4 (44)	0.67 (0.14, 3.17)	0.61		
	Height	166 (10)	161 (10)	0.95 (0.88, 1.03)	0.21		
Lung function	FEV ₁ %pred	56 (25)	42 (22)	0.97 (0.94, 1.01)	0.16		
	RV/TLC (%)	47 (11)	58 (15)	1.074 (0.997, 1.157)	0.06		
	KCOc %pred	75 (20)	58 (30)	0.969 (0.941, 0.999)	0.041	0.97 (0.89, 1.05)	0.40
Nutritional status	BMI (kg/m ²)	28.3 (7.0)	24.7 (7.7)	0.92 (0.82, 1.05)	0.22		
	FFMI (kg/m ²)	18.0 (2.6)	15.8 (3.4)	0.75 (0.55, 1.03)	0.07		
Quadriceps strength & bulk	QMVC (kg)	33 (11)	21 (67)	0.81 (0.68, 0.97)	0.02	0.56 (0.26, 1.21)	0.14
	RF _{CSA} (mm ²)	528 (106)	332 (140)	0.985 (0.973, 0.997)	0.01	0.97 (0.94, 1.00)	0.08
Physical activity & exercise capacity	6MWT (m)	434 (81)	274 (113)	0.979 (0.962, 0.996)	0.015	0.99 (0.96, 1.01)	0.28
	Steps/day	5123 (3138, 7461)	2785 (1268, 5155)	-	-		
Quadriceps biopsy features	% Type II fibres	58 (15)	71 (14)	1.07 (1.00, 1.15)	0.06		
	Type I fibre CSA (µm ²)	4311 (866)	4573 (1467)	1.000 (0.999, 1.001)	0.54		
	Type II fibre CSA (µm ²)	3395 (894)	3132 (1291)	0.999 (0.998, 1.005)	0.52		
	C:Fi	1.60 (0.3)	1.40 (0.4)	0.14 (0.07, 2.81)	0.20		

Table 4.5: Descriptive characteristics of the biopsy cohort, stratified according to SPPB score and logistic regression analyses of the predictors of the SPPB when including muscle biopsy characteristics and additional lung function measurements.

4.3.5 Stratifying patients according to SPPB score

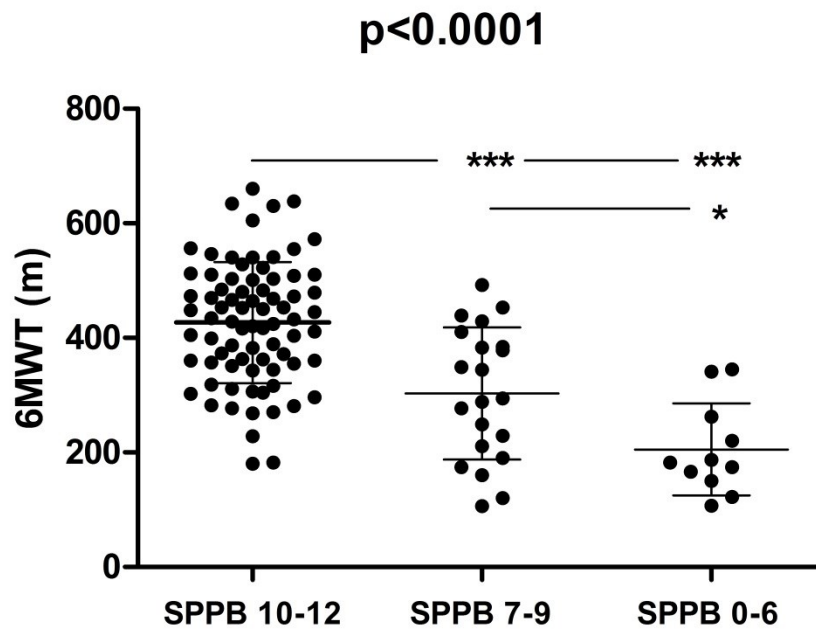
Patients were stratified into 3 groups according to SPPB score, see Table 4.6. 71% of patients had minimal limitation, 19% had limitation and 10% had major limitation (Figure 4.2). There was no difference in the demographic characteristics between the three groups, nor was there a difference in FEV₁%pred, BMI, FFM or FFMI. However, patients identified as having limitation according to SPPB score had reduced QMVC/BMI, QMVC%pred and 6MWT distance (Figures 4.4 & 4.5), and higher MRC dyspnoea and ADO scores. Patients with major limitation also had reduced daily steps, RF_{CSA} and higher SGRQ and BODE scores. Additionally, 6MWT distance was lower in those with major limitation as compared to those with limitation, Figure 4.4.

Of those with biopsy data, only 2 patients had an SPPB score of less than 7, comparisons were therefore made between patients with an SPPB score of <10 (i.e. having at least mild limitation) and those with a score of 10-12. Patients with an SPPB <10 had a higher proportion of type II fibres than those with an SPPB of 10-12, 71 (14)% v 58 (15)%; $p=0.04$, however, there was no difference in either fibre CSA ($p=0.71$) or C:Fi ($p=0.23$); Figure 4.6. The cohort with an SPPB score <10 also had a higher RV/TLC ($p=0.04$).

	SPPB 10-12 (n=77)	SPPB 7-9 (n=21)	SPPB <7 (n=11)	ANOVA p-value
Age (years)	64 (10)	67 (7)	69 (7)	0.09
Gender (m:f)	32:45	13:8	5:6	0.25
Height (cm)	168 (10)	166 (8)	163 (9)	0.11
FEV₁%pred	49 (24)	45 (20)	49 (30)	0.80
BMI (kg/m²)	25.7 (6)	26.7 (7)	26.3 (8)	0.84
FFM (kg)	49 (11)	45 (8)	42 (10)	0.17
FFMI (kg/m²)	16.9 (2.8)	16.4 (2.5)	15.8 (2.6)	0.45
QMVC (kg)	33 (9)	25 (7)**	22 (8)**	<0.0001
QMVC%pred	76 (18)	63 (15)**	56 (20)**	0.0004
QMVC/BMI	1.29 (0.40)	0.98 (0.3)**	0.91 (0.3)**	0.0003
MRC dyspnoea	2.9 (1.2)	3.6 (1.1)*	4.5 (0.8)***	<0.0001
SGRQ	46 (21)	56 (19)	66 (16)**	0.003
Steps/ day	5593 (3855)	3548 (2166)	1817 (2302)*	0.01
6MW (m)	427 (106)	303 (115)***	205 (80)***	<0.0001
RF_{CSA} (mm²)	570 (161)	463 (159)	326 (103)***	0.0002
BODE	3.1 (2.4)	4.6 (2.5)	6.2 (2.6)**	0.0006
ADO	3.9 (1.8)	5.1 (1.7)*	5.8 (1.7)**	0.0007
SPPB score	11.5 (0.7)	8.1 (0.7)	4.1 (1.1)	-

Table 4.6: The characteristics of patients, stratified according to SPPB score: minimal limitation (10-12), mild limitation (7-9) or major limitation (<7).

A)



B)

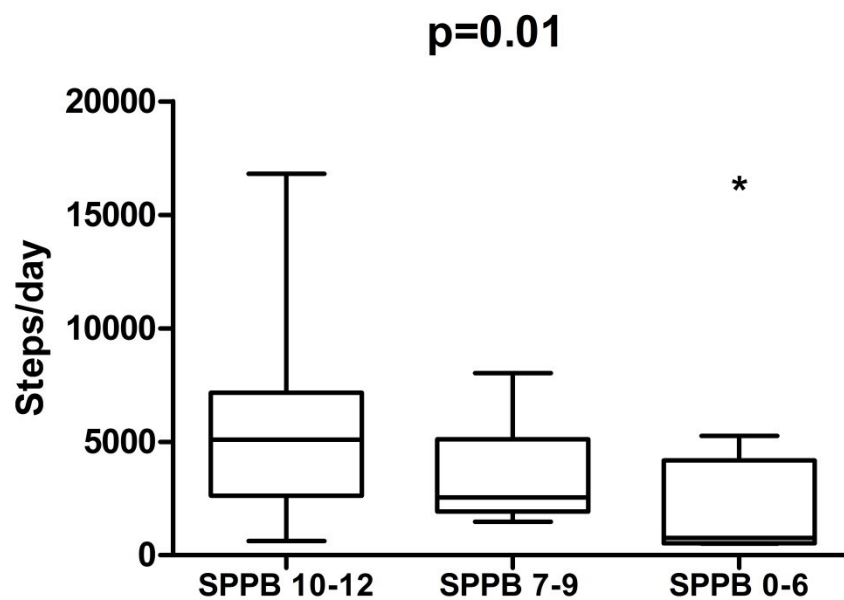
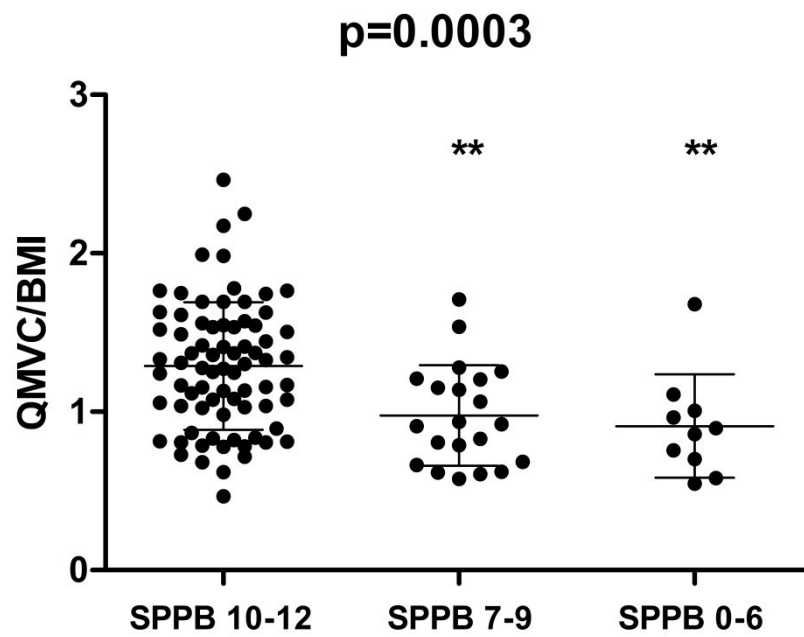


Figure 4.4: Stratifying COPD patients according to SPPB score identifies those with **A)** reduced exercise capacity and **B)** reduced physical activity.

A)



B)

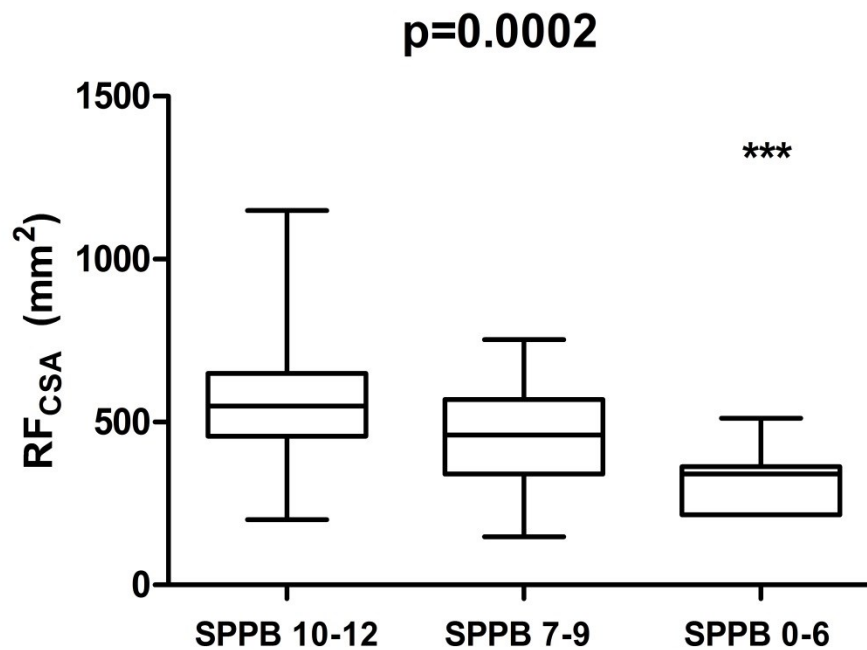


Figure 4.5: Stratifying patients according to SPPB score identifies COPD patients with **A)** reduced quadriceps strength and **B)** reduced muscle bulk.

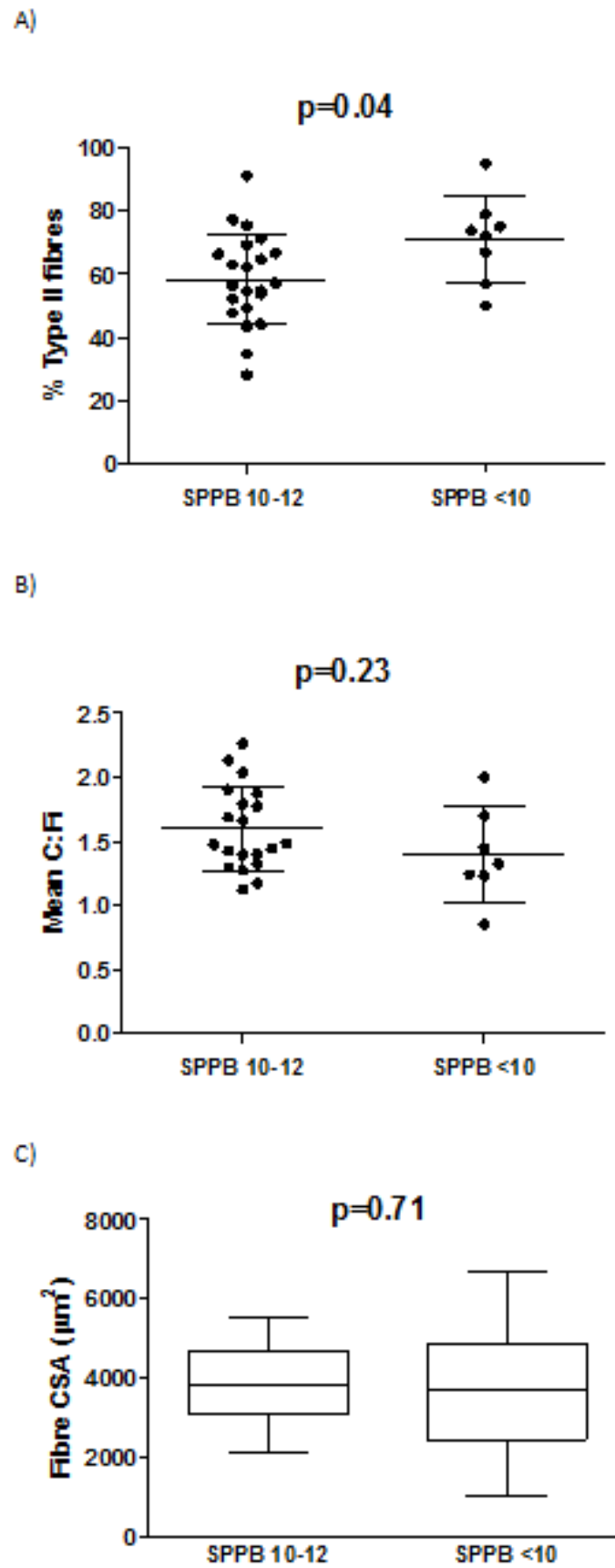


Figure 4.6: *Vastus lateralis* biopsy characteristics in COPD patients stratified according to SPPB score: **A)** fibre preponderance, **B)** capillarity, **C)** fibre size.

4.3.6 Stratifying patients according to FEV₁

Stratifying patients according to FEV₁%pred, identified patients with a reduced BMI, FFM, FFMI, daily steps and 6MWT distance, and higher MRC and SGRQ scores. However, there was no difference in QMVC/BMI, QMVC%pred, RF_{CSA}, SPPB (Table 4.7), or fibre preponderance (Figure 4.7).

	GOLD 1 (n=12)	GOLD 2 (n=35)	GOLD 3 (n=31)	GOLD 4 (n=31)	p-value
Age (years)	63 (11)	67 (11)	66 (9)	63 (6)	0.25
Gender (m:f)	7:5	18:17	12:19	10:21	0.46
Height (cm)	167 (12)	169 (9)	166 (9)	167 (10)	0.68
FEV₁%pred	91 (12)	63 (9)	39 (6)	23 (5)	-
BMI (kg/m²)	26.3 (6)	28.9 (6)	26.6 (9)	23.4 (5)	0.003
FFM (kg)	52 (14)	52 (11)	45 (8)	42 (7)	0.002
FFMI (kg/m²)	18.2 (3)	18.1 (2)	16.3 (2)	15.1 (1.9)	<0.0001
QMVC (kg)	31 (12)	32 (10)	30 (10)	27 (7)	0.26
QMVC%pred	69 (17)	75 (21)	73 (19)	67 (16)	0.31
QMVC/BMI	1.15 (0.5)	1.15 (0.4)	1.22 (0.5)	1.21 (0.3)	0.79
MRC dyspnoea	2.1 (0.8)	2.7 (1.1)	3.1 (1.2)	4.1 (0.9)	<0.0001
SGRQ	30 (22)	44 (20)	53 (18)	61 (17)	<0.0001
Steps/ day	6924 (3025)	6592 (3886)	2925 (3713)	2373 (1567)	<0.0001
6MWT (m)	434 (149)	433 (116)	383 (109)	304 (117)	<0.0001
RF_{CSA} (mm²)	563 (117)	552 (209)	513 (143)	509 (168)	0.77
SPPB score	9.7 (3.4)	10.5 (2.1)	9.9 (2.5)	10.1 (2.5)	0.48

Table 4.7: The characteristics of COPD patients stratified according to GOLD stage.

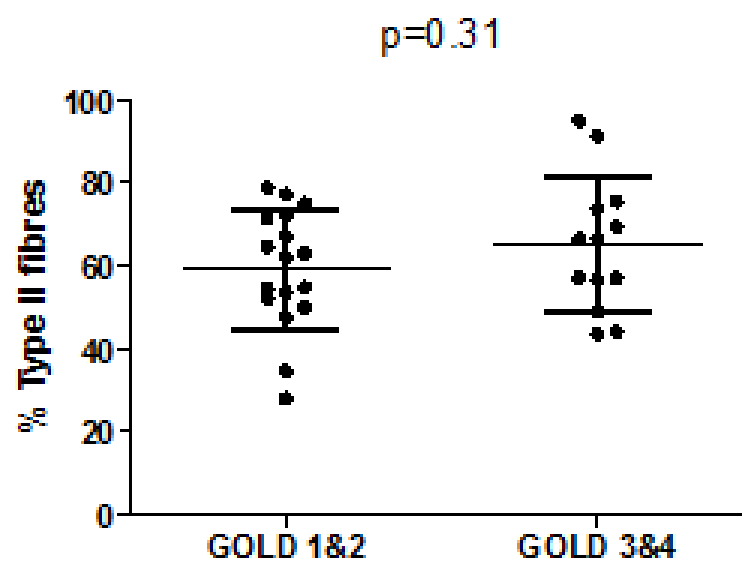


Figure 4.7: *Vastus lateralis* biopsy fibre type stratified by GOLD stage disease severity.

4.4 Discussion

4.4.1 Summary of the findings

The main finding of this study is that the main determinants of the SPPB were measures of peripheral muscle function, particularly strength, rather than lung function as judged by FEV₁. In a biopsy sub-study, patients with a low SPPB score were more likely to have evidence of fibre shift, suggesting a possible role for the test as a biomarker. The data show that the SPPB remains a valid measure of lower extremity function despite the presence of COPD and that SPPB impairment arises primarily because of skeletal muscle dysfunction. The known utility of the SPPB in unselected elderly populations and its relationship to established scoring systems in COPD (such as the BODE index) suggests a role for SPPB for the identification of frail COPD patients.

4.4.2 Significance of the findings

The SPPB has repeatedly been shown to be predictive of future adverse events in general geriatric populations (Guralnik, Simonsick et al. 1994; Guralnik, Ferrucci et al. 1995). In context, the SPPB is not the only functional field test of that has been evaluated in COPD; Puhan *et al.* evaluated the number of times patients could stand from a seated position over 1 minute and demonstrated that this test was predictive of mortality (Puhan, Siebeling et al. 2013). This version of the 'sit-to-stand test' is likely to place a greater emphasis on quadriceps endurance properties than the variant within the SPPB which assesses the time taken to stand 5 times. Of the 100 participants that were able to complete the sit-to-stand test within 60 seconds in this study, the average completion time was 12.5 seconds, in line with results that we have previously reported (Jones, Kon et al. 2013). Given that

quadriceps endurance relates to muscle phenotype (Swallow, Gosker et al. 2007), whether fibre shift or oxidative capacity predicts differential performance in these two tests is of interest.

The SPPB has been repeatedly shown to be reliable, Ostir *et al.* reported an ICC of 0.88-0.92 in more than 1000 women (Ostir, Volpato et al. 2002). Although the reproducibility data presented here is in a much smaller cohort, the SPPB seems to retain good inter-observer reproducibility in COPD, with an even better ICC of 0.99 and no evidence of bias. Although there was a level of bias (-0.45) observed when the SPPB was repeated one week later, whether this is consistent with relevant sub-clinical physiological fluctuation or a limitation in the consistency of the test is difficult to establish in this small cohort. Given that this was by design a stable population, larger reproducibility studies are warranted, as are interventional and exacerbation studies focussing on the ability of the SPPB to detect change.

In COPD, data on the SPPB is currently limited to a single large cohort that focusses on the causes and associations of functional limitation rather than the SPPB. Eisner and colleagues demonstrated that COPD patients have lower SPPB scores (Eisner, Blanc et al. 2008) and that this may predict future disability (Eisner, Iribarren et al. 2011). They also showed that increased lean-to-fat ratio (Eisner, Blanc et al. 2007) and preserved lung function (Eisner, Iribarren et al. 2008) were associated with an improved SPPB score. The participants of the present study were an older group of COPD patients, in fact a cohort closer to those for whom the SPPB was initially developed. Additionally, given our aim to evaluate the SPPB as an assessment tool in patients already diagnosed with COPD, our patients had a wider range of disease severity. This is particularly relevant as nearly a third of the patients Eisner *et al.* reported on were in the 'at risk group' with normal lung function represented by the former GOLD stage 0; under current guidelines these patients would no longer be diagnosed with COPD (Eisner, Blanc et al. 2007; Eisner, Iribarren et al. 2008).

An interesting aspect of the data presented is that while the SPPB detected patients with reduced quadriceps strength and RF_{CSA} , there was only a weak relationship with FFM and none with FFMI in the larger cohort. In fact based on the regression analyses, only quadriceps strength and 6MWT distance were independently related to the SPPB score, the fact that BMI and FFMI were not predictors supports the validity of the SPPB as a specific test of leg dysfunction rather than overall sarcopenia in COPD. This is consistent with the previously reported observation that quadriceps strength is reduced without a reduction in FFMI in patients with early spirometric disease (Shrikrishna, Patel et al. 2012). This interpretation of the data indicates that caution should be applied to using FFMI as a measure of sarcopenia where loss of muscle may be regional (as in COPD) rather than general (as for example in malignant disease).

To my knowledge, this is the first study to report the structural biopsy findings that relate to the SPPB score in any population. Those with SPPB limitation were observed to have an increased proportion of type II fibres. This is an important finding that validates the utility of the SPPB in COPD patients where quadriceps muscle dysfunction is characterised by a shift towards type II fibres, unlike the type I fibre preponderance that is present in healthy older individuals. As reviewed in Chapter 3, our group recently reported that quadriceps fibre preponderance relates to exercise capacity in COPD and established cut-offs of 65% for type II fibres in women and 68% for men (Natanek, Gosker et al. 2013). Therefore, the group with SPPB limitation had a fibre type preponderance consistent with a pathological state, whilst those without limitation did not. Given the results of Chapter 3, where patients identified as having quadriceps fibre shift using these same cut-offs were identified to have a greater risk of mortality, COPD patients with an SPPB score of below 10 may therefore also have an increased risk of mortality. There was no difference in either type I or type II fibre CSA in those with SPPB limitation, consistent with previously reported findings that fibre shift is more relevant than fibre atrophy in determining exercise performance in COPD,

and that fibre CSA is not different except when considering type IIx fibres, which are relatively infrequent (Natanek, Gosker et al. 2013).

Quadriceps muscle capillarity was not different in those with lower SPPB scores as compared to those with better performance. Muscle capillarity is not as well characterised as fibre type in COPD, it remains unclear what C:Fi values are relevant and even whether capillarity changes are universally present. Whittom *et al.* considered quadriceps capillarity to be relatively preserved in COPD (Whittom, Jobin et al. 1998). However, Eliason and colleagues showed that *tibialis* anterior capillarity was impaired in COPD and that irrespective of fibre type, C:Fi related to exercise capacity (Eliason, Abdel-Halim et al. 2010). Despite this, *tibialis* muscle dysfunction in COPD is less studied, and to some extent therefore more controversial than quadriceps dysfunction (Marquis, Debigare et al. 2009; Seymour, Ward et al. 2012). Gouzi *et al.* extended these findings to demonstrate not only that C:Fi related to QMVC and peak oxygen uptake, but there was an impaired capillarity response to pulmonary rehabilitation (Gouzi, Préfaut et al. 2013). Pertinently, these studies biopsied fewer COPD patients than the number reported on presently. Intuitively, the SPPB seems less likely to relate to muscle endurance than strength, which may explain the apparent 'disconnect' with muscle capillarity. This is supported by the fact that although 6MWT distance, which relates to muscle endurance and strength, was a predictor of SPPB score, quadriceps strength was observed to be the major independent predictor (comparative odds ratios of 0.99 (0.983, 0.996) and 0.89 (0.82, 0.96) respectively).

Most patients completed the SPPB within 4 minutes, which is in keeping with other studies (Studenski, Perera et al. 2003). The practical utility of the test is further evidenced by the minimal requirements of a short course, stopwatch and chair. It is therefore attractive as a screening tool that may be applied to community, acute or outpatient settings. When stratifying patients according to SPPB score, the between group differences observed in the 6MWT distances were more than

double the accepted clinically significant minimally important difference of the test (Polkey, Spruit et al. 2013); in fact those most impaired managed less than half the distance that better performers managed. Furthermore, unlike those with minimal limitation, patients with an SPPB score of less than 10 had a mean QMVC/BMI of below 1.2 and a 6MWT distance of below 334m, cut-offs that are predictive of mortality in COPD (Swallow, Reyes et al. 2007; Spruit, Polkey et al. 2012). There were no differences in FEV₁%pred between the groups when stratifying according to SPPB, indicating that these differences occurred irrespective of airflow obstruction. In a separate analysis, stratifying disease severity according to FEV₁%pred did not detect differences in SPPB score or relevant quadriceps parameters including strength, bulk and fibre preponderance.

Given that COPD patients are usually characterised by FEV₁%pred, the relationship with lung function warrants further evaluation. Although in the smaller sub-cohort with additional pulmonary function measures, there was a weak to moderate relationship with FEV₁%pred and with RV/TLC, the relationship with FEV₁%pred was not borne out in the larger cohort. Furthermore, neither FEV₁%pred nor RV/TLC were predictors of the SPPB in the regression analysis; although RV/TLC was higher in those with SPPB limitation and was excluded as being a univariate predictor on account of a p-value of 0.06. Despite this, KCoc% was a univariate predictor of SPPB score and although it was not a predictor after accounting for other relevant variables in the analysis, QMVC and 6MWT distance were not retained as independent predictors in this model either. Gas transfer and lung volumes may provide important information not provided by FEV₁, such as an indication of the degree of emphysema or pathological vascular adaptations. Contextually, both gas transfer and measures of hyperinflation are of prognostic relevance in COPD (Moore, Soler et al. 2010; Boutou, Shrikrishna et al. 2013). However, unlike spirometry, they are not routinely performed in community populations as they require the use of specialist equipment and expertise. In future, larger studies may establish whether the SPPB has any value in identifying patients who have significant emphysema that is disproportionate to the level of airflow obstruction detected by spirometry.

4.4.3 Critique of the Method

A limitation of this study is the cross-sectional design; longer term it will be necessary to confirm that the SPPB is predictive of increased adverse outcomes in COPD beyond the impact of lung function impairment (Boutou, Shrikrishna et al. 2013). This is likely however, as the SPPB has repeatedly been shown to be predictive of future adverse events in general geriatric populations (Guralnik, Simonsick et al. 1994; Guralnik, Ferrucci et al. 1995); conversely in a hospital based cohort we have previously found that FEV₁ provides little prognostic power once values are below 50% of that predicted (Swallow, Reyes et al. 2007). The likely prognostic value of the SPPB is further supported by the associations observed in this study with parameters recognised to be predictive of mortality in COPD, namely quadriceps strength (Swallow, Reyes et al. 2007), functional exercise capacity (Spruit, Polkey et al. 2012), physical activity (Waschki, Kirsten et al. 2011), composite mortality indices (Celli, Cote et al. 2004; Puhan, Garcia-Aymerich et al. 2009) and based on the findings reported in Chapter 3, fibre shift.

Given the hospital based setting of the study it is uncertain how applicable these findings would be to the wider COPD population. By design, patients with a wide range of airflow limitation were recruited so as to broaden the applicability of the study findings. Even so, few patients had a very low SPPB (i.e <7), in fact 40% had the maximal score demonstrating a ceiling effect. It seems likely that the SPPB has more utility as a screening tool to detect those with impairment; certainly in its present guise it would not capture improvement in better functioning patients. The value of identifying patients with lower SPPB scores is demonstrated by the observation that these patients have both physiological and functional impairment and even biopsy evidence of structural abnormality. Consequently, the SPPB does seem to have potential as a global screening tool either

for detection of patients who require closer clinical surveillance or for stratified medicine studies of agents which aim to reverse muscle atrophy or even, based on the biopsy sub-study, type II fibre shift.

Lastly, we have previously demonstrated reliability and construct validity of two components of the SPPB, namely habitual gait speed and the sit to stand test (Kon, Patel et al. 2012; Jones, Kon et al. 2013). Larger studies of the SPPB are required to evaluate whether each of the three tests (and in particular the balance component which is currently unevaluated) adds equal value to the assessment of the COPD patient. The SPPB is scored in whole integers from 0 to 12; however, habitual gait speed and sit to stand performance are initially evaluated as continuous measures. Subsequently, assessment of habitual gait speed or the sit to stand test may have greater utility in assessing deterioration or the response to intervention; both are also likely to have their own disadvantages and given the multiple functional deficits observed in COPD, it remains unclear whether they would have the same global utility as a screening tool in COPD. Other functional tests, that have also been validated in non-selected older populations, may also have clinical utility in the assessment of the COPD patient (Roig, Eng et al. 2010; Butcher, Pikaluk et al. 2012; Mesquita, Janssen et al. 2013), one example is the Timed Up and Go (TUG) test which is predictive of mortality in non-selected populations (De Buyser, Petrovic et al. 2014). The inherent nature of each functional assessment test is likely to differ; subsequently each test would require validation in COPD populations prior to widespread use.

Longer-term studies in larger cohorts may identify which functional assessment test has the greatest utility in the assessment of the COPD patient; based on the findings of this study, the SPPB seems to provide a pragmatic and clinically valid assessment of lower limb structure and function. Consistent with the accrual of biological and functional deficits that occur during the ageing process (Bhasin, Cress et al. 2008), chronological age unsurprisingly relates to SPPB score in non-selected populations

(Guralnik, Ferrucci et al. 2000), it is therefore of interest that in this cohort of COPD patients with a wide range of airflow limitation, chronological age was not independently associated with SPPB score, although quadriceps strength was. Lower SPPB scores are associated with skeletal muscle adaptations including reduced quadriceps strength and bulk, and a fibre type preponderance consistent with a higher risk of mortality in COPD (see Chapter 3) and may be considered the functional manifestation of accelerated biological ageing in susceptible COPD patients. It seems likely, that COPD patients with lower SPPB scores are at greater risk of adverse events, subsequently the biological determinants of a possible accelerated ageing process in COPD patients with skeletal muscle dysfunction warrant further study and this is the focus of Chapters 5 and 6.

4.4.4 Conclusion

The SPPB is a simple and useful tool which relates to measures of quadriceps function in COPD, independent of FEV₁. In fact, on the basis of this study, the only independent determinants of the SPPB in COPD are quadriceps strength and to a lesser extent functional exercise capacity. The SPPB may also have value as a stratification tool, especially in trials that necessitate the identification of participants with functional impairment. In context, the SPPB may be particularly useful in evaluating agents aimed at improving muscle bulk or fibre preponderance, especially given that the latter otherwise requires an invasive test to establish.

It seems relevant that a functional marker of biological ageing, the SPPB, validated as an assessment tool in non-selected populations, is applicable in the assessment of skeletal muscle dysfunction in COPD, in fact more so than FEV₁. Given that lung function and chronological age were not independently associated with SPPB score, but quadriceps strength was, biological markers of ageing may represent a therapeutic target in the management of skeletal muscle dysfunction in COPD.

Chapter 5 : Systemic markers of skeletal muscle dysfunction in COPD

5.1 Introduction

5.1.1 Background

Accelerated biological ageing may be important in the pathophysiology of COPD and indeed may be an aetiological factor in the skeletal muscle adaptations that are observed in the disease. In Chapters 3 and 4 it has been demonstrated that relevant structural and functional quadriceps adaptations occur in patients with COPD independent to chronological age, it follows therefore that other factors may be more relevant, or that susceptible individuals may be predisposed to accelerated biological ageing. As smoking is the main risk factor associated with the development of COPD, smoking may also have a direct influence on the expression of systemic agents; levels of relevant circulating factors in smokers without COPD, or indeed those who have stopped smoking may provide useful information. Potential age-related biomarkers of interest have been highlighted in section 1.1.11 and these are further discussed below, the systemic expression of Klotho and GDF-15 have not been evaluated in COPD populations; furthermore, the relevance of systemic MMP-9 in relation to skeletal muscle function has not previously been assessed in COPD. Evaluating roles for these circulating proteins in COPD may be relevant with regards to identifying potential biomarkers and also potential therapeutic targets.

Klotho knockout mice display an ageing phenotype that includes the development of emphysema, sarcopenia and a reduced life span (Kuro-o, Matsumura et al. 1997; Iida, Kanko et al. 2011). *Klotho* has a humoral role in mineral metabolism and growth factor signalling, it is a co-receptor for fibroblast growth factor-23 (FGF-23) (Kurosu, Ogawa et al. 2006) and influences vitamin D expression (Tsujikawa, Kurotaki et al. 2003). Circulating 1,25(OH)₂D relates to quadriceps strength and 25(OH)D to MHCIIa expression in those without COPD (Jackson, Shrikrishna et al. 2013), although these relationships are not present in patients with COPD. In general populations, circulating *Klotho* levels relate to grip strength (Semba, Cappola et al. 2012) and SPPB score (Crasto, Semba et al. 2012) and are associated with increased mortality (Semba, Cappola et al. 2011). Intriguingly, recent evidence demonstrates that smoking may also influence circulating *Klotho* expression (Lam-Rachlin, Romero et al. 2013).

FGF-23 is a bone derived hormone that belongs to the FGF subfamily of endocrine factors and its actions mediate renal phosphate regulation. Transmembrane *Klotho* significantly enhances the binding affinity of FGF-23 to the FGFRs (Kurosu, Ogawa et al. 2006). FGF-23 deficiency leads to an almost identical phenotype to that observed in *Klotho* deficient mice (Kurosu, Ogawa et al. 2006). The ablation of *Klotho* in mice leads to accelerated ageing in the setting of elevated levels of 1,25(OH)₂D indicating an effect of *Klotho* independent of vitamin D (Kuro-o, Matsumura et al. 1997; Kurosu, Ogawa et al. 2006; Wang and Sun 2009). The *Klotho* mouse model has also been shown to display increased MMP-9 and reduced TIMP-1 inhibitor levels in the lung, although only after the development of emphysema (Funada, Nishimura et al. 2004). This observation is of interest as other data suggests that the balance between MMPs and their endogenous inhibitors may have an important role in the lung remodelling that occurs in COPD (Vernooy, Lindeman et al. 2004; Vignola, Paganin et al. 2004). Furthermore, a role for MMP-9 activity in the skeletal muscle dysfunction of COPD is further supported by the relationship with other systemic manifestations of the disease such as arterial stiffness and skin elasticity (Maclay, McAllister et al. 2012).

TGF- β signalling plays a major role in the pathogenesis of a range of important inflammatory and fibrotic disorders (Leask and Abraham 2004). It is therefore unsurprising that TGF- β signalling has been proposed as a signalling pathway determining muscle mass. GDF-8 (myostatin), is a prominent member of the TGF- β super-family and has a potent inhibitory effect on muscle mass (McPherron, Lawler et al. 1997). GDF-15 is another member of the TGF- β signalling family. Circulating GDF-15 is associated with increased mortality in unselected general populations (Wiklund, Bennet et al. 2010) and in acute (Khan, Ng et al. 2009) and chronic (Kempf, von Haehling et al. 2007) cardiac disease. Bloch *et al.* recently evaluated circulating GDF-15 following cardiothoracic surgery that necessitated an ICU admission; circulating GDF-15 levels remained elevated for up to 1 week post-operatively in those who developed quadriceps muscle wasting compared with levels in patients who maintained quadriceps muscle mass (Bloch, Lee et al. 2013). In that study, a direct atrophic role for GDF-15 on myotubes was also demonstrated. More recently, our group has demonstrated a direct effect of GDF-15 on muscle mass in mice (Jen Lee unpublished observations).

As already discussed in section 1.1.10, increased oxidative stress may result in cellular damage with resultant functional consequences. There is a significant body of evidence supporting systemic inflammation and oxidative stress as having consequences outside of the pulmonary system in COPD; these processes are also relevant to ageing (Kirkwood 2005). The effects of chronological ageing may be accelerated by a failure of cell maintenance or repair. The reduced capacity to protect DNA against oxidative injury is an important aspect of this process (Kirkwood 2005) and may be quantified by the measurement of by-products of DNA damage that are excreted upon DNA repair; 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) is a modified nucleoside base that is commonly studied (Neofytou, Tzortzaki et al. 2012).

5.1.2 Aims and hypotheses

The aim of this study was to identify whether selected circulating markers of ageing are associated with skeletal muscle function in COPD. Specifically, to evaluate a potential role for Klotho, GDF-15 and MMP-9 in the pathophysiology of skeletal muscle dysfunction in COPD the following hypotheses were addressed. Firstly, that serum levels would be altered (elevated in the case of GDF-15 and MMP-9, reduced in the case of Klotho) in current smokers and COPD patients as compared to healthy controls. Secondly, that serum levels relate to important physiological parameters including muscle strength and bulk. Thirdly, given that in the case of Klotho, levels have recently been shown to be reduced in young smokers, the hypothesis that smoking reduces Klotho was further tested by comparing paired circulating Klotho levels before and after attempted smoking cessation. Lastly, that levels of circulating markers of skeletal muscle dysfunction would relate to a circulating marker of DNA damage, namely 8-oxo-dG. To establish that relationships, particularly those with GDF-15 and Klotho were independent to undeclared cardiac disease, serum BNP levels were also measured.

5.2 Methods

5.2.1 Study design and recruitment

Ethical approval was obtained and all participants provided written informed consent prior to study testing within the Royal Brompton & Harefield NHS Foundation Trust. Stable COPD patients were diagnosed according to the GOLD guidelines (Rabe, Hurd et al. 2007) and recruited from outpatient clinics. Controls were recruited via an ethically approved database (REC 10/H1102/36) and were further divided according to smoking status into healthy controls and 'healthy smokers'. Exclusion

criteria included exacerbation within the past 4 weeks, systemic disease including cardiac disease and significant neurological or musculoskeletal limitation to mobilising.

5.2.2 Assessments

All participants underwent venesection prior to the other tests being performed in order to minimise the effects of exercise on the biological samples. Serum samples were stored at -80°C prior to being analysed as a batch. Measurements included spirometry (FEV₁%pred is reported) (Rabe, Hurd et al. 2007), plethysmographic lung volumes (RV/TLC) (Wanger, Clausen et al. 2005; Boutou, Shrikrishna et al. 2013), carbon monoxide diffusing capacity (K_{CO}) (CompactLab system; Jaeger, Wurzburg, Germany) (Macintyre, Crapo et al. 2005), MRC (Mahler D and C. 1988), 6MWT (American Thoracic Society 2002), SGRQ (Jones, Quirk et al. 1992), FFM and FFMI (Steiner, Barton et al. 2002), dominant QMVC/BMI (Swallow, Reyes et al. 2007), RF_{CSA} and daily step count (Shrikrishna, Patel et al. 2012).

5.2.3 Smoking cessation

All current smokers who were willing to enter the smoking cessation sub-study were reviewed in smoking cessation clinic where they were given the usual smoking cessation care by healthcare providers who were not involved with study testing. Where relevant, this comprised counselling and pharmacotherapy in the form of nicotine replacement or varenicline in accordance with local and national guidelines. Participants were contacted by telephone 3 months after entering smoking cessation; those with new medical problems (including exacerbation and treatment of new medical problems) over the intervening period were excluded. 13 participants without new medical problems (8 successfully stopping) were willing to return and had repeat Klotho determinations. Exhaled carbon monoxide measurements were obtained in order to corroborate patient reported

smoking status on repeat assessment. 7 stable patients with COPD (1 current smoker, 6 former smokers) also had serum Klotho measured on two separate occasions 7 days apart.

5.2.4 Serum protein quantification

All samples were stored and treated in the same manner in preparation for each target protein quantification. Serum protein quantification was performed in batches of defrosted samples to avoid different freeze-thaw cycles affecting individual experiments. Testing was performed on separate days on samples that were defrosted on the day of experimentation in the order of MMP-9, Klotho, FGF-23, total vitamin D (25 OH D₂ and D₃), 8-oxo-DG, GDF-15 and finally BNP.

In the case of MMP-9, an activity assay that uses a detection enzyme, in its pro form, was used to quantify the presence of active MMP-9 (Biotrak, GE Healthcare, Amersham, UK). Enzyme linked immunosorbent assay (ELISA) was performed to quantify the other proteins. For the determination of Klotho in serum, the manufacturer's protocol was followed (IBL, Japan) and samples were diluted 3 fold (50 µL of test sample with 100 µL of EIA buffer) prior to Klotho protein quantification. FGF-23 ELISA (EMD Millipore, MA, USA), total vitamin D (DIAsource ImmunoAssays, S.A.), 8-oxo-DG (Trevigen, Maryland, USA), GDF-15 (R&D Systems Inc. Minneapolis, USA) and BNP fragment (Biomedica, Austria) levels were measured as according to the manufacturer's protocol for serum.

5.2.5 Statistical Analysis

Statistical analyses and graphical presentations were performed using GraphPad Prism 5 (GraphPad Software, San Diego, USA) or SPSS version 18 (IBM, USA). Significance was set at a 2-tailed p-value of ≤0.05. Unpaired t-test, Mann-Whitney or Chi-Squared tests were used to compare between two

groups and ANOVA or Kruskal-Wallis with post-hoc correction by Newman-Keuls or Dunn's multiple comparison tests were used to compare 3 groups or more. Association between variables was evaluated by Pearson's and Spearman's rank correlation and by univariate and multivariate analysis. Results are expressed as mean (SD) or median (IQR). Smoking cessation and repeat COPD measurements were analysed using paired t-tests or Wilcoxon.

5.3 Results

5.3.1 Main cross-sectional cohort

Serum MMP-9, Klotho, FGF-23, total vitamin D, 8-oxo-DG, GDF-15 and BNP measurements were made in 87 subjects (25 healthy controls, 12 'healthy smokers' and 50 COPD patients).

The clinical characteristics of the cohort are shown in Table 1. 'Healthy smokers' were on average 12 years younger and had a lower 6MWT distance and higher SGRQ scores than controls, however, smoking history aside, other parameters were not different. As expected, COPD patients had significantly higher smoking pack years, RV/TLC, MRC, SGRQ and lower FEV₁, K_{CO}, 6MWT distance and daily step count than healthy controls. RF_{CSA} measurements were not available in all participants due to the equipment not being available at the time of the patient visit; thus RF_{CSA} data was available in 12 healthy controls, 8 'healthy smokers' and 38 COPD patients. COPD patients had reduced *rectus femoris* muscle bulk as compared to both other groups; $p < 0.0001$. BNP levels were not significantly elevated in either 'healthy smokers' or COPD patients.

	Healthy (n=25)	'Healthy smokers' (n=12)	COPD (n=50)	p-value
Age (years)	63.6 (7)	51.4 (7) ***	64.7 (10)	<0.0001
Gender (M:F)	16:9	4:8	28:22	0.21
Height (m)	1.70 (9)	1.66 (11)	1.64 (23)	0.44
Smoking pack years	0.0 (0, 0)	28 (21, 48) ***	40 (28, 50) ***	<0.0001
% current smokers	0	100	42	<0.0001
FEV₁ (%pred)	106 (13)	100 (10)	53 (26) ***	<0.0001
K_{co} (%pred)	94 (14)	86 (20)	68 (24) **	0.001
RV/TLC (%)	40 (17)	36 (15)	50 (12)	0.001
BMI (kg/m²)	24.9 (4)	28.6 (7)	26.6 (7)	0.24
FFMI (kg/m²)	18.2 (3)	17.8 (3)	17.6 (3)	0.72
QMVC/BMI	1.38 (0.3)	1.39 (0.4)	1.21 (0.5)	0.15
MRC	1.0 (1, 1)	1.7 (1.7)	3.0 (2.1) ***	<0.0001
SGRQ	2 (0, 4)	25 (19) *	46 (24) ***	<0.0001
6MWT (m)	606 (70)	503 (139) * **	392 (107) ***	<0.0001
Steps/day	10775 (3881)	9021 (1444)	4921 (3542) ***	<0.0001

Table 5.1: Clinical and demographic data in the three different cohorts. Data is expressed as mean (SD) or median (IQR). Differences between groups were assessed by Chi-square and ANOVA or Kruskal-Wallis with post hoc analyses by Newman-Keuls or Dunn's multiple comparison test. *, **, *** denote respective significant differences of $p < 0.05$, $p < 0.01$ and $p < 0.001$ as compared to healthy controls.

5.3.2 The determination of active MMP-9 levels in the serum (n=87)

Active serum MMP-9 levels were not determinable in the cohort, as all measurements were below the concentration determined for the first standard (Figure 5.1) and indeed 20 of the 87 (23%) were even lower than the blank control value. All determinations were below 0.5ng/ml, the lowest detection limit of the assay as reported by the manufacturers of the assay (Biotrak, GE Healthcare, Amersham, UK).

The low determinations of active serum MMP-9 levels occurred despite a linear standard curve being achieved (Figure 5.1); although the relationship was not linear at the lowest part of the curve, the exact position where the determinations would have been made. When extending the exposure of the samples to the detection reagent to 2 hours, the standard curve began to plateau, the linearity of the standard curve was lost and 19 (22%) of the determinations remained lower than the blank control value.

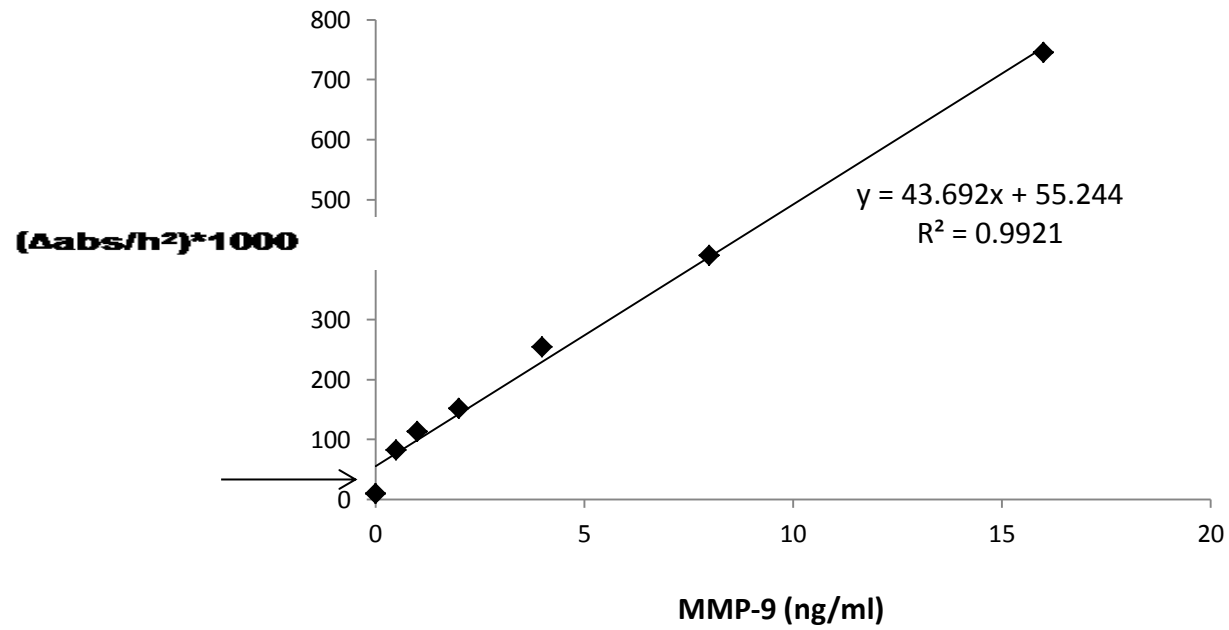
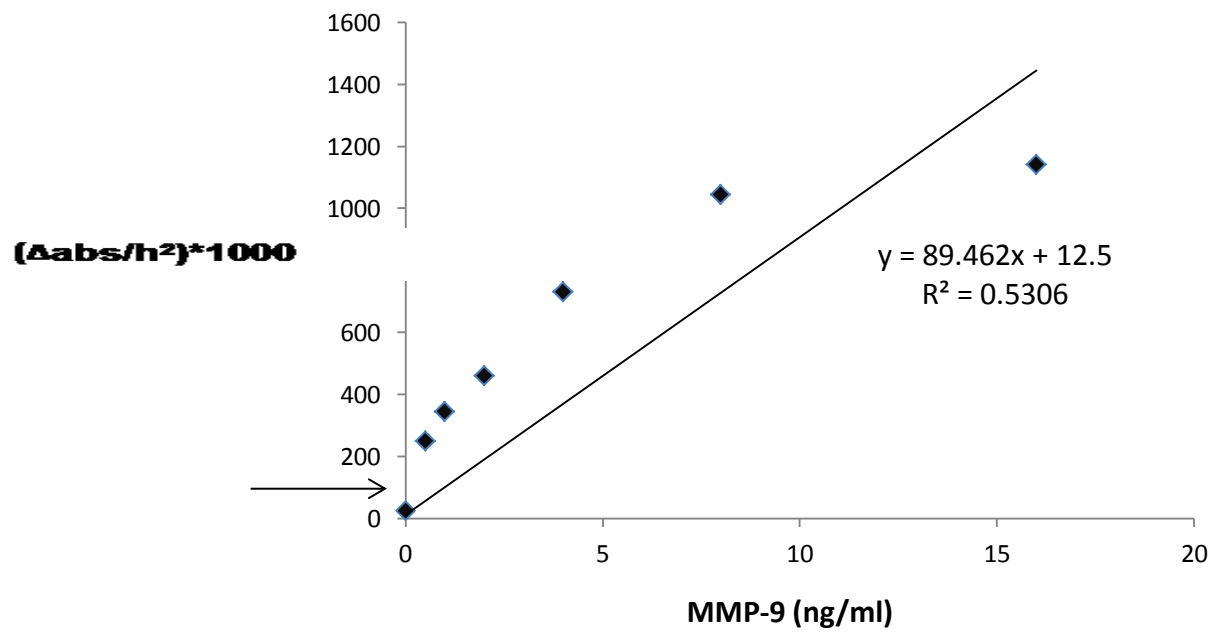


Figure 5.1: MMP-9 control standard curve at 1 hour after exposure (top) and at 2 hours after exposure to the detection enzyme (lower). The arrows show the region of the curve corresponding to sample determinations.

5.3.3 The determination of Klotho levels in the serum (n=87)

Circulating vitamin D, FGF-23 and Klotho levels are shown in Figure 5.2.

FGF-23 and Klotho have not been measured in these cohorts previously. For FGF-23, the reported lowest limit of detection is 3.5 pg/mL (EMD Millipore, MA, USA), whilst all control samples were above this value, a small proportion of 'healthy smokers' and COPD patients had values below this (Figure 3.2). The coefficient of variance was determined to be 12%, comparable to the 11.2% advised by the manufacturer (EMD Millipore, MA, USA). With respect to Klotho determinations, the manufacturers (IBL, Japan) quote the assay to have a measurement range of 93.75 to 6000pg/ml and an intra-assay coefficient of variation of <3.5%, in this cohort all measurements were within the manufacturer's reported range of sensitivity and the co-efficient of variance was measured to be 5.6%.

Vitamin D levels were lower in 'healthy smokers' (17 (10, 23) ng/ml) and COPD patients (16 (11, 26) ng/ml) as compared to never smokers (34 (18, 41) ng/ml); ANOVA $p=0.0009$. FGF-23 levels were low in all cohorts and were not different between healthy controls, 'healthy smokers' and COPD patients ($p=0.50$). COPD patients had reduced serum Klotho levels as compared to never smokers (510 (355, 644) vs 688 (501, 1091) pg/ml; $p=0.003$), but not when compared to 'healthy smokers'; $p=0.51$.

There was no relationship between serum Klotho and either vitamin D or FGF-23 levels (Tables 5.2 & 5.3) in the cohort as a whole or in any of the sub-groups. Vitamin D levels modestly related to age, smoking pack years, FEV₁, MRC, SGRQ and steps/ day, but not the muscle related parameters of BMI, FFMI, RF_{CSA} or QMVC/BMI. Indeed in those with COPD, vitamin D levels only related to age. FGF-23 weakly correlated with BMI and FFMI, and this relationship was slightly stronger when COPD patients were considered alone. Klotho levels correlated with QMVC/BMI and RF_{CSA} in the cohort

taken as a whole, whilst in COPD the only relationship present was a weak correlation with QMVC/BMI (Tables 5.2 & 5.3). Klotho levels also related to QMVC/BMI in the 'healthy smokers' ($r=0.43$, $p=0.02$), but not in those who had never smoked ($r=0.14$, $p=0.43$). Neither FGF-23 nor Klotho levels correlated significantly with other parameters in the cohorts without COPD. There was no relationship between serum Klotho levels and BNP levels.

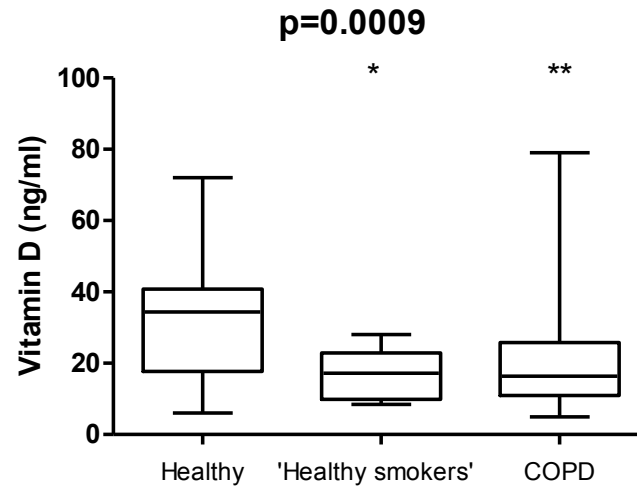
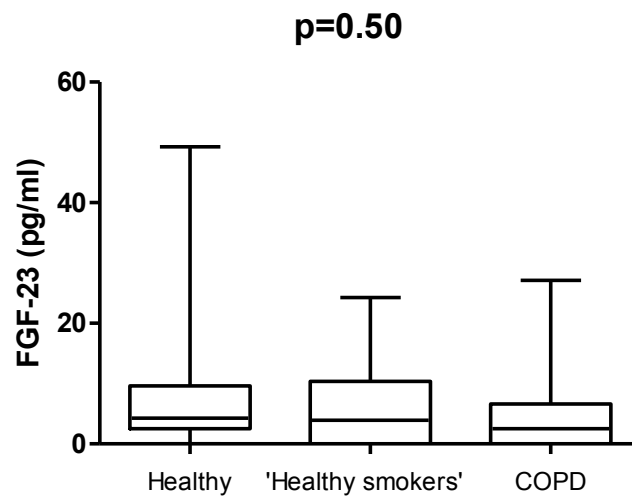
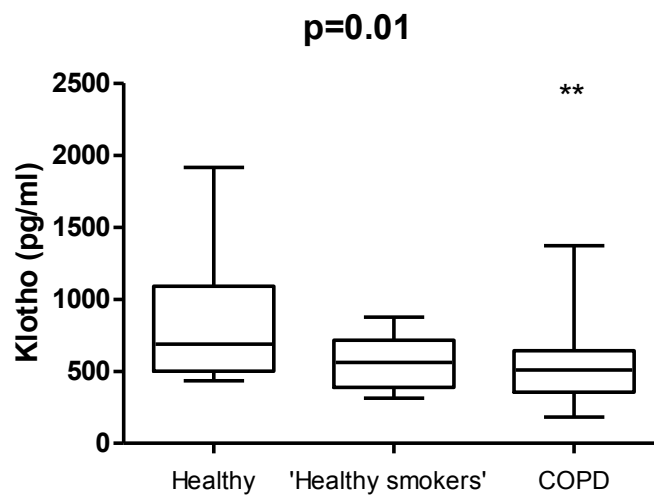


Figure 5.2: Serum vitamin D levels (top), FGF-23 levels (middle) and Klotho levels (lower) in healthy controls (n=25), 'healthy smokers' (n=12) and COPD patients (n=50).

	Mean (SD)	Vitamin D		FGF-23		Klotho	
		r	p-value	r	p-value	r	p-value
Age (years)	63 (10)	0.28	0.0009	-0.09	0.39	-0.18	0.39
Pack years	32 (30)	-0.34	0.002	-0.15	0.19	-0.10	0.36
FEV ₁ (% pred)	76 (32)	0.26	0.01	0.10	0.36	0.20	0.06
KCOc (% pred)	75(25)	0.23	0.15	0.15	0.22	0.08	0.54
RV/TLC (% pred)	46 (14)	0.15	0.96	-0.20	0.10	0.08	0.55
BMI (kg/m ²)	26.4 (6)	-0.10	0.35	0.28	0.01	-0.07	0.54
FFMI (kg/m ²)	17.9 (3)	0.06	0.60	0.22	0.042	0.09	0.41
QMVC/BMI	1.28 (0.4)	0.09	0.43	-0.06	0.60	0.35	0.001
RF _{CSA} (mm ²)	544 (176)	0.07	0.60	0.02	0.89	0.27	0.04
MRC	2.3 (1.3)	-0.37	<0.0001	-0.06	0.57	-0.33	0.002
SGRQ	36 (27)	-0.33	0.005	-0.06	0.65	-0.20	0.11
Steps/day	6974 (4309)	0.35	0.002	0.10	0.40	0.20	0.09
6MWT (m)	466 (144)	0.18	0.10	0.06	0.62	0.37	0.001
Vitamin D (ng/ml)	19 (12, 29)	-	-	-0.02	0.86	0.09	0.39
FGF-23 (pg/ml)	3.6 (0, 7.1)	-0.02	0.86	-	-	0.12	0.28
Klotho (pg/ml)	538 (444, 716)	0.09	0.39	0.12	0.28	-	-

Table 5.2: The relationship between serum vitamin D, FGF-23 and Klotho levels with other relevant parameters in 25 healthy controls (16 male, 9 female), 12 'healthy' smokers (4 male, 8 female), 50 COPD patients (28 male, 22 female). Spearman's rank correlations.

	Mean (SD)	Vitamin D		FGF-23		Klotho	
		r	p-value	r	p-value	r	p-value
Age (years)	65 (10)	0.42	0.003	-0.14	0.33	-0.21	0.15
BMI (kg/m²)	26.6 (7)	0.06	0.67	0.38	0.007	-0.11	0.44
FFMI (kg/m²)	17.6 (3)	0.06	0.67	0.32	0.03	-0.11	0.48
QMVC/BMI	1.2 (0.5)	0.00	1.00	-0.18	0.20	0.31	0.03

Table 5.3: The relationship between serum vitamin D, FGF-23 and Klotho levels in 50 COPD patients (28 male, 22 female). Spearman's rank correlations.

5.3.4 The relationship of Klotho to smoking and QMVC/BMI (n=171)

Given the relationship observed between serum Klotho levels and QMVC/BMI in the main cross-sectional cohort, an extended dataset was established, to identify whether serum Klotho levels are predictive of QMVC/BMI. Furthermore, given that the 'healthy smokers' were significantly younger in the main cross-sectional cohort (n=87) and that Klotho levels fall with age (Crasto, Semba et al. 2012), further analyses were performed on additional samples obtained from other studies within the laboratory to identify whether smoking status influences Klotho levels in an age group at risk of developing COPD. Serum Klotho levels were therefore determined in 105 COPD patients, 30 'healthy smokers' without airflow obstruction (20 current, 10 former smokers) and 36 age-matched never smokers (healthy controls), see Table 5.4 and Figure 5.3; these totals included the patients described in section 5.3.3.

COPD patients had reduced serum Klotho levels as compared to never smokers (566 (440, 678) vs 688 (495, 897) pg/ml; $p=0.006$), but not when compared to 'healthy smokers'; Figure 5.3. Considering smoking status, irrespective of spirometry, the median Klotho levels were 536 (428, 740) pg/ml for current smokers (n=51), 570 (444, 671) pg/ml for ex-smokers (n=84) and 688 (495, 897) pg/ml for never smokers (n=36); $p=0.02$ (Figure 5.4).

Among participants with normal spirometry, Klotho levels were lower in those with a past or current history of smoking as compared to never smokers (523 (438, 716) vs 688 (495, 897) pg/ml; $p=0.03$), despite being on average 10 years younger. When also including COPD patients in the analysis, 'healthy smokers' did not have lower Klotho levels than the controls (Figure 5.3A) unless parametric analysis of log transformed data was performed (Figure 5.3B), as might be considered appropriate

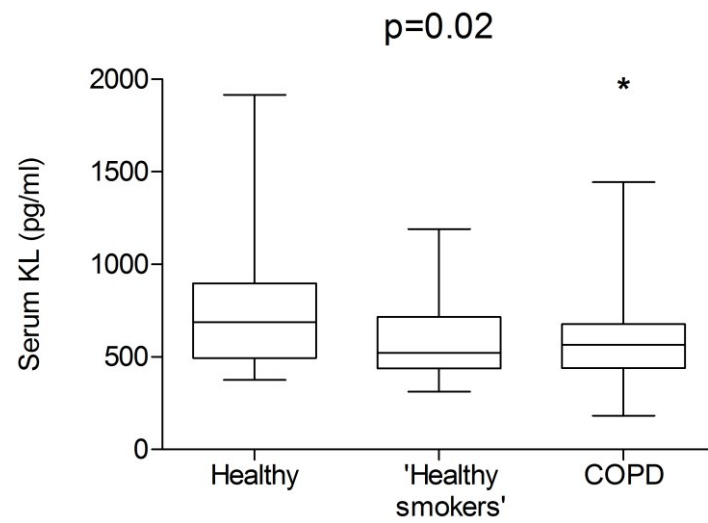
since the data were otherwise not normally distributed. There was no difference in Klotho levels between COPD current and ex-smokers ($p=0.63$, Figure 5.4B).

The relationship between serum Klotho levels and QMVC/BMI seen in the main cross-sectional dataset ($n=87$), was also observed in this larger dataset ($n=171$); see Figure 5.5. Given the evidence of a smoking and disease effect on systemic Klotho levels and the multiple determinants of quadriceps strength, to establish whether serum Klotho levels predicted QMVC/BMI after accounting for other relevant variables, a regression analysis was performed (Tables 5.5 and Table 5.6). In the larger dataset without RF_{CSA} measurements, serum Klotho was retained in a model also including age, gender, COPD diagnosis and FFM, whilst in the dataset including RF_{CSA} , only serum Klotho, gender, RF_{CSA} and FFM were retained.

	Healthy controls (n=36)	'Healthy smokers' (n=30)				COPD (n=105)				Overall p-value
		Current Smokers (n=20)	Former Smokers (n=10)	Mean (SD)	vs control	Current Smokers (n=31)	Former Smokers (n=74)	Mean (SD)	Vs control	
Age (years)	65 (8)	51 (11)	64 (5)	55 (11)	***	63 (10)	65 (9)	65 (9)	NS	<0.0001
Gender (M:F)	21:15	10:10	8:2	18:12	NS	20:11	45:29	65:40	NS	0.90
Pack years	0 (0)	33 (26)	27 (17)	31 (23)	***	52 (25)	39 (19)	43 (22)	***	<0.0001
FEV ₁ %pred	106 (11)	100 (14)	107 (13)	102 (14)	NS	53 (21)	55 (25)	54 (24)	***	<0.0001
BMI (kg/m ²)	25.0 (4)	27.1 (6)	30.9 (4)	28.3 (6)	NS	26.6 (7)	26.3 (6)	26.4 (6)	NS	0.07
FFMI (kg/m ²)	17.9 (3)	18.6 (4)	20.5 (2)	19.3 (3)	NS	18.0 (3)	17.4 (3)	17.6 (3)	NS	0.02
QMVC/ BMI	1.40 (0.4)	1.49 (0.4)	1.33 (0.3)	1.43 (0.4)	NS	1.3 (0.5)	1.20 (0.4)	1.23 (0.4)	NS	0.01

Table 5.4: Demographics of healthy controls, 'healthy smokers' (current and former smokers) and COPD patients (n=171) in the larger dataset in whom serum Klotho levels were determined. Differences between groups were assessed by Chi-square and ANOVA or Kruskal-Wallis with post hoc analyses by Newman-Keuls or Dunn's multiple comparison test. NS, *, ** or *** denote respective p-values of >0.05, ≤0.05, <0.01 and <0.001 as compared to healthy controls. The data are presented as mean ± SD.

A)



B)

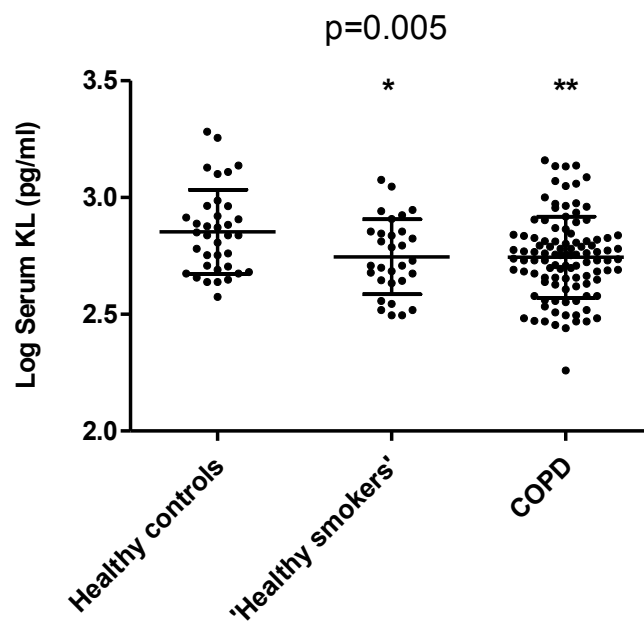
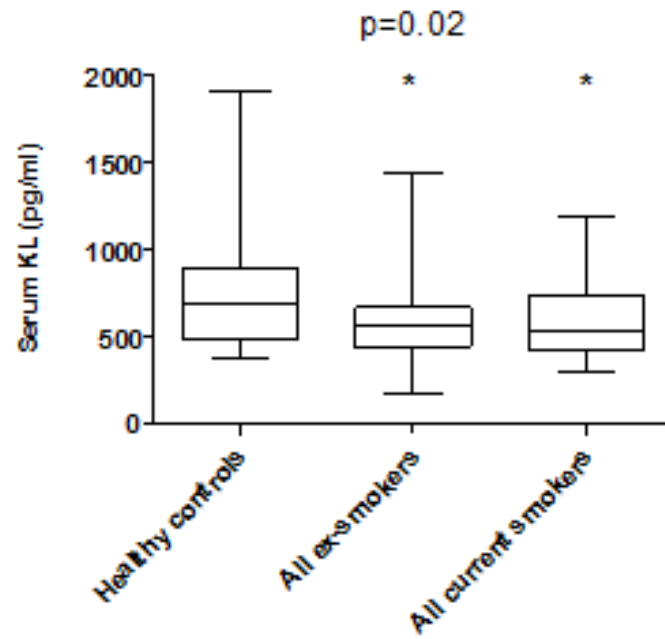


Figure 5.3: Serum Klotho in healthy never smokers (n=36), 'healthy smokers' (n=30) and patients with COPD (n=105). The data in **A)** are the absolute values, log values are presented in **B)**. Overall p-value presented, * denotes significance at $p < 0.05$ as compared to healthy controls.

A)



B)

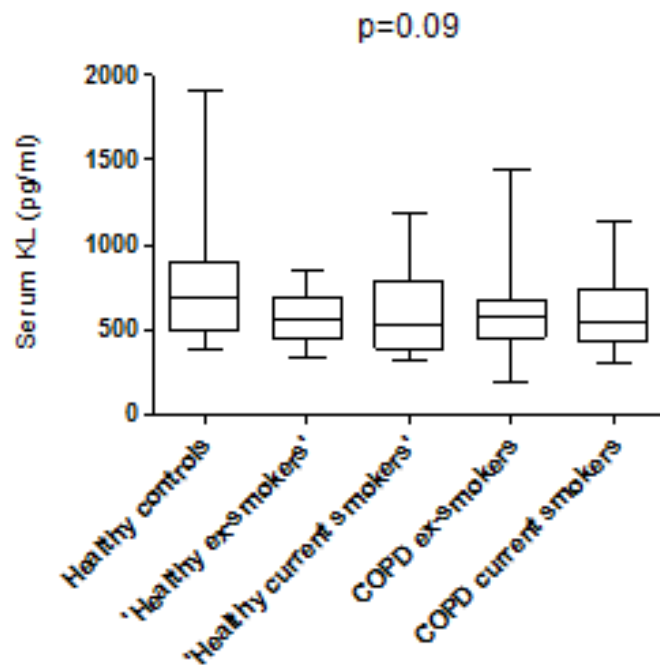


Figure 5.4: Serum Klotho in healthy never smokers (n=36) and in **A)** all current smokers (n=51) and all ex-smokers (n=84) and in **B)** 'healthy ex-smokers' (n=10), 'healthy current smokers' (n=20), COPD ex-smokers (n=74) and COPD current smokers (n=31).

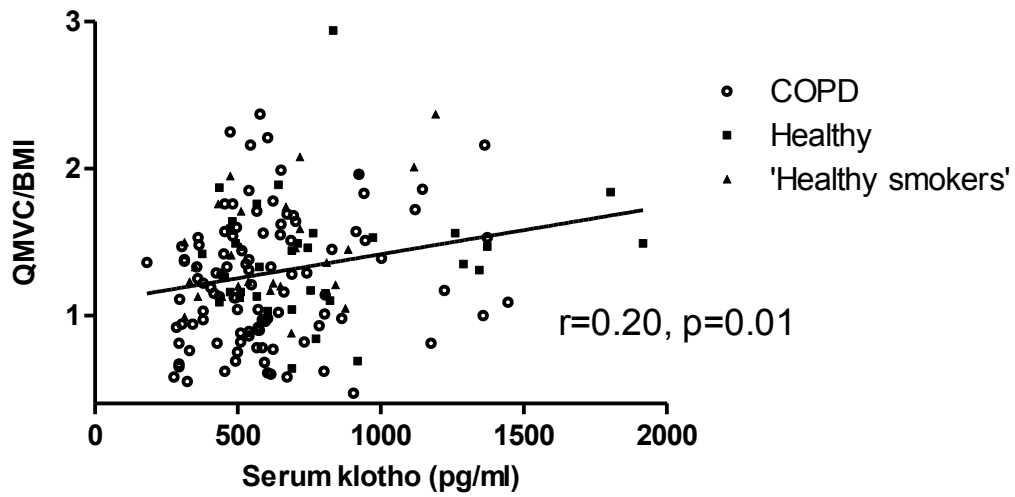


Figure 5.5: XY plot demonstrating the relationship between serum Klotho levels and quadriceps strength in healthy controls (n=36), 'healthy smokers' (n=30) and COPD patients (n=105).

Variable	Univariate regression		Multiple regression		
	Coefficient (95% CI)	p	Coefficient (95% CI)	Standardised coefficient	p
Age	-0.012 (-0.018, -0.005)	< 0.0001	-0.010 (-0.016, -0.004)	-0.24	0.001
Male gender	0.37 (0.25, 0.49)	< 0.0001	0.52 (0.37, 0.66)	0.60	< 0.0001
Serum Klotho	0.0009 (0.0002, 0.0016)	0.007	0.0009 (0.0003, 0.0014)	0.20	0.003
Ever smoked	-0.14 (-0.29, 0.02)	0.09			
COPD	-0.17 -0.29, -0.04)	0.01	-0.14 (-0.25, -0.02)	-0.16	0.022
FEV ₁ (%pred)	0.00 (-0.002, 0.003)	0.58			
FFM	0.007 (0.002, 0.012)	0.01	-0.009 (-0.015, -0.003)	-0.26	0.004

Table 5.5: Univariate and multivariate regression analysis to determine the predictors of QMVC/BMI (n=171).

Variable	Univariate regression		Multiple regression		
	Coefficient (95% Confidence Interval)	p-value	Coefficient (95% Confidence Interval)	Standardised coefficient	p-value
Age	-0.011 (-0.020, -0.002)	0.02	-0.00 (-0.01, 0.01)	0.06	0.999
Male gender	0.30 (0.13, 0.46)	0.001	0.45 (0.18, 0.71)	0.54	0.001
Serum Klotho	0.0011 (0.0004, 0.0019)	0.004	0.001 (0.0001, 0.0021)	0.26	0.03
Ever smoked	-0.13 (-0.33, 0.06)	0.18	-	-	-
COPD	-0.173 (-0.345, 0.002)	0.05	-	-	-
FEV ₁ (%pred)	0.001 (-0.002, 0.004)	0.44	-	-	-
FFM	0.0078 (0.0008, 0.0147)	0.03	-0.013 (-0.024, -0.002)	-0.40	0.03
RF _{CSA}	0.0009 (0.0003, 0.0015)	0.002	0.0009 (0.0003, 0.0015)	0.38	0.005

Table 5.6: Univariate and multivariate regression analysis to determine the predictors of QMVC/BMI in the dataset with additional RF_{CSA} measurements (main cohort).

5.3.5 The effect of smoking cessation on circulating Klotho levels

13 patients were assessed before and after attempted smoking cessation, 11 of whom had exhaled CO levels of 10ppm or more on initial assessment. 8 patients were successful in smoking cessation and returned for post-cessation assessment (Table 5.7); all had stopped smoking for more than 4 weeks and had exhaled CO levels below 10ppm on repeat assessment. Serum Klotho levels increased from 677 (416, 886) to 1098 (771, 1331) pg/ml after successful smoking cessation; $p=0.04$ (Figure 5.6). In those attempting but who were unsuccessful in achieving smoking cessation ($n=5$), Klotho levels before and after the quit attempt were not different; $p=0.81$ (Figure 5.6). There was no difference in the before and after change in Klotho levels between those successful and those unsuccessful in smoking cessation; $p=0.52$. Serum Klotho levels were not different in 7 stable COPD patients when repeated 1 week later (578 (454, 615) to 570 (525, 626) pg/ml; $p=0.84$) (Table 5.8, Figure 5.6). Comparing the differences between repeat measurements in stable COPD patients to those attempting smoking cessation, those successful in smoking cessation had a greater change on repeat assessment (423 ± 469 v -5 ± 68 pg/ml; $p=0.03$). 25 participants did not return for the post-attempt smoking cessation visit, the reasons underlying this are presented in Figure 5.7.

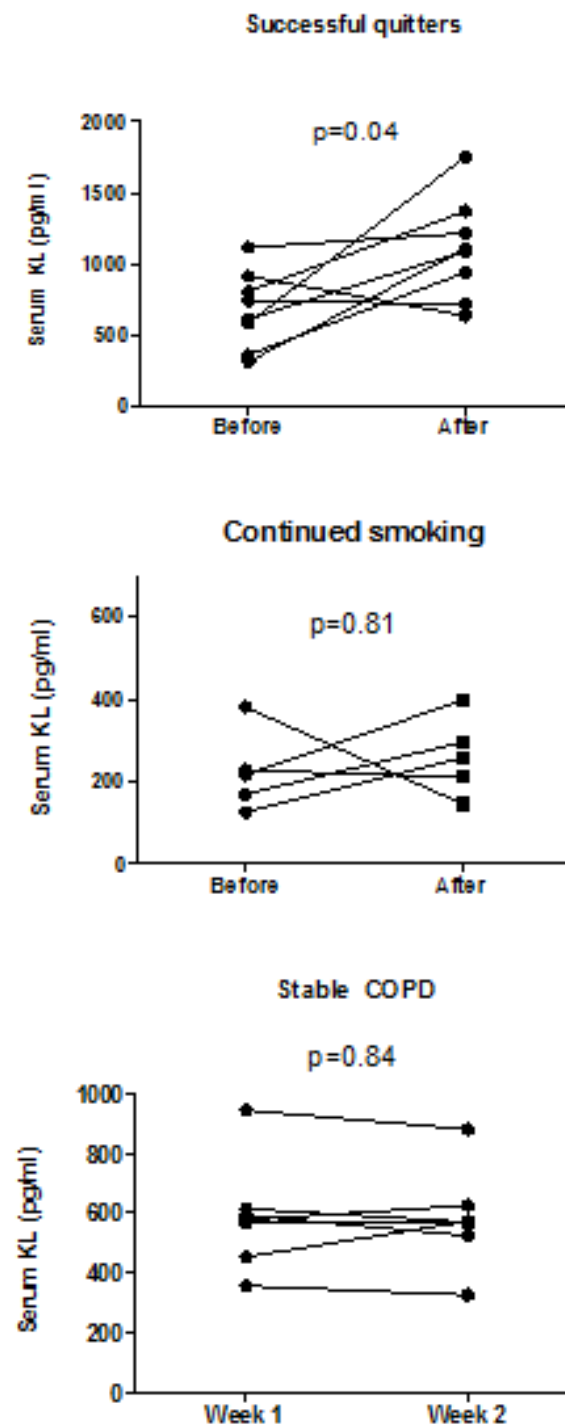


Figure 5.6: Serum Klotho levels: Before and after successful smoking cessation (top), in those unsuccessful in achieving smoking cessation (middle) and stable COPD patients 1 week apart (lower).

	Successful (n=8)	Not successful (n=5)	p-value
Age (years)	59 (12)	64 (9)	0.34
Gender (M:F)	3:5	4:1	0.27
Pack years	35 (14)	71 (48)	0.09
% with COPD	50	100	0.10
CO level (ppm)	14 (6)	19 (13)	0.66
FEV ₁ (%pred)	79 (27)	45 (27)	0.049
K _{co} (% pred)	70 (11)	50 (24)	0.07
RV/TLC (%)	42 (14)	58 (8)	0.04
BMI (kg/m ²)	23.9 (5)	22.6 (5)	0.63
FFMI (kg/m ²)	16.7 (2.4)	17.7 (2.3)	0.45
QMVC/BMI	1.32 (0.4)	1.42 (0.4)	0.67
MRC	1.9 (1.0)	3.4 (1.1)	0.04
SGRQ	29 (25)	53 (23)	0.13
6MWT (m)	543 (61)	374 (167)	0.03
Steps/ day	8084 (3961)	4193 (3275)	0.09
RF _{CSA} (mm ²)	589 (157)	465 (86)	0.38
Serum Klotho (pg/ml)	680 (271)	674 (290)	1.00

Table 5.7: Baseline demographic and physiological data in those completing and those not successful in completing smoking cessation. Differences between groups were assessed by Chi-square and t-test or Mann-Whitney. Data expressed as mean (SD).

Age (years)	71 (6)
Gender (M:F)	6:1
Pack years	39 (14)
% with COPD	100
% current smokers	29
FEV ₁ (%pred)	61 (17)
K _{CO} (% pred)	80 (14)
RV/TLC (%)	49 (6)
BMI (kg/m ²)	27.5 (5)
FFMI (kg/m ²)	17.3 (1.6)
QMVC/BMI	1.50 (0.51)
MRC	2.14 (0.69)
SGRQ	37 (19)
6MWT (m)	494 (118)
Steps/ day	6316 (4157)
Serum Klotho (pg/ml)	587 (183)

Table 5.8: Demographic and physiological data in the 7 stable COPD patients in whom serum Klotho levels were measured 1 week apart. Data expressed as mean (SD).

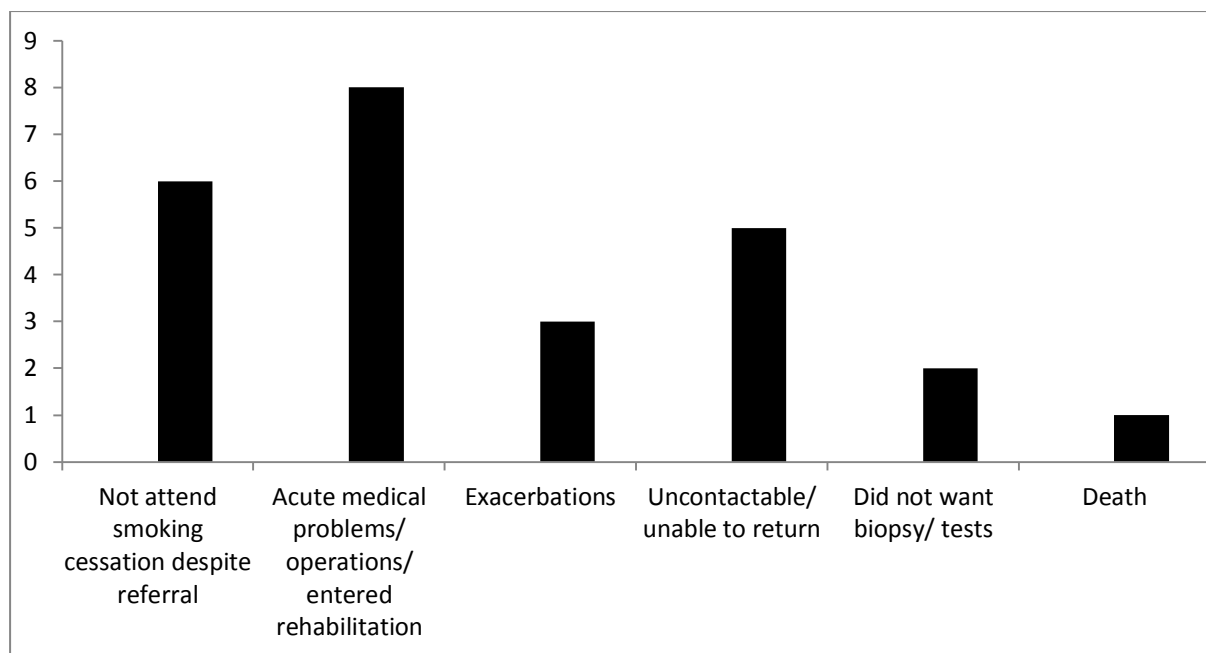


Figure 5.7: The underlying reasons for 25 smokers not returning for repeat assessment after initial assessment and referral to smoking cessation clinic.

5.3.6 The determination of GDF-15 levels in the serum (n=87)

Serum GDF-15 levels were elevated in patients with COPD (1936 (1230, 2969)) pg/mL but not in 'healthy smokers' (841 (594, 1185)) pg/ml, as compared to healthy controls (726 (334, 1199)) pg/ml; $p < 0.0001$ (See Figure 5.8). All values were within the range of detectability reported by the manufacturer of the assay (R&D Systems Inc. Minneapolis, USA), who report the assay to have a coefficient of variance of $< 2.8\%$; in this cohort this was established to be 4.1% . There was no correlation between serum BNP and GDF-15 levels ($r = -0.14$, $p > 0.05$).

RF_{CSA} measures in all three groups are shown in Figure 5.9. In the cohort taken as a whole, serum GDF-15 levels related to age ($r = 0.37$, $p = 0.0001$), FEV₁% ($r = -0.41$, $p < 0.0001$), MRC ($r = 0.49$, $p < 0.0001$), steps/day ($r = -0.54$, $p < 0.0001$), 6MWT distance ($r = -0.54$, $p < 0.0001$) and RF_{CSA} ($r = -0.49$, $p < 0.0001$); Figure 5.11. In COPD, serum GDF-15 levels related to age ($r = 0.45$, $p = 0.001$) and steps/day ($r = -0.40$, $p = 0.006$), whilst the relationship with RF_{CSA} did not quite reach statistical significance, a trend was observed ($r = -0.31$, $p = 0.05$). However, in COPD considered alone there was no relationship between circulating GDF-15 and lung function parameters.

Using the same ultrasound technique, we have previously measured RF_{CSA} in 40 healthy individuals with a similar demographic to the cohort presented in this manuscript, a mean RF_{CSA} of 640 (136) mm² was established (Shrikrishna, Patel et al. 2012). Subsequently, an RF_{CSA} of 368mm² or below was used to identify those with RF_{CSA} atrophy as this value was 2 standard deviations below the mean of the previously published normative data. Using this cut-off, 6 of 38 patients (16%) had evidence of quadriceps atrophy, this cohort had a median serum GDF-15 level of 2919 (2435, 4032) pg/ml. Patients without RF_{CSA} atrophy (n=32) had a lower median serum GDF-15 level of 1756 (1317, 3135) pg/ml (n=32); $p = 0.04$ (See Figure 5.11).

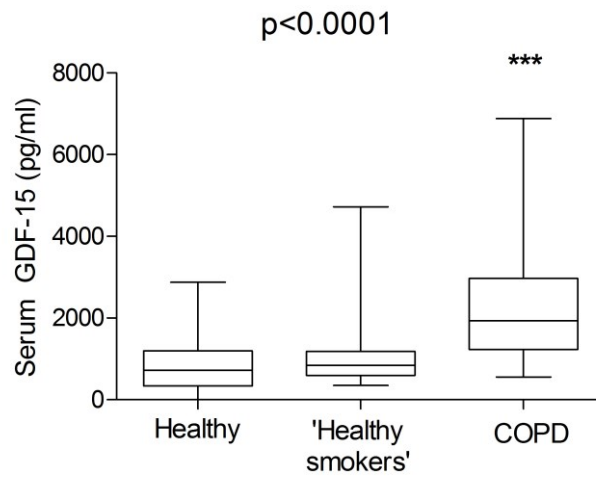


Figure 5.8: Serum GDF-15 levels in healthy controls (n=25), 'healthy smokers' (n=12) and COPD patients (n=50).

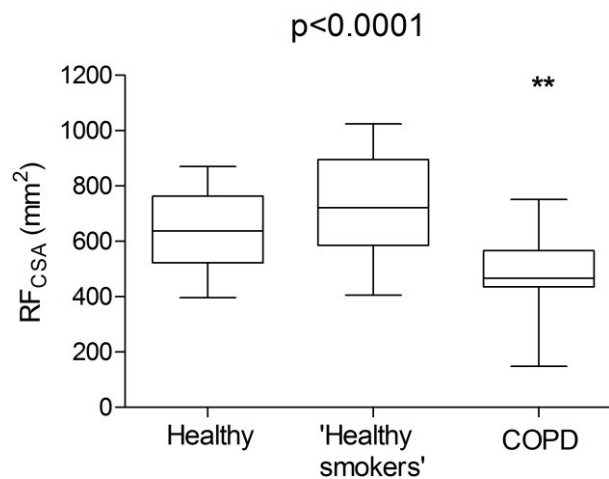


Figure 5.9: RF_{CSA} measurements in healthy controls, 'healthy' smokers' and COPD patients.

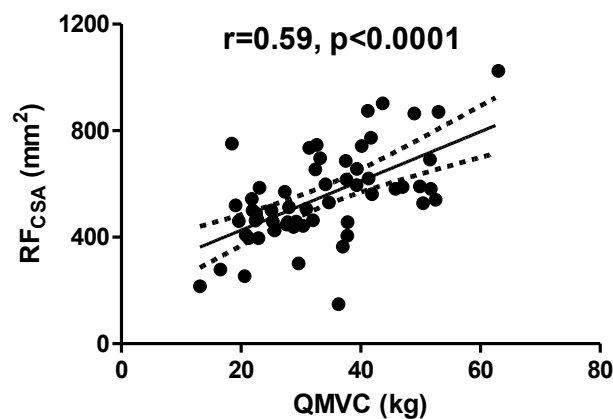
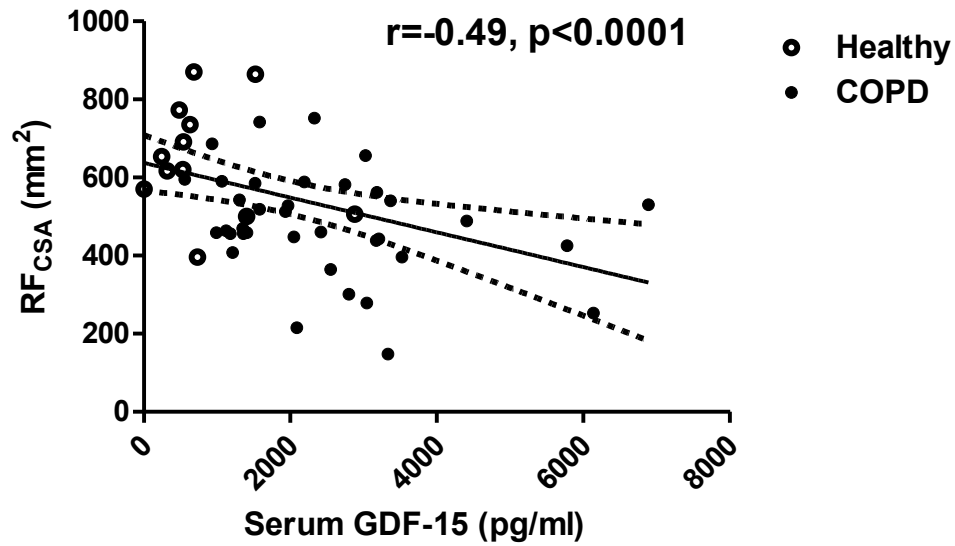


Figure 5.10: An X-Y plot demonstrating the relationship between RF_{CSA} and QMVC.

A)



B)

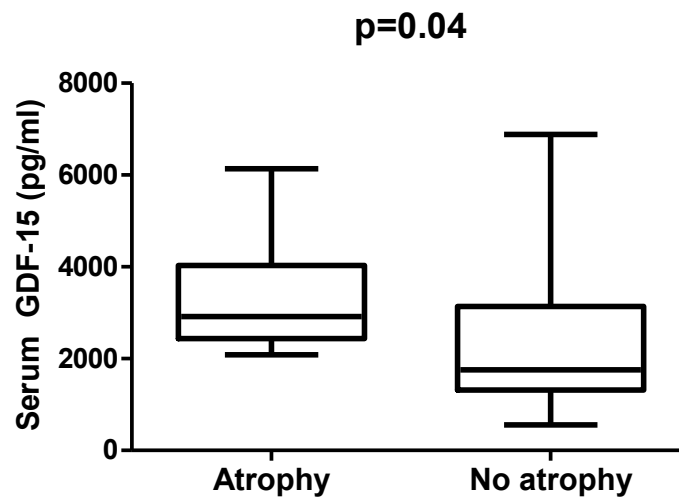


Figure 5.11: A) An X-Y plot demonstrating the relationship between serum GDF-15 and RF_{CSA} in the full cohort and **B)** serum GDF-15 levels in patients with and without *rectus femoris* atrophy.

5.3.7 The predictors of *rectus femoris* cross-sectional area

Univariate predictors of RF_{CSA} included serum GDF-15, age, COPD diagnosis, FEV_1 , RV/TLC, K_{CO} , FFM, BMI, QMVC, MRC, SGRQ, daily steps and 6MWT (Table 5.9). In a multivariate analysis determining the predictors of RF_{CSA} , serum GDF-15 was the only parameter retained (Table 5.9).

Variable	Univariate regression			Multiple regression		
	Coefficient (95% CI)	Standardised coefficient	p	Coefficient (95% CI)	Standardised coefficient	p
Serum GDF-15	-0.045 (-0.073, -0.016)	-0.38	0.003	-0.337 (-0.65, -0.004)	-0.37	0.02
Age	-5.52 (-10.04, -1.000)	-0.31	0.02	-0.24 (-4.4, 4.4)	-0.02	0.99
Current smoker	38.18 (-54.48, 130.8)	0.11	0.41	-	-	-
Gender (male)	85.83 (-3.290, 175.0)	0.25	0.06	-	-	-
COPD	-199.6 (-280.1, -119.2)	-0.55	<0.0001	-10.1 (-150, 129)	-0.03	0.88
FEV₁ (%pred)	2.641 (1.323, 3.958)	0.47	<0.0001	0.960 (-1.54, 3.46)	0.19	0.44
RV/TLC (%)	-3.542 (-6.653, -0.431)	-0.30	0.03	-1.05 (-4.06, 1.97)	-0.70	0.49
K_{co} (%pred)	2.973 (1.172, 4.774)	0.42	0.002	1.53 (-0.98, 4.03)	0.25	0.22
FFM	6.065 (2.665, 9.466)	0.43	0.01	3.79 (-2.35, 9.81)	0.39	0.22
BMI	6.975 (0.406, 13.54)	0.27	0.04	-7.90 (17.66, 1.86)	-0.34	0.11
QMVC	9.251 (5.898, 12.603)	0.59	<0.0001	3.81 (-2.24, 9.86)	0.25	0.21
Steps/day	0.013 (0.004, 0.022)	0.38	0.006	-0.001 (-0.01, 0.11)	-0.04	0.85
6MWT	0.519 (0.196, 0.842)	0.40	0.002	-0.13 (-0.72, 0.46)	-0.10	0.65

Table 5.9: Univariate and multivariate regression analysis to determine the predictors of RF_{CSA} .

5.3.8 The relationship with systemic oxidative stress

There was no difference between the serum levels of 8-oxo-dG measured in healthy controls, 'healthy smokers' and COPD patients (See Figure 5.12). In patients with COPD, serum 8-oxo-dG levels related to BMI ($r=0.45$, $p=0.001$), FFM ($r=0.37$, $p=0.009$) and FFMI ($r=0.40$, $p=0.005$, Figure 5.13), but not to QMVC ($p=0.11$) or RF_{CSA} ($p=0.73$). Across the cohort as a whole, serum 8-oxo-dG related to FGF-23 whilst in COPD, levels related to both FGF-23 and GDF-15 (see Table 5.10 and Figure 5.14).

In a regression analysis to identify the predictors of serum 8-oxo-dG in the whole cohort ($n=87$), serum GDF-15, serum FGF-23, BMI and FFM were the only univariate predictors of serum 8-oxo-dG (see Table 5.11). As may be expected, BMI and FFM had significant colinearity, ($r=0.58$, $P<0.0001$), multivariate analyses were therefore restricted to the inclusion of either BMI or FFMI (see Table 5.11). Serum GDF-15 and FGF-23 were retained as independent predictors of serum 8-oxo-dG in both models and in fact were the only predictors of serum 8-oxo-dG.

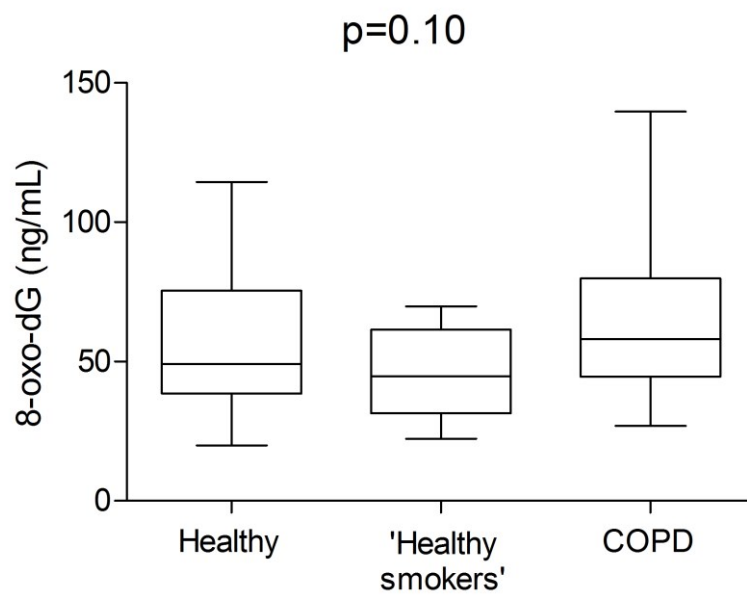


Figure 5.12: Serum 8-oxo-dG in healthy controls (n=25), 'healthy smokers' (n=12) and COPD patients (n=50).

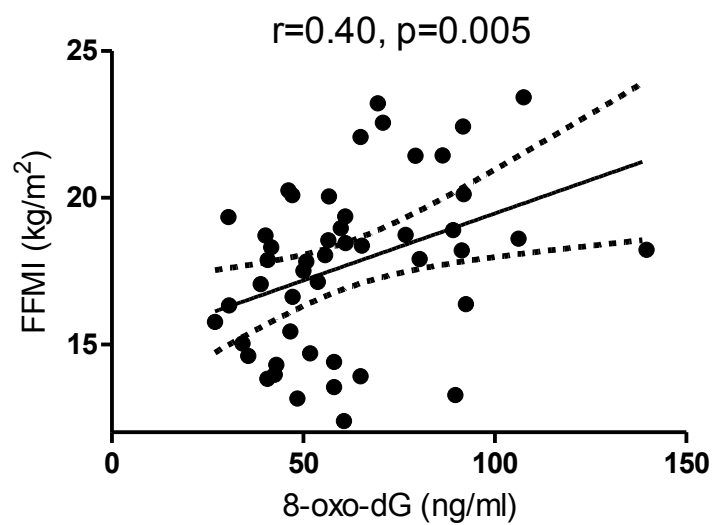


Figure 5.13: An X-Y plot demonstrating the relationship between serum 8-oxo-dG and FFMI in COPD patients.

8-oxo-dG	Vitamin D		FGF-23		Klotho		GDF-15	
	r	p	r	p	r	p	r	p
	0.08	0.44	0.34	0.01	-0.02	0.87	0.18	0.09

8-oxo-dG	Vitamin D		FGF-23		Klotho		GDF-15	
	r	p	r	p	r	p	r	p
	0.04	0.76	0.51	0.0002	0.13	0.39	0.31	0.03

Table 5.10: The relationship between serum 8-oxo-dG and measured serum proteins across the cohort as a whole (n=87, top) and in COPD patients only (n=50, lower).

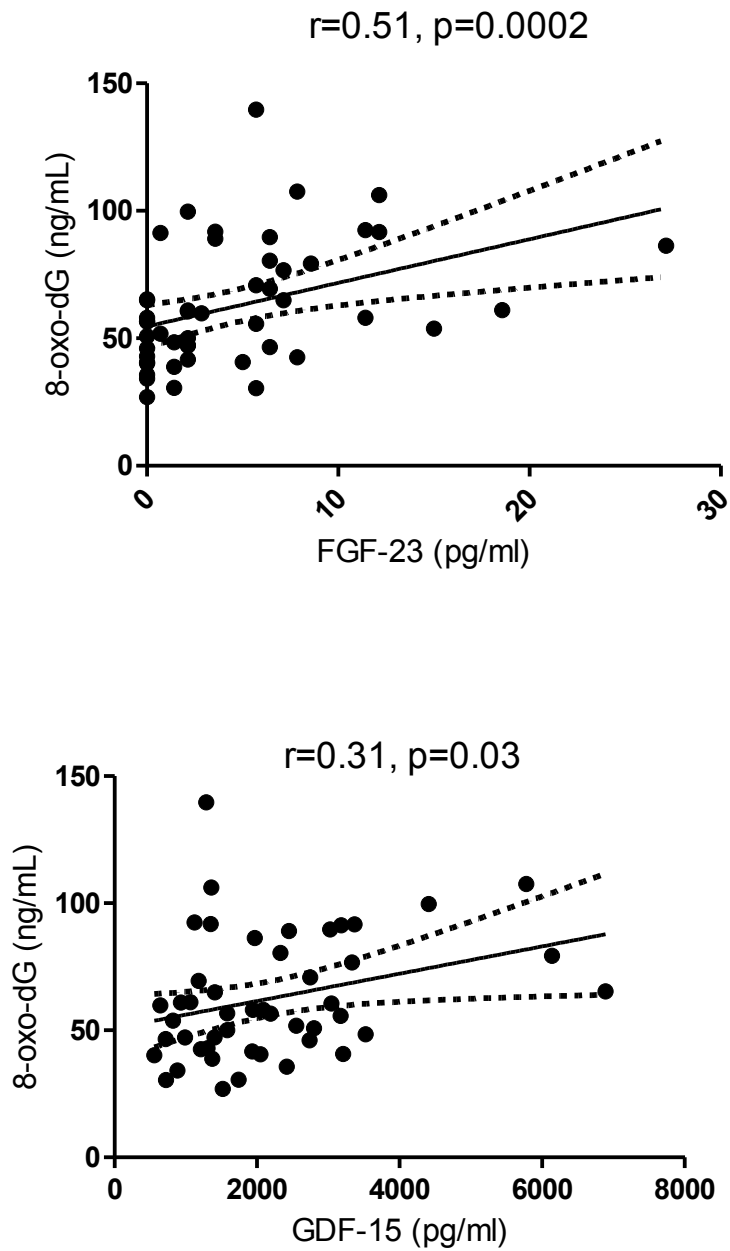


Figure 5.14: An X-Y plot demonstrating the relationship between serum 8-oxo-dG and FGF-23 (top) and with serum GDF-15 levels (lower).

Variable	Univariate regression		Multiple regression		
	Coefficient (95% CI)	p-value	Coefficient (95% CI)	Standardised coefficient	p-value
Serum GDF-15	0.02 (0.0001, 0.008)	0.04	0.04 (0.0001, 0.008)	0.23	0.03
Serum FGF-23	0.82 (0.20, 1.44)	0.01	0.86 (0.25, 1.46)	0.29	0.006
BMI	0.89 (0.10, 1.67)	0.03	0.62 (0.12, -0.16)	0.17	0.12

Variable	Univariate regression		Multiple regression		
	Coefficient (95% CI)	p-value	Coefficient (95% CI)	Standardised coefficient	p-value
Serum GDF-15	0.02 (0.0001, 0.008)	0.04	0.04 (0.0001, 0.008)	0.23	0.03
Serum FGF-23	0.82 (0.20, 1.44)	0.01	0.87 (0.27, 1..46)	0.30	0.005
FFM	0.402 (0.005, 0.799)	0.047	0.26 (-0.02, 0.75)	0.19	0.06

Table 5.11: Univariate and multivariate regression analysis to determine the predictors of serum 8-oxo-dG in a model including BMI (top) and a model including FFM (below).

5.4 Discussion

5.4.1 Summary of the findings

The main findings of this study are that serum Klotho and GDF-15 levels are altered in stable patients with COPD as compared with healthy never smoking controls. Furthermore, both proteins may be relevant to the pathophysiology of skeletal muscle dysfunction in COPD as Klotho levels related to quadriceps strength and GDF-15 levels related to quadriceps bulk as assessed by RF_{CSA}. Lastly, from a mechanistic perspective, both circulating GDF-15 and FGF-23, for which Klotho is a co-receptor related to a systemic marker of oxidative stress.

Additional observations suggest that cigarette smoking may be the factor triggering reduced Klotho expression. Firstly, that smokers with normal spirometry also have lower serum Klotho levels than never smoking controls and secondly that smokers successful in quitting exhibited a rise in serum Klotho as compared to stable COPD patients studied on two occasions. These data indicate that cigarette smoking reduces serum Klotho protein levels even in the absence of COPD. Based on these findings, it may be speculated that reduced Klotho may have a pathophysiological role in the locomotor myopathy of COPD.

5.4.2 Significance of the findings

Ageing is the age-related loss of physiological functions that are necessary for survival. Chronological ageing may therefore be accelerated by impaired homeostasis. As life expectancy and the burdens of COPD continue to rise (Lozano, Naghavi et al. 2012), there is growing interest in the study of age-

related biomarkers that may be involved in the pathophysiology of the disease. Emerging data shows that skeletal muscle dysfunction occurs early on in COPD, in fact even in smokers without airflow obstruction (Montes de Oca, Loeb et al. 2008; Shrikrishna, Patel et al. 2012).

MMPs are a family of zinc endopeptidases that are able to break down the extracellular matrix and are subsequently associated with tissue destruction (Maclay, McAllister et al. 2012). Unexpectedly, serum MMP-9 levels were not quantifiable in this cohort. Whilst a technical error cannot be completely refuted, this seems unlikely given that the control standard curve demonstrated the expected results. Furthermore, this was not a disease related effect as levels were low in all relevant sub-cohorts. The low determinations may have been a consequence of the type of assay performed, the Biotrak assay sought to determine the concentration of active MMP-9, had total MMP-9 or inactive MMP-9 levels been measured, meaningful levels of MMP-9 may have been determined, nonetheless, this alone is not an explanation as others have managed to measure active MMP-9 levels in the serum (Higashimoto, Yamagata et al. 2005; Nikkola, Vihinen et al. 2005). Of interest, in the ECLIPSE cohort (Evaluation of COPD Longitudinally to Identify Surrogate Endpoints), the investigators reported poor reproducibility of MMP-9 measurements taken 3 months apart and concluded as a result that MMP-9 would be of limited use in clinical applications (Dickens, Miller et al. 2011). The fact that in the current study, the specimens were stored with multiple freeze-thaw cycles risked exposing these samples to protein degradation, however, in the case of MMP-9 this is less likely to be relevant as this was the first protein measured. Furthermore, to minimise the effect of freeze-thaw cycles on the interpretations of subsequent protein measurements, determinations for each protein were made in batches on samples that had undergone the same number of freeze-thaw cycles. These suppositions do not provide any further insight into the possible role for MMP-9 in the skeletal muscle dysfunction that occurs in COPD. To address any such role for the MMP-9 system, further measurement of the endogenous MMP inhibitors, such as TIMP-1, would have also been necessary, however, in light of the results obtained this seemed superfluous, especially given

the relative paucity of data on the other age-related proteins evaluated, namely Klotho/FGF-23 and GDF-15, in the COPD literature.

To my knowledge no previous data exists on Klotho levels in COPD. Sotiriou *et al.* showed *Klotho* polymorphisms to be associated with body mass-index but not with FEV₁ in COPD (Sotiriou, Kukuvtis *et al.* 2010); the results from this study were in keeping with this previous observation as there was no significant relationship between FEV₁ and serum Klotho levels. Kim *et al.* also studied *Klotho* polymorphisms and did not show any relationship to disease status; muscle parameters were not measured (Kim, Oh *et al.* 2011).

The potential mechanism of action of Klotho is of interest. Klotho is a co-receptor for FGF-23 which belongs to the fibroblast growth factor (FGF) subfamily and mediates phosphate regulating actions in the kidney through FGF receptors. The transmembrane form of Klotho significantly enhances the binding affinity of FGF-23 to FGF receptors (Kurosu, Ogawa *et al.* 2006). Given that FGF-23 levels were not altered in COPD patients or in smokers, it is interesting that FGF-23 related to and was indeed an independent predictor of serum 8-oxo-dG, a marker of DNA oxidative damage. Despite the relationship of serum Klotho to quadriceps strength, serum Klotho did not correlate with 8-oxo-dG levels. Distinct clinical phenotypes were related to serum determinations of Klotho, FGF-23 and vitamin D. In the whole cohort, systemic Klotho levels related to quadriceps strength, quadriceps bulk, exercise capacity and dyspnoea, and in COPD they related to quadriceps strength. Given the importance of quadriceps strength in COPD (Marquis, Debigare *et al.* 2002; Swallow, Reyes *et al.* 2007), the fact that serum Klotho levels were retained in a multiple regression model determining the predictors of quadriceps strength is of potential clinical significance. Conversely FGF-23, but not Klotho, correlated with BMI and FFMI. This observation is not consistent with the similarity in phenotype developed by mice deficient in FGF-23 and mice deficient in Klotho (Kurosu, Ogawa *et al.* 2006); however, consistent with the findings of the present study, Klotho also exerts actions

independent to FGF-23 (Kurosu, Yamamoto et al. 2005). The assay used to measure FGF-23 levels detects only whole intact FGF-23, the active form; although higher levels may be expected if using a 'c-terminal assay' which measures active and inactive forms, both forms relate, although weakly (Burnett, Gunawardene et al. 2006).

Jackson *et al.* have previously shown that 1,25(OH)₂D relates to quadriceps strength and that 25(OH)D relates to quadriceps MHCIIa expression in those without COPD (Jackson, Shrikrishna et al. 2013). Given that Klotho levels may influence vitamin D expression (Tsujikawa, Kurotaki et al. 2003) and that vitamin D levels were reduced in smokers and COPD patients in this study, it is noteworthy that vitamin D levels did not relate to any muscle specific measures in either the cohort as a whole or in COPD patients considered alone. Given the lack of association with FGF-23 and vitamin D, it seems likely that the influence of circulating Klotho on muscle is independent to these other circulating proteins. Circulating Klotho arises either from direct transcription and secretion by cells or by cleavage of the extracellular domain of the full length transmembrane protein (Matsumura, Aizawa et al. 1998). Therefore, the transmembrane form of Klotho may still be relevant to the effects of FGF-23 as the presence of Klotho within skeletal muscle would significantly enhance the action of circulating FGF-23 in skeletal muscle (Kurosu, Ogawa et al. 2006). Despite this role for Klotho, it remains unknown whether Klotho is expressed in human skeletal muscle and I address this in Chapter 6.

Modulation of Klotho or the effects of reduced Klotho offers a potential therapeutic opportunity in COPD. In this regard, Nagai *et al.* were able to show that, the anti-oxidant α -tocopherol, attenuated the manifestations of oxidative stress in the brain of *Klotho* knockout mice (Nagai, Yamada et al. 2003). Furthermore, α -tocopherol was shown to be safe in a study of over 29,000 smokers; unfortunately measures which might have detected a beneficial effect on skeletal muscle were not obtained (Rautalahti, Virtamo et al. 1997).

GDF-15 levels also related to the levels of oxidative damage assessed by 8-oxo-dG in the serum. In man, GDF-15 expression can be rapidly induced in response to cytokines such as interleukin-1 in several organs (Paralkar, Vail et al. 1998). It has been shown that GDF-15 plays a role in gestational diseases, cancer and renal failure (Breit, Carrero et al. 2012). Although COPD is well recognised to increase the risk of inflammatory stress, minimal data exists regarding GDF-15 in COPD. Nonetheless, it has been observed that human COPD smokers have increased airway epithelium GDF-15 levels as compared to healthy non-smokers, furthermore, cigarette smoke exposure up-regulates GDF-15 expression in airway epithelial cells (Wu, Jiang et al. 2011). To my knowledge, there are no published studies evaluating the systemic levels of GDF-15 in patients with COPD.

Circulating GDF-15 levels were elevated in COPD patients. Various strands of data link GDF-15 to muscle bulk and prognosis in humans. For example, in prostate cancer, GDF-15 levels relate to BMI indicating that GDF-15 may be relevant to the nutritional depletion that is observed in chronic disease (Johnen, Lin et al. 2007). Furthermore, Wiklund *et al.* demonstrated that serum GDF-15 levels are associated with increased all-cause mortality with an OR of 3.38 in 876 males (Wiklund, Bennet et al. 2010). They validated their findings in a twin cohort; serum GDF-15 remained an independent predictor of mortality after adjusting for telomere length, IL-6 and CRP; and directly related to survival independent of genetic background. GDF-15 is additionally of prognostic relevance in specific populations with acute and chronic diseases including acute myocardial infarction, pulmonary embolism and end-stage renal disease. In the longer-term, longitudinal studies may further establish whether circulating GDF-15 levels are predictive of morbidity and mortality in COPD.

Serum GDF-15 related to several important parameters, including age, physical activity and *rectus femoris* muscle bulk. Furthermore, levels were different in patients with and without *rectus femoris*

atrophy, in fact, in a multivariate analysis, GDF-15 was the only predictor of quadriceps muscle atrophy, even though fat free mass and quadriceps strength were included in the analysis. Further studies may validate the use of serum GDF-15 levels as an effective screening tool to identify locomotor muscle atrophy in patients with COPD, and possibly direct patient management, especially if antagonists to the actions of GDF-15 became available for use in humans. At this level, as with Klotho, it is pertinent that GDF-15 expression in skeletal muscle has not been evaluated previously; this is also addressed in Chapter 6.

5.4.3 Critique of the method

Whilst circulating GDF-15 levels related to chronological age, no relationship was observed between Klotho and chronological age, suggesting that other factors such as smoking have a large effect on Klotho expression. Although age, physical activity, muscle bulk and strength are all of prognostic relevance in COPD, associations, such as those demonstrated here with Klotho and GDF-15, do not necessarily show causality.

Senescence can be assessed using a variety of measures, and in particular telomere length is often measured, and these data have recently been reported in COPD (Morla, Busquets et al. 2006; Lee, Sandford et al. 2012; Theriault, Pare et al. 2012). In the present study, rather than engage in a ‘fishing expedition’, I selected circulating markers for study on the basis of literature review (see Introduction) and on prior work from our group particularly in relation to GDF-15 and vitamin D. The markers studied which proved fruitful were further explored by analysis in locomotor and respiratory muscle (see Chapter 6).

Causality is difficult to demonstrate in human studies, this would necessitate functional manipulation of the target protein in man. Nonetheless, it has previously been demonstrated in a longitudinal dataset that smoking negatively impacts skeletal muscle strength in healthy adults (Kok, Hoekstra et al. 2012). In the case of Klotho, the data indicate that serum levels are reduced in smokers who do not have reduced skeletal muscle strength or abnormal lung function, suggesting that a reduction in Klotho levels precedes these features. This is supported by very recent data from Lam-Rachlin *et al.* (Lam-Rachlin, Romero et al. 2013), who found circulating Klotho levels to be lower in pregnant smokers than in non-smoking pregnant women. The concept that reduced Klotho protein may have a pathophysiological role, rather than being epiphenomenon is supported by the increase in serum Klotho following successful smoking cessation, contextually, Hopkinson *et al.* have previously shown that fat free mass improves with smoking abstinence sustained over a longer period of time (Hopkinson, Tennant et al. 2007). Moreover a role for reduced Klotho expression in the pathogenesis of skeletal muscle dysfunction is supported by the fact that *Klotho* knockout mice develop sarcopenia (Iida, Kanko et al. 2011).

The smoking cessation data is a key element of the data supporting a pathophysiological role for altered Klotho expression in the aetiology of skeletal muscle dysfunction in COPD and this warrants further discussion. In particular, while the differences in paired Klotho levels achieved significant differences between successful quitters, as determined by history and corroborated by carbon monoxide measurements, and stable patients studied on two occasions, these differences were not apparent when compared to unsuccessful quitters. This is likely explained by the low number of unsuccessful quitters and, even more importantly the fact that patients attempting unsuccessfully to quit had often reduced and varied their cigarette consumption, rendering interpretation of repeat Klotho levels in this setting challenging.

As has been previously described (Seymour, Ward et al. 2009), there was a relationship between quadriceps strength and *rectus femoris* bulk (Figure 5.10). Whilst Klotho levels related to quadriceps strength and RF_{CSA} in the whole cohort, they did not relate significantly to RF_{CSA} in those with COPD, this is likely explained by the large numbers of smokers also present in the latter cohort and evidence of a smoking attenuation on Klotho. On the basis of the results of the present study and following on from the propositions already discussed, the time course for smoking to have an effect on circulating Klotho levels and a subsequent effect on locomotor muscle bulk remains unclear. Indeed these findings need to be corroborated in larger datasets and future cellular, animal and longitudinal studies may address the further questions posed regarding the sequence of events and the time these take to occur. Whether the same relationships are also observed in other tissues, especially skeletal muscle is also of interest. A further point, which may be particularly relevant in the case of GDF-15, where levels did not relate to quadriceps strength, is that in this cohort, whilst RF_{CSA} area was reduced in those with COPD, quadriceps strength was not significantly different. This may be explained by the fact that for the QMVC to be a true measure of strength this relies upon the patient making a maximal effort, furthermore intramuscular fat and connective tissue may additionally contribute to *rectus femoris* cross-sectional area.

Given that both Klotho and GDF-15 are prognostic markers in cardiovascular disease (Nickel, Kempf et al. 2008; Xue, Fu et al. 2012; Shibata, Fujita et al. 2013; Navarro-Gonzalez, Donate-Correa et al. 2014) and that pulmonary hypertension and ischaemic heart disease are recognised co-morbidities in COPD, the fact that circulating BNP did not relate to either Klotho or GDF-15 levels indicates the reported findings to be independent to the presence of undetected cardiovascular co-morbidity.

5.4.4 Conclusions

This study demonstrates novel findings that may indicate independent pathophysiological roles for two age-related proteins, namely Klotho and GDF-15 in the pathogenesis of COPD. Regarding Klotho, circulating levels are reduced in COPD and smokers, and serum levels relate to quadriceps strength. Given that systemic levels increase after successful smoking cessation, smoking induced suppression of Klotho may be involved in the pathophysiology of skeletal muscle weakness of COPD. The findings of the present study also demonstrate that serum GDF-15 levels are elevated in patients with COPD. Additionally, circulating GDF-15 levels not only relate to the level of oxidative damage present in the circulation, but also to locomotor atrophy; in fact circulating GDF-15 may have a role for identifying patients with locomotor atrophy. In summary, modulation of Klotho and GDF-15 may represent potential therapeutic targets in the skeletal muscle dysfunction observed in COPD, although further studies are required to corroborate this suggestion. Evaluating potential roles for Klotho and GDF-15 expression within skeletal muscle may provide a basis for further studies into the benefit of targeting Klotho or GDF-15 signalling in the skeletal muscle dysfunction of COPD; this is the basis of Chapter 6.

Chapter 6 : The role of Klotho and GDF-15 in the skeletal muscle dysfunction of COPD

6.1 Introduction

6.1.1 Background

The molecular mechanisms underlying the skeletal muscle dysfunction that is observed in COPD are yet to be fully elucidated; however increasing evidence suggests that accelerated biological ageing may have an aetiological role. In this regard, the data presented in Chapter 5 indicate that both Klotho and GDF-15 may have pathophysiological roles in this process, a proposal that is further supported by the literature. The circulating levels of both proteins, which are both associated with mortality in unselected general populations (Wiklund, Bennet et al. 2010; Semba, Cappola et al. 2011), were found to be altered and relate to muscle specific parameters in COPD, however, neither Klotho nor GDF-15 have been quantitatively evaluated in skeletal muscle before, in fact it is unknown whether they are even expressed in human skeletal muscle.

Klotho deficient mice display a 20-30% reduction in *gastrocnemius* fibre diameter (Iida, Kanko et al. 2011) and in unselected human populations, systemic Klotho levels relate to grip strength (Semba, Cappola et al. 2012) and to performance as assessed by the SPPB (Crasto, Semba et al. 2012). In the preceding Chapter, circulating Klotho levels were found to be reduced in COPD and relate to quadriceps strength. Furthermore, consistent with recent data that associates cigarette smoking with lower Klotho levels in other cohorts (Lam-Rachlin, Romero et al. 2013), the data presented in

Chapter 5 are consistent with smoking reducing Klotho expression because levels rose after successful smoking cessation. As previously described, the trans-membrane form of Klotho expressed in tissues acts as a co-receptor for FGF-23 (Kurosu, Ogawa et al. 2006) and whilst Klotho did not relate to FFMI or a systemic marker of oxidative stress, circulating FGF-23 levels did. Furthermore, in rodent models, deficiency in either Klotho or FGF-23 leads to an almost identical ageing phenotype (Kurosu, Ogawa et al. 2006).

Whilst GDF-8, has a potent inhibitory effect on muscle mass (McPherron, Lawler et al. 1997), a role for GDF-15, another member of the TGF- β signalling family, has only recently been established. Our group recently demonstrated circulating GDF-15 levels to be elevated 1 week following complex cardiothoracic surgery in patients developing acute quadriceps wasting (Bloch, Lee et al. 2013), furthermore a direct atrophic role for GDF-15 was confirmed in work on myotubes and most recently in mice. In Chapter 5, it was demonstrated that systemic GDF-15 levels are elevated in COPD, furthermore levels not only related to *rectus femoris* muscle bulk, but were higher in those with established locomotor atrophy. GDF-15 levels also related to the levels of systemic oxidative DNA damage. This may be of relevance as others have demonstrated that although muscle oxidative stress is increased in severe COPD patients, protein ubiquitination is only increased in patients exhibiting muscle atrophy (Fermoselle, Rabinovich et al. 2012).

Barreiro and colleagues have previously reported increased oxidation, manifest as protein carbonylation, to be present in the quadriceps of patients with COPD and that protein carbonylation inversely relates to quadriceps strength (Barreiro, Schols et al. 2008). These authors extended their work to also demonstrate increased protein oxidation to be present in the quadriceps of smokers and in the respiratory and limb muscles of guinea pigs chronically exposed to cigarette smoke in the absence of increased local inflammation, implicating a direct role for cigarette smoke inducing muscle protein oxidative damage (Barreiro, Peinado et al. 2010).

Given these findings, a role for Klotho/ FGF-23 and GDF-15 in human skeletal muscle may provide further insight into the pathophysiological mechanisms underlying skeletal muscle dysfunction in COPD. Furthermore, given the evidence that cigarette smoking reduces systemic levels of Klotho, smoking may also influence Klotho expression in human skeletal muscle. Given the relationships observed between FGF-23 and GDF-15 with systemic oxidative stress, it may be that levels of Klotho/FGF-23 and GDF-15 in muscle relate to oxidative stress, assessed by protein carbonylation, in the skeletal muscle of patients with COPD.

6.1.2 Aims and hypotheses

The aim of this study was to identify whether Klotho and GDF-15 are expressed in human skeletal muscle and whether their expression relates to muscle function. Specifically, to further investigate potential roles for Klotho and GDF-15 in the pathophysiology of skeletal muscle dysfunction in COPD the following hypotheses were addressed. Firstly, that quadriceps Klotho expression is reduced and GDF-15 expression is increased in COPD patients and in smokers. Secondly, that Klotho and GDF-15 expression would also be different (i.e. Klotho reduced and GDF-15 increased) in the quadriceps as compared to the respiratory muscles, which have higher level of contractile activity. Thirdly, that smoking reduces skeletal muscle expression of Klotho, tested by comparing quadriceps Klotho levels before and after attempted smoking cessation, and by measuring *gastrocnemius* Klotho expression in a mouse smoking model. Lastly, that levels of Klotho and GDF-15 would relate to protein carbonylation in the quadriceps muscles of patients with COPD and to relevant physiological parameters such as muscle strength. Given that Klotho is a co-receptor for FGF-23, where possible FGF-23 expression was also evaluated.

6.2 Methods

6.2.1 Cross-sectional and smoking study design and recruitment

Vastus lateralis muscle biopsies were taken as part of the systemic markers study presented in Chapter 5. Biopsies were performed on participants involved in the cross-sectional study and also for those involved in the smoking cessation sub-study, with the exception that biopsies were not repeated on stable COPD patients one week after the initial biopsy. Muscle biopsies were performed one week after the physiological assessments, usually on the day that the physical activity monitor was returned.

6.2.2 Comparative assessment of respiratory and locomotor muscle

Ethical approval was obtained and all participants provided written informed consent prior to study testing within the Royal Brompton & Harefield NHS Foundation Trust (11/H0717/3). Stable COPD patients, diagnosed according to the GOLD guidelines (Rabe, Hurd et al. 2007) were recruited. Exclusion criteria included exacerbation within the past 4 weeks, systemic disease including cardiac disease and significant neurological or musculoskeletal limitation to mobilisation.

COPD patients that were scheduled to have thoracic surgery for non-malignant disease were consented and had physiological testing as previously described for the patients undergoing *vastus lateralis* biopsy and venesection (Chapter 5), with the exception that in place of a percutaneous

vastus lateralis biopsy, surgical *vastus lateralis*, external intercostal and diaphragm biopsies were taken whilst the patients were under general anaesthetic.

6.2.3 Mouse smoking model

GlaxoSmithKline (King of Prussia, USA) developed and implemented the smoked mouse model and provided harvested *gastrocnemius* muscles for further analysis by Jen Lee, as part of her doctoral project undertaken within the Molecular Medicine Department at Imperial College under the supervision of Dr. Paul Kemp. The muscle samples were processed by Dr. Lee in the same manner as described in section 6.2.6, prior to specific Klotho protein determinations being made by myself.

Mouse cigarette smoke exposure

From 3 months of age, female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) received nose-only exposure to air (sham controls, n=9) or 4% cigarette smoke (n=19) from 3R4F cigarettes (College of Agriculture, Reference Cigarette Program, University of Kentucky), for 2 hours/day, 5 days/week, for 77 weeks. Smoke was generated by a Baumgartner-Jaeger CSM 2070i Smoking Machine (CH Technologies Inc., Westwood, NJ, USA) containing a circular head that holds 30 cigarettes and performs one revolution per minute. A 4% concentration of smoke was produced via a 2 second 35 ml puff of smoke taken from each cigarette once per minute, resulting in 1 litre of smoke; this smoke was then mixed with 24 litres of air and delivered to the exposure tower. During exposure to smoke or air, mice were maintained in restraining tubes containing stainless steel nose cone inserts.

6.2.4 RNA extraction, cDNA synthesis and primer validation

RNA extraction and quantification

Muscle samples were homogenised in 500µL TRIzol reagent (Sigma, UK) in 1.4mm ceramic beaded tubes (Stretton Scientific, UK). Samples underwent two 10 second cycles at 5500RPM with a 5 second pause in a Precelly's 24 homogeniser (Peqlab, Erlangen, Germany) prior to centrifugation at 8000RPM (Eppendorf 5810R with a FA 45-30-11 rotor, Hamburg, Germany) for 2 minutes to pellet the insoluble fraction. The supernatant was transferred to a fresh micro-centrifuge tube, 100µl of chloroform added and then vortexed. The samples were then centrifuged for 15 minutes at 10000RPM (Eppendorf 5810R, Hamburg, Germany) at 4°C, to achieve phase separation and the upper aqueous phase (containing RNA) transferred to a fresh micro-centrifuge tube. The RNA was precipitated by addition of 250µl isopropanol followed by incubation on ice for 10 minutes and centrifugation at 10000RPM (Eppendorf 5810R, Hamburg, Germany) for 10minutes, at 4°C. The supernatant was removed and the resultant RNA pellet was washed twice with 500ul of 75% ethanol. The RNA pellet was left to dry on the bench and then re-suspended in 30µl RNase free water. A spectrophotometer (Nanodrop ND1000, Wilmington, USA) was used to quantify the resultant RNA concentration and the purity of the RNA assessed by inspection of the $A_{260/280}$ ratio.

cDNA synthesis

For each sample, 150ng of RNA was added to 11µl of RNase free water and heated to 65°C for 5 minutes and then placed on ice for 2 minutes. A 9µl reaction mixture consisting of 2µl 10x Buffer RT (Qiagen, Hilden, Germany), 1µl 5mM dNTPs, 0.5µl random primers, 0.5µl Omniscript reverse transcriptase (Qiagen, Hilden, Germany), 0.5µl RNase inhibitor, 1µl (0.1M) DTT and 3.5µl of RNase-

free water was added to each sample and the reaction incubated at 42°C for 2 hours. The cDNA was then diluted 1:10 by addition of 180 µL of distilled water stored at -20°C.

Primer validation

Primers were selected from the literature to amplify target genes (Klotho, FGF-23, GDF-15 and GDF-8; Table 6.1) and were validated by Polymerase Chain Reaction (PCR) to ensure that only the regions targeted were amplified. Each PCR reaction contained 3µL of sample cDNA, 2µL of forward/reverse primer mix (2pmol/µL), 10µL of 2x SYBR green (Qiagen, Hilden, Germany) and 5µL of distilled H₂O. PCR was performed using 40 cycles of 10 seconds at 95°C followed by 30 seconds at 60 °C. The products of the PCR were subsequently analysed by electrophoresis on a 2% TBE-agarose gel containing 5µg/ml ethidium bromide per 100ml of gel, derived from an ethidium bromide stock solution of 10mg/ml, to confirm the product size and exclude the presence of primer-dimers which would otherwise affect subsequent RT-qPCR results.

Gene	Forward primer	Reverse primer
Human RPLPO	TCTACAACCCTGAAGTGCTTGATATC	GCAGACAGACACTGGCAACATT
Human Klotho	ACAGAGGTTACAGCATCAGGCG	TTCCTCTTCTTGGTCACAACC
	GCTCTCAAAGCCCACATACTG	GCAGCATAACGATAGAGGCC
	GATAGAGAAAAATGGCTTCCCTC	GGTCGGAAACTGAGACAGAGTGG
Human FGF-23	CACAGCCACAGCCAGGAACAGC	GTCGTACCCGTTTTCCAGCGTCT
Human GDF-15	TGCCCCGCCAGCTACAATC	TCTTTGGCTAACAAAGTCATCATAGGT
Human GDF-8	ACATGAACCCAGGCACTGGT	GGTTGTTTGAGCCAATTTTGC

Table 6.1: Forward and reverse primer sequences for target genes, 3 primers were tested for Klotho RT-qPCR as discussed in results section 6.3.1

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

Real-time quantitative PCR (RT-qPCR), was performed in 96-well fast optical plates sealed with adhesive film (MicroAmp, Optical adhesive film, Applied Biosystems, UK). Each reaction consisted of 10µL SYBR® Green Quantitative Kit (Sigma Aldrich, Poole, UK), 5µL distilled water, 2µL of forward/reverse primer mix (2pmol/µL) and 3µL of sample. RT-qPCR was run using the 7500 Fast Real-time PCR System (Applied Biosystems, UK) with the following programme: 5minutes at 95 °C, followed by 40 cycles of 95 °C for 10 seconds and 60°C for 30 seconds. Each reaction was performed in duplicate and the cycle threshold (C_T) values for technical replicates averaged and exported into Microsoft Excel. Expression levels of Klotho, FGF-23 and GDF-15 were normalised to RPLPO using the $\Delta\Delta C_T$ method (Schmittgen and Livak 2008). Normalised data were log transformed to equalise variance and achieve normally distributed results.

6.2.6 Protein extraction and quantification

Derivation of protein extracts

Muscle biopsy samples were homogenised with lysis buffer (Tris pH 7.4 (50mM), NaCl (250mM), EDTA (5mM), 1% Nonidet P40 (Roche Applied Science)) supplemented with protease and phosphatase inhibitor (Sigma, UK) in a Precelly's machine as described above. Samples were stored at -80°C until protein quantification.

A Bradford assay was performed to measure the derived protein concentration against BSA standards in accordance with the manufacturer's recommendations. A BioRad microtitre plate reader was used to measure the subsequent absorbance at 595nm using Luminex analysis software.

Specific protein quantification

Protein expression levels were determined by ELISA. Assays were performed on separate days for each target protein, on samples that were defrosted on the day of experimentation. ELISAs were performed using commercially available kits according to the manufacturer's instructions, first for Klotho (IBL, Japan), then FGF-23 (EMD Millipore, MA, USA) and finally for protein carbonylation (Biocell, Papatoetoe, NZ). Since in the case of Klotho and FGF-23, skeletal muscle protein determinations had not previously been made, the amount of total protein to be added for each reaction was determined experimentally. Subsequently, Klotho and FGF-23 determinations were performed on muscle protein extracts which were all diluted with EIA buffer to a standardised total protein concentration of 3mg/ml (which was the highest possible standardised concentration across the range of samples). In the case of protein carbonylation, as according to the manufacturer's protocol, sample determinations were made from a total of 250 µg of protein derivative. To determine *gastrocnemius* Klotho protein expression in the smoked mouse cohort and mouse controls, an ELISA was performed as according to the manufacturer's protocol (Cusabio, Wuhan, China) on protein lysates standardised to 3mg/ml total protein concentration.

6.2.7 The immunohistochemical detection of Klotho

Immunohistochemistry was performed on mouse *tibialis anterior* and human *vastus lateralis* muscle sections. Serial muscle sections (10 µm) were cut at -20°C such that the majority of fibres were in transverse section, and stored at -80°C until analysis. For analysis, sections were thawed at room temperature and fixed for 10 minutes in 4% PFA. The slides were then washed three times for 3 minutes in PBST (0.1%), followed by a blocking step with 5% milk diluted in TBST for 30 minutes.

Sections were incubated with rabbit anti-Klotho antibody [diluted 1:100] (Aviva Systems Biology, San Diego, CA, USA) at 4°C overnight. The sections were then washed a further 3 times in PBST as previously described and incubated with secondary antibody diluted in PBST [diluted 1:500] (AlexaFluor568 goat anti-rabbit IgG, Molecular Probes, Invitrogen, Breda, the Netherlands) protected from light in a humidification box for one hour at room temperature. After a further wash step, sections were incubated with DAPI diluted 1:10000 in PBST for 15 minutes at room temperature and a further wash step implemented. Fluoromount aqueous mounting medium (Sigma-Aldrich, St. Louis, MO, USA) and a coverslip were applied and the sections were stored in the dark at 4°C. Epifluorescence signal was recorded using a Nikon Eclipse 800 microscope with a DXM 1200 camera (Nikon Instruments Europe BV, the Netherlands) and imaging software (VLOCITY V; PerkinElmer, Waltham, Massachusetts, USA).

6.2.8 Statistical Analysis

Statistical analyses and graphical presentations were performed using GraphPad Prism 5 (GraphPad Software, San Diego, USA) or SPSS version 18 (IBM, USA). Significance was set at a 2-tailed p-value of ≤ 0.05 . Muscle Klotho protein levels were normalised to the total protein content obtained. Log values are provided for RT-qPCR data unless otherwise stated. Chi-square and ANOVA or Kruskal-Wallis test with post hoc analyses by Newman-Keuls or Dunn's multiple comparison tests were used to assess for differences between healthy controls, 'healthy smokers' and COPD patients. Spearman's rank correlation was used to assess the relationship between protein levels and other measurements. Smoking cessation measurements were analysed using Wilcoxon pairs analysis.

6.3 Results

6.3.1 The quadriceps expression of Klotho

As described in the methods (section 2.15), biopsy samples were divided to allow RNA and protein quantification from samples. Adequate RNA was extracted to allow RT-qPCR determinations from 83 participants and sufficient protein was extracted to make protein determination from 94 *vastus lateralis* biopsies (the characteristics of the relevant dataset are presented in Table 6.2).

To determine the relative mRNA expression of Klotho and FGF-23 from RNA extracts, qPCR primers were selected from the literature. The initial qPCR results revealed that both Klotho and FGF-23 primers produced PCR products of predicted molecular weight (Klotho 357 base pairs, FGF-23 247 base pairs), suggesting that the mRNA transcripts for both genes are readily detectable in skeletal muscle. However, owing to the formation of an additional non-specific low molecular weight product, consistent with a primer dimer, in approximately a third of the samples tested it was not possible to quantitatively compare expression levels of Klotho and FGF-23 by SYBR RT-qPCR (as identified by the presence of 2 melt curves during RT-qPCR amplification and confirmed by electrophoresis of the products obtained, see Figure 6.1). Despite several attempts to eliminate the formation of primer dimers in these samples, including in the case of Klotho, testing 3 different primer sequences (see Table 6.1) and optimising the annealing temperatures of each primer pair over a temperature gradient, quantifiable determinations were not possible for either Klotho or FGF-23 due to repeated primer dimer formation. Moreover, the qPCR results obtained were not reproducible, as the primer dimers did not always occur in the same samples. Subsequently protein measurements were used to evaluate the expression of Klotho and FGF-23 in skeletal muscle.

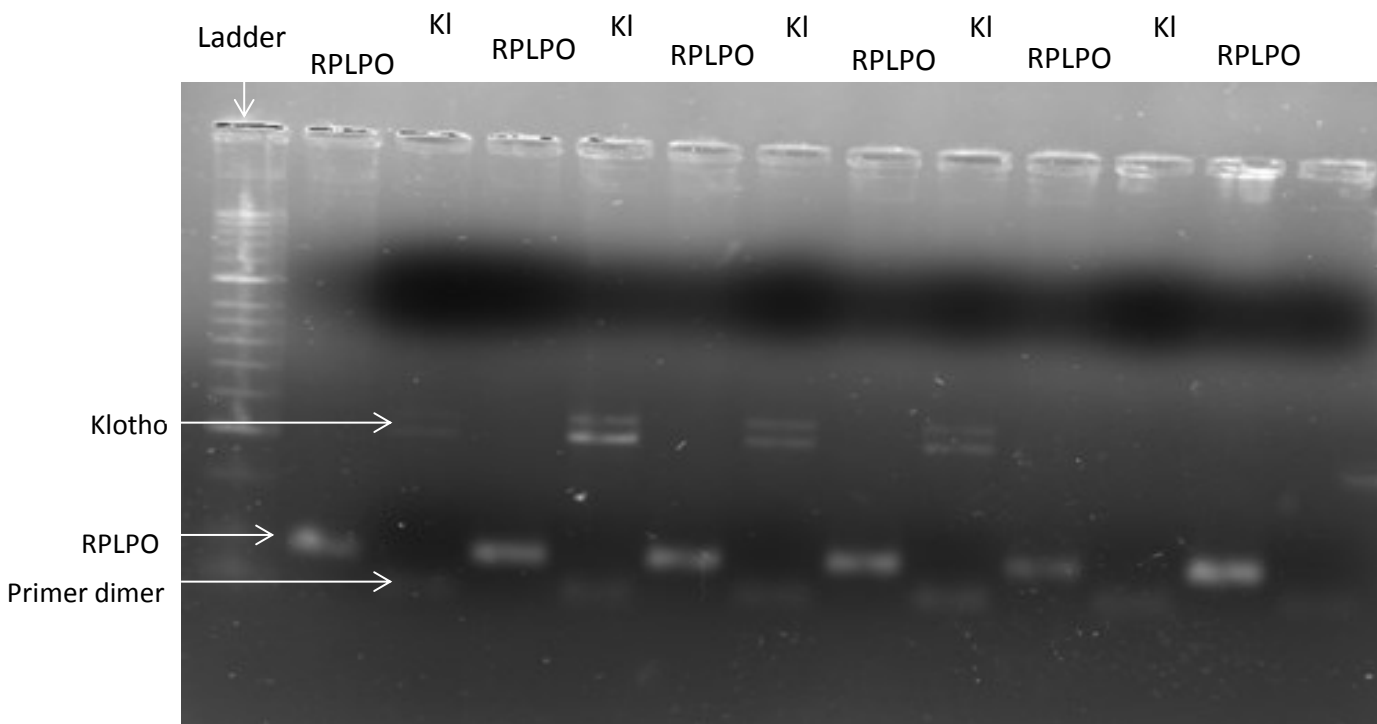


Figure 6.1: An electrophoretic gel demonstrating the PCR products when adding primers targeting the house-keeping gene RPLPO and Klotho, the primers alternate as according to both genes being tested with different participant samples. As demonstrated by the arrows on the left hand side, the resultant product when testing for RPLPO is in the region expected as compared to the corresponding position on the ladder (which indicates the molecular weight of the product). Alternatively, when primers targeting Klotho are tested, 3 products are visible for most samples tested, 2 in the expected region corresponding to a Klotho PCR product and 1 corresponding to primer dimer formation. The dual band product occurring in the Klotho region is likely to represent alternative RNA splicing as previously described (Matsumura et al., 1998; Shiraki-Iida et al., 1998).

The characteristics of the cohort in whom vastus lateralis protein determinations were made

Protein determinations were made in 94 participants (15 healthy, 14 'healthy smokers' and 65 COPD patients), the characteristics of the cohort are presented in Table 6.2. Although 'healthy smokers' were on average 12 years younger than healthy controls, age was not different in controls and COPD patients. Furthermore, in the setting of being younger, RF_{CSA} was greater in 'healthy smokers' than in controls, although COPD patients had a lower RF_{CSA} than controls. There was no difference in gender, BMI, FFMI, QMVC/BMI or *vastus lateralis* Klotho levels between controls, 'healthy smokers' and COPD patients (Tables 6.2 and Figure 6.2). In this cohort, FGF-23 levels were very low and all but one determination was lower than the lowest standard concentration, subsequently the FGF-23 determinations were not analysed further.

There were no significant correlations between quadriceps Klotho levels and other relevant parameters, in COPD, 'healthy smokers' or controls. In patients who had serum and *vastus lateralis* Klotho levels measured, there was no correlation between the two ($r=0.13$, $p=0.23$). Immunohistochemistry was available to provide fibre type data from *vastus lateralis* biopsies in 65 participants (9 healthy, 13 'healthy smokers' and 43 COPD patients). There was no correlation between *vastus lateralis* Klotho levels and type II fiber % either in the cohort as a whole or in COPD patients considered alone ($r=0.04$, $p=0.95$ and $r=-0.20$, $p=0.21$ respectively).

The presence of Klotho protein within skeletal muscle was confirmed by immunohistochemistry which revealed Klotho to be localised to the muscle fibre membrane (Figure 6.3), confirming that the Klotho protein measured is not likely explained by the influx of Klotho protein from the circulation or other tissue sources obtained during the muscle biopsy.

n=94	Healthy (n=15)	'Healthy smokers' (n=14)	COPD (n=65)	P-value
Age (years)	63 ± 9	51 ± 6 **	63 ± 10	0.0003
Gender (M:F)	12:3	6:8	46:19	0.07
Pack years	0 ± 0	37 ± 27***	45 ± 23***	<0.0001
% current smokers	0	100	45	<0.0001
FEV ₁ (%pred)	106 ± 13	98 ± 10	49 ± 24***	<0.0001
BMI (kg/m ²)	26.8 ± 4	29.3 ± 8	26.4 ± 7	0.45
FFMI (kg/m ²)	19.2 ± 3	19.1 ± 4	17.8 ± 3	0.10
QMVC/BMI	1.50 ± 0.4	1.40 ± 0.4	1.21 ± 0.4	0.04
RF _{CSA} (mm ²)	670 ± 123	745 ± 145***	506 ± 157**	<0.0001

Table 6.2: Demographics and physiological data in healthy never smokers, 'healthy smokers' and COPD patients (n=94) in whom *vastus lateralis* Klotho measurements were made. Differences between groups were assessed by Chi-square and ANOVA or Kruskal-Wallis. *, ** or *** denote significant difference at ≤0.05, <0.01 and <0.001 respectively as compared to healthy controls. The data are presented as mean ± SD or median (IQ range).

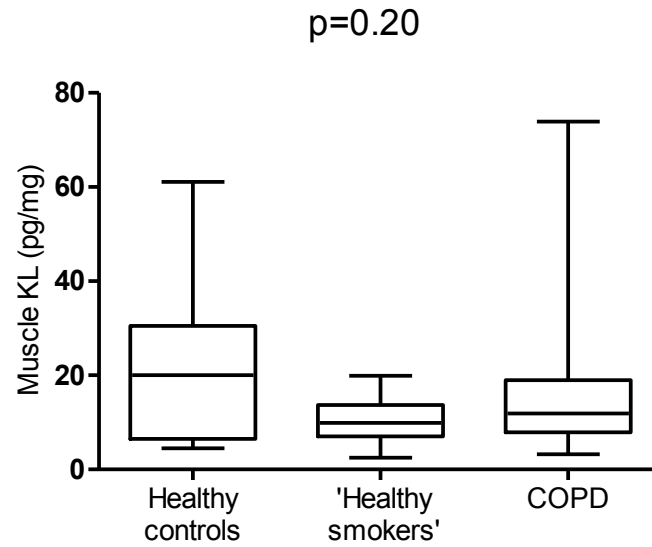


Figure 6.2: Box and whiskers plots demonstrating *vastus lateralis* Klotho levels in healthy never smokers (n=15), 'healthy smokers' (n=14) and COPD patients (n=65),

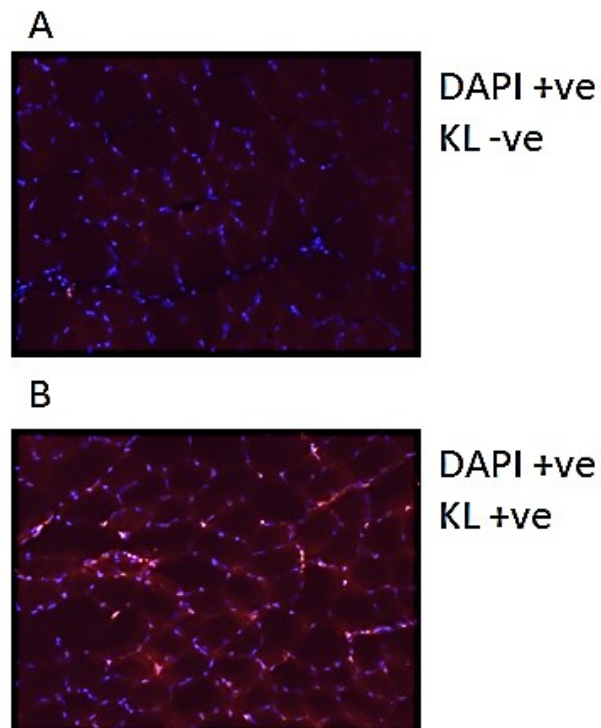


Figure 6.3: Immunohistochemistry of mouse *tibialis* anterior muscle sections **A**) in a control with DAPI stain only (blue), and **B**) with Klotho (red) and DAPI staining (blue).

6.3.2 The effect of smoking on the expression of Klotho

Current smokers, irrespective of spirometric status, had lower quadriceps Klotho protein levels than never-smoking controls (9.5 (5.7, 13.7) v 20.0 (6.5, 30.5) pg/mg; $p=0.04$) and as compared to all non-current smokers (9.5 (5.7, 13.7) v 13.9 (9.4, 21.7) pg/mg; $p=0.0008$); see Figure 6.4A. Furthermore, when limiting the analysis to patients with COPD (see Table 6.3 for characteristics), current smokers had lower Klotho levels than former smokers (9.2 (5.5, 15.1) v 13.9 (9.9, 21.4) pg/mg; $p=0.005$) (Figure 6.4B).

Smoking cessation sub-study

As described in Section 5.3.5, and with the participant characteristics demonstrated in Table 5.7, 13 patients had entered the smoking cessation study. Before and after *vastus lateralis* biopsies were performed on 12 of these (7 successful in cessation, 5 who were not) as one patient successful in smoking cessation did not want a repeat biopsy.

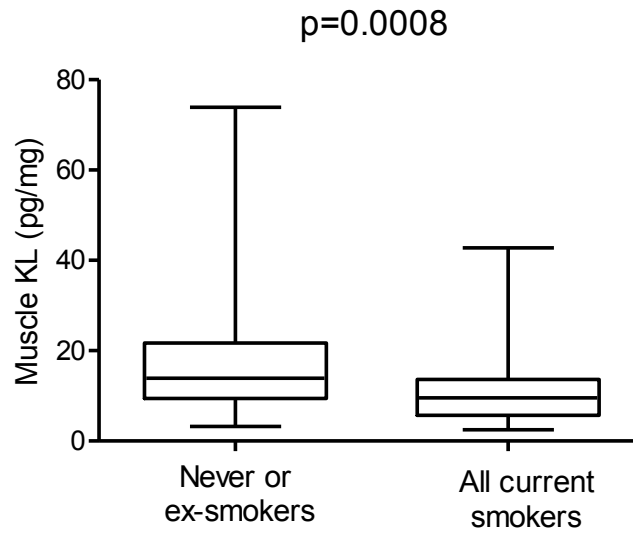
Vastus lateralis Klotho protein levels did not change after successful smoking cessation 32.6 (19.1, 52.5) to 56.7 (37.6, 83.3) pg/mg; $p=0.38$ ($n=7$; Figure 6.5A), or in those attempting but unsuccessful in achieving smoking cessation; $p=0.31$ ($n=5$; Figure 6.5B). Furthermore, there was no difference in the before and after change in Klotho levels between those successful and those unsuccessful in smoking cessation; $p=0.88$.

Mice exposed to smoke ($n=19$) had lower *gastrocnemius* Klotho levels than mice exposed to sham air ($n=9$); $p=0.005$ (Figure 6.6).

	COPD former smokers (n=36)	COPD current smokers (n=29)	P-value
Age (years)	64 ± 11	62 ± 10	0.45
Gender (M:F)	26:10	20:9	0.79
Pack years	38 ± 19	52 ± 26	0.002
FEV ₁ (%pred)	47 ± 26	50 ± 21	0.67
BMI (kg/m ²)	26.8 ± 7	25.8 ± 7	0.57
FFMI (kg/m ²)	17.8 ± 3	17.7 ± 3	0.88
QMVC/BMI	1.13 ± 0.4	1.31 ± 0.5	0.10
RF _{CSA} (mm ²)	504 ± 155	510 ± 163	0.54

Table 6.3: Demographics, physiological data and *vastus lateralis* Klotho levels in COPD current smokers and former smokers (n=65). Data are expressed as mean ± SD or median (IQR).

A)



B)

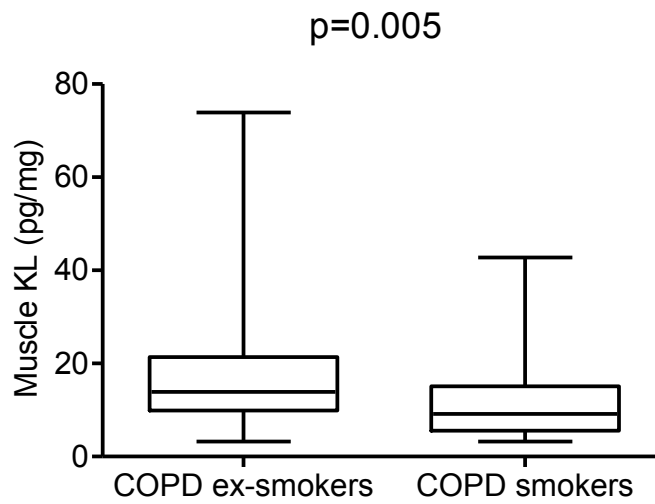
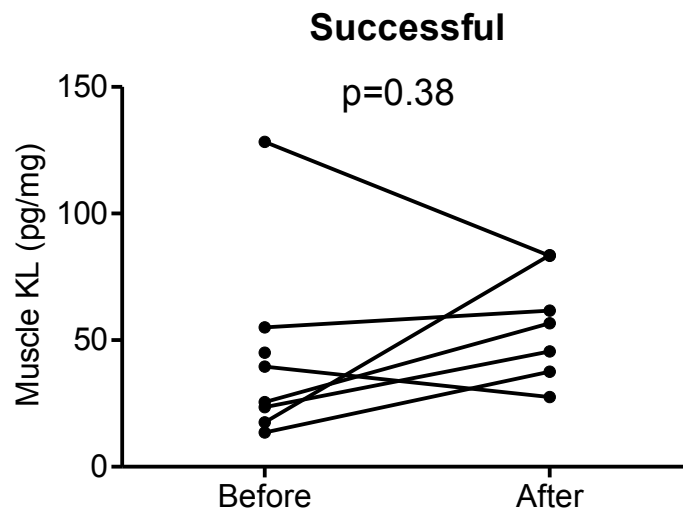


Figure 6.4: Box and whiskers plots demonstrating *vastus lateralis* Klotho levels in **A)** non-current smokers (healthy controls and ex-smokers; n=52) and current smokers (n=42), and **B)** COPD ex-smokers (n=36) and COPD current smokers (n=29).

A)



B)

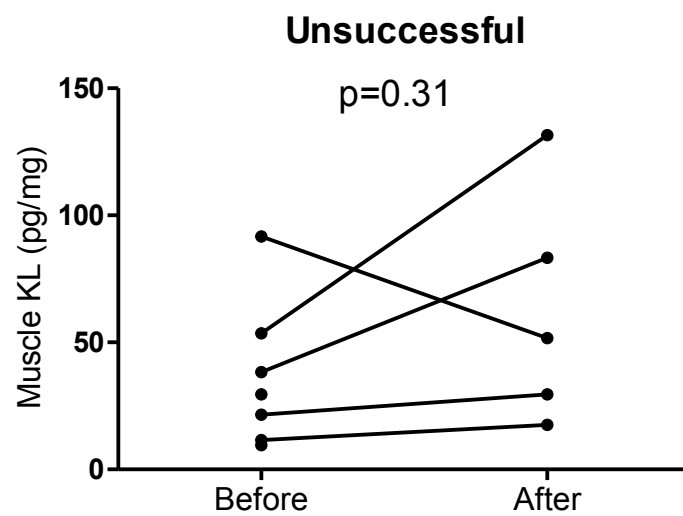


Figure 6.5: *Vastus lateralis* Klotho levels before and after attempted smoking cessation in **A)** those successful in quitting ($n=7$) and **B)** those unsuccessful in smoking cessation ($n=5$).

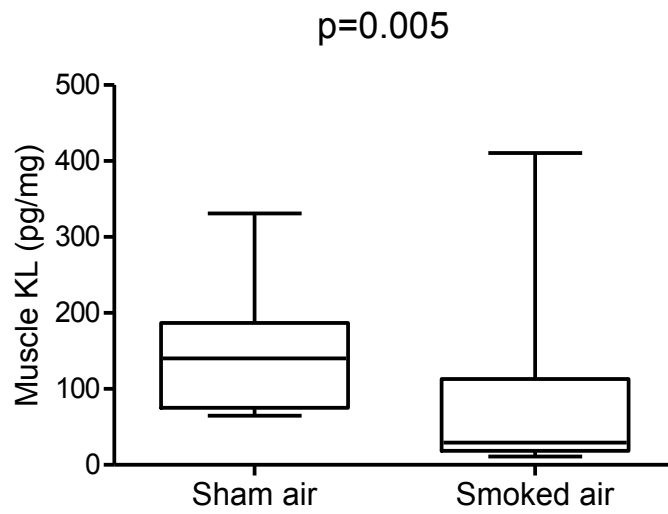


Figure 6.6: Box and whiskers plots demonstrating Klotho levels in the *gastrocnemius* of mice exposed to either sham air (n=9) or smoked air (n=19) daily over a 77 week period.

6.3.3 Klotho expression in respiratory and locomotor muscles

In the 12 COPD patients undergoing thoracic surgery, 7 underwent lung volume reduction surgery, 4 underwent bullectomy and 1 had surgery for the resection of a lung nodule (subsequently confirmed benign on histology), the characteristics of the cohort are presented in Table 6.4.

Sufficient protein was extracted to determine Klotho protein levels from 12, 11 and 9 *vastus lateralis*, external intercostal and diaphragm biopsies respectively. *Vastus lateralis* levels (13.9 (10.0, 20.0) pg/mg) were lower than diaphragm (17.8 (17.0, 22.5) pg/mg) and external intercostal levels (25.6 (17.2, 33.3) pg/mg) levels, $p=0.001$ (Figure 6.7). Where sufficient protein was extracted from paired quadriceps and respiratory muscle samples obtained from the same patients, levels in the quadriceps were lower than in the diaphragm ($n=9$, $p=0.03$) or intercostals ($n=11$, $p=0.002$).

Parameter	Mean (SD)
Age (years)	57 (9)
Gender (M:F)	7:5
Pack years	40 (13)
CO level (ppm)	7.8 (8.7)
FEV ₁ (% pred)	41 (25)
K _{CO} (% pred)	40 (15)
RV/TLC (%)	61 (6)
BMI (kg/m ²)	24.0 (6)
FFMI (kg/m ²)	16.8 (3)
QMVC/BMI	1.15 (0.5)
MRC	3.8 (1.2)
SGRQ	54 (25)
6MWT (m)	353 (98)
Steps/ day	4658 (3915)
RF _{CSA} (mm ²)	527 (243)

Table 6.4: Demographics and physiological data in the 12 COPD patients undergoing thoracic surgery for non-malignant disease. Data expressed as mean (SD).

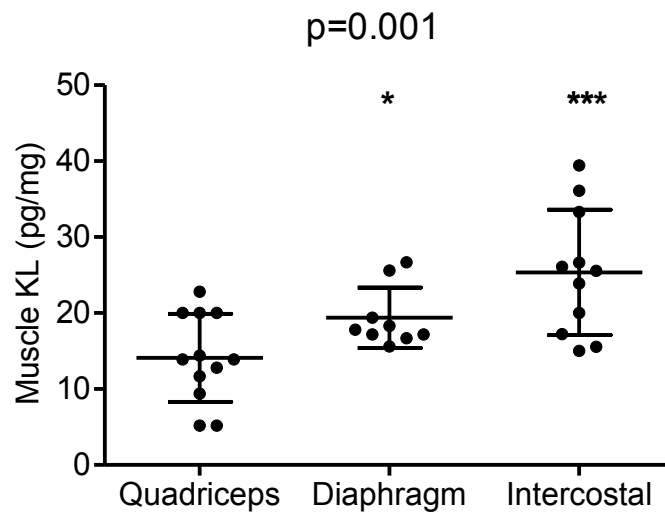


Figure 6.7: Comparative Klotho protein levels in the quadriceps, intercostal and diaphragm muscles of COPD patients. The lines represent the mean and SD.

6.3.4 Quadriceps Klotho protein and local oxidative stress

Protein carbonyl determinations were available in 68 participants (7 healthy, 14 'healthy smokers' and 47 COPD patients). Levels were not different between the groups; ANOVA $p=0.36$ (Figure 6.8). Furthermore, levels were not different in current smokers as compared to all participants who were not current smokers ($p=0.88$), or as compared to healthy controls ($p=0.86$).

Across the cohort as a whole, there was no significant relationship with FFMI, QMVC, QMVC/BMI, or RF_{CSA} , however, protein carbonyls did positively correlate with *vastus lateralis* Klotho protein levels ($r=0.30$, $p=0.01$).

In patients with COPD, protein carbonyls positively correlated with *vastus lateralis* Klotho protein ($r=0.34$, $p=0.02$), Figure 6.9, but not with clinical muscle parameters.

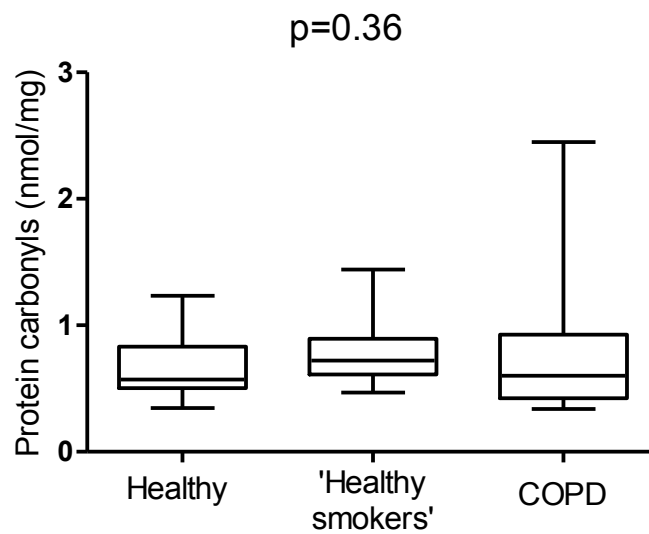


Figure 6.8: *Vastus lateralis* protein carbonylation in healthy (n=7), 'healthy smokers' (n=14) and COPD patients (n=47).

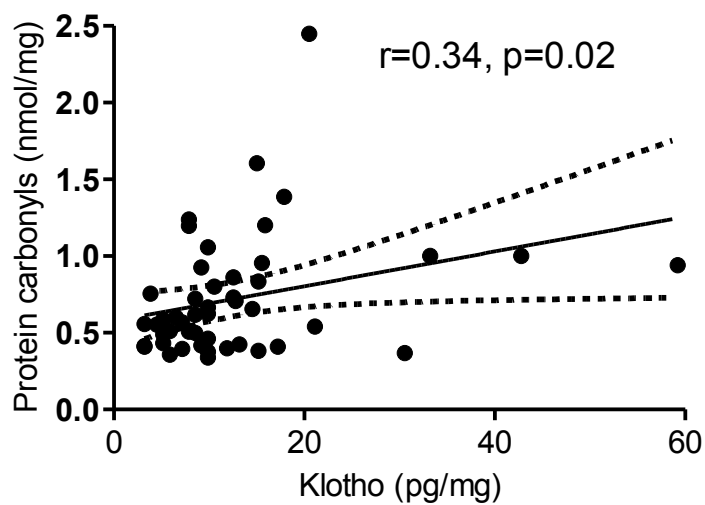


Figure 6.9: An X-Y plot demonstrating the relationship between vastus lateralis protein carbonyls and Klotho protein in COPD patients (n=47).

6.3.5 Klotho protein in established skeletal muscle dysfunction

Given the observation that *vastus lateralis* Klotho protein levels positively relate to protein carbonylation and that Klotho levels are lower in those currently smoking, Klotho expression in human skeletal muscle is likely to be complex, indeed given the relationships observed between circulating levels and QMVC/BMI, *vastus lateralis* levels may also be altered in COPD patients with established skeletal muscle dysfunction. In order to evaluate this, Klotho levels and protein carbonyls were compared in patients with established skeletal muscle dysfunction manifest either as a loss of whole body muscle mass (FFMI) or muscle strength (QMVC/BMI).

47 COPD patients had *vastus lateralis* protein carbonyl determinations; these patients also had Klotho protein measurements (Figure 6.10). 12 patients had an FFMI consistent with cachexia ($<15\text{kg/m}^2$ in females and $<16\text{kg/m}^2$ in males), whilst 35 had preserved nutritional status (Schols, Soeters et al. 1993). Patients with a reduced FFMI had increased *vastus lateralis* Klotho levels as compared to those with preserved FFMI ($p=0.04$), however, whilst protein carbonylation tended to be increased in this cohort, this was not statistically significant ($p=0.08$); Figure 6.10A. 24 patients had evidence of muscle weakness (QMVC/BMI <1.20) and 23 had preserved strength. COPD patients with reduced strength had increased *vastus lateralis* protein carbonylation ($p=0.04$) as compared to those with preserved strength, however, Klotho levels were not significantly different between the two groups ($p=0.12$); Figure 6.10B. COPD patients with both reduced QMVC/BMI and FFMI ($n=6$) had higher levels of both protein carbonylation and Klotho protein as compared to those without reduced FFMI and QMVC/BMI (41).

In the larger cohort ($n=65$), the same analyses demonstrated Klotho levels to be increased in both those with a reduced FFMI and those with reduced QMVC/BMI, Figure 6.11.

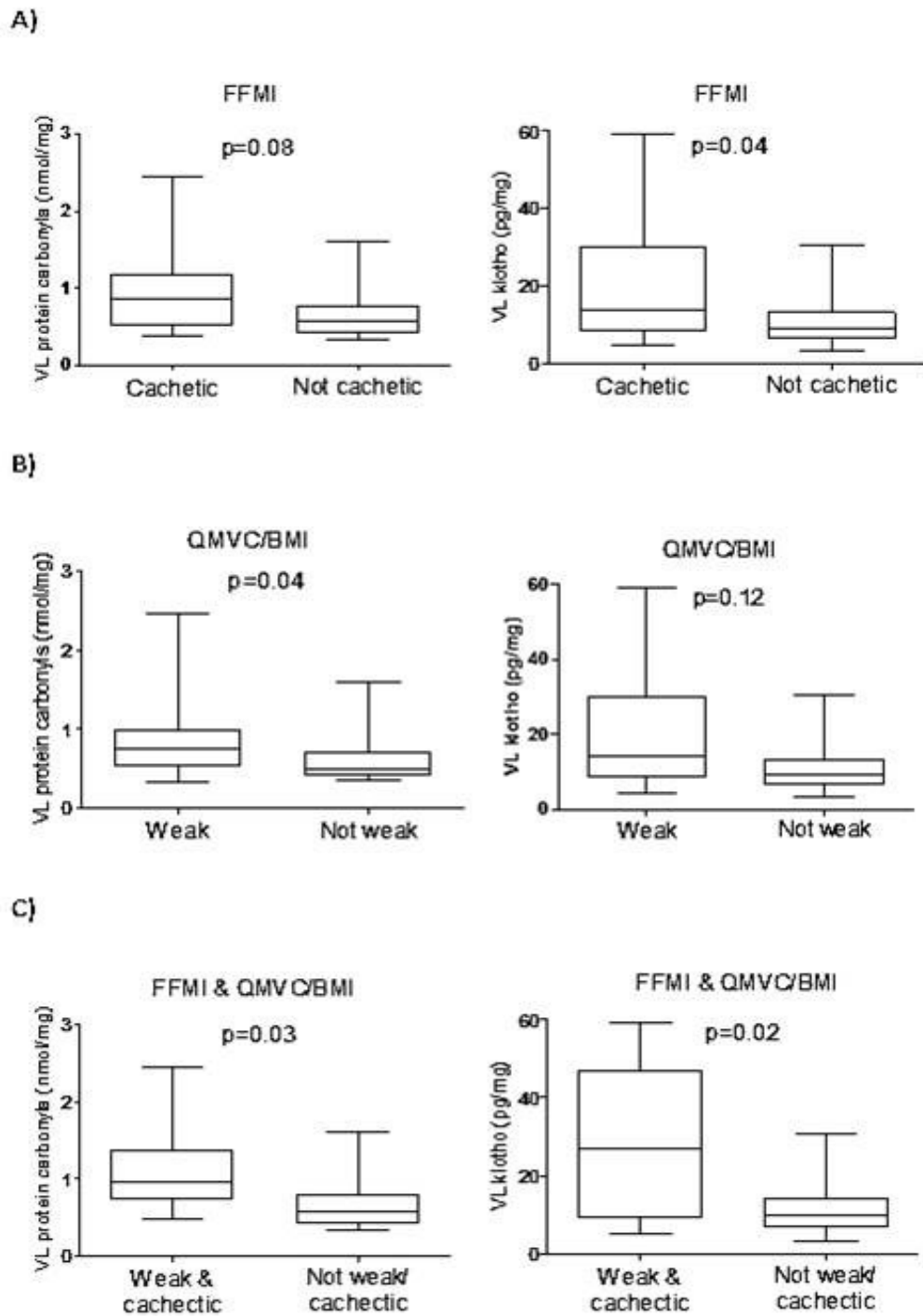
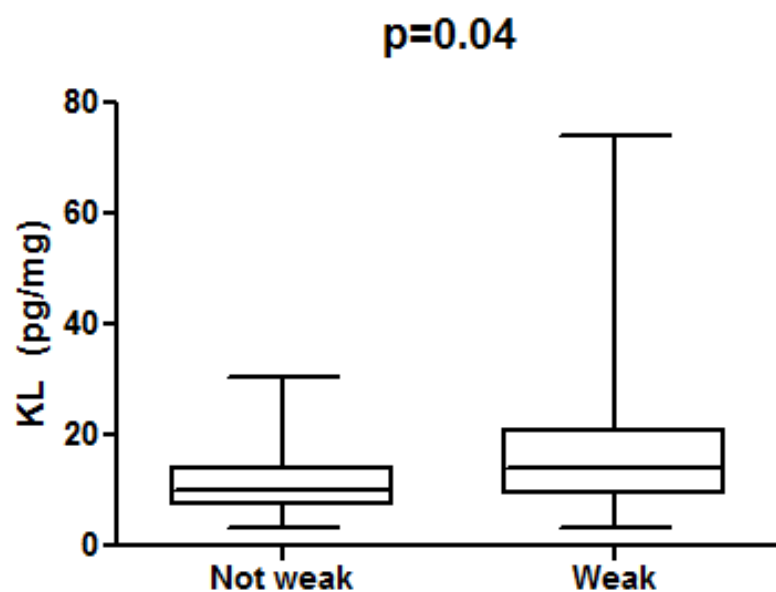


Figure 6.10: *Vastus lateralis* protein carbonylation (left panel) and Klotho levels (right panel) in COPD patients **A)** with (n=12) or without (n=35) reduced FFMI, **B)** with (n=24) or without (n=23) reduced QMVC/BMI and **C)** with (n=6) or without (41) reduced FFMI and QMVC/BMI.

A)



B)

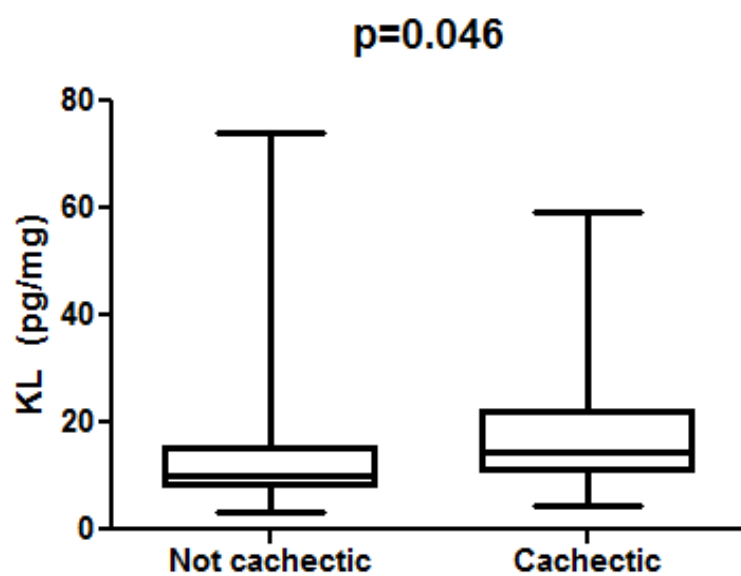


Figure 6.11: Data from the larger cohort ($n=65$) in whom *vastus lateralis* Klotho levels were available, with patients dichotomized according to **A)** $QMVC < 1.20$ or > 1.20 and **B)** $FFMI < 15/16 \text{ kg/m}^2$ or $FFMI > 15/16 \text{ kg/m}^2$.

Since Klotho protein was unexpectedly increased in the quadriceps of patients who had developed either weakness or muscle atrophy, further immunohistochemistry was performed to identify if Klotho was associated with centralised nuclei and as demonstrated in Figure 6.12, this was confirmed to be the case.

Given that a colleague in the Molecular Medicine Department, Jen Lee, was working on electroporated mouse *gastrocnemius* tissue, a process observed to confer extensive tissue damage followed by subsequent regeneration; immunohistochemistry was also performed by myself on muscle sections provided by Jen Lee. This analysis confirmed significant Klotho expression to be present in damaged skeletal muscle tissue and each regenerating fibre to express Klotho co-localised to a centralised nucleus (Figure 6.13).

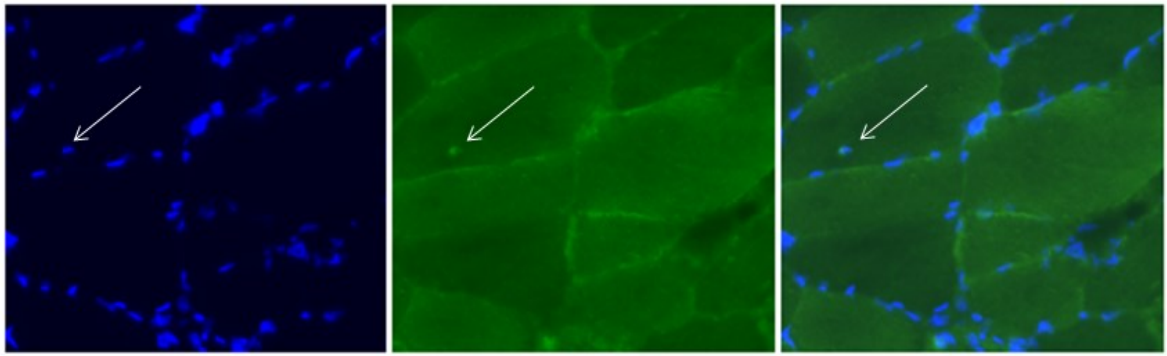


Figure 6.12: Immunohistochemistry of a human *vastus lateralis* muscle section with blue DAPI nuclear staining only (left panel) and with Klotho staining green (centre panel) and a merged image with both Klotho and DAPI staining demonstrating Klotho to co-localize to an intracellular nucleus (right panel). The arrow highlights the presence of an intracellular nucleus.

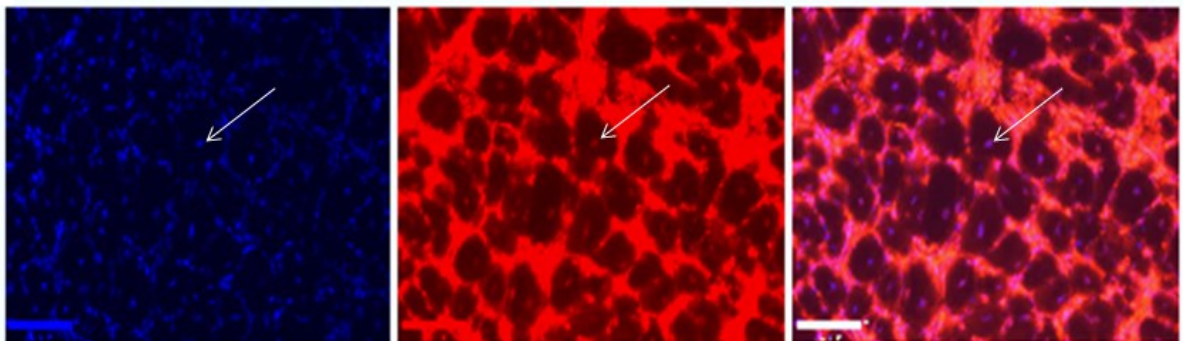


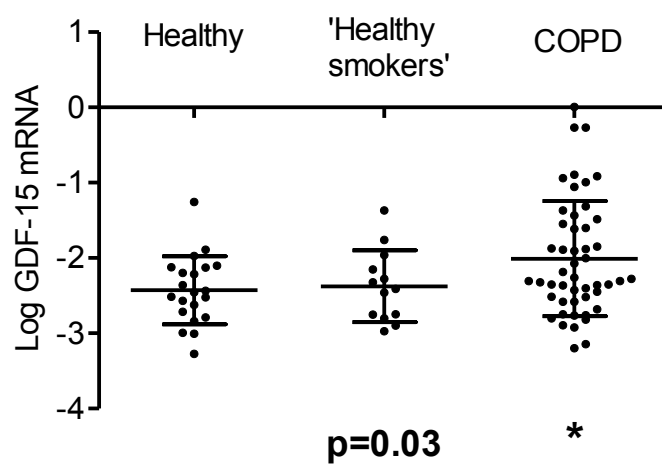
Figure 6.13: Immunohistochemistry of electroporated mouse *gastrocnemius* muscle section with blue DAPI staining (left panel), Klotho staining in red (centre panel) and a merged image demonstrating widespread Klotho expression in regenerating tissue (right panel). Different colours are present for Klotho staining in human and mouse muscle due to the availability of secondary antibodies. The arrow highlights an example of one of numerous intracellular nuclei. (Muscle section provided by Jen Lee, staining by the author).

6.3.6 The quadriceps expression of GDF-15

Sufficient tissue was available to evaluate GDF-15 transcript levels from 83 biopsies (21 healthy controls, 13 'healthy smokers' and 49 COPD patients); the demographics were not different from the cohort presented in Table 5.1. GDF-15 mRNA levels were higher in the quadriceps of COPD patients, than healthy controls, although GDF-15 mRNA expression was not different in 'healthy smokers'; ANOVA: $p=0.03$ (Figure 6.14A). Quadriceps GDF-8 expression was also elevated in COPD patients as compared to healthy subjects, but again was not elevated in 'healthy smokers', ANOVA $p=0.009$ (Figure 6.14B). There was no relationship between serum GDF-15 levels and quadriceps mRNA transcripts ($r=0.10$, $p=0.35$), however, quadriceps GDF-8 and GDF-15 mRNA levels related, $r=0.59$, $p<0.0001$ (Figure 6.15).

Quadriceps GDF-15 mRNA levels did not relate to any other parameters, including protein carbonyls, either in the full cohort or in those with COPD. Univariate predictors of muscle GDF-15 transcript levels included current smoking status, COPD diagnosis, $FEV_{1\%}$, K_{CO} , MRC and 6MWT; however, none were retained in a multivariate analysis (Table 6.5). Given the number of variables and the collinearity between them, a further multivariate analysis only containing current smoking status and the presence of COPD, identified both of these variables to independently predict quadriceps GDF-15 mRNA expression (Table 6.6).

A)



B)

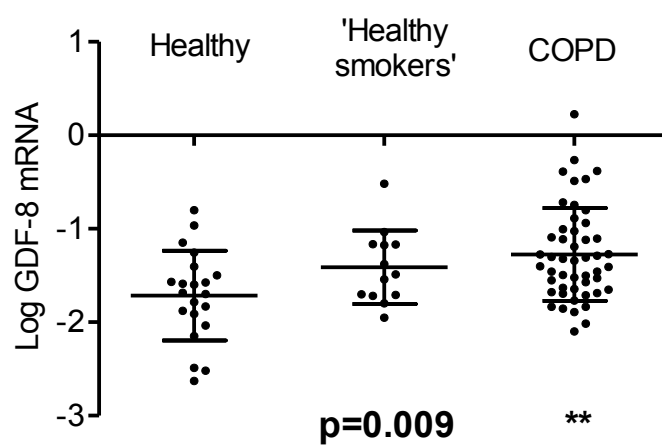
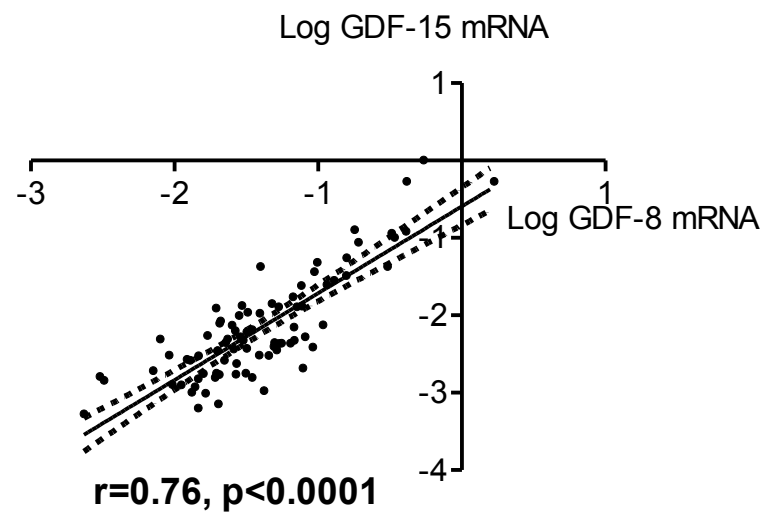


Figure 6.14: The mRNA expression of **A)** GDF-15 and **B)** GDF-8 within the quadriceps muscle of healthy controls, 'healthy smokers' and COPD patients. Data are normalised to RPLPO, the lines represent the mean and SD.

A)



B)

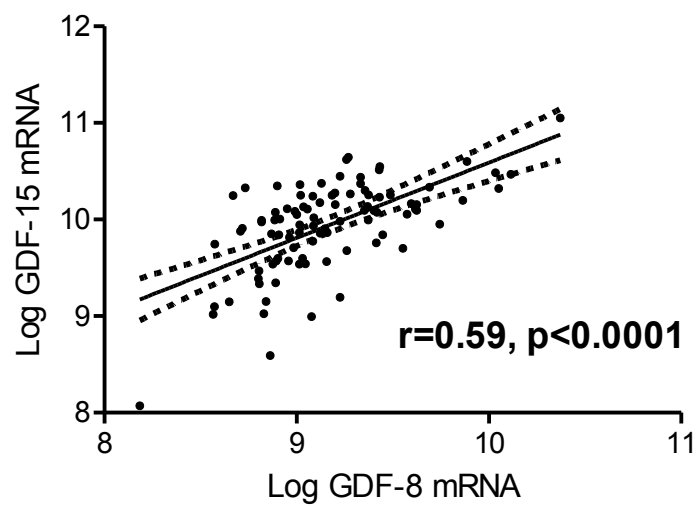


Figure 6.15: X-Y plots demonstrating the relationship between GDF-15 mRNA and myostatin mRNA levels when, **A)** normalised to RPLPO and **B)** not normalised to RPLPO.

Variable	Univariate regression			Multiple regression	
	Coefficient (95% CI)	Standardised coefficient	p	Coefficient (95% CI)	p
Age	-0.01 (-0.02, 0.01)	-0.12	0.29	-	-
Current smoker	0.31 (0.02, 0.61)	0.23	0.04	-	-
Gender (male)	-0.03 (-0.33, 0.28)	-0.02	0.86	-	-
COPD	0.40 (0.11, 0.69)	0.29	0.01	-	-
FEV₁ (%pred)	-0.006 (-0.100, -0.001)	-0.28	0.01	-	-
K_{co} (%pred)	-0.07 (-0.12, -0.01)	-0.27	0.02	-	-
6MWT	-0.001 (-0.002, 0.000)	-0.25	0.03	-	-
Steps/ day	-0.00 (-0.76, 0.45)	-0.09	0.45	-	-
RF _{CSA}	-0.00 (-0.00, 0.00)	-0.15	0.26	-	-

Table 6.5: Regression analysis to identify the predictors of quadriceps GDF-15 mRNA expression, when including all univariate predictors. None were retained as independent predictors.

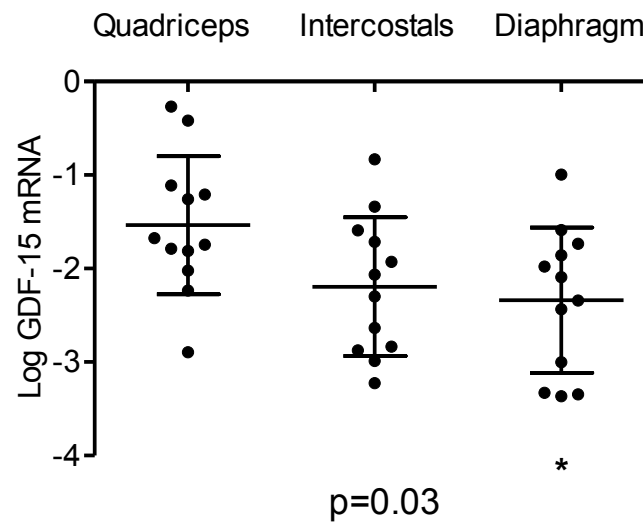
Variable	Univariate regression			Multiple regression		
	Coefficient (95% CI)	Standardised coefficient	p	Coefficient (95% CI)	Standardised coefficient	p
Current smoker	0.31 (0.02, 0.66)	0.23	0.04	0.30 (0.01, 0.58)	0.21	0.04
COPD	0.40 (0.11, 0.69)	0.29	0.01	0.39 (0.10, 0.67)	0.28	0.009

Table 6.6: A multivariate analysis limited to current smoking status and COPD, demonstrating both independently predict quadriceps GDF-15 transcript expression.

6.3.7 GDF-8 and GDF-15 expression in respiratory & locomotor muscles

Sufficient RNA was extracted for RT-qPCR to be performed to evaluate expression in all 3 tissues, in all 12 patients. Comparing locomotor with respiratory muscle, GDF-15 mRNA levels were elevated in the quadriceps (-1.54 ± 0.7), as compared to the diaphragm (-2.34 ± 0.8), Figure 6.16A, although GDF-8 expression was not different in respiratory and locomotor muscles; $p=0.76$ (Figure 6.16B). Intercostal muscle GDF-15 and GDF-8 expression was not different as compared to quadriceps or diaphragm expression (Figure 6.16).

A)



B)

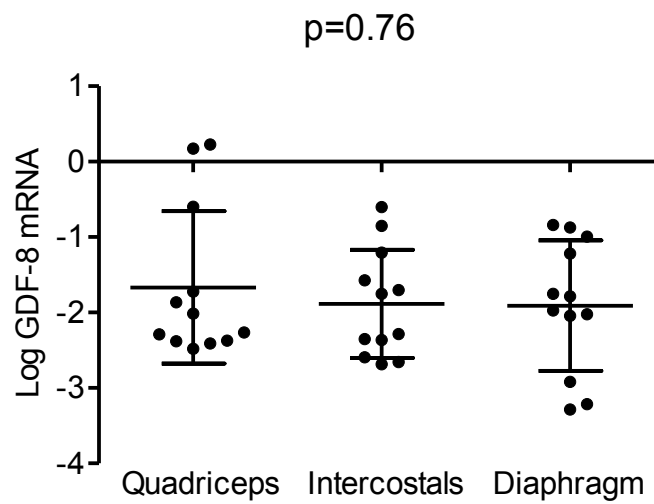


Figure 6.16: The comparative mRNA expression of **A)** GDF-15 and **B)** GDF-8 within the quadriceps, intercostals and diaphragm muscles of COPD patients. The data presented are normalised to RPL0, the lines represent the mean and SD.

6.4 Discussion

6.4.1 Summary of the findings

This study is the first to evaluate Klotho and GDF-15 expression in human skeletal muscle. Additional observations implicate a role for Klotho in the pathophysiology of the skeletal muscle dysfunction observed in COPD. Firstly, evidence that cigarette smoking may be a factor inhibiting Klotho expression was corroborated by the observation that levels are reduced in the quadriceps of human smokers and also that smoke exposed mice had lower *gastrocnemius* levels of Klotho than mice exposed to air under the same circumstances. The relatively higher Klotho levels observed in respiratory muscle suggests that contractile activity may attenuate this effect. It is interesting that in contrast to the serum data (Chapter 5), quadriceps Klotho levels were not lower in COPD. Consistent with this observation, quadriceps Klotho levels were demonstrated to have a positive correlation with protein carbonylation within the muscle. Further evidence for a role for Klotho signalling in the skeletal muscle of COPD patients was demonstrated by the fact that quadriceps Klotho levels were actually increased in patients with either nutritional depletion or muscle weakness. Lastly, immunohistochemistry confirmed Klotho to be localised not only to the muscle fibre membrane but also to centralised nuclei, a hallmark of regenerating muscle fibres. A role for Klotho in regeneration was supported by extensive Klotho protein being visualised in damaged mouse tissue with multiple regenerating fibres expressing Klotho co-localised to a centralised nucleus.

Whilst GDF-15 was also demonstrated to be expressed in skeletal muscle, a potential pathophysiological role for the expression of this protein in skeletal muscle in the development of skeletal muscle dysfunction in COPD is seemingly less clear. Levels were not altered in smokers, but were increased in the quadriceps of COPD patients compared to controls. In patients with COPD,

unlike GDF-8 transcripts, GDF-15 transcripts were reduced in the respiratory muscles as compared to the locomotor muscles; despite this observation, in the larger cohort in whom quadriceps transcripts were measured, there was no relationship with other relevant parameters such as muscle bulk. Nonetheless, in a regression analysis, smoking and COPD were observed to have independent effects on *vastus lateralis* GDF-15 mRNA expression.

6.4.2 Significance of the findings

As observed in Chapter 5, consistent with the theory that COPD is a disease characterised by accelerated ageing, the expression of biological markers of ageing may be altered in smokers and patients with COPD and these adaptations may have functional consequences. To my knowledge there have been no specific studies of the expression of either Klotho or GDF-15 in human skeletal muscle. In this study, it has been demonstrated that not only are both expressed in human muscle, but that levels are altered by the presence of smoking or COPD, pertinently in the setting of these risk factors, chronological age did not relate to either Klotho protein or GDF-15 mRNA expression in muscle.

Higher Klotho protein levels were observed in respiratory muscle groups than in the quadriceps. In health the respiratory muscles exhibit a similar fibre type composition to the quadriceps, however, in COPD this changes so that the diaphragm has more type I fibres (Stubbings, Moore et al. 2008) and the quadriceps fewer (Natanek, Gosker et al. 2013). The external intercostal muscles also display features of adaptation to the chronically elevated mechanical load (Sanchez, Brunet et al. 1988; Ribera, N'Guessan et al. 2003). These data may be interpreted as local factors such as contractile load attenuating the deleterious effect of systemic insults such as smoking on Klotho levels. Similarly, GDF-15 mRNA expression was elevated in the quadriceps as compared to the respiratory

muscles. Despite a positive relationship being observed between GDF-8 and GDF-15mRNA levels in the quadriceps, GDF-8 levels were not altered in the locomotor muscles as compared to respiratory muscle expression, indicating that a pathophysiological role for altered GDF-15 expression in skeletal muscle may be independent to GDF-8 expression. Pertinently, at least in the quadriceps, neither Klotho nor GDF-15 expression related to fibre type preponderance or measures of whole body physical activity, so expression may not be explained by contractile burden alone. Although there was no reduction in quadriceps Klotho levels in those with COPD compared to those without; levels were reduced in the quadriceps of smokers. Furthermore, when the analysis was confined to COPD patients, current smokers had lower levels than former smokers, again suggesting a direct effect of smoking. Quadriceps GDF-15 expression was not altered in smokers, but expression was elevated in patients with COPD. In the cohort as a whole, a regression analysis identified smoking status, but not age to be a predictor of GDF-15 expression; furthermore, this was independent to the effect conferred by the presence of COPD.

Given that, especially in the case of Klotho, smoking affects expression, these data add further credence to the argument that smoking may trigger skeletal muscle dysfunction in COPD through a process of accelerated ageing. This is of potential clinical relevance as modulation of both Klotho and GDF-15 signalling in skeletal muscle may offer potential therapeutic opportunities. Nonetheless, exact roles for these proteins in human skeletal muscle need to be established, as especially in the case of Klotho, expression of these proteins is clearly complex.

Unlike circulating Klotho, *vastus lateralis* protein levels were not seen to rise on successful smoking cessation. This may be a consequence of skeletal muscle expression taking longer to respond to smoking cessation; alternatively other factors may also be relevant in affecting skeletal muscle Klotho expression. Consistent with this suggestion, it was an unexpected observation that Klotho levels were in fact elevated in COPD patients with established skeletal muscle dysfunction manifest

either as a pathological loss of fat free mass or quadriceps strength. It was hypothesised that increased Klotho levels in this setting may be a physiological response, the concept that Klotho signalling may have a role in regeneration was supported by a physical association with satellite cells, which are the hallmark of regenerating fibres. These findings were corroborated in another mouse model (formed to determine the general response to muscle injury as part of the overall doctoral project of Jen Lee). In the study of electroporated mouse *gastrocnemius* tissue, a process observed to confer extensive tissue damage followed by subsequent regeneration; immunohistochemistry confirmed significant Klotho expression and each regenerating fibre to express Klotho co-localised to a centralised nucleus. Nonetheless, based on the current data, it remains to be ascertained whether Klotho signalling has an active role in this scenario or is simply an epiphenomenon, or indeed if other factors such as FGF-23 are more relevant given that Klotho levels are elevated in the setting of a sarcopenic phenotype. An alternative proposition may be that up-regulated Klotho expression in this setting may even be pathological; however, other data presented elsewhere in this thesis and in the literature do not support this concept.

Evidence against elevated Klotho in this setting being an epiphenomenon or a pathological response comes from the observation that Klotho may influence muscle regeneration by influencing signalling pathways that are recognised to be altered in senescence, such as the Wnt signalling pathway. Brack *et al.* demonstrated that the regenerative potential of skeletal muscle in mice declines with age and is associated with increased tissue fibrosis as a consequence of satellite cells converting to a fibrogenic lineage as they begin to proliferate; this change is mediated by factors in the systemic environment of older animals including altered Wnt signalling (Brack, Conboy et al. 2007). Mice deficient in Klotho exhibit decreased stem cell number and increased progenitor cell senescence, furthermore, not only does Klotho suppress Wnt biological activity in cell culture, but Klotho-deficient mice display increased Wnt signalling and Klotho counters the accelerated cellular senescence conferred by Wnt (Liu, Fergusson et al. 2007).

The transmembrane form of Klotho acts as a co-receptor for FGF-23, significantly enhancing FGF-23 binding to FGF receptors (Kurosu, Ogawa et al. 2006). FGF-23 levels in muscle were so low that meaningful determinations could not be made; consistent with the findings in the serum, Klotho also exerts actions independent to FGF-23 (Kurosu, Yamamoto et al. 2005). Nonetheless, given that the relationship between serum FGF-23 and systemic levels of oxidative stress was similar to the positive relationship observed between quadriceps Klotho levels and protein carbonylation, a marker of oxidative stress in muscle, an integrated role for Klotho and FGF-23 in affecting skeletal muscle phenotype cannot be refuted.

Based on data from other models, putative mechanisms by which Klotho may influence skeletal muscle function in COPD include autophagy, the ubiquitin-ligase pathway, oxidative stress and apoptosis. Whilst the autophagy-lysosomal pathway is activated in the masseter and tongue of *Klotho* deficient mice, more relevantly to COPD patients, it is not up-regulated in the *gastrocnemius* (Iida, Kanko et al. 2011). Furthermore, the ubiquitin-proteasomal pathway is unaffected in any of these muscle groups within *Klotho* deficient mice (Iida, Kanko et al. 2011), consistent with our group's observation that ubiquitin E3 ligases are not up-regulated in the quadriceps of stable COPD patients (Natanek, Riddoch-Contreras et al. 2013). A more plausible proposal is that the protective effects of Klotho occur through the attenuation of oxidative stress. Yamamoto *et al.* showed that recombinant Klotho protected cells from oxidative stress *in vitro* and that manganese superoxide dismutase was increased in the muscle of *Klotho* over-expressing mice consistent with a protective effect *in vivo*. However, they did not measure Klotho levels in muscle, indeed the mouse model used over-expressed *Klotho* in the brain and testis suggesting that effects on muscle gene expression were through circulating Klotho (Yamamoto, Clark et al. 2005). Barreiro and colleagues have demonstrated that oxidative stress is increased in both the diaphragm and *gastrocnemius* of mice exposed to cigarette smoke (Barreiro, del Puerto-Nevado et al. 2012) and also in the quadriceps of

smokers and COPD patients, in whom it relates to skeletal muscle dysfunction (Barreiro, Peinado et al. 2010). However, having demonstrated that smoking has an attenuating effect on Klotho protein expression, this alone does not explain the findings, especially as smokers have elevated levels of oxidative stress (Barreiro, Peinado et al. 2010).

In this study, protein carbonyls were found to positively relate to Klotho levels, this may be explained by smoking and oxidative stress affecting Klotho by independent mechanisms, or indeed that the increased Klotho levels in the setting of elevated oxidative stress may be a protective mechanism, a situation analogous to the unexpected observation of elevated quadriceps Klotho protein levels in patients with sarcopenia. It may well be that a basal level of Klotho is present within each myofibre and this level is attenuated by smoking, and possibly other currently unidentified factors, and this attenuating effect is superceded by other stimuli which result in the upregulation of Klotho including muscle wasting and elevated oxidative stress, both of which seem to occur together in the same cohort of patients. As already discussed, the specific molecular trigger for an upregulation in Klotho remains to be established, as is whether the elevation in Klotho is functional. These findings are difficult to prove in man, however, interventional studies and further animal and cell work may further elucidate the relevance of altered Klotho expression in muscle. In this regard other proteins such as FGF-23 and Wnt may also be relevant and also altered under different conditions; this currently remains supposition and cell work would be important in addressing these questions.

Vastus lateralis GDF-15 mRNA expression was elevated in patients with COPD. Whilst levels were not elevated in current smokers, in a regression analysis, smoking was also observed to affect GDF-15 expression. However, aside from the elevated expression of GDF-15 in locomotor muscles as compared to the respiratory muscles, direct evidence for a pathophysiological role for altered GDF-15 expression in skeletal muscle was not demonstrated. No relationship was observed with relevant

physiological parameters such as muscle bulk or strength, suggesting that a precise role for GDF-15 expression within skeletal muscle remains unclear. Nonetheless, this is the first study to demonstrate that GDF-15 is expressed in skeletal muscle and that there is evidence of both a disease and smoking effect on GDF-15 expression. Of relevance, airway epithelial GDF-15 expression has also been demonstrated to be elevated in COPD smokers, furthermore, cigarette smoke exposure up-regulates GDF-15 expression in airway epithelial cells (Wu, Jiang et al. 2011). When this is considered in the context of the results presented in Chapter 5, which demonstrated that circulating GDF-15 levels are also elevated in COPD and may identify patients with locomotor atrophy, further work to elucidate a role for GDF-15 in skeletal muscle is warranted. A pathophysiological role for GDF-15 in skeletal muscle is supported by the data previously reported by our group that demonstrates GDF-15 to have a direct atrophic effect on myotubes and that systemic levels rise in patients developing acute locomotor muscle wasting following cardiothoracic surgery (Bloch, Lee et al. 2013).

4.4.3 Critique of the Method

As previously discussed, whilst the data reported here support pathophysiological roles for Klotho and to a lesser extent also GDF-15 signalling within skeletal muscle, direct relationships would require functional manipulation in man and longitudinal follow up. Nonetheless, the smoked mouse model confirms a smoking effect on Klotho expression in muscle. A pathophysiological role for Klotho expression in the development of skeletal muscle dysfunction is further provided by the work of others, which confirms that Klotho deficient mice also develop sarcopenia (Iida, Kanko et al. 2011) and a shortened life-span (Kuro-o, Matsumura et al. 1997).

Klotho and FGF-23 mRNA expression could not be quantified in human skeletal muscle due to probable primer dimer formation; a scenario in which primer molecules have hybridized to each

other because of strings of complementary bases in the primers. DNA polymerase amplifies the primer and this product competes for PCR reagents with the genuine product in future rounds of PCR. Such dimers are therefore likely to inhibit the amplification of the intended target for PCR amplification and subsequently prevent accurate quantification. In this setting, as the expression of Klotho and FGF-23 were also very low, the results were uninterpretable, especially as the effect was not restricted to the same samples on each occasion. Whilst this may have led to the interpretation that Klotho and FGF-23 are too lowly expressed in human skeletal muscle to be meaningful, and in the case of FGF-23 this seems plausible, protein quantification of Klotho was possible. To confirm that the Klotho protein quantified in the biopsy extracts was of muscular origin, immunohistochemistry was performed which confirmed Klotho to be present in skeletal muscle.

The heterogeneity of COPD as a disease and the development of skeletal muscle dysfunction in this cohort appear to be important considerations when evaluating biological findings in cross-sectional studies, highlighting the importance of validating findings in other populations. Consistent with this, Barreiro *et al.* observed protein carbonylation to be elevated in COPD patients as compared to healthy controls, and an inverse relationship to be present between quadriceps strength and protein carbonylation. These findings were not reproduced in this cohort, even when also limiting the analysis to patients with severe disease (Barreiro, Schols et al. 2008). However, the findings of the present study are likely to be relevant as they include data on a large cohort of patients who have undergone muscle biopsies and extensive phenotyping; contextually the relationship reported by Barreiro *et al.* was observed in 10 COPD patients. In addition to sample size, another aspect highlighted by the present data is the role of stratifying patients. Molecular pathways are intertwined and subject to a milieu of different agonistic and antagonistic stimuli; the balance of which may alter in different environments, such as exposure to cigarette smoke or physical inactivity during an exacerbation. As consistent with the current data, it seems likely that different molecular targets and signalling pathways may have different physiological or pathophysiological roles in

different scenarios, subsequently validating findings and further delineating these different roles in animal and cell models is relevant, as is replicating these findings in other human populations. Furthermore, identification of the downstream targets of the relevant signalling pathways, such as Wnt signalling, would further validate a role for Klotho or GDF-15 in the pathogenesis of skeletal muscle dysfunction in COPD.

6.4.4 Conclusions

To my knowledge, the present study is the first to evaluate the expression of two independent biological correlates of ageing, namely Klotho and GDF-15, in skeletal muscle. In COPD, quadriceps expression of GDF-15 is elevated as compared to expression in controls and as compared to the diaphragm. Klotho protein levels are reduced in the quadriceps of human smokers and also smoke exposed mice, with relatively higher Klotho levels in respiratory muscle. Although quadriceps Klotho levels were not lower in COPD, Klotho levels were unexpectedly observed to positively correlate with muscle protein carbonylation. Furthermore, quadriceps Klotho levels were actually elevated in patients with established skeletal muscle dysfunction, consistent with this, immunohistochemistry confirmed Klotho to be expressed in regenerating muscle fibres. Modulation of the expression of Klotho and GDF-15, or down-stream pathways, may provide therapeutic options in patients with skeletal muscle dysfunction in COPD.

Chapter 7 : General Discussion

7.1 Summary of the findings presented in this thesis

Several novel findings are presented in this thesis. Firstly, the change in quadriceps fibre proportion that occurs in COPD is associated with an increased risk of mortality, and in those with more severe lung function impairment (i.e. GOLD stage III or IV), quadriceps strength is the most relevant muscle parameter that impacts mortality. Secondly, relevant markers of skeletal muscle dysfunction, including fibre shift and reduced muscle strength, may be detected by a simple physiological tool, the SPPB. At a pathophysiological level, it is therefore relevant that circulating proteins that are both predictive of mortality in general populations, Klotho and GDF-15 (Wiklund, Bennet et al. 2010; Semba, Cappola et al. 2012), were observed to have altered expression in COPD and relate to markers of skeletal muscle function in COPD. Lastly, given these positive findings, it was demonstrated that not only are Klotho and GDF-15 both expressed in human skeletal muscle, but that levels are altered by contractile burden and either cigarette smoking or the presence of COPD. Additionally, there was also evidence to support a role for Klotho signalling in muscle regeneration.

7.2 Significance of these findings

The predictive properties of fibre shift and quadriceps strength in COPD were observed to be independent of chronological age; furthermore, SPPB score and circulating Klotho levels were not associated with age. Circulating Klotho and GDF-15 levels related to quadriceps strength and bulk independent to the effects of chronological age, additionally when measured in the quadriceps, neither Klotho nor GDF-15 expression related to age. Given that both Klotho and GDF-15 are

associated with ageing (Kuro-o, Matsumura et al. 1997; Vila, Riedl et al. 2011; Crasto, Semba et al. 2012; Berberoglu, Aktas et al. 2014), the finding that cigarette smoking affects the expression of Klotho, and to a lesser extent GDF-15, is of potential pathophysiological relevance. Smoking is associated with accelerated ageing (Valdes, Andrew et al. 2005); these findings would support the theory of accelerated biological ageing, possibly through the mechanism of cigarette smoking, as having a role in the locomotor skeletal muscle dysfunction that occurs in COPD. Although loss of strength or muscle bulk was not observed in smokers without COPD, this was complicated by the significantly younger age of this cohort. Nonetheless, it has previously been demonstrated that skeletal muscle dysfunction occurs early on in the disease process, including in smokers without airflow obstruction (Montes de Oca, Loeb et al. 2008; Shrikrishna, Patel et al. 2012) and that smokers are at risk of losing strength (Kok, Hoekstra et al. 2012) which may improve with smoking cessation (Hopkinson, Tennant et al. 2007). The literature also supports the concept that GDF-15 may be increased and Klotho reduced by smoking in other cohorts. Wu *et al.* observed that human smokers have increased airway epithelium GDF-15 levels and demonstrated that cigarette smoke exposure up-regulates GDF-15 expression in airway epithelial cells (Wu, Jiang et al. 2011). Furthermore, findings of smoking attenuating Klotho are supported by other very recent data; young pregnant smokers were observed to have lower circulating Klotho levels than non-smoking counterparts, which is consistent with the concept that circulating levels may be reduced by smoking even at an age where COPD is rarely established (Lam-Rachlin, Romero et al. 2013). In this context, the effects of chronic Klotho suppression may therefore gradually accrue, along with chronological age, over a prolonged time period in susceptible individuals who continue to smoke.

Ageing is a complex process that is manifest at genetic, molecular, cellular, organ and system levels. Given that chronological age cannot be altered, but is recognised to be associated with physiological decline and worse outcomes, it seems pertinent that altered expression of age-related markers are relevant in the skeletal muscle dysfunction of COPD, either as biomarkers of the disease or as

therapeutic targets. The fact that fibre shift is associated with increased mortality in an outpatient COPD population is of clinical relevance. The detection of fibre shift may not only provide important prognostic information to the individual COPD patient, but also guide potential therapy such as referral for pulmonary rehabilitation. Furthermore, the detection of fibre shift is likely to be important in clinical trials that are aimed at addressing the burden of skeletal muscle adaptations. In this regard, loss of quadriceps strength is also important and indeed seems to be more relevant in predicting mortality than fibre shift in those with more advanced airflow obstruction. The detection of fibre shift necessitates an invasive procedure and the use of specialist skills and facilities, indeed measurement of fibre shift and to a lesser extent quadriceps strength are not readily practised. It is therefore significant that fibre shift and indeed loss of quadriceps strength may be detected by a simple functional tool, the SPPB. The SPPB is readily performed in the assessment of older non-selected populations and may be easily adopted into both routine clinical practice and when stratifying COPD patients prior to entry into clinical trials. Given the clinical relevance of the skeletal muscle adaptations that occur in COPD, the molecular adaptations that are associated with them are of particular interest as manipulation of target pathways may offer a therapeutic opportunity.

It seems likely that there is an ongoing interaction between environmental and biological factors and the balance between the two culminates in the skeletal muscle phenotype observed, an altered balance conferred by additional stressors is likely to herald the pathophysiological skeletal muscle adaptations that occur in COPD. In this regard smoking, physical activity and chronological age are all likely to be relevant, a concept supported by the data presented in this thesis and the literature.

Serum GDF-15 levels were demonstrated to be elevated in patients with COPD, and quadriceps GDF-15 expression was elevated as compared to controls and as compared to expression in the diaphragm. Given that systemic levels related to the systemic levels of oxidative damage, and that circulating levels inversely related to quadriceps muscle bulk, GDF-15 may have a role in the

pathophysiological adaptations observed in the skeletal muscle of COPD patients. Indeed given that circulating levels were higher in those with established atrophy, GDF-15 may have a potential role as a biomarker. Despite a role for GDF-15 in the development of myopathy being supported by the work in our group and the literature, it is nonetheless surprising that levels did not relate to quadriceps strength given its functional relevance and strong relationship with quadriceps bulk. Given that GDF-15 levels were only altered in those with established airflow limitation, based on the current data, it remains unclear whether GDF-15 has an active role in the pathophysiological skeletal muscle adaptations observed in COPD, especially since quadriceps GDF-15 expression did not relate to muscle bulk. Nonetheless, the lower expression of GDF-15 in respiratory than locomotor muscle, indicates that contractile activity influences GDF-15 skeletal muscle expression.

It is of particular interest that Klotho levels are altered by smoking, systemically and within skeletal muscle, and that at least in the case of systemic Klotho, this effect was even observed in smokers with normal spirometry. As higher Klotho protein levels were observed in muscles with a greater level of contractile activity, it seems plausible that the effects of smoking and physical activity may counteract one another in influencing Klotho expression, at least in skeletal muscle. Systemic Klotho levels improved with smoking cessation, which is associated with not only well-recognised global benefits (Taylor, Hasselblad et al. 2002), but also a gain in muscle mass (Hopkinson, Tennant et al. 2007). The association of circulating Klotho levels with quadriceps strength may represent circulating Klotho to be a marker of global 'well-being', especially given that transmembrane Klotho is predominantly expressed in the kidney and the parathyroid glands. Nonetheless, given that Klotho is expressed in skeletal muscle and cigarette smoking was observed to have an attenuating effect on expression within the quadriceps, cigarette smoking may have a role in the pathological muscle adaptations that occur in COPD; whether this is a direct or indirect effect will require the work of further studies. Indeed on the basis of the data presented in this thesis, a role for Klotho in the skeletal muscle of COPD patients seems more complex than that of GDF-15. The finding of elevated

Klotho levels in the quadriceps of patients with established loss of muscle or strength was unexpected. In this scenario, increased Klotho expression may be interpreted as an attempted protective mechanism, which would be consistent with the observed association with regenerating muscle fibres. Despite this, quadriceps Klotho levels also related positively with the level of local oxidative stress, a scenario incongruent with the literature. The 'oxidative stress theory' is characterised by accumulative oxidative damage conferred by the generation of reactive oxidative species, it is believed that this impacts the ageing process and contributes to physiological impairment and a reduction in life span. The suppression of oxidative stress in muscle is therefore likely to be beneficial, the fact that elevated Klotho levels alone do not suppress oxidative stress in the muscle of COPD patients indicates that either other factors are also relevant, or indeed more relevant, or alternatively that Klotho levels are not sufficiently raised to suppress the process. Whether the presence of Klotho in this setting is functional remains to be established. Given the observed association with a sarcopenic phenotype and elevated oxidative stress, it is plausible that Klotho up-regulation in this setting is associated with the generation of myofibres with abnormal functional capacity or reduced longevity. Promoting myogenesis and attenuating local oxidative stress are relevant goals in COPD; contextually, a detailed understanding the relevance of Klotho and GDF-15 signalling may be profitable and warrant further study.

7.3 Limitations and future work

As is evidenced by the fibre mortality and Klotho work, the heterogeneity observed in the pulmonary adaptations that occur in COPD are also reflected in the skeletal muscle adaptations that occur. Different results may be observed in different populations; whilst fibre shift was the only muscle parameter predictive of mortality across the range of airflow obstruction, the relevance of this was superseded by the functional relevance of quadriceps weakness in those with GOLD stage III and IV

disease. Furthermore, Klotho levels were observed to be elevated in the quadriceps muscle of weak COPD patients in an extended dataset of 65 patients, although there was no difference observed when the analysis was performed in 47 patients with additional protein carbonylation data, this is perhaps a consequence of the many factors that may affect Klotho expression in muscle. This highlights two important limitations to the work presented, firstly the mostly cross-sectional nature of the studies and secondly sample size.

Whilst the mortality study required a longitudinal follow-up, the patients were only evaluated at one time point. A prospective study with multiple longitudinal assessments and additional physical activity measurements would certainly have been preferable; however, the findings of this study are strengthened by being derived from a large multinational cohort with a long follow up time period. The SPPB study and, for the most part, the molecular studies were all cross-sectional in nature. It is only possible to identify associations from these studies and causality cannot be demonstrated, this is pertinent to some of the complex observations seen with Klotho. Nonetheless, in the case of Klotho the finding of a smoking effect was validated in an animal model, which corroborated the human findings. Given the positive and interesting results presented, it is appropriate to validate the findings of these studies in different cohorts. Pertinent to this fact, these studies were performed on stable COPD outpatients, who are likely to express a different phenotype to those who are exacerbating frequently or indeed to those who are under primary care management. Furthermore, it seems likely that as with the different trajectories that are observed in the progression of the lung pathology that occurs in patients with COPD or at risk of COPD, skeletal muscle adaptations are also likely to occur at different rates in different individuals, perhaps even more so given that muscle adaptations may be reversed. Different results may have been observed if evaluating patients at different time-points throughout their illness.

Sample size calculations were not performed in the studies presented in this thesis, this is a consequence of the fact that the data presented are the first relevant data as Klotho and GDF-15 have not been measured in patients with COPD previously and there is limited data on the SPPB in this cohort. Nonetheless, these studies have been performed on large cohorts who have undergone a muscle biopsy and indeed in the case of Klotho and GDF-15 are larger than most comparable studies in the literature. It is nevertheless important that these findings are validated in larger cohorts. Furthermore, at a clinical level, it remains to be established whether reversal of fibre shift or achieving an improved SPPB score translates to relevant benefits to COPD patients. Additionally, it remains to be ascertained whether the functional manipulation of Klotho and GDF-15 expression is of clinical relevance. Longitudinal studies would provide a further insight into the predictive properties of the SPPB, GDF-15 and Klotho in COPD. Additionally, further clinical studies evaluating the response to intervention such as pulmonary rehabilitation or the changes that occur during COPD exacerbations would also be of value.

Given the complexity of the molecular biology observed in skeletal muscle, it is unlikely that one pharmacological therapeutic target is likely to reverse all of the pathological adaptations that occur in COPD patients. It seems increasingly likely that specific therapies may be appropriate to particular disease phenotypes. In the case of Klotho and GDF-15, further work is warranted with a view to identifying actions on candidate pathways and also the response to manipulation of the functional expression of these proteins. Furthermore, although these proteins are associated with mortality in general populations and relate to chronological age in these populations, they are clearly influenced by other factors such as smoking in COPD, it is of interest to see whether these markers relate to other markers of ageing such as telomere length. In this regard, it has previously been demonstrated that cigarette smoke exposure enhances telomere shortening in circulating lymphocytes, furthermore, there is a dose-effect relationship between exposure to tobacco smoking and telomere length (Morla, Busquets et al. 2006). Studies targeting those at risk of developing COPD may be

especially fruitful; specifically work in smokers without established COPD is relevant. The observation that Klotho levels rise on successful smoking cessation is particularly interesting, however, data were only available in a relatively small number of patients, whether levels remain elevated and whether this is related to an improvement in muscle strength remains to be determined. Achieving successful smoking cessation, as evidenced by the smoking cessation study presented in this thesis remains challenging. Manipulation of systemic Klotho in smokers may represent an interesting future therapeutic strategy; clearly a significant body of work would be required to demonstrate this.

Whilst the multiple factors influencing the expression of circulating Klotho limit its use as a biomarker in COPD, further studies clarifying potential protective and regenerative roles for Klotho in skeletal muscle, rather than Klotho being a simple bystander or even pathophysiological in certain circumstances are relevant. Further animal work in addition to cell work would validate the molecular findings presented and also provide a platform for in depth analysis of the role of Klotho and GDF-15 signalling in the setting of relevant conditions such as cigarette smoke exposure and exposure to muscle damage.

Although currently marketed drugs, specifically 3-hydroxy-3-methylglutaryl CoA reductase inhibitors (statins), angiotensin II receptor blockers and PPAR- γ agonists have all been shown to augment endogenous Klotho (Hu, Kuro-o et al. 2012), more efficient methods for augmenting Klotho levels or function would be attractive. Strategies for augmenting Klotho in humans are currently under development (Abraham, Chen et al. 2012). Given that the signalling pathways and receptors that Klotho acts upon are not yet fully understood, finding Klotho mimetics is not feasible at present. Restoration of endogenous Klotho may be possible by preventing degradation, or increasing DNA transcription or protein expression. Alternatively, supplementation with exogenous Klotho, either locally or systemically, may be another option.

Secreted Klotho has been demonstrated to possess weak glycosidase activity *in vitro* and through this activity can mediate the activation of transmembrane transient receptor potential vanilloid type isoform-5 (TRPV5) (Chang, Hoefs et al. 2005; Cha, Ortega et al. 2008), influencing the influx of calcium, which may be relevant to the associations observed between circulating Klotho and quadriceps strength. Furthermore, Klotho has also been demonstrated to inhibit TGF- β 1 signalling *in vitro* (Doi, Zou et al. 2011), which may be relevant given the data presented in this thesis and a role for myostatin in skeletal muscle dysfunction (Thomas, Langley et al. 2000; Langley, Thomas et al. 2002; Man, Natanek et al. 2010). Secreted Klotho has also been demonstrated to inhibit Wnt which may be relevant to a role in regeneration (Brack, Conboy et al. 2007; Liu, Fergusson et al. 2007) and IGF-1 signalling which may be relevant to inhibiting oxidative stress (Yamamoto, Clark et al. 2005). Based on the findings reported in this thesis, up-regulation of Klotho alone does not appear to adequately attenuate oxidative stress in the quadriceps of COPD patients or prevent the development of a sarcopenic phenotype; for these reasons approaches which aim to enhance Klotho sensitivity may also be of value. Admittedly in a very large cohort, others have demonstrated that α -tocopherol, attenuates the manifestations of oxidative stress (Rautalahti, Virtamo et al. 1997); the same agent ameliorates the oxidative stress that occurs in *Klotho* knockout mice (Nagai, Yamada et al. 2003). Given the heterogeneity of the skeletal muscle dysfunction that occurs in COPD, other factors may therefore require consideration when considering the therapeutic modulation of Klotho expression.

Given that Klotho exerts many of its actions through an interaction with FGF-23, modulation of both Klotho and FGF-23 may provide a more rewarding therapeutic option. In this regard, further studies elucidating precise roles for both Klotho and FGF-23 would be required, however, given that myotube diameter is increased by Klotho-FGF23 fusion polypeptide treatment, this strategy is an attractive option (Razzaque 2010). Clinical studies to identify those that are most likely to benefit

from a Klotho/FGF-23 agonist in COPD would require detailed consideration given that no differences in circulating levels were seen when stratifying according to functional measures of sarcopenia, which is likely explained by the large numbers of smokers studied and the attenuating effect of smoking on Klotho expression. Further studies of larger COPD cohorts carefully stratified into current smokers and former smokers may identify reduced circulating Klotho levels in specific COPD phenotypes, likely those with reduced quadriceps strength, that would benefit from intervention. Those at particular risk of developing muscle dysfunction in the future, for example, 'healthy smokers' or COPD patients with maintained physical function but suffering recurrent exacerbations, would seem most likely to benefit from clinical trials of a Klotho agonist. Since local Klotho expression was actually elevated in COPD patients with established skeletal muscle dysfunction, this cohort may require a different therapeutic strategy such as the use of a Klotho-FGF23 agonist, or the additional myogenic stimulus of exercise training in addition to Klotho augmentation.

Although functional GDF-15 manipulation is not currently possible in humans, an antibody against GDF-15 has been used in mice, where it can ameliorate the weight loss caused by GDF-15 over expressing tumours (Tsai, Husaini et al. 2012). Infusion of human recombinant GDF-15 in GDF-15 knockout mice reduces body weight (Tsai, Macia et al. 2013). GDF-15 antagonism is currently under development by industry and subsequently may eventually prove possible in humans (Ebner, Steinbeck et al. 2014). It therefore seems plausible that GDF-15 antagonism may present a potential therapeutic option for cachectic COPD patients who have elevated circulating GDF-15 levels.

Whilst this thesis has particularly focussed on the skeletal muscle aspects of COPD, age-related markers are also likely to be relevant to other systemic aspects of the disease. Furthermore, other age-related markers may also be relevant to the pathophysiological adaptations that occur in COPD. In this regard, recent work on sirtuins is of interest, dietary supplementation with the SIRT1-

activating molecule SRT1720 increased mouse life span by 9% and was not only associated with improved muscle function and co-ordination, but also improved insulin sensitivity and lipid profile (Mitchell, Martin-Montalvo et al. 2014). The study of age-related molecules remains an exciting area of work which may potentially provide global therapeutic benefits to the COPD patient. In future, as is currently necessitated when treating other conditions, such as hypertension, the management of skeletal muscle dysfunction in COPD is likely to require a number of different modalities.

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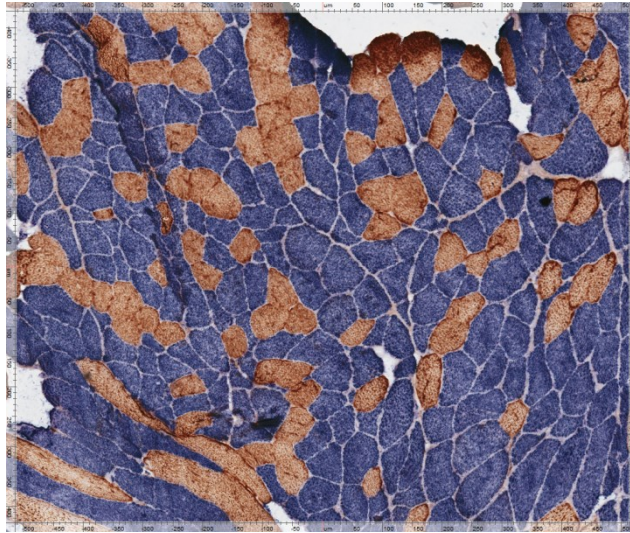
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Example of an immunohistochemistry image used in fibre type analyses

Used to identify fibre type proportions and myofibre cross-sectional area (purple stain: type I fibres, brown stain: type II fibre)



Characteristics of the full dataset in whom mortality was assessed

The core dataset and additional data available (including the numbers in whom data were available) for the study presented in Chapter 3, where the association of fibre type to mortality was assessed.

	Parameter	Number with available data	Number/ Mean (SD)/ Median (IQR)
Core dataset	Age (years)	392	65.9 (8)
	Male gender	392	73
	FEV ₁ %predicted	392	41.6 (28.0, 61.0)
	% type II fibres	392	66.6 (56.0, 75.0)
	Fibre shift (yes:no)	392	45
	Time between biopsy and survival status check (days)	392	1699 (1100, 2199)
	% survivors	392	73.3
Additional data	FEV ₁ (L)	324	1.13 (0.79, 1.75)
	BMI (kg/m ²)	320	25.1 (22.1, 28.7)
	FFMI (kg/m ²)	204	17.0 (15.0, 18.4)
	QMVC (kg)	281	28.0 (23.0, 34.0)
	QMVC/BMI	218	1.15 (0.90, 1.36)
	MT _{CSA} (cm ²)	144	85.4 (26.5)
	TLCOc (%pred)	209	47.5 (29.5, 59.9)
	RV/TLC (%)	205	60.1 (48.9, 73.4)