# Exercise intolerance and mitochondrial dysfunction in patients with Chronic Obstructive Pulmonary Disease

**A thesis submitted for the degree of Doctor of Philosophy**

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## Dedication

My parents and sister have provided a life time of unwavering love, support and encouragement and to them the magnitude of my appreciation is beyond words.

I would like to dedicate this thesis to my wife Hana-Fatimah in all that she has done for me; her boundless love and inspiration have been invaluable.

## Abstract

The aim of this thesis was to evaluate mechanisms that are of potential interest in the pathogenesis of Chronic Obstructive pulmonary Disease (COPD) and associated symptoms; and subsequently to identify targets that may be relevant in the management of patients with structural and functional abnormalities of the pulmonary and skeletal muscle systems.

Dynamic hyperinflation (DH) is a recognised feature in the aetiology of dyspnoea and exercise limitation in COPD and strategies to improve DH by offsetting intrinsic positive end expiratory pressure (PEEPi) may improve exercise performance. Here the role of the upper airway was evaluated using continuous laryngoscopy during exercise. Increased laryngeal narrowing in COPD patients was associated with markers of disease severity such as lung function, hyperinflation indices and exercise performance, suggesting that patients may use this strategy to create PEEPi thus reducing flow limitation. This novel insight supports potential therapeutic benefits of upper airway strategies to improve gas exchange and act as a gateway to optimising tissue oxygen delivery.

Oxygen uptake ($VO_2$) plays a vital role in maintaining mitochondrial homeostasis and is the measured parameter at the end of a metabolic gas exchange cardio-pulmonary exercise test. Mitochondria play a crucial role in maintaining the cellular oxidant-antioxidant balance. Mitochondrial function in airway and muscle compartments was evaluated in COPD patients and controls; and there was found to be evidence of airway mitochondrial dysfunction in patients with COPD compared to control subjects and a strong association with clinical phenotype. Similar changes were found in the pulmonary system of mice exposed to ozone and these were partially reversed with a mitochondria targeted

antioxidant. Skeletal muscle mitochondrial function was not impaired in COPD despite a comparable oxidative stress burden to that found in the pulmonary system. Targeting the mitochondria may provide therapeutic options for airway related symptoms in patients with COPD.

## Contents

Declarations of originality and copyright.....2

Acknowledgements.....3

Dedication.....4

Abstract.....5

List of Tables and Figures.....13

**Chapter 1: Introduction**.....17

1.1 Chronic obstructive pulmonary disease – an overview.....17

1.2 Exercise intolerance and cardio-pulmonary exercise testing in COPD.....21

1.3 The upper airway in COPD.....24

1.4 The pathogenesis of pulmonary manifestations of COPD.....27

1.5 Extra-pulmonary manifestations of COPD.....30

1.6 Skeletal muscle dysfunction in COPD.....32

1.7 Mitochondria.....35

1.8 The role of mitochondria in COPD.....37

1.9 The spill-over theory in COPD.....39

1.10 Study aims and hypothesis.....40

1.11 Overall research rationale.....42

**Chapter 2: Methods**.....43

2.1 Human Studies.....44

2.1.1 Subject recruitment.....44

2.1.2 Inclusion and exclusion criteria.....44

2.1.3 COPD Subjects……..44

2.1.4 Healthy control subjects……45

2.2 Subject assessments……..47

2.2.1 Anthropometric measures……..48

2.2.2 Pulmonary function testing……..48

2.2.3 Spirometry……48

2.2.4 Lung volumes and gas transfer……49

2.2.5 COPD assessment tool (CAT) questionnaire……..49

2.2.6 Modified Borg scores for dyspnoea and leg fatigue……..50

2.2.7 Short physical performance battery (SPPB)……50

2.2.8 Quadriceps maximal voluntary contraction……..52

2.2.9 Six minute walk test……52

2.2.10 Cardio-pulmonary exercise testing (CPET)……53

2.3 Invasive sampling techniques……54

2.3.1 Fibre-optic bronchoscopy and endo-bronchial biopsy……..54

2.3.2 Percutaneous muscle biopsy of the vastus lateralis muscle……54

2.4 Molecular techniques for the study of mitochondria and cytoplasm……..56

2.4.1 Sample processing……56

2.4.2 Measurement of mitochondrial membrane potential……..58

2.4.3 Mitochondrial reactive oxygen species detection……..58

2.4.4 Cytoplasmic reactive oxygen species detection……..59

**Chapter3: The role of the upper airway in COPD**……60

3.1 Introduction and aim……60

3.2 Methods……62

3.2.1 Study population……62

3.2.2 Study design and procedures……62

3.2.3 Laryngeal movement analysis……65

3.2.4 Statistical analysis……67

3.3 Results……68

3.3.1 Subject characteristics……68

3.3.2 Cardio-pulmonary exercise test measurements……68

3.3.3 Glottic and supraglottic narrowing……69

3.4 Discussion……83

3.4.1 Summary of results……83

3.4.2 Critique of methods……84

3.4.3 Significance of findings……86

3.4.4 Conclusion……88

**Chapter 4: Mitochondrial dysfunction in airway and muscle compartments in COPD – association to clinical phenotype**……89

4.1 Introduction and aim……89

4.2 Methods……94

4.2.1 Study population……94

4.2.2 Study design and procedures……94

4.2.3 Mitochondrial functional analysis and ROS evaluation……95

4.2.4 Statistical analysis……96

4.3 Results……97

4.3.1 Subject characteristics……97

4.3.2 Airway mitochondrial function and mitochondrial and cellular ROS Levels……97

4.3.3 Airway mitochondrial function and phenotypic associations……98

4.3.4 Muscle mitochondrial function and mitochondrial and cellular ROS levels……99

4.3.5 Muscle mitochondrial function and phenotypic associations……99

4.4 Discussion……111

4.4.1 Summary of results……111

4.4.2 Critique of methods……112

4.4.3 Significance of findings……114

4.4.4 Conclusion……116

**Chapter 5: Mitochondrial dysfunction in a murine model of COPD**……117

5.1 Introduction and aim……117

5.2 Methods……121

5.2.1 Murine Studies……121

5.2.2 The ozone model of mitochondrial dysfunction……121

5.2.3 Murine measures of airway hyper-responsiveness……122

5.2.4 Bronchoalveolar lavage and collection of lung and muscle tissue from mice.....122

5.2.5 Total and differential cell counts and IL-6 and GM-CSF levels.....123

5.2.6 Mitochondrial and cytoplasmic analysis and ROS detection.....123

5.2.7 Statistical analysis.....125

5.3 Results.....126

5.3.1 Ozone induce lung inflammation.....126

5.3.2 Mitochondrial function is impaired in the lungs of ozone exposed Mice.....126

5.3.3 Effects of MitoQ on ozone induced inflammation, mitochondrial membrane potential, mitochondrial and cellular ROS levels in mouse lung tissue.....127

5.3.4 Effects of MitoQ on mitochondrial membrane potential, Mitochondrial and cellular ROS levels in mice muscle tissue.....127

5.3.5 The functional significance of MitoQ – ozone induced airway hyper-responsiveness.....127

5.4 Discussion.....139

5.4.1 Summary of Results.....139

5.4.2 Critique of methods.....140

5.4.3 Significance of findings.....142

5.4.4 Conclusion.....143

**Chapter 6: Discussion**.....144

6.1 General discussion.....144

6.2 Study limitations.....148

6.3 Future work.....149

**References**……151

**Appendix**……161

Publications arising from this thesis……161

## List of Tables and Figures

**Table 1.1** The global initiative for chronic obstructive lung disease - classification of severity (Rabe, Hurd et al. 2007)……20

**Table 3.1 –** Subject Characteristic and pulmonary function……..71

**Table 3.2 –** Peak Exercise data……73

**Table 3.3 –** Laryngeal narrowing at rest and peak exercise……75

**Table 4.1 –** Subject Characteristics: Data shown as mean (SD) or median (range).p<0.05 from A/B *, p<0.01 from A/B **, p<0.05 from C $^{\#}$, p<0.01 from C $^{\#\#}$……..100

**Figure 2.1 –** Graphical representation of the assessments undertaken by patients for the SPPB and how points are awarded for each component. The maximum possible score is twelve and the minimum is zero (Riskowski, Hagedorn et al. 2012)……51

**Figure 2.2 –** An example of a protein assay standard curve. The Y axis denotes the known concentration of BSA. The X axis denotes the optical density reading for a known volume of sample extracted from fresh tissue. The equation (Y=) allows calculation of the protein concentration of the extract……57

**Figure 3.1 –** Graphical representation of a subject undertaking cycle-ergometry with continuous laryngoscopy – (Courtesy of B Panchasara)……64

**Figure 3.2 –** Example cases illustrating dynamic expiratory laryngeal narrowing and method for calculation of glottic and supra-glottic narrowing ratio……66

**Figure 3.3 –** Laryngeal narrowing ratio at rest and peak exercise at glottic level (A) and supraglottic level (b)……76

**Figure 3.4 –** Inter-observer agreement of the glottic narrowing ratio at rest (A) and peak exercise (B)……77

**Figure 3.5 –** Relationship between $FEV_1$ % predicted and glottic narrowing ratio (GNR) at rest (A) and peak exercise (B)……78

**Figure 3.6 –** Relationship between $FEV_1$ % predicted and Supraglottic narrowing ratio (SGNR) at rest (A) and peak exercise (B)……79

**Figure 3.7 –** Relationship between $VO_2$ % predicted and Glottic narrowing (GNR) at peak exercise in COPD subjects (A) and Supraglottic narrowing (SGNR) at peak exercise in COPD subjects (B)……80

**Figure 3.8 –** Relationship between respiratory duty cycle and glottis narrowing at rest (A) and peak exercise (B)……81

**Figure 3.9 –** Relationship between peak glottic narrowing ratio (GNR) and dynamic hyperinflation index (DHI) in controls (•), patients with mild–moderate COPD (□) and patients with severe COPD (▪). Data are presented as mean values with error bars representing 1 SEM. *p<0.05 from controls……82

**Figure 4.1 –** Inhalation of noxious substances drives inflammation and ROS production - the spill over of which contributes to systemic features of COPD (adapted from Barnes & Celli 2013)……93

**Figure 4.2 – (**A) Airway mitochondrial membrane potential of the never smokers (A), Ex-smokers/GOLD I (B) and GOLD II patients (C). (B) Airway mitochondrial reactive oxygen species levels of the never smokers (A), Ex-smokers/GOLD I (B) and GOLD II patients (C). p<0.05*, p<0.01 #.....101

**Figure 4.3 –** Airway whole cell reactive oxygen species levels of the never smokers (A), Ex-smokers/GOLD I (B) and GOLD II patients (C). p<0.05*……102

**Figure 4.4 –** Western blot analysis demonstrating a reduction in expression of mitochondrial complex proteins I, IV and V in the GOLD II patients……102

**Figure 4.5 –** (A) Correlation between airway mitochondrial membrane potential and % predicted $FEV_{1.}$ (B) Correlation between airway mitochondrial membrane potential and % predicted $TLCO_c$……103

**Figure 4.6 –** (A) Correlation between airway mitochondrial membrane potential and six-minute walk distance (6MW) in metres. (B) Correlation between airway mitochondrial membrane potential and weight adjusted peak oxygen uptake ($VO_2$ peak ml/min/kg)……104.

**Figure 4.7 –** No correlation is seen between airway mitochondrial membrane potential and quadriceps muscle strength (quadriceps maximal voluntary contraction – QMVC in Kg)….105

**Figure 4.8 –** (A) Quadriceps muscle mitochondrial membrane potential does not differ between groups of never smokers (A), Ex-smokers/GOLD I (B) GOLD II (C) and GOLD III/IV patients. (B) Quadriceps muscle mitochondrial reactive oxygen species levels of the never smokers (A), Ex-smokers/GOLD I (B), GOLD II (C) and GOLDIII/IV patients (D). p<0.01 #……106

**Figure 4.9 –** Quadriceps muscle whole cell reactive oxygen species levels of the never smokers (A), Ex-smokers/GOLD I (B), GOLD II patients (C) and GOLD III/IV patients(D). p<0.05*p<0.01 #.....107

**Figure 4.10 –** No correlation is seen between quadriceps muscle mitochondrial membrane potential and quadriceps muscle strength (quadriceps maximal voluntary contraction – QMVCin Kg)……107

**Figure 4.11 –** No correlation is seen between quadriceps muscle mitochondrial membrane potential and % predicted $FEV_1$ (A) and % predicted $TLCO_{c.}$ (B)……108

**Figure 4.12 –** No correlation is seen between quadriceps muscle mitochondrial membrane potential and six-minute walk distance (6MW) in metres (A) and weight adjusted peak oxygen uptake ($VO_2$ peak ml/min/kg) (B)……109

**Figure 4.13 –** High Power Transmission Electron Microscopy images of human vastus lateralis muscle at 10000 x magnification show a reduction in number and distribution of mitochondria and an increase in the number of auto-phagasomes in the COPD patient…..110

**Figure 5.1 –** Ozone exposed mice showed marked increases in total BAL cell counts compared with those in air exposed control mice after 1 and 6-weeks. $p<0.01$ #.....129

**Figure 5.2 –** Ozone exposed mice have considerably higher cell counts for neutrophils (A) and macrophages (B) when compared to air exposed mice. $p<0.01$ #.....130

**Figure 5.3 –** Ozone exposed mice have considerably higher cell counts for lymphocytes (A) and eosinophils (B) when compared to air exposed mice. $p<0.05$*, $p<0.01$ #.....131

**Figure 5.4 –** 1 and 6-week ozone exposure induces an increase in the pro-inflammatory cytokine GM-CSF. $p<0.01$ #.....132

**Figure 5.5 –** 1-week ozone exposure induces an increase in the pro-inflammatory cytokine IL-6. No significant difference was seen in the 6-week ozone exposure group. $p<0.01$ #.....132

**Figure 5.6 –** Mitochondrial membrane potential tested in mitochondria isolated from mouse lung tissue was reduced in the 1 and 6- week ozone exposed mice. $p<0.01$ #.....133

**Figure 5.7 –** ATP production in mouse lung tissue was reduced in both 1 and 6-week ozone exposed mice when compared to the air exposed control groups. $p<0.05$*, $p<0.01$ #.....133

**Figure 5.8 –** MitoQ treatment resulted in a reduction in ozone induced total BAL cell counts compared with dTPP alone. $p<0.05$*, $p<0.01$ #.....134

**Figure 5.9 –** MitoQ treatment resulted in restoration of mitochondrial membrane potential in ozone exposed mice lung tissue compared with dTPP alone. $p<0.05$*, $p<0.01$ #.....134

**Figure 5.10 –** MitoQ treatment resulted in a reduction of mitochondrial ROS levels in ozone exposed mice lung tissue compared with dTPP alone. $p<0.05$*, $p<0.01$ #.....135

**Figure 5.11 –** MitoQ treatment resulted in a reduction of cellular ROS levels in ozone exposed mice lung tissue compared with dTPP alone. $p<0.05$*, $p<0.01$ #.....135
**Figure 5.12 –** MitoQ treatment had no effect on mitochondrial membrane potential in ozone exposed mice muscle tissue compared with dTPP alone.....136
**Figure 5.13 –** MitoQ treatment had no effect on the mitochondrial membrane potential in ozone exposed mice muscle tissue compared with dTPP alone. A difference was observed in mitochondrial ROS levels between the Air/MitoQ and the Ozone/dTPP group. $p<0.05$*, $p<0.01$ #.....136
**Figure 5.14 –** MitoQ treatment had no effect on cellular ROS levels in ozone exposed mice muscle tissue compared with dTPP alone.....137
**Figure 5.15 –** Airway hyper-responsiveness to acetylcholine was greater in the ozone exposed mice at 1 and 6 weeks compared to air groups with lower concentrations of aerosolized acetylcholine inducing a 100% increase in lung resistance. $p<0.01$ #.....138
**Figure 5.16 –** Treatment with MitoQ resulted in a significant improvement in AHR compared to control treated mice with higher concentrations of aerosolized acetylcholine required to induce a 100% increase in lung resistance (log $PC_{100}$). $p<0.05$*, $p<0.01$ #.....138

# Chapter 1: Introduction

## 1.1 Chronic Obstructive Pulmonary Disease – an overview

Chronic obstructive pulmonary disease (COPD) is a major global health problem and a leading cause of mortality throughout the world. It is a debilitating disease that significantly impacts function and healthcare use. It is typically associated with smoking and is characterised by airflow obstruction with minimal reversibility (Rabe, Hurd et al. 2007), however despite this bronchodilators remain the mainstay of drug treatment. Skeletal muscle dysfunction is a common extra-pulmonary manifestation of COPD and pulmonary rehabilitation, involving endurance and resistance training, is an effective treatment strategy (Maltais, Decramer et al. 2014).

### *Symptoms and diagnosis*

Patients with COPD will typically present with features of chronic bronchitis, emphysema and reactive airways disease (Garcia-Aymerich, Gomez et al. 2011). Breathlessness is the commonest pulmonary symptom however occurs later in the disease process. Cough may predominate as a feature of inflammation and is typically worse in the mornings. Wheeze and variable symptoms occur more frequently in patients with hyper-reactive airways akin to asthma – this is usually temporary and more common when suffering an infective exacerbation. Physical signs tend only to be present when patients have established and more advanced disease and include increased respiratory rate and accessory muscle use. Early features may include weakness of the quadriceps muscles and a reduction in daily activity (Seymour, Spruit et al. 2010) – these are not routinely screened for in clinical

practice and unfortunately only become apparent when clinically detectable cachexia and low muscle mass of more advanced disease are a feature (Debigare, Cote et al. 2001).

The diagnosis of the condition remains based on the Global Initiative for Obstructive Lung Disease (GOLD) guidelines. The threshold is a post bronchodilator ratio of forced expiratory volume in 1 second ($FEV_1$) to forced vital capacity (FVC) of less than 0.7. Disease severity is sub classified according to $FEV_1$ values measured against predicted values for age, height and gender **(Table 1.1)**. An $FEV_1$/FVC ratio of less than 0.7 for diagnosis is slightly controversial because we know that this ratio decreases with age and so perhaps using a ratio below the standard residual for the lower limit of normal would be more appropriate.

***Epidemiology***

Epidemiological studies have shown that the worldwide prevalence of COPD is increasing, mostly due to the increased diagnosis in the developing world where smoking rates are also increasing and where the current and past inhalation of noxious agents such as biomass fuels still remains a significant problem (Barnes. 2000) (Decramer, Janssens et al. 2012). Overall moratlity in the developed world is thought to be on the decline due in part to restrictions placed on smoking and improved education about harmful effects (Gershon, Wang et al.2010) – COPD still remains the third leading cause of death worldwide behind cancer and cardiovascular disease (Decramer, Janssens et al. 2012). Problems with early diagnosis exist and often by presentation well established disease is a feature. In Europe, in particular, there is a high risk of under diagnosis in women with mild to moderate symptoms (Ancochea, Miravitlles et al. 2013).

In the context of similar smoking histories only a minority of individuals develop COPD and we have over time understood genetic elements and susceptibility profiles that coupled with environmental triggers lead to disease. A well-established genetic cause of COPD alpha 1 antitrypsin deficiency accounts for 1-2% of all diagnoses and genome wide association studies have identified other chromosomal locations associated with COPD susceptibility (Lomas and Silverman 2001). The exact molecular and cellular mechanisms at play that contribute to the pathophysiology of COPD are still poorly understood and the concept of a single natural history in COPD is one that has quite rightly been challenged. COPD is a far more heterogeneous condition than initially thought, even when simply subdivided into clinical phenotype. A number of patients exhibit significant small airways involvement and parenchymal lung destruction in the form of emphysema. These individuals tend to suffer with breathlessness early in the course of their illness due to air trapping, dynamic hyperinflation and alveolar over distension with an increased diaphragmatic load (O'Donnell, Revill et al. 2001). The majority of patients will present with a more indolent phenotype characterised by airway inflammation and chronic bronchitis.

***Current therapies***

Pharmacological therapies that exist for COPD remain unchanged for many years. The most effective interventions that we have, specifically pulmonary rehabilitation involving endurance and resistance training, are indeed non-pharmacological, suggesting novel molecular targets are limited. As such pulmonary rehabilitation and smoking cessation remain the most effective approach (Cote and Celli 2005) (Postolache, Nemes et al. 2015). Oxygen therapy provides symptomatic relief and survival benefit for those with hypoxia and

a recent study has shown the benefit of non-invasive ventilation in selected patients with hypercapnic respiratory failure (Murphy, Rehal et al. 2017). Treating exacerbations early is very important as these events play a significant role in health status, disease progression and survival (Wedzicha 2015). The most promising novel therapy developed more recently is again a non-pharmacological strategy in the form of bronchoscopic lung volume reduction targeting emphysema (Davey, Zoumot et al. 2015). In developing new therapies it is of interest to consider how the respiratory dynamics of the airways disease cause exercise intolerance and also identify common molecular and cellular changes of the pulmonary and skeletal muscle systems.

| Stage | Lung function Criteria – $FEV_1/FVC < 0.70$ |
|---|---|
| Stage I – mild | $FEV_1$ - greater than 80% predicted |
| Stage II – moderate | $FEV_1$ - between 50% and 80% predicted |
| Stage III – severe | $FEV_1$ - between 30% and 50% predicted |
| Stage IV – very severe | $FEV_1$ – less than 30% predicted or less than 50% predicted with chronic respiratory failure |

**Table 1.1** The global initiative for chronic obstructive lung disease - classification of severity (Rabe, Hurd et al. 2007).

## 1.2 Exercise intolerance and cardio-pulmonary exercise testing in COPD

COPD is a condition of progressive dyspnoea and exercise intolerance. It is well recognised that systemic manifestations of the disease are the cause of significant morbidity and mortality (Swallow, Reyes et al. 2007; Barnes and Celli 2009). Exercise intolerance in COPD is therefore a multifactorial issue. In early disease where skeletal muscle dysfunction has been demonstrated before the onset of dyspnoea (Shrikrishna, Patel et al. 2012) poor exercise capacity may be the result of the directly deleterious effects of oxidative stress on muscle (Powers, Smuder, et al. 2011). In more advance disease where gas exchange is now sub optimal the aforementioned changes will be greater given poor oxygen transport and delivery and this will further embarrass an already failing skeletal muscle system due to delivery-utilisation mismatch (Medeiros, Fernades et al. 2015).

The pathology of systemic manifestations that contribute to exercise intolerance may simply be a result of shared pathological features such as an accelerated aging process (MacNee 2016), or it may be the result of spill over of oxidative stress and pro-inflammatory mediators from the pulmonary system (Barnes and Celli 2009). The lung pathology is generally regarded irreversible but muscle dysfunction through training can improve and this remains a dedicated treatment strategy (Troosters, Casburi at al. 2005).

Cardio-pulmonary exercise testing allows an assessment of the contributory factors to exercise intolerance in patients with COPD – precisely one of the reasons it is used to assess fitness for surgery in this population group. It has been shown that peak oxygen uptake ($VO_2$) is predictive of adverse events (Guazzi, Adams et al. 2012). Understanding the physiology behind peak oxygen uptake and the variables that contribute to this single end

point parameter will allow us to better understand the mechanisms of exercise intolerance in COPD.

Metabolic gas exchange cardio-pulmonary exercise protocols are a specialised form of exercise tolerance testing. Data are derived from the non-invasive procedure that involves the acquisition of expired concentrations of oxygen and carbon dioxide during progressive exercise (Datta, Normandin et al. 2015). It provides the most accurate measure of cardio-respiratory and muscle fitness. If we consider COPD to be a pulmonary disease of ineffective gas exchange with muscle dysfunction early in the course of the illness which then progresses to more advanced disease with greater inactivity, significant deconditioning (Troosters, van der Molen et al. 2013) and cor-pulmonale with an impaired cardiac output, then we can better understand parameters of interest when interpreting physiological data.

Ventilatory inefficiency with an elevation of the $VE/VCO_2$ slope will be more profound when there is emphysematous destruction of the alveolar-capillary bed. It will also be apparent with alveolar over distension that occurs secondary to hyperinflation – an important mechanism of breathlessness. Effectively the minute ventilation required, to clear carbon dioxide and allow adequate oxygenation, will increase and this will place greater stress on an already failing system. Oxygen delivery-utilization dynamics will be negatively affected and this will be reflected by a falling oxygen pulse and early anaerobic threshold. Both these parameters are already likely to be affected given the early muscle dysfunction that we know occurs. Further cardiac dysfunction as part of the multi-system disease that COPD is will lead to greater problems with oxygen delivery and subsequently leave less available for muscle uptake, this will further increase the rate of fiber shift and muscle atrophy (Natanek, Gosker et al. 2013). In the absence of any current pharmacological therapies that

can treat or reverse the pathogenesis of the disease we currently look to optimization of operational lung mechanics to help improve gas exchange. This is the key to ensuring adequate oxygen delivery and carbon dioxide clearance the benefits of which ensure maintenance of key homeostatic mechanisms such as mitochondrial function and anaerobic metabolism. Looking at the upper airway to help optimize these lung mechanics has potential.

### 1.3 The role of the upper airway in COPD

The pulmonary hallmarks of COPD are inflammation of the airways and destruction of the lung parenchyma (emphysema). Progressive expiratory flow limitation and loss of elastic recoil properties of the lung lead to lung hyperinflation (Calverley and Koulouris 2005) such that at the end of expiration the lungs have not fully emptied before the start of inspiration. This leads to alterations of the respiratory duty cycle - and breathing pattern particularly on exercise may be erratic as problems are accentuated when the respiratory duty cycle is shorter and the drive to breathe is even greater. Over time hyperinflation increases the load on the respiratory muscles which become weakened (Kawagoe, Permutt et al. 1994). Hyperinflation will also lead to alveolar over-distension which along with emphysematous destruction contributes to progressive V/Q mismatching. Dynamic hyperinflation on exercise can be detected as an increase in end expiratory lung volume and a reduction of inspiratory capacity (O'Donnell, Guenette et al. 2012). Dynamic hyperinflation will contribute to dyspnoea and exercise insufficiency. As hypoxemia develops with progressive V/Q mismatching a less favourable environment for efficient mitochondrial work ensues contributing to skeletal muscle fibre changes (Kent, Mitchell et al. 2011). As this occurs patients enter a cycle of decline with progressive inactivity and increasing skeletal muscle dysfunction (O'Donnell, Revill et al. 2001).

Methods to overcome dynamic hyperinflation are limited. Lung volume reduction is helpful in optimising lung mechanics (Martinez, de Oca et al. 1997). Bronchodilators are largely poor in there effect to improve hyperinflation indices through improvement in expiratory flow limitation. Methods to allow airways to remain open for longer will inhibit expiratory airways collapse and allow better lung emptying which corresponds to improved exercise

performance (Hawkins, Johnson et al. 2002). Studies that have looked at the use of positive pressure ventilation during exercise in patient with COPD have shown promise, however the feasibility and practicality of use of often cumbersome machines mean they are not employed in routine practice. The interest in improving exercise performance using physiological methods centres around the knowledge that exercise is beneficial at a molecular and cellular level in COPD patients to improve muscle composition. Favourable muscle kinetics as dependent upon muscle fibre type will regulate respiration in patients with COPD through anaerobic metabolism (Casaburi 2006) - methods to optimise tissue oxygen delivery via improved ventilation will be of benefit.

The upper airway has not been extensively studied in COPD; however it is known that laryngeal muscle control varies for different functions. With regards to respiration and breathing there is thought to be both voluntary and reflex activity of the vocal cords although electromyography of these muscles is difficult (Ludlow 2005). Interestingly with hyperventilation the adductor muscles of the glottis become more active causing a reduction in glottis aperture (Kuna, Insalaco et al. 1993).

Upper airway dysfunction and abnormal vocal cord closure has been recognised as a pathological condition that is often a co-existent pathology in asthma – noted typically as closure on inspiration (Ayres and Mansur 2011) rather than an expiratory phenomenon which may have beneficial effect. Laryngeal closure has been identified at rest in patients with COPD; Higenbottam and Payne studied 34 patients with obstructive lung disease and observed expiratory narrowing of the glottis at rest (Higenbottam and Payne 1982). The laryngeal response to physical exertion in patients with COPD has never been studied. The occurrence of laryngeal narrowing during the expiratory phase of the respiratory duty cycle

may be relevant and by increasing intrinsic positive end expiratory pressure (PEEPi) may serve to splint the airways and may be an independent or supplementary mechanism to optimise operational lung mechanics. This will enable improved gas exchange, better oxygen carriage and a subsequent improvement of oxygen delivery-utilisation at the level of the muscle.

### 1.4 The pathogenesis of pulmonary manifestations of COPD

COPD results from maladaptive inflammatory responses to inhaled particles and gases in the lung (Fletcher and Peto 1977). Cigarette smoke through its contained toxins destroys the normal structure of the airways and lung through chronic inflammation. These changes are seen, but to a lesser degree in smokers without COPD suggesting an exaggerated response in those that develop clinically significant disease (Barnes 2000). Damage to lung tissue is for the most part irreversible and smoking cessation does not allow already established damage to reverse. Smoking elicits airway inflammation but not all smokers develop clinically significant COPD. The pathogenesis of COPD involves recruitment of inflammatory cells and induction of oxidative stress, both of which results in airway remodelling and parenchymal lung destruction (Chung 2005).

Airspace inflammation appears to be different in susceptible, as opposed to non-susceptible smokers and involves a predominance of T lymphocytes, neutrophils and macrophages (Angelis, Porpodis et al. 2014). Frequent exacerbations will increase the inflammatory load and lead to more rapid progression of disease (Wedzicha 2015). Aside from inflammation other important aspects of COPD pathogenesis centre on the oxidant-antioxidant balance and premature cellular senescence (Decramer, Janssens et al. 2012). Damage to airway epithelium is perhaps the first factor (Thorley and Tetley 2007) which triggers a cascade of detrimental events, through both innate and adaptive immune responses. Over time the ability to regulate the immune system and allow adequate and appropriate regeneration is lost. Senescence occurs more rapidly and cellular structure and function are lost – emphysema and cellular ageing share some key features (MacNee 2005).

Amplification of inflammation is seen in patients with COPD compared to unaffected or mildly affected smokers. This inflammation persists even after smoking cessation, and immune dys-regulation remains a feature. The amplified response in susceptible smokers may ultimately result in mucous hyper secretion (bronchitis), tissue destruction (emphysema) and disruption of normal repair mechanisms leading to small airway fibrosis (bronchiolitis) (MacNee 2005).

The inflammatory cell type in COPD is different to that in asthma accounting for the limited reversibility of the condition (Fabbri, Romagnoli et al. 2003). Neutrophils have a significant role in the development of COPD due partly to their ability to mediate tissue destruction through the release of elastases. In asthma eosinophils have a predominant role in airway epithelium and smooth muscle and account for steroid responsive phenotypes. Individuals in which cigarette smoke leads to eosinophilic inflammation may have a more favourable outcome. The airways of COPD sufferers exhibit increased numbers of goblet cells, mucous gland hyperplasia and alveolar wall destruction (Thorley and Tetley 2007). Activated macrophages within airway epithelium release mediators and chemotactic factors such as IL-6 and IL-8 which have pro-inflammatory effects (Chung 2005). These aid the recruitment of further inflammatory cells from the systemic circulation and cause generalised inflammation. Alongside this reactive oxygen species are produced in abundance and the ability of the cellular responses to neutralise them is lost (MacNee 2005). Proteolytic enzymes that are secreted, especially matrix metalloproteinase-9 and matrix metalloproteinase-12, in response to cell damage perpetuate the destruction of tissue (Houghton 2015) .

Bronchial biopsies of COPD patients show a predominance of CD8 T lymphocytes and there is a correlation between the numbers of lymphocytes the extent of tissue destruction and the clinical picture with regards to severity of airflow obstruction (MacNee 2005). Airways smooth muscle cells also play an important role in the cytokine and oxidative stress response. Cigarette smoke can directly stimulate airway smooth muscle cells to release IL-8 and in turn airway smooth muscle cells stimulated by Tumour Necrosis Factor-alpha trigger and perpetuate the cell mediated Th2 response contributing to a vicious cycle of events (Di Stefano, Caramori et al. 2002). This is further proof that inflammatory burden is important to the severity of the disease and targeting exacerbations will provide an effective treatment strategy.

The interplay between inflammation, oxidative stress and tissue death with scarring needs to be further elucidated as molecular targets need to focus on a central effector. Mitochondria are multifunctional organelles whose role within cells is linked to all the aforementioned mechanisms.

### 1.5 Extra-pulmonary manifestations of COPD

The effects beyond the lung are well documented in COPD; several other organ systems are known to be defective. COPD is associated with conditions such as cardiovascular disease, diabetes, osteoporosis and metabolic syndrome; more so than would be expected by chance (Barnes 2010). It stands to reason that the underlying mechanisms are similar and that smoking is a driving factor contributing to inflammation beyond the lung (Chung 2005). Higher levels of circulating cytokines and inflammatory mediators have been demonstrated in the blood of COPD patients particularly during an active exacerbation (Barnes 2010). Combatting these other aspects of disease is tough as the primary focus for treatment in these patients and targeted therapy has been directed towards the lung. More recent trials have looked at the direct effects of muscle targeted drug therapies on functional status and performance in COPD patients, the results of which are yet to be published.

Cardiovascular disease is not an unexpected finding in the context of shared risk factors - similar underlying mechanisms of inflammation and oxidative stress are contributory. Other relevant considerations include hyperinflation and chronic hypoxia. Hyperinflation will contribute to a rise in thoracic pressure and directly impair right ventricular filling. Coupled with hypoxia which contributes to pulmonary vascular remodelling combined cardio-pulmonary failure is seen in the later stages of disease. It is recognised that up to 50% of patients with advance COPD who are being considered for lung volume reduction surgery suffer from pulmonary hypertension (Thabut, Dauriat et al. 2005). It is the early deleterious effects of poor oxygen delivery due to impaired cardiac function that will contribute to the systemic cycle of decline.

It has been established that aging is a contributor to many of the processes described and much work into this has been done looking at molecular mechanisms in the airways of COPD patients (Ito and Barnes 2009). We know from independent work in other fields that mitochondrial dysfunction is a common mechanism found in diabetes, osteoporosis and cardiovascular disease (Duncan 2011) and indeed important in the aging process. Mitochondria have been shown to contribute to cellular senescence, chronic inflammation and the age-related decline in stem cell activity (Bratic and Larsson 2013). How this relates to COPD is relatively unknown.

### 1.6 Skeletal muscle dysfunction in COPD

Skeletal muscle dysfunction which is frequently observed in COPD patients is a complicated and multifactorial process which contributes to poor disease prognosis irrespective of lung function (Patel, Natanek et al. 2014). A key deleterious aetiological factor is deconditioning secondary to reduced physical activity (Troosters, van de Molen et al. 2013). Contributory factors are the well-recognised direct deleterious effects of smoking and alongside this the theory of oxidative stress spill over from the pulmonary system contributing to systemic inflammation (Barnes 2010), and a reduction in peripheral capillary density (Jobin, Maltais et al. 1998). Hypoxia that occurs due to alveolar-capillary dysfunction will impact muscle oxygen delivery, and along with co-existent cardiac morbidity contributing to a poor cardiac output, will cause oxygen delivery-utilisation mismatch at the level of the muscle (Gea, Pauscual et al. 2015). The subsequent changes alter the balance between muscle atrophy and hypertrophy and effect muscle composition (Sanders, Kneppers et al. 2016). This leads to the well-recognised consequences that we see in both early and late disease - most notably a reduction in power and endurance (Allaire, Maltais et al. 2004).

It has been established that skeletal muscle dysfunction in COPD is characterised by the loss of muscle mass and a change in fibre distribution (Natanek, Gosker et al. 2013). We see a reduction in the proportion of oxidative fibres and an increase in the proportion of glycolytic anaerobic fibres (Gosker, van Mameren et al. 2002). These changes appear early in susceptible individuals and have been shown independent of lung function and age to be a predictor of mortality (Patel, Natanek et al. 2014). Earlier studies demonstrated that quadriceps femoris strength was significantly lower in patients with moderate to severe COPD in spite of having a greater proportion of glycolytic (power) fibres, thus demonstrating

that the combination of overall muscle atrophy and fibre shift will affect both endurance and power leading to a distinctly un-favourable phenotype. Hence training programmes targeting both power (to increase overall muscle bulk) and endurance (to increase the amount of oxidative fibres) are of benefit (Lepsen, Jorgensen et al. 2015) (Vogiatzis, Nanas et al. 2010).

Aside from the theoretic spill over of inflammatory mediators and oxidative stress to muscle, patients with a progressive decline in lung function and ensuing ventilatory inefficiency will over time exhibit chronic hypoxia and hyper-capnia. Hypoxia will adversely affect skeletal muscle and contribute to further changes that have been mentioned. This has been shown as an increase in glycolytic enzyme activity and a decrease in oxidative enzyme activity (Howald, Pette et al. 1990). Exercise performance is known to improve in hypoxic patients with COPD who are given supplemental oxygen (Jakobsson and Jorfeldt 1995). Perhaps more importantly is the long-term effect of hyper-capnia as increased carbon dioxide concentrations are associated with a marked reduction in adenosine triphosphate (ATP) levels (Fiaccadori, Del Cante et al. 1987) thus suggesting that mitochondrial irregularity is a contributory mechanism to skeletal muscle dysfunction in COPD. Limited work has been done in chronic patients to look at novel intrinsic mechanisms that help to optimise lung emptying and clearance of carbon dioxide. The use of the upper airway to do this (akin to purse lip breathing) may be of benefit.

Overtime patients with COPD will exhibit distinctly lower levels of physical activity than healthy counterparts (Moy, Danilack et al. 2012). This perpetuates the cycle of decline as with inactivity comes greater deconditioning and further alterations to skeletal muscle. In

turn this leads to increased symptoms at lower levels of work as anaerobiosis will contribute to greater neuro-ventilatory dissociation.

Further contributory mechanisms include therapies that are used in patients with COPD. In particular corticosteroids which for many years have been a principle adjunct to alleviate symptoms and inflammation during acute exacerbations. Corticosteroids have a direct deleterious effect on muscle (Decramer, de Bock et al. 1995), in certain groups they are used long term at low doses as a treatment strategy. It has been shown that corticosteroids at an average dose of greater than 4mg daily contributes to quadriceps muscle weakness in patients with obstructive airways disease (Decramer, Lacquet et al. 1994). Thank fully more recent research has highlighted the population subgroups, such as patients with an eosinophilic phenotype, that may benefit from corticosteroids and so their use has been rationalised in clinical practice (Bafadhel, McKenna et al. 2012).

Pulmonary rehabilitation (PR) is to date the most effective treatment for patients with functional limitation secondary to COPD and improves symptomatology and quality of life (Cote and Celli 2005). In both an acute setting post exacerbation and in a more stable chronic setting PR has been shown to improve quadriceps strength and walking distance (Seymour, Moore et al. 2010). The exact mechanisms of skeletal muscle dysfunction should be further investigated and common links to the airways should be sought in view of the systemic nature and heterogeneity of the disease.

## 1.7 Mitochondria

Mitochondria have for many years since their identification been thought of as the power house of a cell. While true they play a much more important role being the main intrinsic producer of reactive oxygen species (ROS) (Murphy 2009) through oxidative phosphorylation resulting in the production of superoxide as oxygen is split. They are also the main neutraliser of whole cell ROS from both endogenous and exogenous sources. If we understand that oxidative stress is an imbalance between ROS production and cellular ability to clear this then we see that cells with poorly functioning mitochondria will be at higher risk of damage and destruction. Aside from this they are vital in allowing progression from G1 – S phase of the cell cycle (Mandal , Guptan et al. 2005) (Van den Bogert , Muus et al. 1988) and thus preventing premature apoptosis and necrosis of cells in limbo. While lung parenchyma is of significant interest in the histopathology of COPD with a more emphysematous radiological phenotype it is the structural airway cells that act as a protective barrier and are destroyed first.

The structure of individual mitochondria plays a crucial role in function. Alterations thus affect overall capability and efficiency. Mitochondria are the main site of adenosine triphosphate (ATP) synthesis in cells. They comprise a matrix which contains the enzymes required for oxidative phosphorylation and the folded cristae of the inner mitochondrial surface enable greater surface area for these reactions. The outer membrane is relatively permeable to allow molecules including ROS to flow between the cellular cytoplasm and the mitochondria themselves (Sherratt 1991). The inner membrane space houses key enzymes and complexes. Under normal physiological states ROS act as important signalling molecules to ensure the effective function of mitochondria.

Within the mitochondria the electron transport chain (ETC) is the primary site for ROS production. The ETC is a series of complex proteins that allow the transfer of electrons from donors to acceptors via reduction and oxidation (REDOX) which occur simultaneously (Turrens 2003). This process is coupled with the transfer of protons across the inner mitochondrial membrane. The proton gradient that is created drives the synthesis of ATP (Dimroth, Kaim et al. 2000). ROS production will occur under normal conditions as molecules are split and free radicals are generated. The ability of the mitochondria to deal with this is usually good as they are not over burdened by exogenous stressors and the enzyme superoxide dismutase (SOD) found in the mitochondria and cytoplasm functions to clear mitochondrial and cellular ROS. Under more pathological conditions or at times of higher demand when turnover is greater we see an imbalance in the mechanism occur and with persisting damage SOD activity is reduced (Vaziri, Dicus et al. 2003).

A late response to excessive ROS accumulation is mitochondrial membrane rupture, an earlier response manifests as a reduction in mitochondrial membrane potential (MMP) and in the activity of complex proteins. This destructive process can be further exacerbated by excessive ROS that accumulates within the cellular cytoplasm as a by-product of oxidative stress. This can enter mitochondria causing further swelling and membrane rupture (Ly, Grubb et al. 2003: Crompton 1998). Transition pore opening is regulated by MMP and matrix ph. The probability of pore opening and mitochondrial damage increases with a decrease in MMP (Ly, Grubb et al. 2003). The transition pore remains tightly closed during normal mitochondrial function and maintenance of a proper MMP is essential for the survival of the cell (Gottlieb 2001). Disruption of MMP by oxidative stress leads to poor function and simultaneous cell death (Satoh, Enokido et al 1997).

### 1.8 The role of mitochondria in COPD

Most of the current pharmacological treatments in COPD fail to prevent progression of disease and attenuate disease severity. A better mechanistic understanding of the condition is required to develop disease modifying therapies. Current theories in COPD are that of the oxidant-antioxidant balance, exaggerated inflammation and accelerated cellular senescence – the mitochondria play a central role in these processes (Murphy 2009) (Mandal , Guptan et al. 2005). Perhaps the most pertinent role of mitochondria is the influence they have over the ROS balance within cells. They are themselves known to be the site of ROS production and neutralisation, but in a disease free state the ROS generated are key regulators of the immune system and cell cycle progression. COPD is a disease charterised by an excessive oxidative stress burden (Kirkham and Barnes 2013), but little is known as to how mitochondrial function is affected by oxidative stress and how mitochondrial dysfunction contributes to oxidative stress in COPD patients.

In skeletal muscle of COPD patients demonstration of mitochondrial density change and increased ROS levels has been reported (Meyer, Zoll et al. 2013) in line with the reduced proportion of type I fibres that Natanek and colleagues demonstrated (Natanek, Gosker et al. 2013). The functional significance of this is early lactic acidosis and fatigue which contributes to greater inactivity which in turn perpetuates a poor skeletal muscle phenotype (Maltais, Simard et al. 1996). There is evidence that excessive mitochondrial ROS production in skeletal muscle in COPD patients exists and has been presented by Peunte-Maestu and colleagues in 2012. They studied skeletal muscle in both the resting and exercise states and showed that exercise stimulates mitochondrial ROS production which is unsurprising given that greater metabolic demand is placed on the mitochondrial

respiratory chain for ATP production. The pertinent results were they were able to show a threefold increase in mitochondrial ROS in biopsy samples from COPD patients when compared to controls - how this relates to mitochondrial function remains uncertain.

Work in the airways has been more limited and the study of mitochondrial function has generally been undertaken in cell culture lines. Culture of epithelial cells by Hoffman and colleagues for the purpose of mitochondrial study in COPD has been undertaken however it has involved the use of bronchial epithelium growth medium, fibronectin and collagen – the mitochondrial functional effects of which are unknown. They were able to show that cigarette smoke exposure up regulated the activity of super oxide dismutase (SOD) and identified this as a marker of excess oxidative stress, however did not directly assess for mitochondria derived ROS. They also demonstrated the detrimental effect of cigarette smoke exposure on mitochondrial ATP production (Hoffman, Zarrintan et al. 2013). When considering the role of mitochondria in COPD these limited studies have not evaluated direct mitochondrial function or shown an association with markers of mitochondria derived ROS.

### 1.9 The spill-over theory in COPD

In COPD two main sources of inflammation and oxidative stress may be considered; lungs and skeletal muscle. There are multiple theories about mechanisms that drive defective processes but there is no consensus on which theory is correct. There is a long-standing debate whether local inflammation that arises in the pulmonary system spills over into the circulation and aggregates in skeletal muscle to cause subsequent deleterious effects; or rather there is enhanced production of inflammatory mediators and oxidative stress within the skeletal muscle itself. The notion that COPD is a pulmonary disease and skeletal muscle dysfunction is a secondary consequence rests upon this debate. Findings such as the early defective muscle phenotype in COPD (Seymour, Spruit et al. 2010) go against the spill over theory and suggest that both the pulmonary and skeletal muscle systems are two effected organs in what is actually a systemic disease state.

The spill-over theory is itself difficult to prove however general consensus may be leaning toward this as the more likely mechanism. Many inflammatory cytokines and systemic markers of inflammation are elevated in the systemic circulation of patients with COPD however the finding more recently that a circulating mediator produced exclusively in the lungs (surfactant protein D) is found at higher levels in the systemic circulation of COPD patients provides more evidence in support of the spill-over theory (Barnes 2010). No conclusions can be drawn as to how the elevation of this mediator in the circulation effects skeletal muscle as the simultaneous study of skeletal muscle was not undertaken. There have been no studies that look at both pulmonary and skeletal muscle tissue from the same group of COPD patients – this may be of benefit when trying to prove or disprove the spill-over hypothesis.

### 1.10 Study aims and hypothesis

The pulmonary manifestations of COPD contribute to functional limitation and the symptom of dyspnoea is a limiting factor for many patients. As exercise performance worsens patients enter a well-established cycle of decline that comes with physical inactivity. Dynamic hyperinflation is a key mechanism that contributes to dyspnoea and hypoxemia - the functional consequences of which are aberrant cellular processes downstream as ventilation worsens. The effects of the upper airway on exercise have not been previously evaluated in patients with COPD. Thus the first hypothesis tested was that laryngeal narrowing during exercise may be a mechanism to increase intrinsic positive end expiratory pressure (PEEPi) and allow greater expiratory flow; and in Chapter 3 I evaluate dynamic movements of the larynx in patients with COPD and determine relationships with static and dynamic lung function measures and exercise tolerance.

Mitochondria are potentially a major central effector in the pathogenesis of COPD. Changes in mitochondrial density have been demonstrated in skeletal muscle. The interplay between the pulmonary and skeletal muscle systems is well recognised in COPD and the knowledge that optimising respiratory dynamics improves exercise performance demonstrates this at a pathophysiological level. The aim of Chapter 4 is to explore hypothesis number two; that if oxidative stress arises primarily in the pulmonary system as a result of mitochondrial dysfunction, and spills over to cause non pulmonary manifestations (in this case skeletal muscle dysfunction) then greater evidence of mitochondrial dysfunction will be present in the airways; and that the degree of mitochondrial dysfunction will be associated with the severity of the clinical phenotype. I sought to do this by the measurement of mitochondrial membrane potential and

mitochondrial and cellular ROS levels in airway and skeletal muscle compartments of a group of phenotypically well characterised ex-smokers and never smokers stratified according to $FEV_{1.}$ It is the first time that both pulmonary and skeletal muscle compartments have been studied in the same group of patients with COPD.

The aim of chapter 5 was to establish a murine model of COPD with inducible mitochondrial dysfunction. Previous work from Professor Chung's group had shown that using an ozone model mouse develop airway inflammation, airway hyper-responsiveness and alveolar destruction with emphysema similar to that seen in patients with COPD. I first examined whether mitochondrial dysfunction was present in this model, and if so wished to study the role of mitochondrial dysfunction underlying the various COPD features described above and importantly test the effects of a mitochondria targeted antioxidant. For the first time I looked to evaluate the effects on the skeletal muscle system in mice exposed to ozone as a way to further test the spill-over hypothesis of COPD in an animal model. The hypothesis being that ozone exposure will induce mitochondrial changes in the pulmonary and muscle compartments of mice and that mitochondrial targeted antioxidant therapy would abate this.

### 1.11 Overall research rationale

The overall research strategy for this thesis is based on the upper airway having potential to control and improve downstream alveolar-capillary ventilation in patients with COPD by exerting functional changes that may act to increase PEEPi as the respiratory duty cycle shortens and thus optimise lung mechanics by helping to offset the problem of dynamic hyperinflation. This will improve gas exchange and optimise oxygenation and will be seen downstream as improved oxygen delivery to tissues in the airway and muscle which may help to improve the reactive oxygen species profile of these tissues by optimising cellular metabolism. The measurable variables to demonstrate this will be markers of oxidative stress and mitochondrial function - which is dependent on its environment (Yang, Harison et al. 2007). As we hypothesise that mitochondrial function will be negatively affected in the lung and muscle systems of COPD patients the next stage of the work presented herein is to assess mitochondria as a potential therapeutic target for disease modification in an animal model.

## Chapter 2: Methods

This thesis comprises three human observational studies that were approved by the London research ethics committee and a murine study which was performed within the legal framework set out under a project license granted by the British Home Office, United Kingdom under the Animals Scientific Procedures Act 1986.

The first study allowed the assessment of laryngeal function during exercise (11/LO/1404); recruitment and assessment were aided by Dr Manuel Baz and Dr James Hull and the detailed applicable methods are presented in Chapter 3.

Studies two and three enabled the assessment of mitochondrial function in both airway and skeletal muscle compartments and they ran parallel to one another with subjects simultaneously recruited into both: 11/LO/1852 - Oxidative stress and innate immune dysfunction as drivers of airway and lung remodelling in COPD; and (12/LO/0088) - Deep phenotyping of the quadriceps muscle in patients with COPD. The methodological sequence for these studies is presented in Chapter 4.

The assessment of mitochondrial dysfunction in a murine model of COPD is presented in Chapter 5 and the detailed methodology is presented their in.

This chapter thus will detail how subjects were recruited into studies; inclusion and exclusion criteria; subject assessments such as anthropometric measures and measures of lung function and exercise capacity; Invasive techniques used to sample airway and skeletal muscle tissue; and molecular techniques for the study of mitochondria and cytoplasm.

## 2.1 Human Studies

### 2.1.1 Subject Recruitment

Subjects with a confirmed diagnosis of COPD and healthy controls were recruited from the Royal Brompton Hospital muscle and airways laboratory databases. They were also identified from the Royal Brompton Hospital COPD clinic. Studies were explained in detail and patient information sheets were given to potential recruits who subsequently provided written informed consent if they wished to participate. Study participants were free to withdraw at any time and continue to receive best clinical care.

### 2.1.2 Inclusion and exclusion criteria

Study subjects with COPD had a comparable ex-smoking history to one another and no current smokers were recruited. Healthy subjects were either ex-smokers or never smokers.

### 2.1.3 COPD subjects

Inclusion Criteria

- Males or females aged 39-79 years
- Evidence of COPD as measured by lung function **(Table 1.1)** and respiratory symptoms
- An ex-smoking history of at least 5-pack years

Exclusion Criteria

- Patients unable to give consent
- History of coexisting pulmonary pathology or laryngeal disease
- History of cardiovascular or metabolic disease
- Respiratory infection or hospitalisation for any cause in the preceding four weeks
- Contraindication to biopsy for research purposes (e.g. coagulopathy or use of anticoagulant medicines)
- Joint or neurological disease which would preclude exercise
- Use of long-term domiciliary oxygen or ventilatory support

### 2.1.4 Healthy control subjects

Inclusion Criteria

- Males or females aged 39-79 years
- No evidence of COPD as measured by lung function **(Table 1.1)** and respiratory symptoms
- An ex-smoking history of at least 5-pack years or those who have never smoked

Exclusion Criteria

- Patients unable to give consent
- History of coexisting pulmonary pathology or laryngeal disease
- History of cardiovascular or metabolic disease
- Respiratory infection or hospitalisation for any cause in the preceding four weeks

- Contraindication to biopsy for research purposes (e.g. coagulopathy or use of anticoagulant medicines)
- Joint or neurological disease which would preclude exercise
- Use of long-term domiciliary oxygen or ventilatory support

## 2.2 Subject assessments

All patients enrolled had a full clinical history documented and clinical examination undertaken. Measures of height and weight were obtained from which body mass index was calculated (BMI). Smoking history was recorded in pack years and subjects undertook baseline lung function testing for spirometry, lung volume and gas transfer measures. The COPD assessment tool (CAT) questionnaire was completed by all, as were the modified Borg scores for breathlessness and leg fatigue pre- and post- exercise testing. Subjects recruited for the purpose of laryngeal assessment during exercise (11/LO/1404), undertook incremental cycle-ergometer exercise testing with a nasal endoscope in situ with continuous video recording of the larynx (see Chapter 3). Subjects recruited into studies 11/LO/1852 and 12/LO/0088 were more extensively phenotyped over two days; short physical performance battery protocol (SPPB), quadriceps maximal voluntary contraction (QMVC), six-minute walk test (6MWT) and incremental cycle-ergometer cardio-pulmonary exercise testing were undertaken by all. On the third and final day patients underwent fibreoptic bronchoscopy with endo-bronchial biopsies followed by percutaneous muscle biopsy of the vastus lateralis, in those who had an $FEV_1$ of less than one litre only a muscle biopsy was performed.

### 2.2.1 Anthropometric measures

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 was calculated using these measurements - BMI = weight (kg) / Height $(m)^2$.

### 2.2.2 Pulmonary function testing

Pulmonary function testing was undertaken by respiratory physiologists of the Royal Brompton and Harefield NHS foundation trust in the biomedical research facility at the Royal Brompton Hospital site.

### 2.2.3 Spirometry

Spirometry was measured for all subjects according to the American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines. 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 ($FEV_1$) and forced vital capacity (FVC) achieved were recorded. The predicted normal values were expressed in accordance with the British Thoracic Society guidelines (British Thoracic Society 1997).

### 2.2.4 Lung volumes 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).

### 2.2.5 COPD assessment tool (CAT) questionnaire

The CAT questionnaire is a simple to use patient-completed quality of life instrument that covers the impact of symptoms in **COPD**. It has been validated and provides a standardised measure of health status in patients with COPD (Jones, Harding et al. 2009). GlaxoSmithKline is the copyright owner of the test and it compromises eight questions which are each given a score from zero to five. The maximum test score therefore being forty and the minimum being zero. The questions within the test are listed below.

- I never cough → I cough all the time (0-5)
- I have no mucous → My chest is full of mucous (0-5)
- My chest does not feel tight at all → My chest feels very tight (0-5)
- When I walk up a hill or flight of stairs, I am not breathless → When I walk up a hill or flight of stairs, I am very breathless (0-5)

- I am not limited doing any activities at home → I am very limited doing activities at home (0-5)
- I am confident leaving my home despite my lung condition → I am not confident at all leaving my home because of my lung condition (0-5)
- I sleep soundly → I don't sleep soundly because of my lung condition (0-5)
- I have lots of energy → I have no energy at all (0-5)

### 2.2.6 Modified Borg scores for dyspnoea and leg fatigue

The modified Borg scores were recorded pre- and post-exercise in all study participants before and after six-minute walk tests and cardio-pulmonary exercise testing. They are a useful tool for the assessment of breathlessness and leg fatigue and are based on Gunnar Borg's original dyspnoea scale. They provide a rating of perceived limitation before and after physical exertion. The scoring scale runs from zero to ten with zero being no breathlessness or leg fatigue at all. They can help to determine the reason a subject terminates exercise.

### 2.2.7 Short physical performance battery (SPPB)

The SPPB is a group of measures that combines the results of the four-metre gait speed, chair stand and balance tests. It has been used as a predictive tool for possible disability and can aid in the monitoring of function in chronic obstructive pulmonary disease (Patel, Mohan et al. 2014). The three components assessed have a maximum score of four points each. The test was also applied to healthy control subjects. A graphical representation of the scoring system is shown in **Figure 2.1** (Riskowski, Hagedorn et al. 2012).

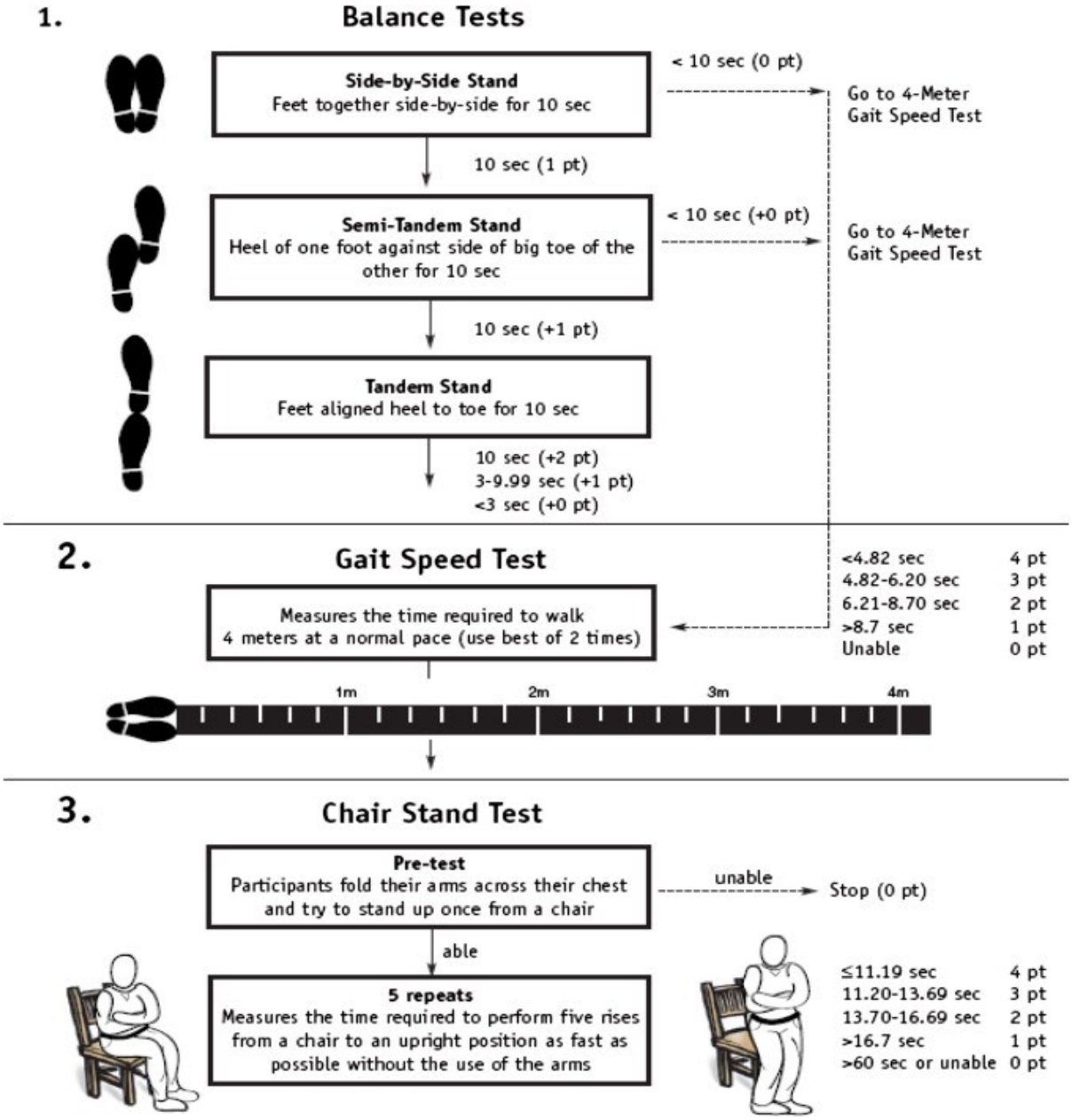



**Figure 2.1 -** Graphical representation of the assessments undertaken by patients for the SPPB and how points are awarded for each component. The maximum possible score is twelve and the minimum is zero - (Riskowski, Hagedorn et al. 2012)

### 2.2.8 Quadriceps maximal voluntary contraction

Quadriceps maximum voluntary contraction was measured as described by Edwards *et al.* (Edwards, Young et al. 1977). Subjects were seated upright on a couch and placed their dominant leg into a strain gauge which was fixed comfortably around their ankle. 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.

### 2.2.9 Six-minute walk test (6MWT)

A 6MWT was performed in accordance with the American Thoracic Society guidelines (American Thoracic Society 2002) over a thirty-metre track. Subjects were seated for ten minutes before starting the route and during this period completed the modified Borg scores for dyspnoea and leg fatigue. Pre-test transcutaneous oxygen saturation (SaO2) and heart rate were recorded with an N-200E Nellcor oximeter (Nellcor, Covidien-NellcorTM Boulder, USA). Standardised instructions were given to each subject prior to starting the test such that each subject was asked to walk as far as they could in the six minutes by making laps of the thirty-metre track. They were instructed not to run or jog and that should they experience extreme dyspnoea or chest pain they should stop immediately. Each

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. The modified Borg scores were completed by each subject again at the end of the test as were $SaO_2$ and heart rate measures.

### 2.2.10 Cardio-pulmonary exercise testing (CPET)

A symptom limited metabolic gas exchange ten-watt incremental exercise test was performed on a cycle ergometer (Ergoselect 100, Ergoline, Bitz, Germany). Metabolic measurements were collected using a mouthpiece (Masterscreen 85 CPX metabolic cart, CareFusion, Basingstoke, UK) and analysed using JLAB software (JLAB Lab Manager version 5.32, Jaeger, CareFusion, Basingstoke, UK). Simultaneous recording of heart rate, blood pressure and electrocardiogram traces were also obtained. Severe dyspnoea, chest pain, arrhythmias or pre-syncope were indications for immediate termination of the test. Following a two-minute rest period and one-minute of unloaded cycling, gradual work was undertaken with a smooth increment of ten watts every sixty seconds with subjects being asked to maintain a speed of 55-65 revolutions per minute (RPM). Measurements were taken breath-by-breath of oxygen uptake ($VO_2$), carbon dioxide production ($VCO_2$), minute ventilation (VE), respiratory rate (BF) and tidal volume (VT). These were averaged in the final thirty seconds of loaded cycling for the peak exercise values, with the exclusion of aberrant breaths

## 2.3 Invasive sampling techniques

### 2.3.1 Fibre-optic bronchoscopy and endo-bronchial biopsy

Fibreoptic bronchoscopies were performed under sedation, where required, according to standard clinical practice at the Royal Brompton Hospital and in line with2 local ethics committee approval guidance for study 11/LO/1852. As the majority of patients were ex-smokers the endobronchial anatomy was examined to ensure no pathology was present. Four to six Endobronchial biopsies were then taken from the right middle and lower lobe bronchi. One biopsy specimen per subject was stored in Dulbecco modified Eagle medium for later airway smooth muscle culture. The majority of biopsy tissue obtained was stored in phosphate buffered saline on ice for immediate post procedure extraction and subsequent study of mitochondria and cytoplasm as detailed later in this chapter (section 2.5).

### 2.3.2 Percutaneous biopsy of the vastus lateralis muscle

Using the technique described by Bergstrom in 1962 a percutaneous needle biopsy of the Vastus lateralis was obtained from the dominant side at least one week after but within one month of any form of exercise or strength testing. The procedure was performed under sterile conditions with the patient supine in a lateral position with their knee flexed by thirty degrees. The skin was cleaned with 2% chlorhexidine/ 70% isopropyl. Five millilitres of 2% lignocaine were then injected into the skin and subcutaneous tissue. A sub centimetre incision was made and the Bergstrom needle inserted through the subcutaneous tissues and through the fascia of the muscle. A three-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 the procedure repeated with the needle cutting further pieces of muscle tissue at the same pass. The majority of muscle was stored in phosphate buffered saline on ice for immediate post procedure extraction and subsequent study of mitochondria and cytoplasm as detailed later in this chapter (section 2.5). Where large enough samples were obtained some muscle was placed into cryovials and snap frozen in liquid nitrogen then stored at $-80^0$ c and some was stored in 2.5% glutaraldehyde for electron microscopy at a later time.

## 2.4 Molecular techniques for the study of mitochondria and cytoplasm

This section details the molecular study of human tissue samples. The same techniques were used for mouse samples obtained following terminal anaesthesia (Chapter 5). It documents how the mitochondrial and cytoplasmic fractions were extracted from fresh tissue and their subsequent study.

### 2.4.1 Sample processing

Fresh samples kept in phosphate buffered saline on ice were processed on the same day as they were obtained. Intact mitochondria and cytoplasm were isolated using a Mitochondrial Isolation Kit for Tissue (Thermo-Scientific, Waltham) exactly as described by the manufacturer this involved initial make up of buffers A and C using reagents provided within the kit. 10 mg of Bovine Serum Albumin (BSA) was added to 2500 µl Reagent A from the kit with half a protease inhibitor tablet (making buffer A), half a protease inhibitor tablet was then added to Reagent C (making buffer C). The samples were weighed and added separately to 800 µl of buffer A in a dounce homogeniser apparatus. Five dounces are undertaken with douncer A and 20 with douncer B. The homogenised samples were then added to reagent C in an Eppendorf and gently mixed. The first centrifugation spin at 2,500 rpm for 10 minutes at 4 degrees Celsius isolates the nuclear pellets which are re-suspended in phosphate buffered saline. The supernatant was then spun at 10,500 rpm for 15 minutes at 4 degrees Celsius. The pellets obtained are the mitochondrial pellets and these were washed with phosphate buffered saline then re-suspended in Hanks balanced salt solution (HBSS) for subsequent study. The supernatant is the cytoplasmic component. Protein concentrations of the cytoplasmic and mitochondrial fragments were then calculated to

ensure molecular results obtained are correct for quantity. This was done against a known concentration of Bovine serum albumin (BSA) using reagents obtained from Sigma Aldrich and exactly following their guidelines (Sigma, UK). The protein standard BSA was diluted in water to a stock of 2 mg/ml and then gradually diluted further down to 0 mg/ml. 5 µl of each of the mitochondrial or cytoplasmic fragments were then loaded for each sample into a clear ninety-six well plate alongside varied concentrations of the BSA standard. The Bradford assay was used to allow the detection of an absorbance shift of brilliant blue from 460 nm in its free state to 595 nm if complexed with proteins. The absorbance of the unknow protein samples was related to samples with a defined concentration that were measured in parallel. The derived equation obtained was then used to calculate protein concentration per µl of the samples to be studied and an example is shown from one of the samples below **(Figure 2.2)**. They were then frozen at -80 degrees Celsius for subsequent study.

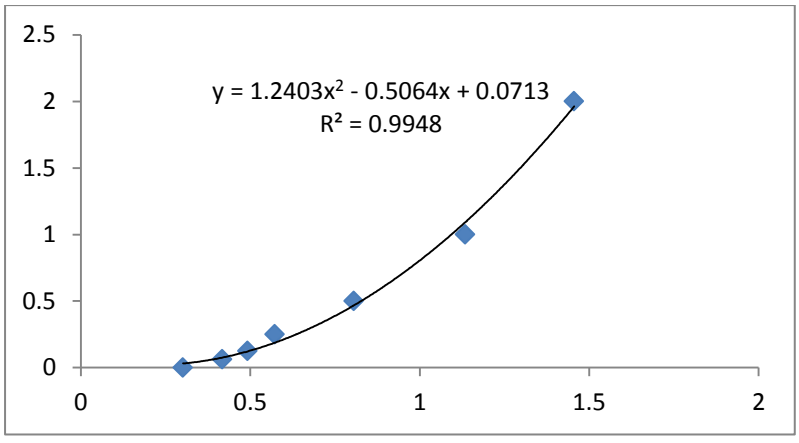



**Figure 2.2:** An example of a protein assay standard curve. The Y axis denotes the known concentration of BSA. The X axis denotes the optical density reading for a known volume of sample extracted from fresh tissue. The equation (Y=) allows calculation of the protein concentration of the extract.

### 2.4.2 Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi m$) was measured in intact isolated mitochondria using the cationic dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Invitrogen) according to the manufacturers guidelines (Thermo-Scientific, Waltham). 5 µl of each sample of standardized concentrations of intact mitochondria, which were processed as documented in 2.5.1, were incubated with 3 µl of JC-1 for thirty minutes at thirty-seven degrees Celsius with five percent carbon dioxide. JC-1 monomers emit green fluorescence but when they enter live mitochondria they form J-aggregates which emit red fluorescence. An increase in the ratio between red and green fluorescence is an indicator of $\Delta\Psi m$ and was determined using a fluorescence micro plate reader (BioTek instruments).

### 2.4.3 Mitochondrial reactive oxygen species detection

Mitochondrial reactive oxygen species activity was measured with MitoSOX$^{TM}$ Red (Invitrogen) exactly as described by the manufacturer. MitoSOX$^{TM}$ Red is redox-sensitive fluorescent probe that is selectively targeted to the mitochondria. 5 µl of each sample of standardized concentrations of intact mitochondria, which were processed as documented in 2.5.1, were incubated with 5 µl of MitoSOX for thirty minutes at thirty-seven degrees Celsius with five percent carbon dioxide and red fluorescence was determined at 510/580 nm wavelengths using a fluorescence micro plate reader (BioTek instruments).

### 2.4.4 Cytoplasmic reactive oxygen species detection

Cytoplasmic reactive oxygen species were detected as a marker of whole cell ROS content. This was done using 5 µl of each sample of standardized concentrations of cytoplasmic supernatant, which were processed as documented in 2.5.1. They were incubated with 3 µl of 2'-7'-dichlorofluorescin diacetate (DCF) stain (Sigma) which is a cell permeable non-fluorescent probe that when de-esterified by oxidation products turns into the highly fluorescent 2'-7'-dichlorofluorescin, which was detected using a fluorescence micro plate reader according to manufacturer's guidelines (Sigma Aldrich, UK).

# Chapter 3: The role of the upper airway in COPD

## 3.1 Introduction and Aim

Dynamic hyperinflation (DH) and in particular the loss of inspiratory reserve volume are recognised features in the aetiology of dyspnoea and exercise limitation in COPD (O'Donnell, Revill et al. 2001). Strategies to reduce DH including lung volume reduction and inhaled treatments improve exercise performance. Patients with COPD regularly pursed lip breathe as a mechanism to generate intrinsic positive end expiratory pressure (PEEPi) and this has been shown to increase expiratory flow time and tidal volume (Bianchi, Giglotti et al. 2004). There is limited knowledge as to how optimising respiratory mechanics by implementing upper airway manoeuvres increases peak oxygen consumption and how this may benefit patients at a cellular level. Laryngeal closure has been identified at rest in patients with COPD; Higenbottam and Payne studied 34 patients with obstructive lung disease and observed expiratory narrowing of the glottis at rest (Higenbottam and Payne 1982). The laryngeal response to physical exertion in patients with COPD has never been studied. The occurrence of laryngeal narrowing during the expiratory phase of the respiratory duty cycle may be relevant and by increasing PEEPi could serve to splint the airways akin to pursed lip breathing, and indeed may be an independent or supplementary mechanism to optimise operational lung mechanics.

The aim of this chapter is to evaluate dynamic movements of the larynx in patients with COPD and relationships with static and dynamic lung function measures and exercise tolerance. The ultimate goal is to determine if upper airway compensatory mechanisms improve exercise performance in patients with COPD. The overall hypothesis is that

laryngeal narrowing during expiration will be more prevalent in patients with COPD than healthy controls, and that it would become more pronounced on exercise and relate to dynamic hyperinflation indices.

## 3.2 Methods

### 3.2.1 Study Population

Subjects with confirmed COPD on the basis of an adequate smoking history of greater than five pack years, respiratory symptoms and lung function were recruited from the royal Brompton hospital muscle lab database. Exclusion criteria were a history of cardiac disease, known laryngeal disease, and the use of oxygen or ventilatory support. Patients were also excluded if they had another physical reason (e.g. severe arthritis) that limited their ability to exercise. Healthy subjects were recruited into the control arm.. They were free from respiratory, cardiac and metabolic disease, had normal lung function and no physical functional impairment.

### 3.2.2 Study design and procedures

Study subjects attended the laboratory for clinical assessment and relevant history details were obtained. Lung function was undertaken and any COPD patients with an exacerbation in the preceding month were excluded. Anthropometric assessments were recorded as well as a CAT questionnaire. Patients and controls then undertook an incremental maximal cycle-ergometer cardiopulmonary exercise test (CPET) with cardiorespiratory measurements. Operational lung volumes were determined from inspiratory capacity (IC) manoeuvres performed every 2 minutes to calculate the dynamic hyperinflation index (DHI) (Bauerle, Chrusch et al. 1998). Study subjects had their ventilation monitored breath by breath. Using real time respiratory data to help obtain accurate inspiratory capacity values instruction were given to subjects such that at the end of expiration they were required to

inhale as deeply as possible for their next breath. This was repeated at 2-minute intervals for the duration of the test. During the CPET the larynx was visualised by placing a fibreoptic nasendoscope (Olympus ENF-VQ, Olympus, Japan) in the posterior nasopharynx, this was then secured with specialist headgear **(Figure 3.1)**.

**Figure 3.1 –** Graphical representation of a subject undertaking cycle-ergometry with continuous laryngoscopy – (Courtesy of B Panchasara).

### 3.2.3 Laryngeal movement analysis

Image stills at end inspiration and end expiration were taken from continuous video recording obtained during the CPET. Breathing cycle was confirmed from mouth piece pneumotacograph flow data. An expiratory narrowing ratio was calculated for the glottic (distance between the vocal cords at their midpoint) and supra glottic apertures (distance between the medial aspect of the arytenoids) **(Figure 3.2)**. The ratio was taken as 1 – (aperture measurements at end expiration divided by the aperture measurements at end inspiration). Thus a narrowing ratio of 1 = complete narrowing and 0 = no narrowing at all. Narrowing scores were determined independently using the timed image data by another observer and myself. The accuracy was determined by plotting inter-observer agreement as the mean of our individual scores that were obtained as an average over five breaths at rest and at peak exercise (Higenbottom and Payne 1982). **(Figure 3.4).**

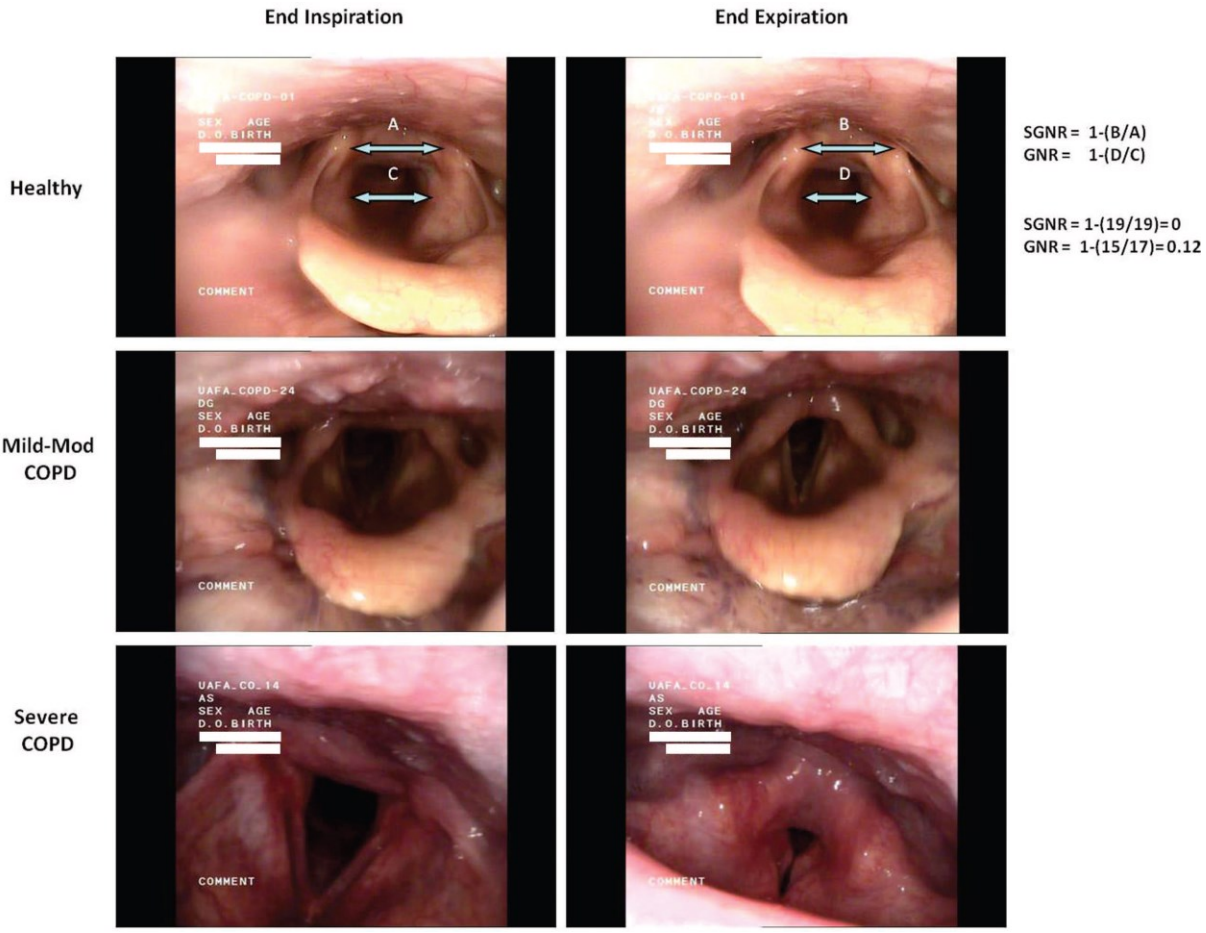



**Figure 3.2 -** Example cases illustrating dynamic expiratory laryngeal narrowing and method for calculation of glottic and supra-glottic narrowing ratio.

### 3.2.4 Statistical analysis

Data were normally distributed and are presented as mean ± SEM. A formal power calculation was not undertaken as no intervention was being tested. Numbers required to demonstrate a between group difference were based on work conducted by Higenbottam and Payne in 1982 in which 34 subjects with varying degrees of airflow obstruction had laryngeal visualisation. Groups were analysed as healthy, mild – moderate COPD (GOLD I and II), and severe – very severe COPD (GOLD III-IV). Differences in continuous variables were assessed using t tests and one-way analysis of variance with a Tukey post hoc test or the non-parametric equivalent. Correlations that follow were assessed by Pearson or Spearman's rank methods. Statistical calculations were made with Graphpad Prism V5.0. Significance is reported at $p<0.05$.

## 3.3 Results

### 3.3.1 Subject characteristics

34 subjects were recruited with technically acceptable data being obtained in 30, inadequate data was due to poor laryngeal visualisation (n=2) and inability to complete the test (n=2). Patients and controls were well matched for age, sex, height and BMI (all $p>0.05$). COPD patients had a higher symptom burden as assessed by the CAT questionnaire. CAT score in healthy subjects was 4 (0-9) and COPD subjects 19 (4-31), $p<0.001$. COPD patients also had impaired pulmonary function ($p<0.01$) and baseline oxygen saturations ($p<0.05$) (**Table 3.1 and Table 3.2).**

### 3.3.2 Cardio-pulmonary exercise test measurements

As expected, COPD patients demonstrated a lower peak work rate than healthy subjects: 60±8 watts vs 133±15 watts ($p<0.001$), and a lower peak $VO_2$ than healthy subjects: 13.7±0.8 ml/min/kg vs 24.6±1.8 ml/min/kg ($p<0.001$). COPD patients demonstrated evidence of dynamic hyperinflation with a reduction in inspiratory capacity compared with controls **(Table 3.2).** Patients with COPD exhibited a higher dynamic hyperinflation index, as calculated from the previous equation described by Bauerle and colleagues in 1998 (Bauerle, Chrusch et al. 1998), than healthy controls **(Table 3.3 and Figure 3.9).**

### 3.3.3 Glottic and Supraglottic laryngeal narrowing

**Rest**

In healthy subjects, there was a median percentage reduction in GNR during expiration of 10 %. This closure was more pronounced in patients at 50 % ($P<0.001$). A similar relationship was apparent at the supraglottic level ($P<0.01$). Predominant closure overall was at the glottic level **(Table 3.3 and Figure 3.5)**. There was no relationship between either GNR or SGNR and age, height, BMI (all $P>0.05$). Likewise, there was no difference in GNR or SGNR between males and females.

There was a correlation between the resting GNR and the $FEV_1$ in the whole cohort ($r=0.71$, $p<0.001$) and the COPD subjects alone ($r=-0.53$, $p<0.05$) **(Figure 3.5).** This correlation was not seen between $FEV_1$ and SGNR in COPD patients **(Figure 3.6)**. In all subjects a correlation was seen between markers of gas trapping and GNR. For RV/TLC and GNR ($r=0.44$, $p<0.05$), RV % predicted and GNR ($r=0.48$, $p<0.05$). Gas transfer (TLCO%) also correlated with GNR ($r=-0.57$ and $p<0.01$).

A high level of inter-observer agreement for GNR scoring measures ($r=0.93$, $P<0.0001$) was observed **(Figure 3.4).**

**Exercise**

Exercise GNR and SGNR data are shown in (**Table 3.3)**. Exercise GNR was inversely related to peak ventilation in all subjects ($r=-0.55$, $P<0.01$) and patients ($r=-0.71$, $P<0.001$) and peak exercise VT ($r=-0.58$, $P<0.01$ and $r=-0.55$, $P<0.05$ respectively). Likewise exercise GNR was

inversely related too peak $VO_2$ (% predicted) in all subjects (r=-0.65, p<0.0001) and patients alone (r=-0.58, p<0.01). A similar relationship was apparent for SGNR **(Figure 3.7)**.

Exercise respiratory duty cycle related to exercise GNR for all subjects (r=-0.69, P<0.001) and patients (r=-0.62, P<0.01) **(Figure 3.8).**

Patients with COPD and a significant exercise GNR (i.e. > 0.5) were no more likely to stop secondary to dyspnoea or report a higher peak exercise dyspnoea BORG score (P>0.05). Peak exercise GNR related to DHI if all subjects were considered together (r=0.45, P<0.05) but not if COPD patients were considered alone (r=0.24, P=0.35) **(Figure 3.9)**. There was no relationship between peak GNR and oxygen saturation and no significant difference in peak GNR between patients who de-saturated, i.e. $SpO_2$<94% (n=6).

**Table 3.1 – Subject Characteristic and pulmonary function**

| | Healthy (n=11) | Mild-Mod COPD (n=8) | Severe COPD (n=11) |
|---|---|---|---|
| **Age (yr)** | 60 ± 1 | 61 ± 2 | 60 ± 3 |
| **Sex (M:F)** | 6:5 | 3:5 | 6:5 |
| **Height (cm)** | 172 ± 3 | 166 ± 3 | 168 ± 3 |
| **Weight (kg)** | 75 ± 5 | 84 ± 6 | 69 ± 3 |
| **BMI ($kg/m^2$)** | 25 ± 1 | 30 ± 2 | 24 ± 1$^{\#}$ |
| **CAT (x/30)** | 4 ± (0-9) | 12 ± (4-24) * | 21 ± (11-31) **$^{\#}$ |
| **FEV1 (L)** | 3.1 ± 0.3 | 1.9 ± 0.2 * | 0.9 ± 0.1**$^{\#}$ |
| **FEV1 (%pred)** | 104 ± 5 | 72 ± 3 ** | 32 ± 2 **$^{\#\#}$ |
| **FVC (L)** | 4.2 ± 0.3 | 3.1 ± 0.4 | 2.7 ± 0.2* |
| **FVC (%pred)** | 114 ± 4 | 96 ± 4 * | 79 ± 5 ** |
| **$FEV_1$/FVC** | 74 ± 2 | 62 ± 3 * | 32 ± 2 **$^{\#\#}$ |
| **TLC (L)** | 6.9 ± 0.5 | 6.7 ± 0.8 | 7.8 ± 0.3 |
| **TLC (%pred)** | 110 ± 3 | 113 ± 5 | 138 ± 3 **$^{\#\#}$ |
| **RV (L)** | 2.4 ± 0.2 | 3.0 ± 0.3 | 4.4 ± 0.3 **$^{\#}$ |
| **RV (%pred)** | 109 ± 5 | 137 ± 13 | 203 ± 7.5 **$^{\#\#}$ |
| **RV/TLC (%)** | 35 ± 2 | 46 ± 4 | 56 ± 3 ** |
| **TLCOc (% predicted)** | 98 ± 6 | 74 ± 5 * | 43 ± 4 **$^{\#}$ |

**Table 3.1 -** Data shown as mean and SEM or median and range - * p<0.05, **p<0.01 from healthy controls, $^{\#}$p<0.05, $^{\#\#}$p<0.01 from mild-moderate COPD. Lung volume and gas transfer data only available in 21 subjects. BMI, body mass index; CAT' COPD assessment tool; RV, residual volume; TLC total lung capacity; $TLCO_c$, transfer coefficient for carbon monoxide corrected for haemoglobin

**Table 3.2 – Peak Exercise data**

| | Healthy | Mild -Moderate COPD | Severe COPD |
|---|---|---|---|
| **Reason for stopping**<br>**Breathing (B), Legs (L)** | B = 2<br>L = 9 | B = 5<br>L = 3 | B = 10<br>L = 1 |
| **Dyspnea Borg (x/10)** | 3 (1-5) | 4 (2-5) | 4 (3-5) |
| **Load (W)** | 133 ± 15 | 82 ± 13* | 44 ± 8** |
| **VO2 (L/min)** | 1.85 ± 0.19 | 1.31 ± 0.14* | 0.83 ± 0.15** |
| **VO2 (ml/min/kg)** | 24.6 ± 1.8 | 15.9 ± 1.7** | 12.0 ± 0.4** |
| **VO2 % predicted** | 100 ± 6 | 77 ± 5* | 49 ± 4**$^{\#\#}$ |
| **HR (b/m)** | 147 ± 7 | 131 ± 7 | 107 ± 4**$^{\#}$ |
| **HR reserve** | 8 ± 5 | 18 ± 4 | 33 ± 3**$^{\#}$ |
| **VE (L/min)** | 66 ± 10 | 46 ± 6 | 28 ± 2** |
| **BR** | 49 ± 5 | 41 ± 3 | 16 ± 6**$^{\#\#}$ |
| **SpO2 (%)** | 98 ± 0 | 98 ± 1 | 94 ± 1**$^{\#\#}$ |
| **VT peak (L)** | 2.15 ± 0.24 | 1.56 ± 0.22 | 1.11 ± 0.08 ** |
| **$T_I/T_{Tot}$** | 0.45 ± 0.07 | 0.41 ± 0.02* | 0.36 ± 0.01**$^{\#}$ |
| **IC PEAK (L)** | 3.44 ± 0.27 | 2.52 ± 0.28* | 1.99 ± 0.15 ** |
| **Delta IC (%IC rest)** | -0.35 ± 2.90 | 12.10 ± 3.54 | 15.26 ± 4.08** |
| **DHI** | -0.01 ± 0.06 | 0.46 ± 0.18 | 0.96 ± 0.27** |

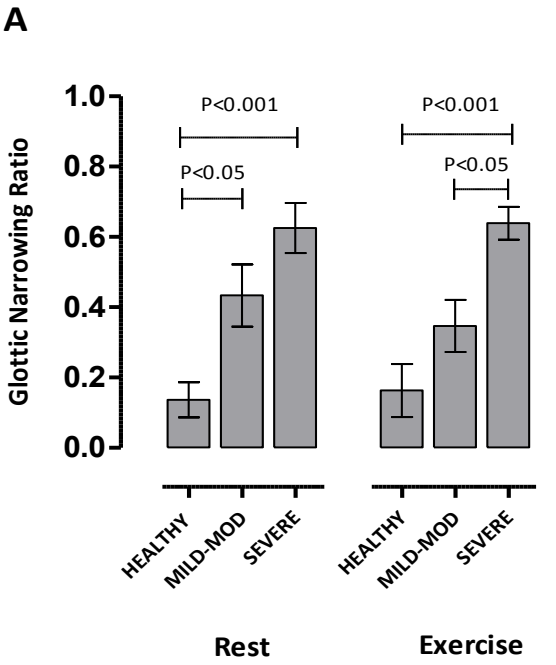
**Table 3.2** - Data shown as mean and SEM or median and range - * $p<0.05$, **$p<0.01$ from healthy controls, $^{\#}p<0.05$, $^{\#\#}p<0.01$ from mild-moderate COPD. Operational lung volume data available in 7 patients with mild-moderate COPD and 10 patients with severe COPD. BR, breathing reserve; DHI dynamic hyperinflation index; HR, heart rate; IC, inspiratory capacity; $SpO_2$, peripheral oxygen saturation; $T_I/T_{TOT,}$ respiratory duty cycle; VE, minute ventilation; $VO_2$ oxygen consumption; VT tidal volume.

**Table 3.3 – Laryngeal narrowing at rest and peak exercise**

| GNR | Healthy | Mild -Moderate COPD | Severe COPD |
|---|---|---|---|
| Rest | 0.14 ± 0.05 | 0.43 ± 0.09 * | 0.63 ± 0.07 ** |
| Exercise | 0.16 ± 0.08 | 0.35 ± 0.08 | 0.64 ± 0.05 **# |
| Change | 0.02 ± 0.09 | −0.09 ± 0.05 | −0.01 ± 0.07 |

| SGNR | Healthy | Mild -Moderate COPD | Severe COPD |
|---|---|---|---|
| Rest | 0.14 ± 0.03 | 0.31 ± 0.10 | 0.36 ± 0.05*# |
| Exercise | 0.10 ± 0.05 | 0.20 ± 0.06 | 0.43 ±0.07** # |
| Change | −0.04 ± 0.07 | −0.11 ± 0.08 | −0.07 ±0.07 |

**Table 3.3** – Data shown as mean ± SEM. *p<0.05, * *p<0.01 from healthy, #p,0.05 from mild-moderate COPD



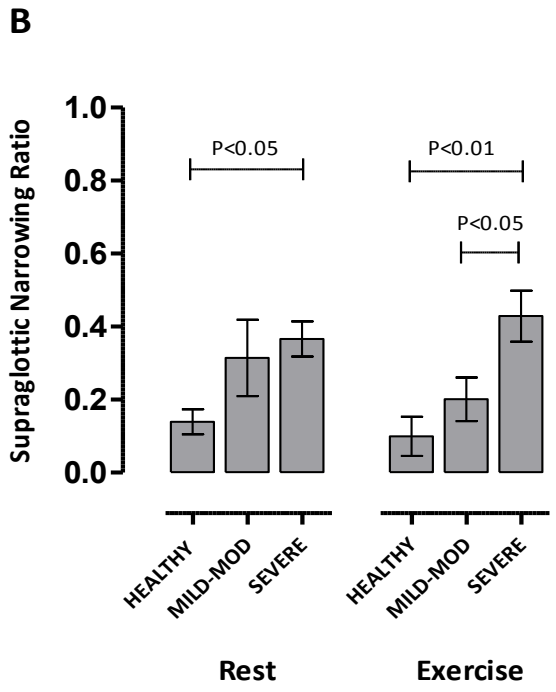




**Figure 3.3 –** Laryngeal narrowing ratio at rest and peak exercise at glottic level (A) and supraglottic level (b)

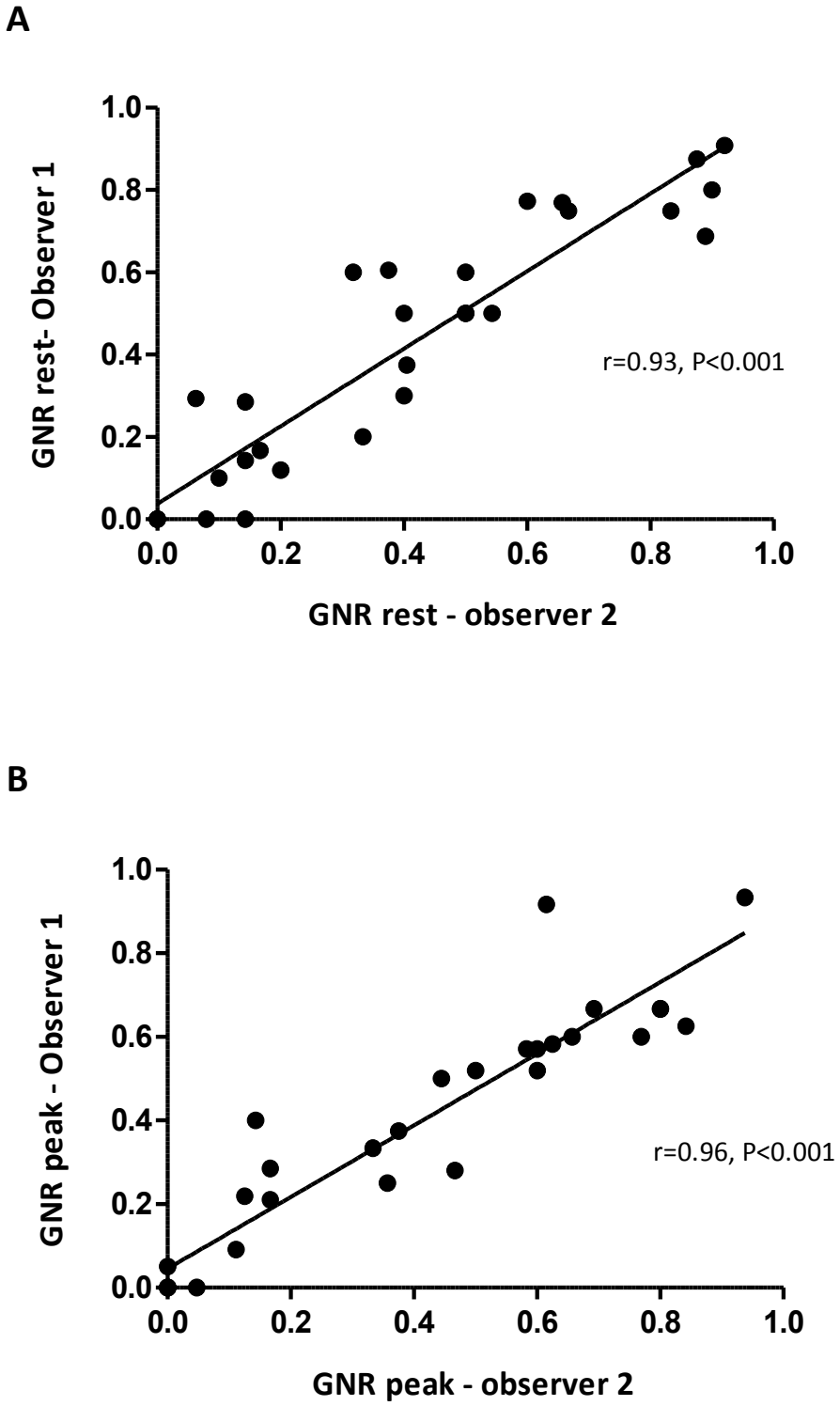



**Figure 3.4 –** Inter-observer agreement of the glottic narrowing ratio at rest (A) and peak exercise (B)

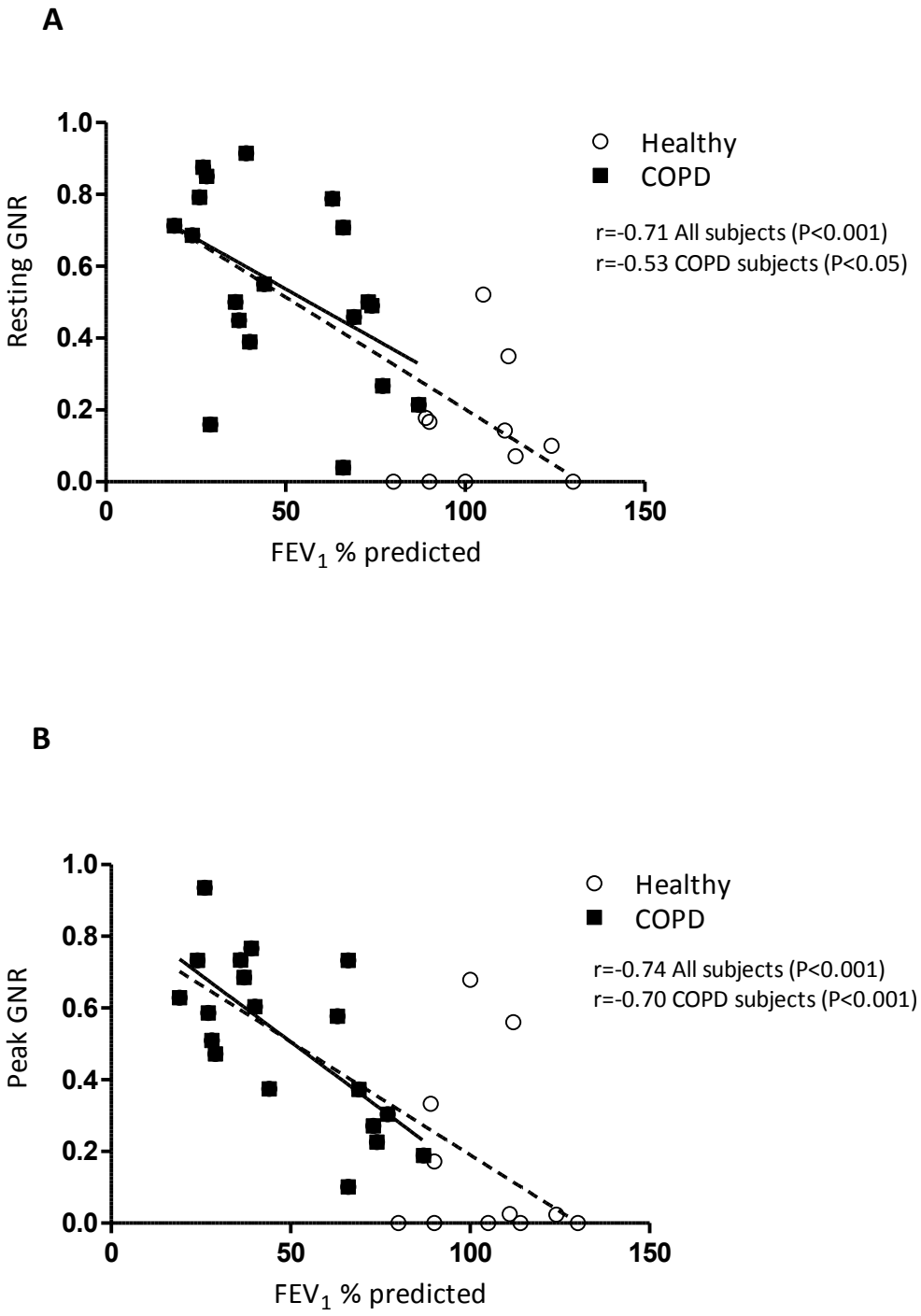



**Figure 3.5** – Relationship between $FEV_1$ % predicted and glottic narrowing ratio (GNR) at rest (A) and peak exercise (B)

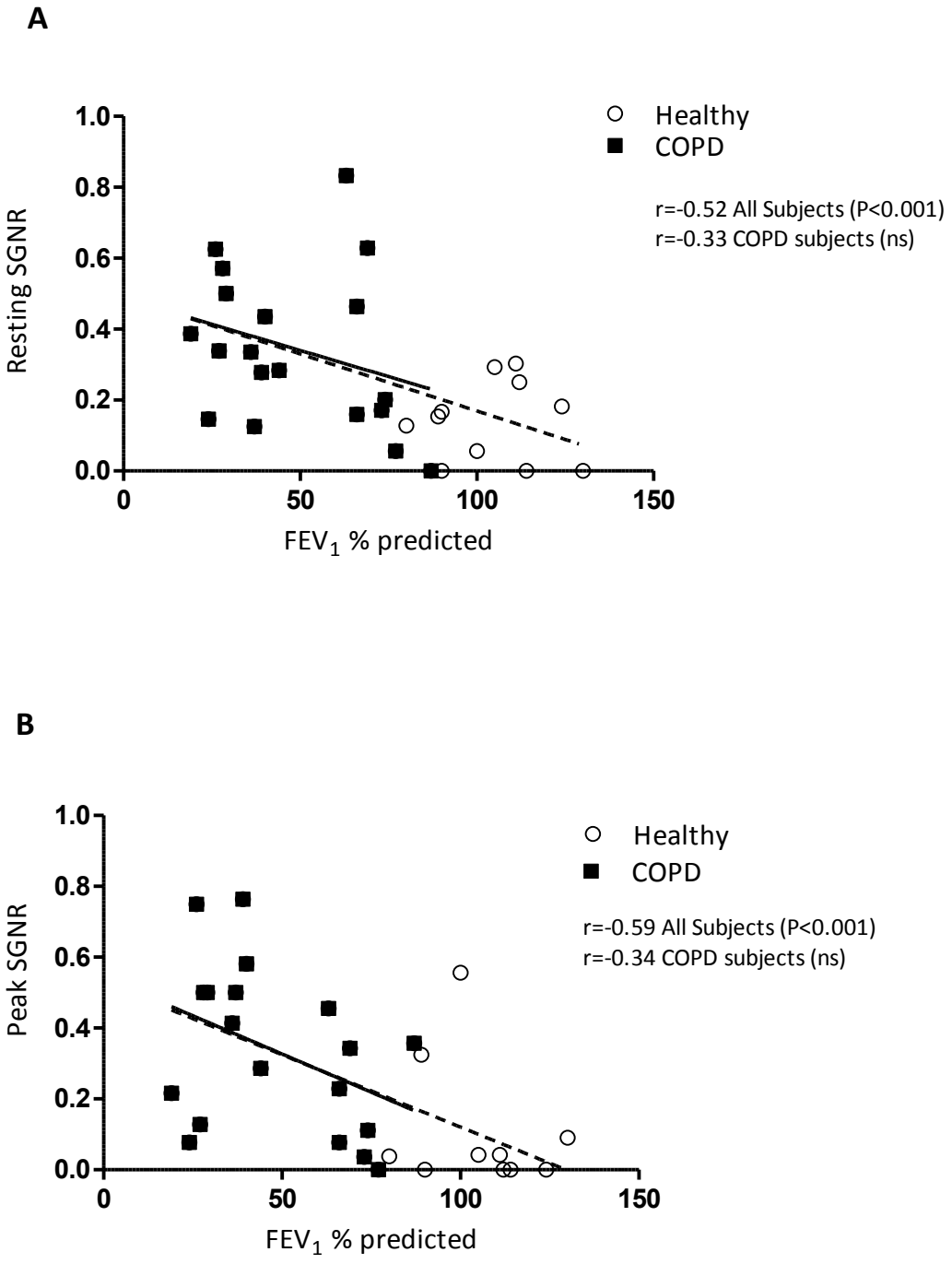



**Figure 3.6** -– Relationship between $FEV_1$ % predicted and Supraglottic narrowing ratio (SGNR) at rest (A) and peak exercise (B)

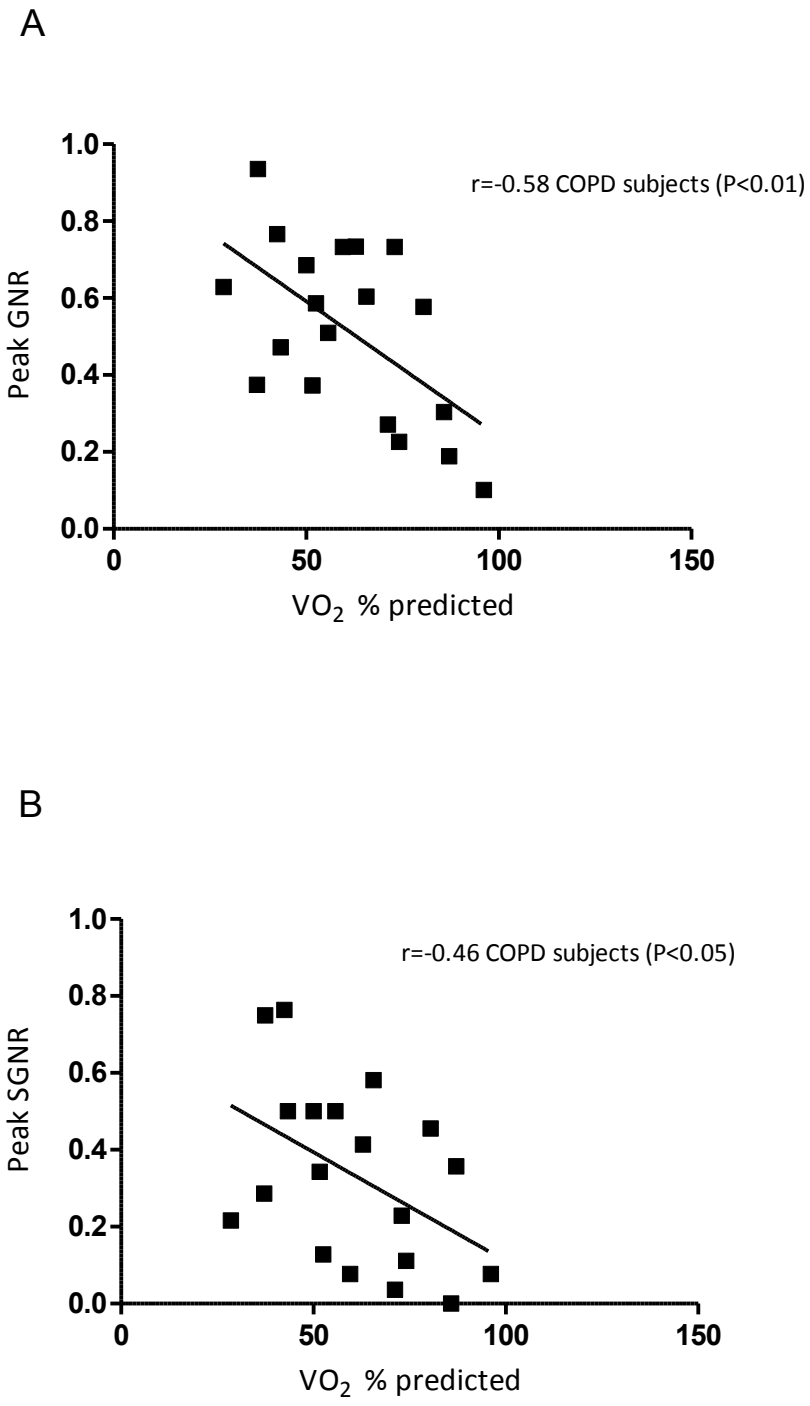



**Figure 3.7 -** Relationship between $VO_2$ % predicted and Glottic narrowing (GNR) at peak exercise in COPD subjects (A) and Supraglottic narrowing (SGNR) at peak exercise in COPD subjects (B)

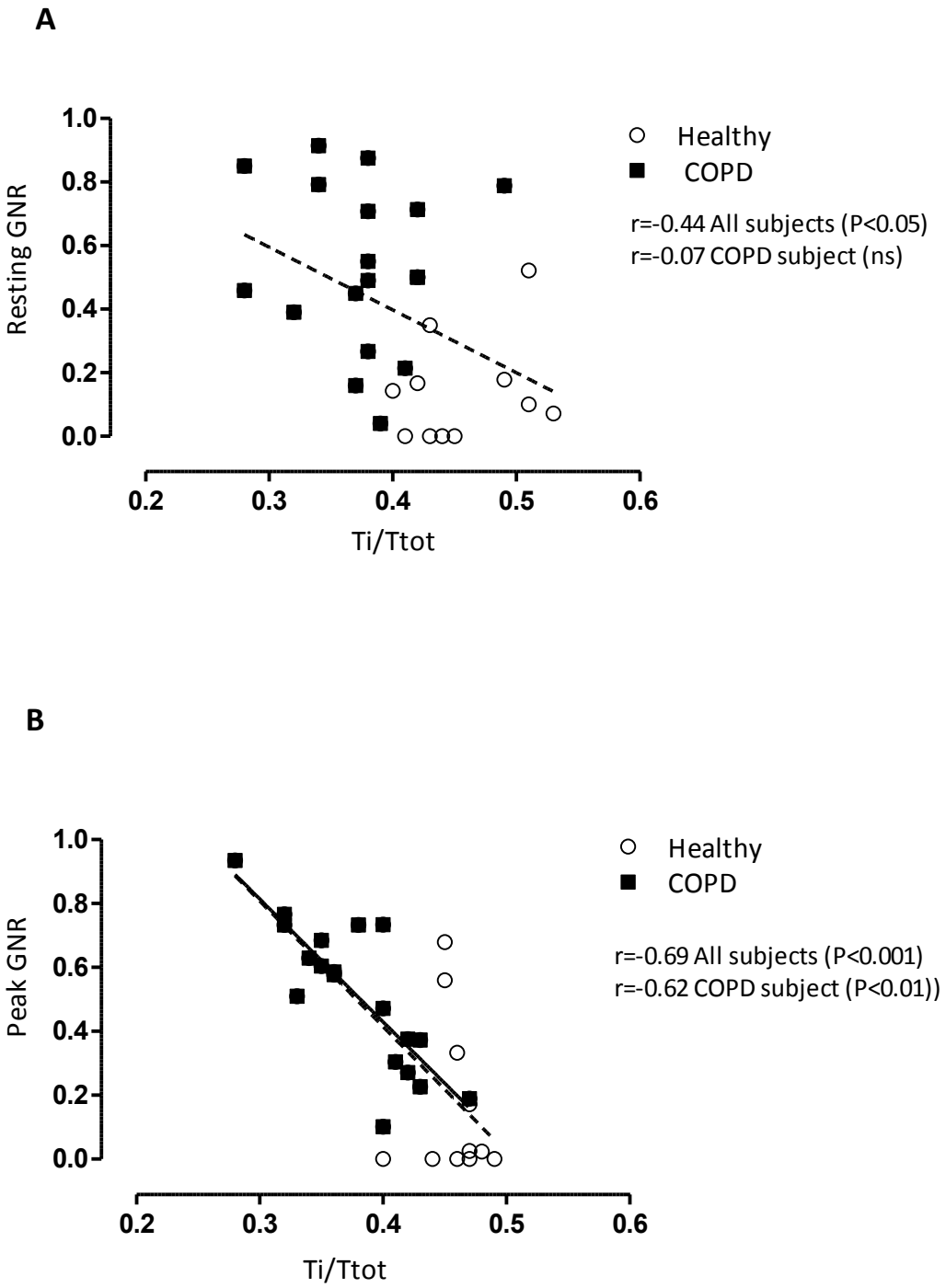



**Figure 3.8 –** Relationship between respiratory duty cycle and glottis narrowing at rest (A) and peak exercise (B)

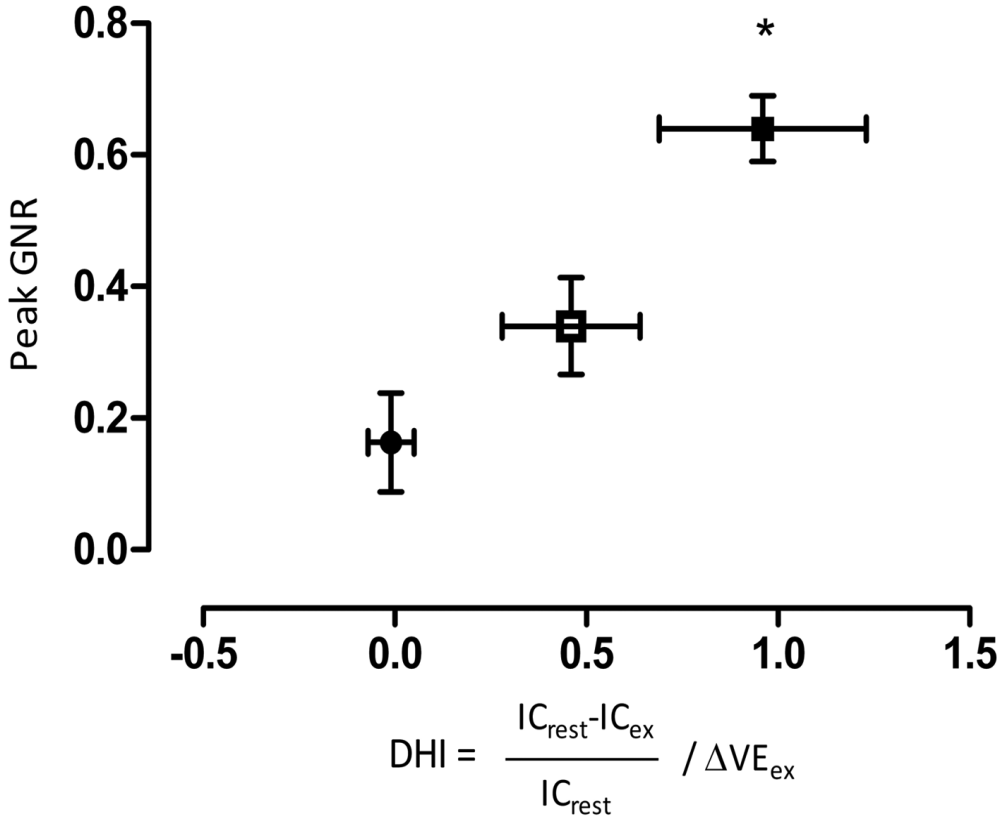



**Figure 3.9** - Relationship between peak glottic narrowing ratio (GNR) and dynamic hyperinflation index (DHI) in controls (•), patients with mild–moderate COPD (□) and patients with severe COPD (▪). Data are presented as mean values with error bars representing 1 SEM. *p<0.05 from controls.

## 3.4 Discussion

### 3.4.1 Summary of results

These results show that laryngeal assessment on exercise in patients with advanced COPD is a feasible technique. I have demonstrated that laryngeal narrowing predominantly originates at the level of the glottis - supraglottic narrowing may act as an adjunct. I have also shown that glottic narrowing relates to indices of dynamic hyperinflation and that the relationship between glottic narrowing and the respiratory duty cycle is more pronounced during exercise.

As expected I have shown that dynamic hyperinflation is more prevalent in patients with advanced COPD in comparison to milder disease. These results indicate that dynamic laryngeal narrowing during expiration is prevalent in patients with COPD and is directly related to lung function indices of disease severity and poor exercise performance ($VO_2$ Peak). The mechanistic rationale in this case is the relationship between glottis narrowing and dynamic hyperinflation. An obvious argument is the effect of dynamic hyperinflation on cardiac physiology with increased right ventricular afterload and subsequent reduction in pulmonary flow (Gagnon, Guenette et al. 2014). This will manifest as a reduction in cardiac output which will impair oxygen delivery/utilisation and thus lead to a reduction in $VO_2$ Peak above what may be expected given that these patients do still have a degree of available ventilatory reserve, albeit strictly speaking are ventilatory limited.

### 3.4.2 Critique of methods

The finding that laryngeal narrowing is associated with disease severity in the resting state and that this does not change considerably with exercise was perhaps unexpected. However, it suggests that for patients with severe disease the larynx is acting to provide optimal flow even at rest. A significant problem with this study was that all patients had a CLE performed in conjunction with a metabolic gas exchange cycle-ergometer test, and in order to do this they simultaneously had a mouth piece in situ which would abolish their ability to purse lip breathe. I cannot therefore answer the question as to whether laryngeal narrowing is a useful adjunct to pursed lip breathing as it may be that it is a strategy that patients employ due to an inability to pursed lip. The observation that laryngeal narrowing was prevalent at rest alleviates these concerns to a degree however the use of a facemask with a hole created to insert the nasendoscope rather than a mouth piece connected to the pneumotactograph would have been an obvious way to ensure the validity of these findings.

While a relationship between laryngeal narrowing and disease severity has been demonstrated its possible benefits to improve exercise performance have not. In order to do this a further study would need to be conducted in which many more patients with similar disease severity and hyperinflation indices are recruited and studied. If indeed those exhibiting greater laryngeal narrowing, despite similar lung function indices, performed better on exercise testing then the hypothesis could be answered. Against the theory that it may be beneficial to exercise performance is the finding that although dynamic

hyperinflation occurs in this group of patients the laryngeal aperture does not tighten further in response to this.

### 3.4.3 Significance of these findings

The hallmarks of COPD are inflammation of the airways and destruction of the lung parenchyma or emphysema. Progressive expiratory flow limitation and loss of elastic recoil properties of the lung lead to progressive lung hyperinflation (Calverley and Koulouris 2005) such that at the end of expiration the lungs have not fully emptied before the start of inspiration. This leads to alterations of the respiratory duty cycle - and breathing pattern particularly on exercise may be erratic as problems are accentuated when the respiratory duty cycle is shorter and the drive to breathe is even greater. Over time hyperinflation increases the load on the respiratory muscles which become weak (Kawagoe, Permutt et al. 1994). Hyperinflation will also lead to alveolar over-distension which along with emphysematous destruction contributes to progressive V/Q mismatching (O'Donnell, Guenette et al. 2012). These mechanisms will contribute to dyspnoea and exercise insufficiency as COPD advances, as well as hypoxemia and therefore a less favourable environment for efficient mitochondrial work leading to skeletal muscle fibre type changes (Kent, Mitchell et al. 2011), once this occurs patients have entered a well-established cycle of decline.

Dynamic hyperinflation clearly leads to dyspnoea and limitations in functional capacity in COPD such that patients with COPD will characteristically show poor exercise tolerance which manifests as a reduction in peak oxygen uptake and decreased endurance (O'Donnell, Revill et al. 2001). Methods to overcome dynamic hyperinflation are limited. Novel bronchoscopic lung volume reduction surgery is perhaps the most exciting therapy in this field and allows optimisation of lung mechanics (Davey, Zoumot et al. 2015), as open

techniques have been shown to do (Martinez, de Oca et al. 1997). Bronchodilators are poorly effective for improving hyperinflation indices through reduction in expiratory flow limitation. Methods to allow airways to remain open for longer will inhibit expiratory airways collapse and allow better lung emptying which corresponds to improved exercise performance. One such study that investigated the potential benefits of proportional assist ventilation as a strategy to improve exercise performance in severe COPD was able to demonstrate an improvement in peak work rate and interestingly reported a decrease in plasma lactate for an equivalent work load in the intervention group (Hawkins, Johnson et al. 2002). Feasibility and practicality of the use of devices mean they are not employed in routine practice. The handheld Vita-Breath device developed by Philips and studied in a small group of COPD patients has shown promise and demonstrated improvements in exercise tolerance during six minute walk testing when compared to patients using sham devices. The interest in improving exercise performance using physiological methods centres around the knowledge that exercise is beneficial at a molecular and cellular level in COPD patients. Lactic acid production due to anaerobic metabolism will directly stimulate respiration in patients with COPD leading to early fatigue (Casaburi 2006) thus strategies to optimise tissue oxygen delivery via improved ventilation may have beneficial downstream effects that will delay attainment of anaerobic threshold - the larynx may possibly be a target for intervention to improve ventilatory dynamics in patients with COPD.

### 3.4.4 Conclusion

These results show laryngeal narrowing during expiration is prevalent in patients with COPD and is directly related to disease severity, respiratory duty cycle and exercise capacity. The reduction in laryngeal aperture arises predominantly from narrowing at the glottic level and was evident during quiet breathing at rest. Supraglottic narrowing may act as an adjunct to what is occurring at the level of the vocal cords. During exercise glottis narrowing relates to markers of hyperinflation and the dynamic relationship between GNR and the respiratory duty cycle becomes even closer. Symptomatically it has been noted that patients with a higher exercise GNR (>0.5) were more likely to stop exercising due to dyspnoea, this is not unexpected given the relationship with disease severity and dynamic hyperinflation. In contrast to the findings here - in exercise induced laryngeal obstruction, a pathological condition, narrowing is predominantly at the supraglottic level (Heimdal, Roksund et al. 2006).

Whether cellular feedback mechanisms have any impact on glottic function is yet to be evaluated however it may be that chemoreceptor responses to cellular respiration and blood carbon dioxide levels impact upon the neurophysiological control of breathing and airway dynamics.

# Chapter 4: Mitochondrial dysfunction in airway and muscle compartments in COPD – association to clinical phenotype

## 4.1 Introduction and Aim

Breathlessness and consequent exercise intolerance is a key feature of COPD, which has been postulated, may lead to a downward cycle of deconditioning and secondary locomotor muscle weakness (Polkey and Moxham 2011). Airflow obstruction causes expiratory flow limitation which can cause air trapping – and emphysematous destruction of lung parenchyma causes a loss of elastic recoil which also contributes to airflow obstruction and early airway closure which leads to hyperinflation (O'Donnell, Revill et al. 2001). On exercise as the respiratory cycle shortens, reducing time available for expiration the phenomenon worsens.

In Chapter 3 I investigated the role of the upper airway in patients with COPD and was able to show an association between exercise tolerance, respiratory duty cycle, dynamic hyperinflation and laryngeal narrowing. Novel non pharmacological therapies, for example with endo-bronchial lung volume reduction, improve $FEV_1$ and reduce dynamic hyperinflation, and this has progressed towards routine clinical use (Davey, Zoumot et al. 2015). However despite much research over the past decades pharmacological treatments for COPD remain largely the same suggesting that key therapeutic targets have yet to be identified. Reversing the pathological features that cause airflow obstruction and parenchymal lung destruction which are thought to results from inflammation and altered tissue repair would offer one approach to this – thus there is a need to develop drug therapies that target these mechanisms (Barnes 2003).

Oxidative stress and inflammation are closely related pathophysiological processes. Inflammation is a response to harmful stimuli that involves the activation of the innate immune system and cytokine mediators leading to changes in tissue ultra-structure that exert pathological effect. In the case of COPD this is a local process of the pulmonary system that leads to proliferation of smooth muscle cells contributing to airway narrowing, as well as overt tissue destruction of the lung parenchyma (Chung and Adcock 2008). Skeletal muscle dysfunction in COPD is a prevalent and clinically important manifestation and the knowledge that it occurs early in the course of disease (Natanek, Gosker et al. 2013) suggests the process is not driven solely by disuse secondary to inactivity. One candidate hypothesis is that the systemic manifestations of COPD are driven by the spill over of inflammatory molecules and Reactive Oxygen Species (ROS) that are generated in the lung **(Figure 4.1)**, this is supported by the finding by some investigators that circulating inflammatory markers in the blood stream of subjects with COPD are known to be higher than healthy controls (Eagan, Ueland et al. 2010).

The noxious stimuli, that drive the oxidant-antioxidant imbalance in favour of ROS accumulation and inflammation, are most commonly cigarette smoking and other environmental air pollutants such as biomass fuels, ozone and diesel fumes (Kirkham and Barnes. 2013). Once COPD is established smoking cessation does slow disease progression however it does not prevent on-going mechanisms of inflammation and ROS production (Louhelainen, Ryita et al. 2009). On-going high intracellular ROS levels are likely due to the presence of dysfunctional mitochondria which are known to be a potent source of intracellular ROS from within the electron transport chain. In vitro assessment using airway

smooth muscle cells has shown that pro inflammatory mediators that are elevated in COPD such as tumour necrosis factor α (TNF-α) and interferon Y contribute to the production of greater amounts of mitochondrial derived ROS. Analysis has confirmed that this is due to dysfunction of specific mitochondrial complex proteins in particular Complex I (NADH: ubiquinone oxido-reductase) and Complex III (Cytochrome c) that traverse both oxidised and reduced states to ensure the overall efficiency of ATP production and cellular functioning is maintained (Aguilerra-Aguirre, Basci et al. 2009). Unfortunately once this endogenous cycle is activated it can be very difficult to stop as higher oxidative stress levels are independently associated with the up regulation of redox sensitive cellular targets, such as transcription factor nuclear factor-kβ (NF-kβ) and p38 mitogen-activated protein kinase, that go on to induce further inflammation. Elevated levels of NF-kβ are known to correlate with the severity of airflow obstruction in patients with COPD (Di Stefano, Caramari et al. 2002). Continued exposure to noxious agents such as cigarette smoke will serve to drive these processes at a more rapid rate given lipid soluble components in cigarette smoke are known to induce mitochondrial production of ROS in lung epithelial cells which act as a first line defensive barrier (van der Toorn, Rezayat et al. 2009). The pro-oxidative and pro-inflammatory modulation that occurs as a cascade of events is difficult to turn off once it has begun. One mechanism for this is phagocytosis of highly inflamed necrotic cells with a significant oxidative stress burden; however this process is also defective in COPD (Kirkham and Barnes. 2013).

Historically the mitochondria are the overall drivers of cell function as they are the energy producing centres. They catalyse oxidation of metabolites to drive production of ATP by the process of oxidative phosphorylation (Dimroth, Kaim et al. 2000). The mitochondrial

electron transport chain is responsible for generating a proton positive force to drive ATP synthesis and also maintain mitochondrial membrane potential which regulates the movement of mitochondrial and cellular ROS in order to maintain a favourable oxidant-antioxidant balance (Ly, Grubb et al. 2003). Even under normal conditions ROS production will occur due to the generation of free radicals as a by-product of oxidative phosphorylation (Turrens 2003). The ability of the mitochondria to deal with this is usually good as they are not over burdened by exogenous stressors and the enzyme superoxide dismutase (SOD) found in the mitochondria and cytoplasm functions to clear mitochondrial and cellular ROS. Under more pathological conditions or at times of higher demand when turnover is greater we see an imbalance in the mechanism occur and with persisting damage SOD activity will reduce (Vaziri, Dicus et al. 2003).

The aim of this chapter is to explore the hypothesis that if oxidative stress arises primarily in the pulmonary system as a result of mitochondrial dysfunction, and spills over to cause non pulmonary manifestations (in this case skeletal muscle dysfunction) then greater evidence of mitochondrial dysfunction will be present in the airways; and that the degree of mitochondrial dysfunction will be associated with the severity of the clinical phenotype. I sought to do this by the measurement of mitochondrial membrane potential and mitochondrial and cellular ROS levels in airway and skeletal muscle compartments of a group of phenotypically well characterised ex-smokers and never smokers stratified according to $FEV_{1.}$

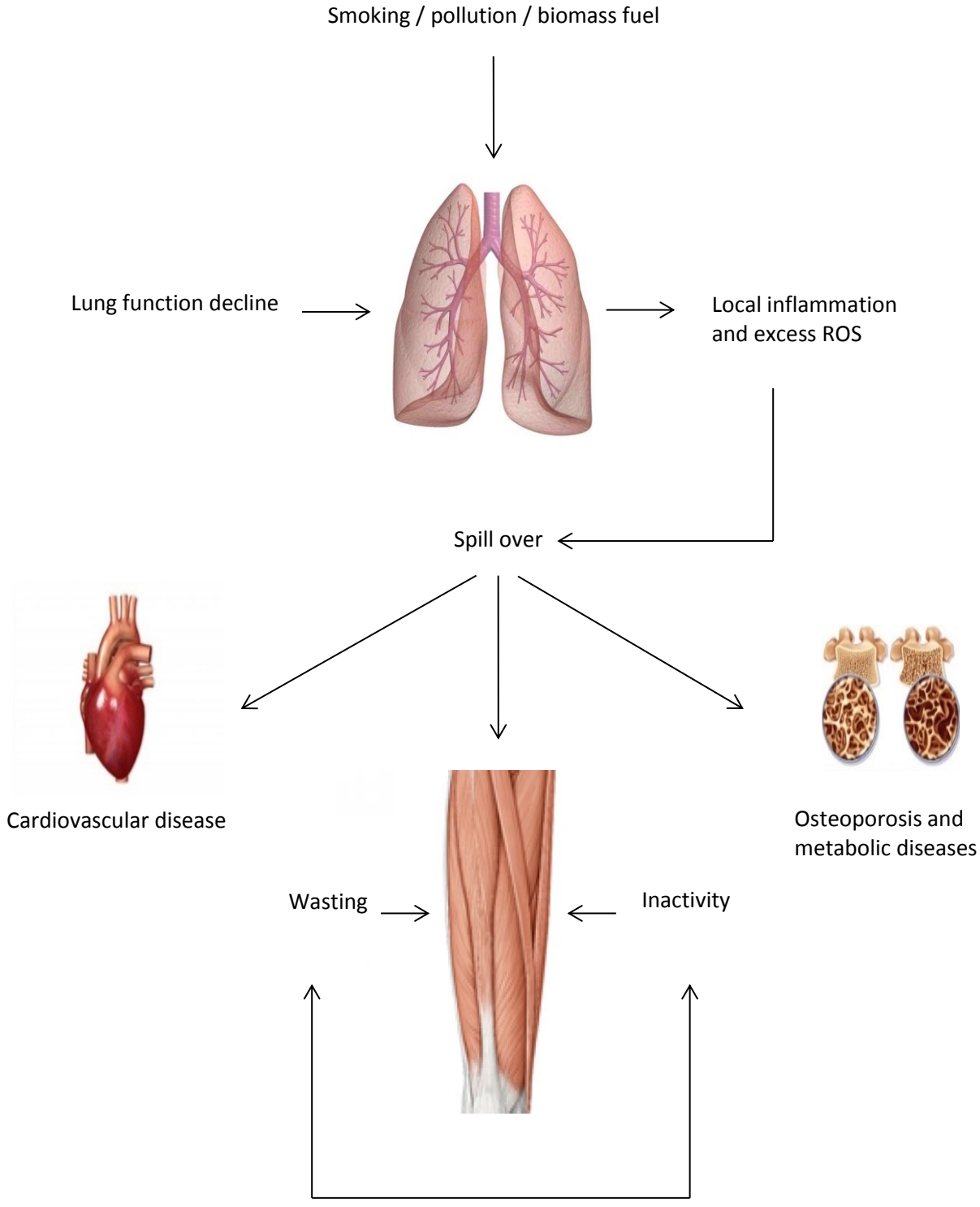



**Figure 4.1 -** Inhalation of noxious substances drives inflammation and ROS production - the spill over of which contributes to systemic features of COPD (adapted from Barnes & Celli 2013)

## 4.2 Methods

### 4.2.1 Study Population

Subjects with confirmed COPD and healthy controls were recruited from the royal Brompton hospitals Muscle and Airway Laboratory databases. Study subjects were recruited into two observational trials that ran parallel to each other. Both were ethically approved by the local research ethics committee; 11/LO/1852 "Oxidative stress and innate immune dysfunction as drivers of airway and lung remodelling in COPD: the MRC-ABPI;" 12/LO/0088 "Deep phenotyping of the quadriceps muscle in patients with COPD." Patients who gave informed consent were extensively phenotyped and went on to undertake paired endobronchial sampling at bronchoscopy and quadriceps vastus lateralis biopsy on the same day (methods as described in Chapter 2). According to ethical committee safety criteria those with an $FEV_1$ < 1 Litre did not undergo bronchoscopy so had a muscle biopsy only.

### 4.2.2 Study design and procedures

Subjects were recruited and stratified according to $FEV_1$ into 4 groups and were either ex-smokers or never smokers **(Table 4.1).** No current smokers were recruited into this study. They attended the laboratory for clinical assessment and relevant history details were obtained. Lung function was undertaken and any patients with a documented exacerbation or symptoms of a respiratory tract infection in the preceding month were excluded. Patients and controls then undertook an incremental maximal cycle-ergometer cardiopulmonary exercise test (CPET) with cardiorespiratory measurements, six-minute walk

test and quadriceps strength measurements. These phenotypic measures were conducted over two visits. Paired endobronchial sampling at bronchoscopy and quadriceps vastus lateralis biopsy were undertaken on a separate occasion on the same day within 1 month of phenotypic characterisation. According to ethics committee safety criteria those with an $FEV_1$ < 1 Litre did not undergo bronchoscopy so had a muscle biopsy only. The detailed methods for recruitment and characterisation are documented in Chapter 2.

### 4.2.3 Mitochondrial functional analysis and ROS evaluation

Intact mitochondria and cytoplasm were isolated using a Mitochondrial Isolation Kit for Tissue (Thermo-Scientific, Waltham) as described by the manufacturer as detailed in Chapter 2 (2.5.1).

Briefly, mitochondrial membrane potential ($\Delta\Psi m$) was measured in intact isolated mitochondria using the cationic dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Invitrogen). Intact mitochondria were incubated with 3µM JC-1 for 30 min at 37°C with 5% carbon dioxide. JC-1 monomers emit green fluorescence but when they enter live mitochondria they form J-aggregates which emit red fluorescence. An increase in the ratio between red and green fluorescence is an indicator of $\Delta\Psi m$.

Mitochondrial ROS activity was measured with MitoSOX$^{TM}$ Red (Invitrogen), a redox-sensitive fluorescent probe that is selectively targeted to the mitochondria. Intact isolated mitochondria were incubated with 5µM MitoSOX for 30min at 37°C and 5% carbon dioxide and red fluorescence was determined at 510/580nm using a fluorescence plate reader.

Whole cell ROS was detected in the cytoplasm by 2'-7'-dichlorofluorescin diacetate (DCF) staining (Sigma). DCF is a cell permeable non-fluorescent probe that when de-esterified by oxidation products turns into the highly fluorescent 2'-7'-dichlorofluorescin, which is detected according to manufacturer's guidelines.

The exact methods are as detailed in Chapter 2 (2.5)

### 4.2.4 Statistical analysis

Data is expressed as mean (SD). Kruskal-Wallis test for ANOVA was used for multiple comparisons of different groups. Differences in continuous variables were assessed using t tests and one-way analysis of variance with a Tukey post hoc test or the non-parametric equivalent. Correlations that follow were assessed by Pearson or Spearman's rank methods. Statistical analysis was performed with Graphpad Prism 5 software (GraphPad Software, San Diego, Calif). Significance is reported at $p<0.05$.

## 4.3 Results

### 4.3.1 Subject characteristics

Twenty-five subjects were recruited with technically acceptable data being obtained in all. 19 had endobronchial biopsies and vastus lateralis muscle biopsy. 6 subjects had vastus lateralis biopsy alone and these were from Group D – patients with GOLD Stage III and GOLD Stage IV COPD who did not meet ethics committee safety criteria for bronchoscopy. The mean age of the study subjects was not significantly different between the groups. There was no difference in lung function and exercise performance between the healthy never smokers (Group A) and ex-smokers with an $FEV_1$ >80% predicted (Group B). Patients with more significant airflow obstruction (Group D – GOLD III and GOLD IV) demonstrated poorer exercise performance and higher symptom burden than those with more minimal airflow obstruction (Group C – GOLD II) and controls (Groups A and B). Unsurprisingly the most striking differences were between $VO_2$ peak and six-minute walk distance; $p<0.01$ for both when comparing GOLD III and GOLD IV patients to controls and $p<0.05$ when compared to GOLD II patients. GOLD II patients also demonstrated a reduction in $VO_2$ Peak and six-minute walk distance when compared to controls, $p<0.01$ for both **(Table 4.1).**

### 4.3.2 Airway mitochondrial function and mitochondrial and cellular ROS levels

Patients with GOLD stage II COPD demonstrated a significant reduction in mitochondrial membrane potential when compared to control subjects ($p<0.01$) **(Figure 4.2 A)**. They also had significantly higher levels of mitochondrial reactive oxygen species when compared to "healthy" ex-smokers ($p<0.01$) and controls ($p<0.05$) **(Figure 4.2 B)**. When looking at the

overall cellular ROS burden it was significantly higher in the GOLD stage II patients when compared to the healthy control groups ($p<0.05$ for both) **(Figure 4.3)**. Western blot analysis demonstrated a reduction in the expression of mitochondrial complex proteins I, IV and V in the GOLD II patients compared to the two control groups **(Figure 4.4).**

### 4.3.3 Airway mitochondrial function and phenotypic associations

In considering all subjects together a correlation was seen between airway mitochondrial membrane potential and lung function parameters; specifically, for $FEV_1$ % predicted and membrane potential ($r=0.74$, $p<0.01$) and $TLCO_c$ % predicted and membrane potential ($r=0.64$, $p<0.01$) **(Figure 4.5)**. Mitochondrial membrane potential also correlated with phenotypic markers of exercise capability; specifically, for six-minute walk distance and membrane potential ($r=0.48$, $p<0.05$) and for peak $VO_2$ ($r=0.67$, $p<0.01$) **(Figure 4.6)**. No correlation was seen between mitochondrial membrane potential and quadriceps muscle strength ($r=0.13$, $p=0.57$) **(Figure 4.7)**.

### 4.3.4 Muscle mitochondrial function and mitochondrial and cellular ROS levels

There were no significant differences between mitochondrial membrane potential in the vastus lateralis muscle between all the study groups **(Figure 4.8 A)**. There were significantly higher mitochondrial ROS levels in the quadriceps muscle of GOLD II patients and GOLD III/IV patients when compared to the control groups ($p<0.01$) **(Figure 4.8 B)**. Whole cell ROS levels were also significantly higher in the quadriceps muscle of GOLD II and GOLD III/IV patients when compared to the control groups; specifically for GOLD II patients and "healthy" ex-smokers ($p<0.05$), for GOLD II patients and healthy never smokers ($p<0.01$). For GOLD III/IV patients compared to both control groups ($p<0.01$) **(Figure 4.9)**.

### 4.3.5 Muscle mitochondrial function and phenotypic associations

Overall there was no association between muscle mitochondrial function and quadriceps muscle strength and membrane potential ($r=0.05$, $p=0.81$) **(Figure 4.10)**. There was also no association between muscle mitochondrial function and lung function or exercise parameters; specifically for $FEV_1$ % predicted and membrane potential ($r=0.08$, $p=0.71$) and for $TLCO_c$ % predicted and membrane potential ($r=0.12$, $p=0.57$) **(Figure 4.11)**; for Six-minute walk distance and membrane potential ($r=0.03$, $p=0.90$) and for peak $VO_2$ ($r=0.03$, $p<0.89$) **(Figure 4.12)**. Higher power Transmission Electron Microscopy showed a difference in the muscle ultra-structure between a patient with advanced COPD and a healthy never smoker. It demonstrated changes in the architecture of the muscle fibres, a reduction in mitochondrial density and a higher proportion of auto-phagasomes in the COPD patient **(Figure 4.13).**

| Mean(SD) | Never Smoker N=5 Group A | Ex- smokers/ GOLD I N=8 Group B | GOLD II N=6 Group C | GOLD III & IV N=6 Group D |
|---|---|---|---|---|
| **Muscle Biopsy** | N=5 | N=8 | N=6 | N=6 |
| **Endobronchial Biopsy** | N=5 | N=8 | N=6 | N=0 |
| **Age (years)** | 61 (6) | 63 (5) | 64 (3) | 64 (7) |
| **Female: Male** | 1:4 | 3:5 | 4:2 | 1:5 |
| **PYH** | 0 (0) | 36 (16) | 34 (12) | 40 (6) |
| **$FEV_1$ (%Pred)** | 100 (9) | 103 (11) | 71 (5) * | 30 (11) **## |
| **TLCOc (%Pred)** | 90 (14) | 80 (10) | 67 (8) * | 38 (13) **## |
| **6MW (m)** | 657 (18) | 631 (55) | 493 (66) ** | 364 (65) **## |
| **VO2 Peak ml/kg/min** | 28 (3) | 25 (3) | 17 (3) ** | 12 (4) **# |
| **QMVC (absolute)** | 34 (6) | 41 (11) | 31 (8) | 32 (6) |
| **QMVC/BMI** | 1.5 (0.1) | 1.6 (0.4) | 1.1 (0.3) * | 1.4 (0.2) |
| **BMI** | 24 (5) | 25 (4) | 29 (7) | 23 (3) # |
| **SPPB** | 12 | 12 (11-12) | 11 (10-12) | 11 (9-12) |
| **CAT score** | 2 (0-6) | 8 (2-15) | 16 (4-23) | 24 (20-26) **# |

**Table 4.1 –** Subject Characteristics: Data shown as mean (SD) or median (range). $p<0.05$ from A/B *, $p<0.01$ from A/B **, $p<0.05$ from C #, $p<0.01$ from C##

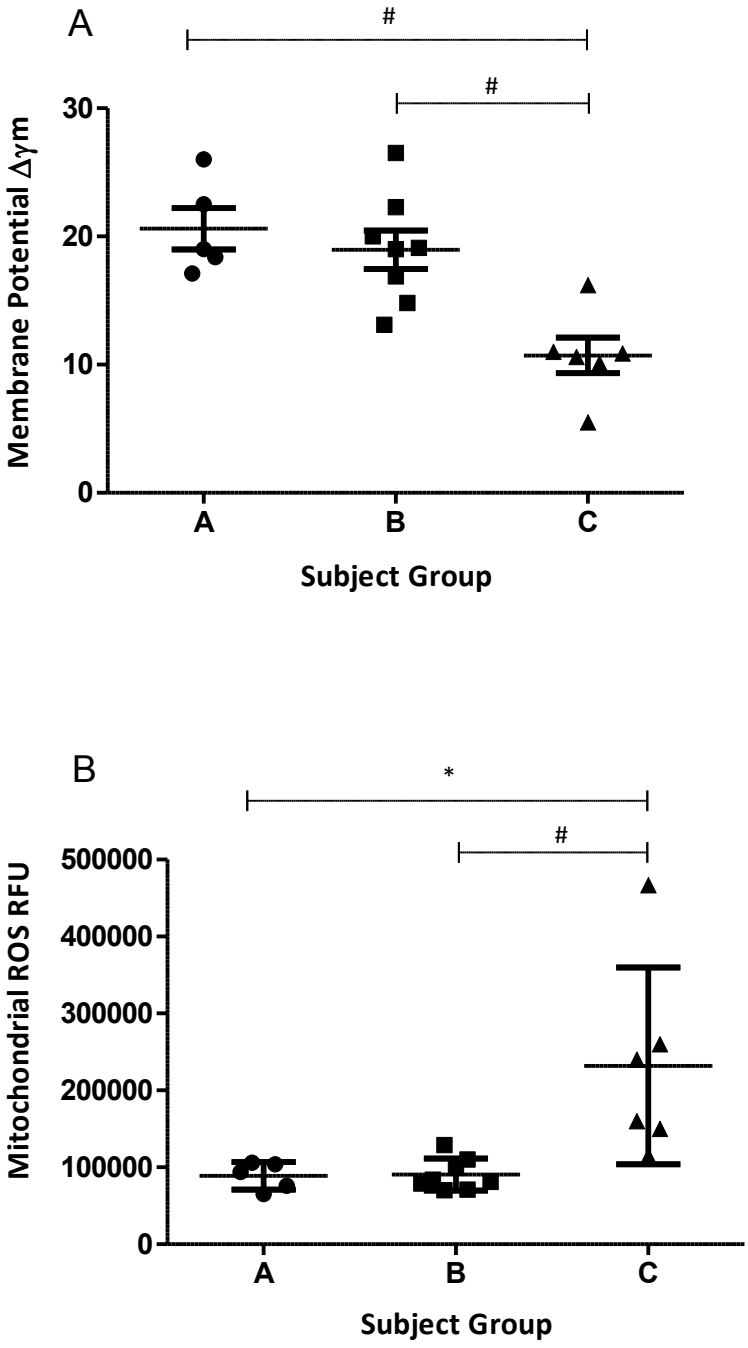



**Figure 4.2 – (A)** Airway mitochondrial membrane potential of the never smokers (A), Ex-smokers/GOLD I (B) and GOLD II patients (C). **(B)** Airway mitochondrial reactive oxygen species levels of the never smokers (A), Ex-smokers/GOLD I (B) and GOLD II patients (C). $p<0.05$*, $p<0.01$ #

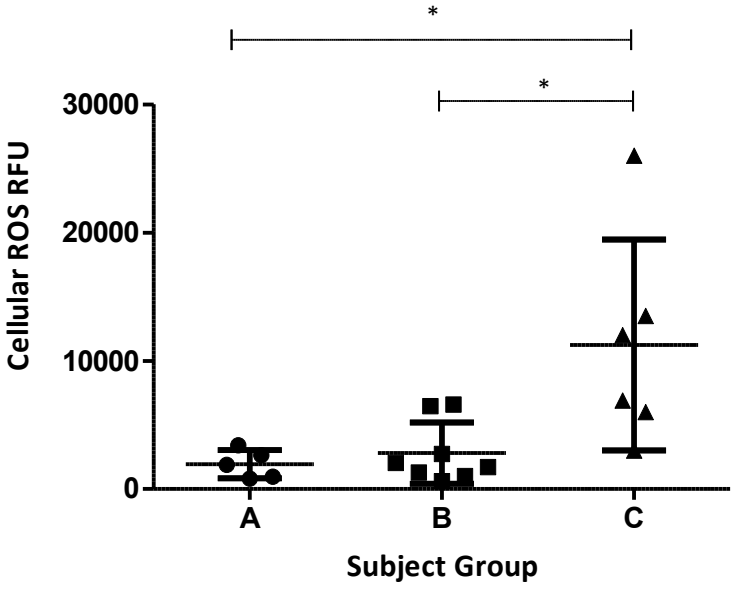



**Figure 4.3 –** Airway whole cell reactive oxygen species levels of the never smokers (A), Ex-smokers/GOLD I (B) and GOLD II patients (C). $p<0.05$*

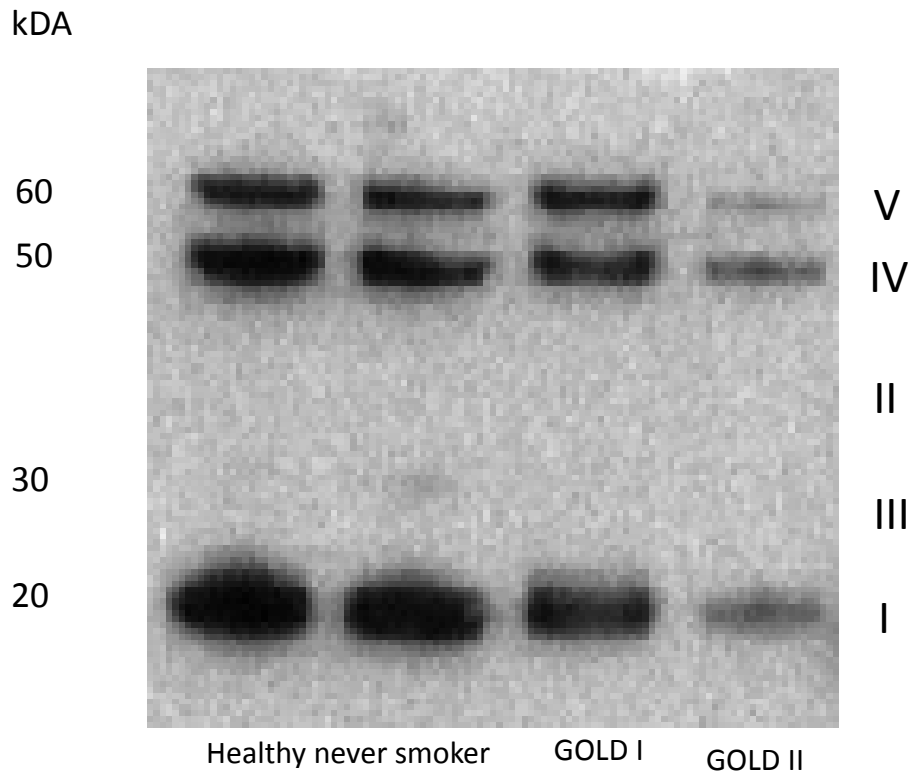



**Figure 4.4 –** Western blot analysis demonstrating a reduction in expression of mitochondrial complex proteins I, IV and V in the GOLD II patients.

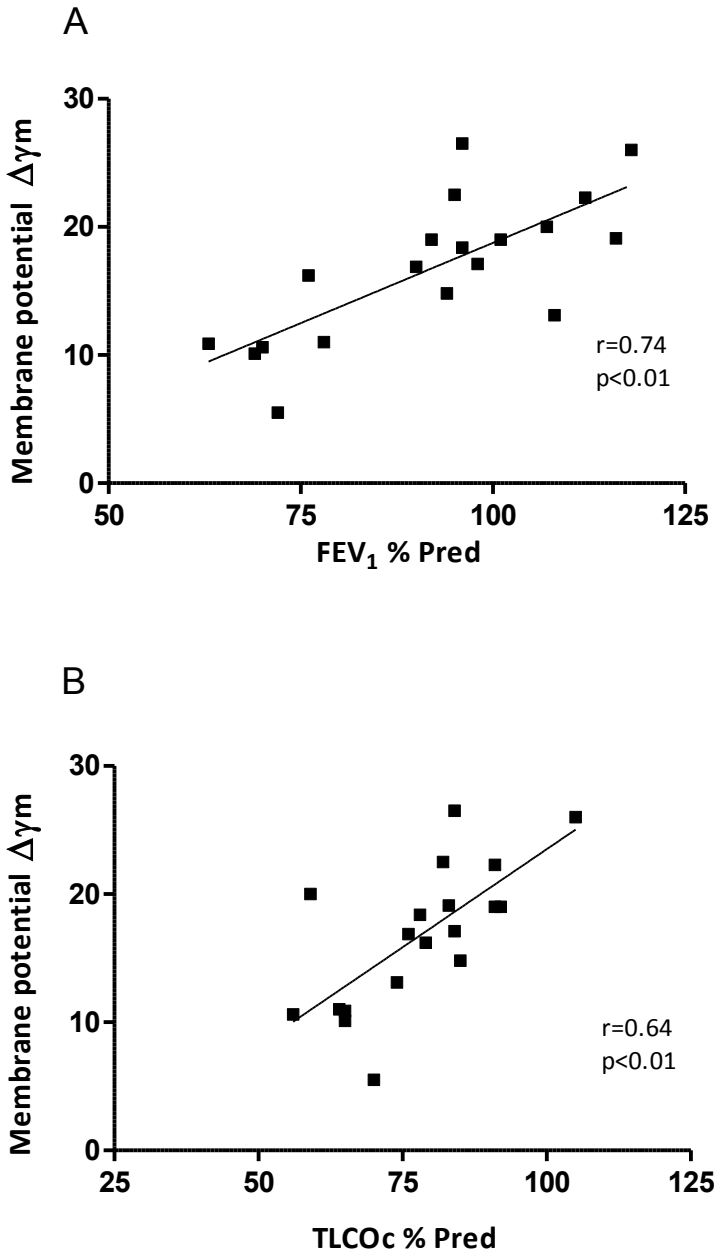



**Figure 4.5 – (A)** Correlation between airway mitochondrial membrane potential and % predicted $FEV_{1.}$ **(B)** Correlation between airway mitochondrial membrane potential and % predicted $TLCO_c$

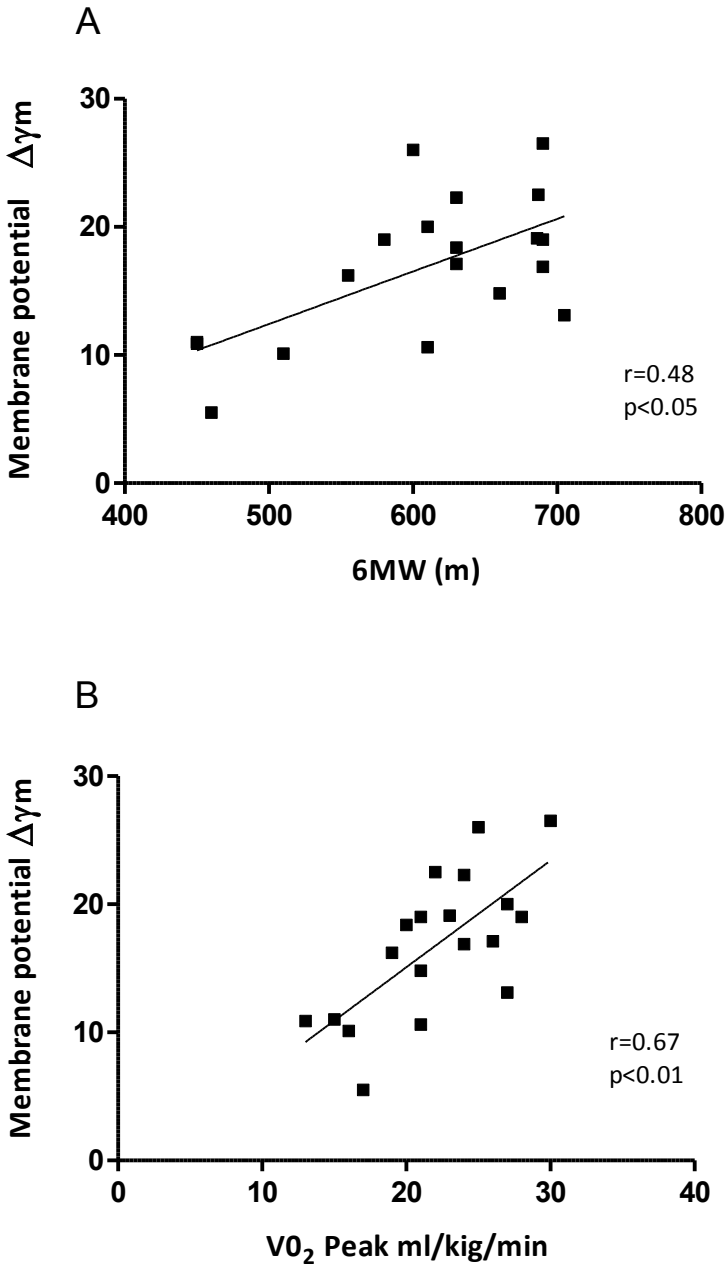



**Figure 4.6 – (A)** Correlation between airway mitochondrial membrane potential and six-minute walk distance (6MW) in metres. **(B)** Correlation between airway mitochondrial membrane potential and weight adjusted peak oxygen uptake ($VO_2$ peak ml/min/kg).

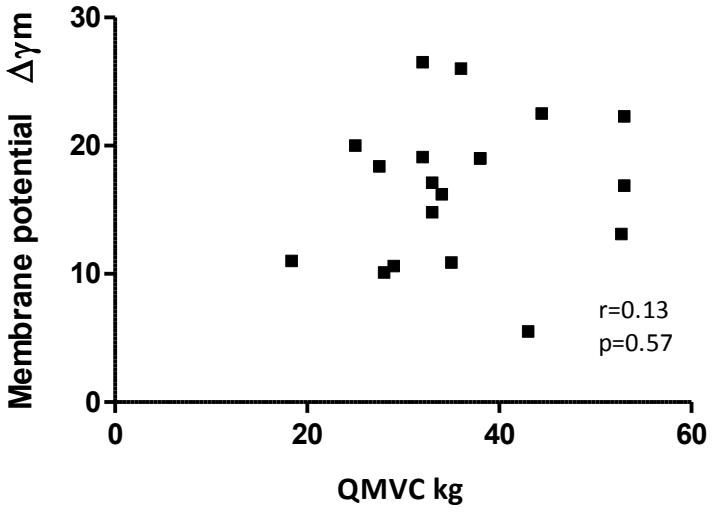



**Figure 4.7 –** No correlation is seen between airway mitochondrial membrane potential and quadriceps muscle strength (quadriceps maximal voluntary contraction – QMVC in Kg)

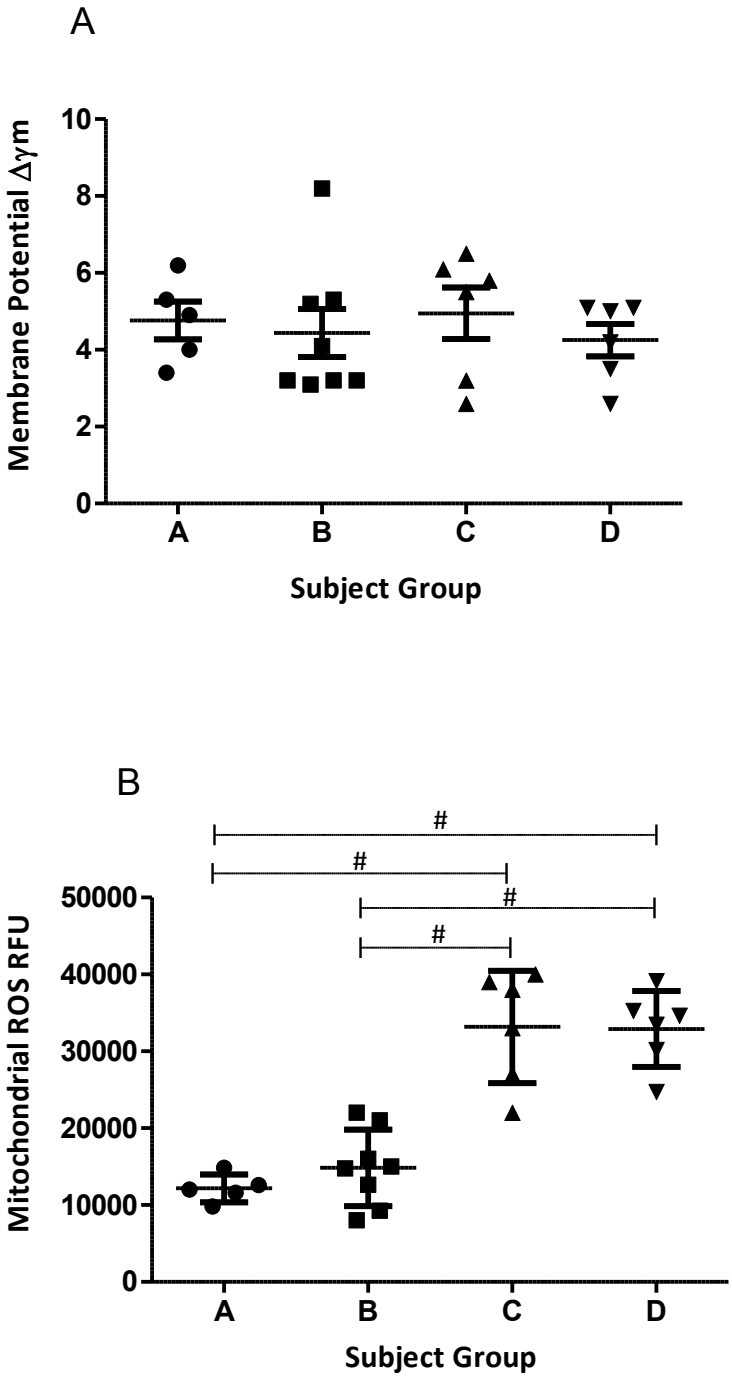



**Figure 4.8 – (A)** Quadriceps muscle mitochondrial membrane potential does not differ between the groups of never smokers (A), Ex-smokers/GOLD I (B) GOLD II (C) and GOLD III/IV patients. **(B)** Quadriceps muscle mitochondrial reactive oxygen species levels of the never smokers (A), Ex-smokers/GOLD I (B), GOLD II (C) and GOLDIII/IV patients (D). $p<0.01$ #

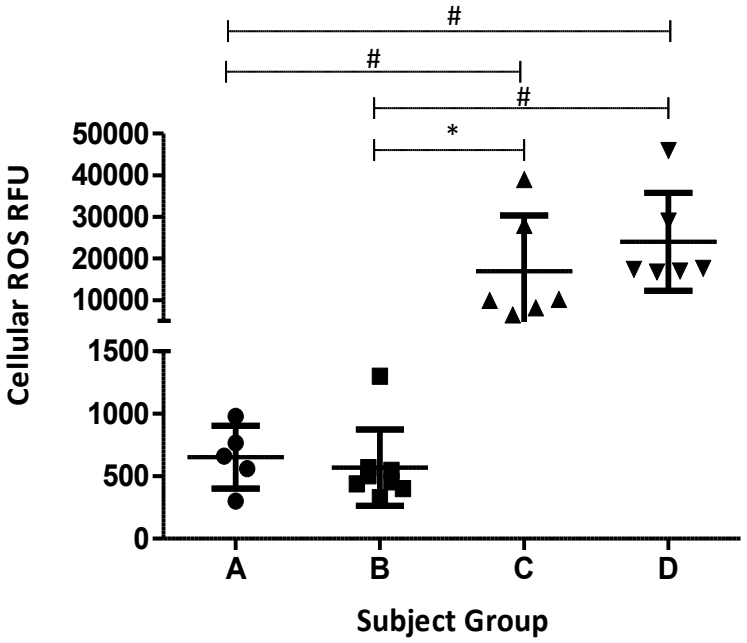
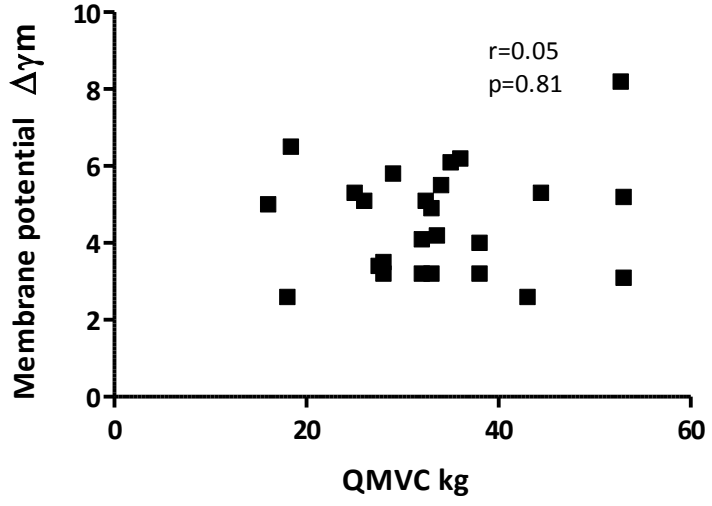
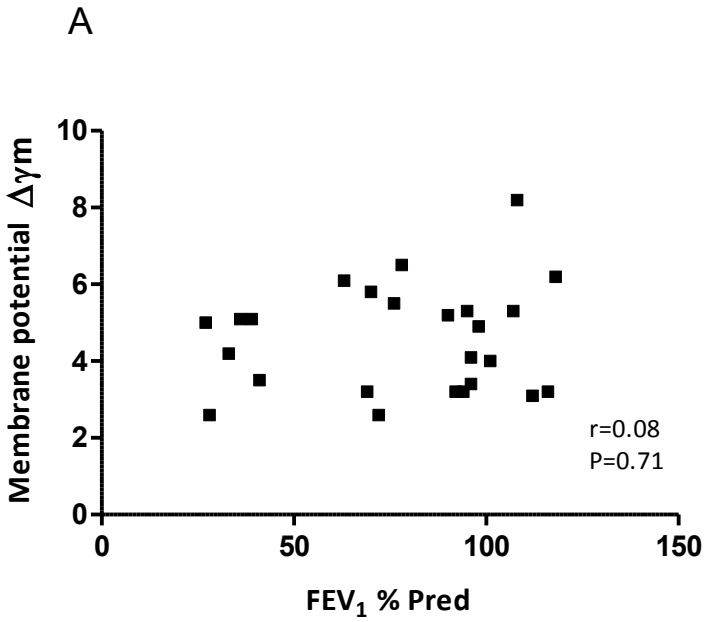



**Figure 4.9 –** Quadriceps muscle whole cell reactive oxygen species levels of the never smokers (A), Ex-smokers/GOLD I (B), GOLD II patients (C) and GOLD III/IV patients(D). $p<0.05$*$p<0.01$ #




(Amended)

**Figure 4.10 -** No correlation is seen between quadriceps muscle mitochondrial membrane potential and quadriceps muscle strength (quadriceps maximal voluntary contraction – QMVC in Kg.



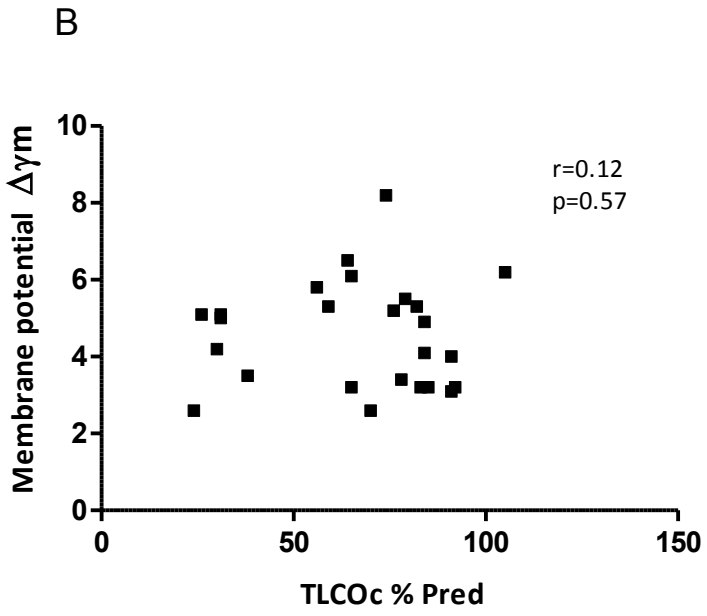
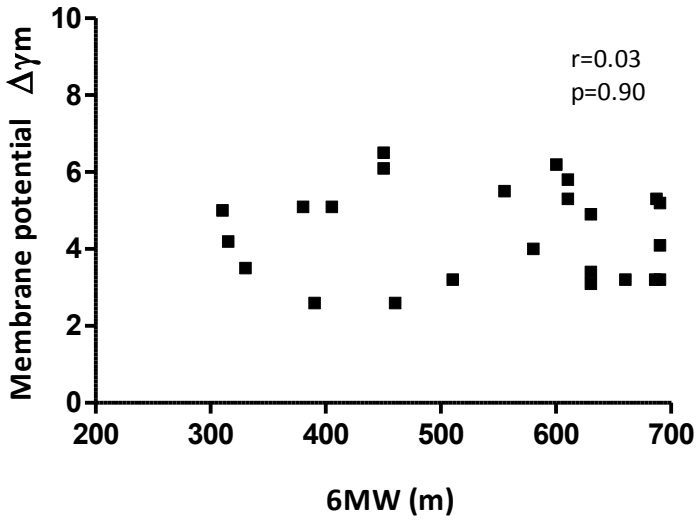




**Figure 4.11 -** No correlation is seen between quadriceps muscle mitochondrial membrane potential and % predicted $FEV_1$ **(A)** and % predicted $TLCO_{c.}$ **(B)**



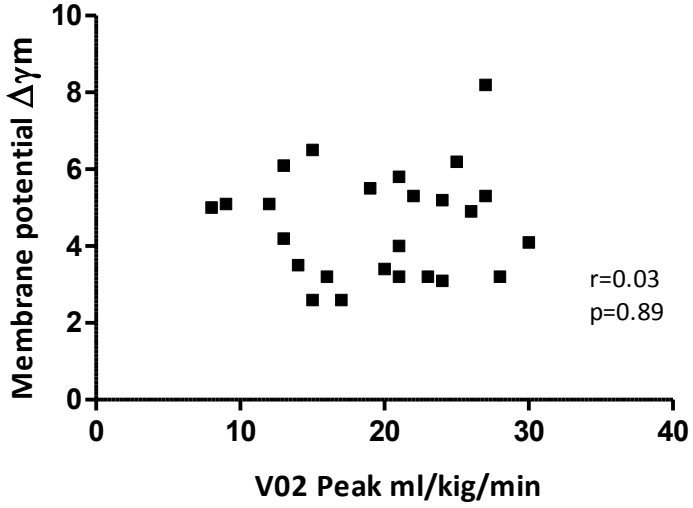




**Figure 4.12 -** No correlation is seen between quadriceps muscle mitochondrial membrane potential and six-minute walk distance (6MW) in metres **(A)** and weight adjusted peak oxygen uptake ($VO_2$ peak ml/min/kg) (**B).**

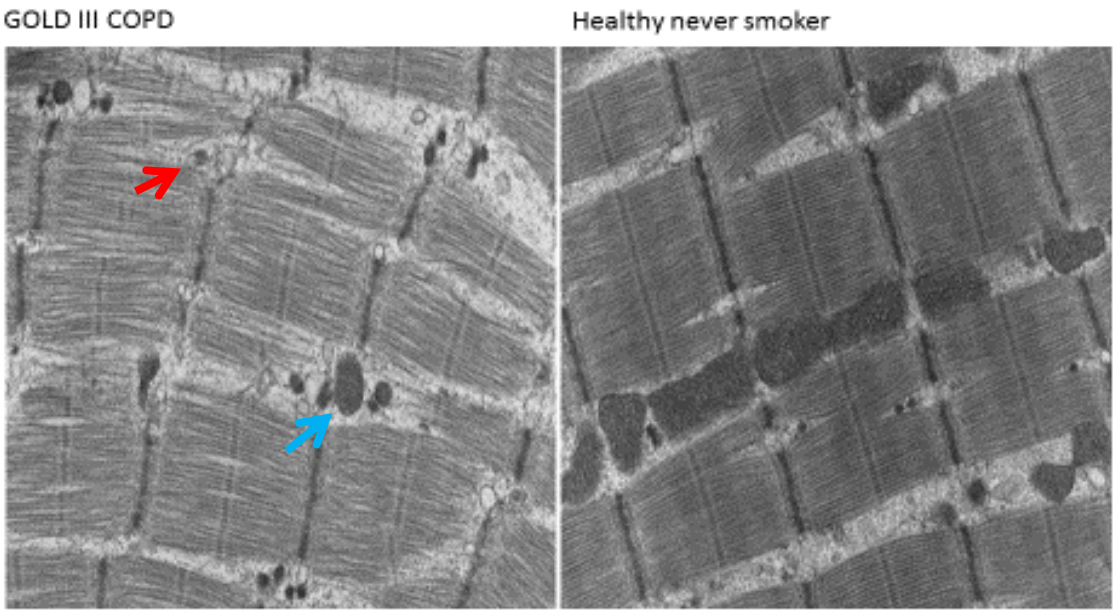



**Figure 4.13** - High Power Transmission Electron Microscopy images of human vastus lateralis muscle at 10000 x magnification show a reduction in number and distribution of mitochondria (blue arrow) and an increase in the number of auto-phagasomes (red arrow) in the COPD patient.

## 4.4 Discussion

### 4.4.1 Summary of results

In airway tissue I observed a more favourable mitochondrial membrane potential in the control population and "healthy" ex-smoker's and this was significantly higher than patients with moderate COPD (GOLD II). This effect is independent of smoking history, given that all study groups were ex-smokers with a similar pack year smoking history. I have also been able to show that mitochondrial and cellular ROS levels are significantly higher in patients with moderate COPD than the control group who have never smoked and the "healthy" ex-smoking group. I have been able to demonstrate that these changes may be modulated by electron transport chain complex protein activity **(Figure 4.4)**. The findings that the airway mitochondrial profile correlated with markers of disease severity such as lung function and exercise performance suggest that mitochondrial dysfunction conveys a less favourable overall clinical phenotype.

In skeletal muscle, patients with COPD exhibited higher levels of mitochondrial and cellular ROS than control subjects and "healthy" ex-smokers. There was no difference in mitochondrial membrane potential observed between any of the studied groups. No correlation between the ROS profile of muscle and clinical phenotype was noted and given there was no difference between the mitochondrial membrane potential of the groups, as expected no correlation was seen with clinical phenotype. Data from one of the COPD patients was obtained from high power transmission electron microscopy and compared to one of the control subjects. This suggests that changes may be due to differences in mitochondrial density rather than overall mitochondrial function.

### 4.4.2 Critique of methods

Although I have been able to demonstrate that mitochondrial membrane potential as a functional marker in airway tissue is negatively affected in COPD, I have not been able to correlate this with an actual outcome marker such as reduced ATP production. The reason for this is the small extractable quantity of mitochondria from tissue isolation of fresh endo-bronchial biopsies. While a criticism this must be considered in the context that for the first time this study investigated mitochondrial profile in airway tissue that is independent of cell culture. Previous work (Hoffman, Zarrintan et al. 2013) has evaluated the role of mitochondrial structure and function in airway epithelial cells that were obtained from COPD cell culture lines. Culture has involved the use of bronchial epithelium growth medium, fibronectin and collagen – the mitochondrial functional effects of which are unknown. When considering the airway phenotype and noting the association with markers of disease severity it may have been worthwhile determining if these changes mirrored that which is occurring within alveolar tissue through the study of alveolar macrophages. That being said I do not think that it was necessary but may in the future if we are to consider mitochondrial targeted therapies in COPD based on airway (bronchitic) or alveolar (emphysematous) profiles.

When considering the features in skeletal muscle the initial hypothesis of this study must be considered. In it I stated that if oxidative stress arises primarily in the pulmonary system as a result of mitochondrial dysfunction, and spills over to cause non pulmonary manifestations (in this case skeletal muscle dysfunction) then greater evidence of mitochondrial dysfunction will be present in the airways. The finding that mitochondrial function in the muscle is unchanged across all the studied groups means that this hypothesis

has not been proven, and it suggests that there are other mechanisms at the level of the muscle which have not been studied here. A possible explanation for this is the skeletal muscle fibre shift of mitochondria enriched type I fibres to relatively mitochondria deplete type II fibres (Natanek, Gosker et al. 2013). What has not been examined in this work is how the overall muscle fibre type proportion relates to these findings and this is a clear weakness and was in part due to the majority of muscle tissue being needed for mitochondrial study. Self-evidently no conclusive suppositions can be made or proven from electron microscopy data comparing the muscle ultra-structure of one COPD patient to one healthy control, although I hope the images are of interest.

The finding that ROS levels are generally higher in airway and muscle compartments in those with disease compared to controls is not a new finding. The study of both tissue types in the same patient is novel, but again in reference to the hypothesis - quantifying a difference between ROS levels across the two compartments (airway and skeletal muscle) is not possible based on these results. In the absence of true muscle mitochondrial dysfunction in COPD patient's the mitochondrial and cellular ROS levels are still elevated to a comparable degree as that which is found within airway tissue possibly disproving the hypothesis. However without the simultaneous study of circulating ROS levels we are unable to prove or disprove the spill over theory - the collection of peripheral blood mononuclear cells that were extracted from blood samples in this cohort are yet to be studied but will hopefully shed further light on this question. As it stands these findings point to COPD being a more generalised oxidative state and may add weight to the theory that COPD is a systemic inflammatory process in which the pulmonary and muscle systems are two of the affected organs.

### 4.4.3 Significance of findings

Although the hypothesis remains un-answered, the finding that mitochondrial ROS levels are elevated in the airway tissue of COPD patients, and this is associated with a reduction in mitochondrial membrane potential, and a poorer clinical phenotype does raise the question regarding mitochondria targeted antioxidant therapy in COPD being of greater benefit than non-specific cellular antioxidants such a N acetyl cysteine which have been trialled previously (Li, Wiegman et al. 2013). The overall association between mitochondria and inflammation could potentially be another avenue of study and in particular the association with airway TGF-β which has previously been shown to regulate superoxide dismutase (Michaeloudes, Sukhar et al. 2011) a potent antioxidant within the mitochondria but more importantly as an inducer of IL-6 release which triggers airway smooth muscle proliferation through inflammation (Michaeloudes, Sukhar et al. 2011).

The findings in skeletal muscle are far better characterised within the literature and provide an explanation for the results presented in this chapter – they centre on skeletal muscle atrophy and fibre type proportion. An early study demonstrated that forty per cent of patients with COPD had limitations due to leg fatigue before breathlessness stopped or limited exercise performance (Killian, Leblanc et at. 1992). This finding raised the question of early onset anaerobic metabolism. Since then a study in 2010 by Seymour and colleagues was able to show that one third of COPD patient's exhibit skeletal muscle weakness and this is independently associated with a poorer clinical phenotype (Seymour, Spruit et al. 2010). When considering follow up work these changes can be explained by muscle fibre shift and proportional atrophy, with a lower number of mitochondria enriched type I (endurance) fibres and a greater proportion of type II mitochondria deplete fibres albeit of reduced

density (Natanek, Gosker et al. 2013). The demonstration of definitive mitochondrial density change and increased ROS levels in skeletal muscle has been reported (Meyer, Zoll et al. 2013) in line with the reduced proportion of type I fibres that Natanek and colleagues demonstrated (Natanek, Gosker et al. 2013). The functional significance of this is early lactic acidosis and fatigue which contributes to greater inactivity which in turn perpetuates a poor skeletal muscle phenotype (Maltais, Simard et al. 1996), and which can be attenuated if non-invasive ventilation is used to reduce lactataemia (Polkey, Hawkins et al. 200). The excessive mitochondrial ROS production in skeletal muscle in COPD patients that has been presented in this chapter is in keeping with previous work in this field by Peunte-Maestu and colleagues (2012). They studied skeletal muscle in both the resting and exercise states and showed that exercise stimulates mitochondrial ROS production which is unsurprising given that greater metabolic demand is placed on the mitochondrial respiratory chain for ATP production. The pertinent results as applicable to the findings within this chapter are the analysis of mitochondrial function and mitochondrial ROS in skeletal muscle, obtained by biopsy, in the rested state. They were able to show a threefold increase in mitochondrial ROS in biopsy samples from COPD patients when compared to controls. Other data which was less well reported but is of significant interest was the finding that mitochondrial oxygen consumption of individual mitochondria between the control group and COPD group was not significantly different (Peunte-Maste, Tejedar et al. 2011). This this corroborates the findings presented here that actual mitochondrial function as evidenced by membrane potential is no difference in COPD patients compared to controls.

### 4.4.4 Conclusion

This is the first study in COPD that has investigated airway and muscle biology in the same cohort of patients but the overall hypothesis of this study evaluating the spill over theory has unfortunately not been answered, although it was not clearly supported by this data. The skeletal muscle changes I observed, in the form of elevated mitochondrial and cellular ROS levels in patients with COPD when compared to controls, have been described previously in more detailed studies and suggest that muscle fibre changes are a key regulator of mitochondrial bio-energetics due to changes in density but that mitochondria are intrinsically normal. The findings that airway mitochondrial function is impaired in patients with COPD and is associated with an elevated oxidative stress burden are novel and have not previously been described. The association with a poorer clinical phenotype suggest that mitochondria targeted therapies in particular mitochondria targeted anti-oxidants may be of benefit in COPD. Work is needed with appropriate animal models to determine if there is any role for mitochondrial agents in the treatment of COPD.

# Chapter 5: Mitochondrial dysfunction in a murine model of COPD

## 5.1 Introduction and Aim

COPD is characterized by chronic inflammation and the development of airflow limitation due to airway remodelling and destruction of lung tissue (Vestbo, Hurd et al 2013). Tobacco smoke and ozone exposure induce local inflammation and enhance oxidative stress in the lung affecting the oxidant-antioxidant balance, which is thought to be one of the major driving mechanisms in the development of COPD (Chung and Adcock 2008). The subsequent airway wall remodelling which involves aberrant tissue repair, epithelial metaplasia and sub mucosal gland hypertrophy leads to an increase in airway smooth muscle mass and contributes to the airflow obstruction that is characteristic of the condition (Chung 2015).

Production of reactive oxygen species (ROS) in the mitochondria occurs as a by-product of oxidative phosphorylation at the mitochondrial electron transport chain (Mailloux, McBride et al. 2013). Mitochondria function to produce adenosine triphosphate, the energy currency of cells. They are involved in calcium metabolism which plays an important role in muscle contraction and also apoptosis (Camougrand and Rigoulet 2001). Mitochondria can sense danger signals and induce inflammation by activating and controlling the innate immune system (Mills and O'Neill 2014). The effects of ROS on mitochondrial DNA repair are another important factor given that this negatively impacts protein synthesis within cytoplasmic ribosomes. Without the regeneration of these proteins that are transported into the mitochondria, and regulated by membrane potential, the complexes of the electron transport chain cannot re-form after they have been damaged (Osiewacz HD and Bernhardt

D 2013). The clearance of ROS is thus important to allow adequate mitochondrial regeneration. Normally, ROS are cleared by the actions of several antioxidants found within the cytoplasm and mitochondria themselves - such as glutathione, and enzymes including catalases, peroxidases and superoxide dismutase (SOD) (Rahman I and MacNee W 2012). When the antioxidants present fail to up regulate sufficiently, for example in response to oxidative stress from cigarette smoking or ozone exposure, increased levels of ROS accumulate and can negatively influence redox sensitive cellular processes most importantly the transfer of electrons within the electron transport chain. The subsequent reduction in ATP synthesis down regulates energy dependant cellular function including cell cycle progression from G1-S phase which is governed by mitochondrial bio-energetics, the result of which is programmed cell death (Osiewacz and Bernhardt 2013). Maintaining the integrity of the mitochondria is paramount in the neutralisation and clearance of ROS.

It has been postulated that the relatively low efficacy of cellular antioxidants such has N-acetyl cysteine is as a consequence of their low penetrance to mitochondria and to overcome these limitations mitochondria targeted antioxidants have been developed to accumulate at therapeutically effective concentrations within mitochondria (Smith, Porteous et al. 2003). One such molecule is MitoQ which was designed in the 1990's by Michael P Murphy and Robin J Smith. The antioxidant component is ubiquinone (an antioxidant of the mitochondrial electron transport chain) and it is covalently linked to a lipophilic cation, triphenylphosphonium (dTPP) which allows extensive accumulation within the mitochondria in vivo (Murphy and Smith 2007).

The growing interest into the efficacy of these mitochondria targeted antioxidants as a treatment for disorders partially driven by oxidative stress, such as chronic kidney disease,

Parkinson's disease and Hepatitis C induced liver dysfunction, has led to animal and human studies some of which have been encouraging. Using an animal model in 2011 Ishikawa and colleagues tested the effect of ubiquinone alone in hemi-nephrectomised rats. Rats that were fed with a ubiquinone supplemented diet showed lower levels of ROS and better renal function (Ishikawa, Kawarazaki et al. 2011). The use of MitoQ in a murine model of kidney disease has been shown to abate the detrimental effects of oxidative stress and restore mitochondrial membrane potential and ATP production (Xiao, Xu et al. 2017). Ubiquinone alone has also been studied in murine models of Parkinson's disease where mitochondrial dysfunction and in particular mitochondrial DNA damage is thought to be a major factor. Encouraging results in mice did not translate to humans; a placebo-controlled study looking at 40mg and 80mg of oral MitoQ did not find any significant difference in time to clinical worsening of symptoms over a twelve month period; it did importantly demonstrate a good safety and tolerability profile (Snow, Rolfe et al. 2010). The second human trial to date investigating oral MitoQ has been in patients with hepatitis C induced liver damage. It found that 40mg and 80mg doses reduced transaminase levels over a 28 day treatment period when compared to placebo; this effect was independent of changes in viral load and was thought to be mediated by oxidative stress (Gane, Weilert et al. 2010).

In chapter 4, I showed that mitochondrial membrane potential is reduced in patients with COPD while mitochondrial ROS levels and cellular ROS levels were elevated in these. Inevitably COPD patients had poorer physical performance than controls but the phenotypic associations raise the hypothesis that mitochondrial targeted therapy may be of benefit in COPD. The aim of this chapter is to study mitochondrial function in a murine model of COPD induced by repeated exposures to the gaseous oxidant, ozone (Triantataphyllopoulos,

Hussain et al. 2011). Ozone is an environmental pollutant that is associated with worsening symptoms in patients with asthma and COPD (Eiswerth, Douglass Shaw, et al. 2005). No previous studies have reported alterations in mitochondrial function in ozone murine models. Previous work from Professor Chung's group showed that using the ozone model mice develop airway inflammation, airway hyper-responsiveness and alveolar destruction with emphysema similar to that seen in patients with COPD (Li, Wiegman et al. 2013) (Triantataphyllopoulos, Hussain et al. 2011). I first examined whether mitochondrial dysfunction was present in this model, and if so wished to study the role of mitochondrial dysfunction underlying the various COPD features described above and importantly test the effects of the mitochondrial targeted antioxidant MitoQ.

## 5.2 Methods

### 5.2.1 Murine studies

The murine study presented in this chapter was performed within the legal framework of the United Kingdom under a project license granted by the British Home Office, United Kingdom under the Animals (Scientific Procedures) Act 1986. This section details the Ozone model of mitochondrial dysfunction and the concurrent use of the mitochondrial targeted antioxidant MitoQ.

### 5.2.2 The ozone model of mitochondrial dysfunction

Pathogen free male C57BL/6 mice (6 weeks old; Harlan, Wyton, United Kingdom) were housed within maximiser filter topped cages (Maximiser, Theseus caging system, Hazleton, USA) in the Biosciences facilities of Imperial College, under controlled temperature at twenty degrees Celsius with air humidity of 40-60%. They were maintained comfortably in a twelve-hour light and twelve-hour dark daily cycle with food and water supplied ad libitum. The protocols used in the study were approved by the Animal Ethics Committee of Imperial College and were fully compliant with the United Kingdom Home Office regulations stipulated within the project license. Mice were exposed to ozone (model 500 Ozoniser, Sander, Wuppertal, Germany) mixed with air (3 parts per million ozone concentration) for three hours and ozone concentration was continuously monitored using an ozone probe (ATi Technologies, Ashton-Under-Lyne, UK). Ozone exposure was undertaken twice per week (every third day) for one to six weeks. Two groups of control mice were exposed to air over the same period. In a concurrent model the effects of MitoQ were tested by

administering MitoQ at 5mg/kg intraperitoneally or the control dTPP alone twice per week (every third day) to both air and ozone exposed mice.

### 5.2.3 Murine measures of airway hyper-responsiveness

After the last exposure to ozone mice were anaesthetised and tracheotomized and placed into a whole-body plethysmography box (eSpira forced manoeuvres system, EMMS, Hants, United Kingdom). Pulmonary airway resistance ($R_L$) was calculated from derived measures of volume (whole-body plethysmography values) and transpulmonary pressure (assessed via an oesophageal catheter, EMMS, Hants, United Kingdom). Mice were ventilated at a standard frequency every minute with a tidal volume of 250µl. $R_L$ was recorded for three minutes after increasing concentrations of aerosolised acetylcholine (4-256 mg/ml) (Sigma, United Kingdom) were administered. $R_L$ was expressed as a percentage change from baseline measures and the concentration of acetylcholine concentration required to increase $R_L$ by 100% from baseline was calculated ($PC_{100}$). This value was logarithmically transformed (log $PC_{100}$) given the overall lung capacity of the mouse is small at 1ml. Log $PC_{100}$ was taken as a measure of airway hyperresponsiveness to acetylcholine (Li, Wiegman et al. 2013).

### 5.2.4 Bronchoalveolar lavage and collection of lung and muscle tissue from mice

Following terminal anaesthesia with phenobarbitone twenty-four hours after the last exposure to ozone, mice were lavaged with two millilitres of phosphate buffered saline via a one-millimetre diameter endotracheal tube. The retrieved bronchoalveolar lavage aliquots were pooled and centrifuged at four degrees Celsius at a speed of 1500 rpm for ten minutes

and the supernatant was collected and stored at -80 degrees Celsius and was later used for the purpose of cell counts and IL-6 and GM-CSF levels as detailed in the next section. For the purpose of this study sections of the right lung and right vastus lateralis muscle were then obtained and stored in phosphate buffered saline on ice for immediate extraction and subsequent study of mitochondria and cytoplasm as detailed later in this chapter **(section 2.5)**.

### 5.2.5 Total and differential cell counts and IL-6 and GM-CSF levels

The cell pellets that were obtained and frozen from lavage fluid were resuspended in 500 µl of phosphate buffered saline and kept on ice. Total and differential cell counts were obtained from cytospin preparations stained by Diff-Quick method (Gentaur, Kampenhout, Belgium) and measured according to manufacturer guidelines. Diluted cell suspensions of 20 µl of sample and 20 µl of cell specific stains were loaded onto a standard haemocytometer. At least 500 cells were counted per mouse and identified as macrophages, neutrophils, lymphocytes and eosinophils according to standard morphology and staining under x400 magnification. IL-6 and GM-CSF levels in BAL fluid were measured with commercial ELISA kits (R&D Systems Europe Ltd, Abingdon, United Kingdom).

### 5.2.6 **Mitochondrial and cytoplasmic analysis including ROS detection**

From the murine lung and muscle tissue obtained intact mitochondria and cytoplasm were isolated using a Mitochondrial Isolation Kit for Tissue (Thermo-Scientific, Waltham) as described by the manufacturer. Mitochondrial membrane potential ($\Delta\Psi m$) was measured in using the cationic dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine

iodide (JC-1, Invitrogen). Mitochondria were incubated with 3µM JC-1 for 30min at 37°C and 5% $CO_2$. JC-1 monomers emit green fluorescence but when they enter live mitochondria they form J-aggregates which emit red fluorescence. An increase in the ratio between red and green fluorescence is an indicator of $\Delta\Psi$m.

Mitochondrial ROS activity was measured with MitoSOX$^{TM}$ Red (Invitrogen), a redox-sensitive fluorescent probe that is selectively targeted to the mitochondria. Intact isolated mitochondria were incubated with 5µM MitoSOX for 30min at 37°C and 5% $CO_2$ and red fluorescence was determined at 510/580nm using a fluorescence plate reader or by flow cytometry.

Adenosine triphosphate (ATP) levels were quantified with a bioluminescence ATP determination assay (Molecular probes, Eugene, Ore).

Whole cell ROS was detected in the cytoplasm by 2'-7'-dichlorofluorescin diacetate (DCF) staining (Sigma). DCF is a cell permeable non-fluorescent probe that when de-esterified by oxidation products turns into the highly fluorescent 2'-7'-dichlorofluorescin, which is detected according to manufacturer's guidelines.

### 5.2.7 Statistical analysis

Data are expressed as mean ± Standard Deviation. The Kruskal-Wallis test for ANOVA was used for multiple comparisons of different groups. The Mann-Whitney test was subsequently used when results on the Kruskal-Wallis test were significant. Power calculations determined that 6 mice per group have 80% power to detect significant difference in lung parameters. Statistical analysis was performed with Graphpad Prism 5 software (GraphPad Software, San Diego, Calif). Significance is reported at $p<0.05$.

## 5.3 Results

### 5.3.1 Ozone induced lung inflammation in mice

Ozone exposed mice showed marked increases in total BAL cell counts compared with those in air exposed control mice after 1 and 6-weeks **(Figure 5.1).** They also demonstrated an increase in specific cell type subsets. Ozone exposed mice had considerably higher cell counts for neutrophils and macrophages when compared to air exposed mice **(Figure 5.2 A and B),** this was also true for both lymphocyte and eosinophil cell types **(Figure 5.3 A and B).** 1 and 6-week ozone exposure also induced an increase in the pro-inflammatory cytokine GM-CSF **(Figure 5.4).** 1-week ozone exposed mice also had significantly higher levels of IL-6 than the air exposed control group, but the difference between IL-6 levels in the 6-week ozone exposure group was not significantly different when compared to the air exposed group **(Figure 5.5)**.

### 5.3.2 Mitochondrial function is impaired in the lungs of ozone exposed mice

Mitochondrial membrane potential measured in mitochondria isolated from mouse lung tissue was reduced in the 1 and 6- week ozone exposed mice **(Figure 5.6).** ATP production was also reduced in both the 1 and 6-week ozone exposed mice when compared to the air exposed control groups **(Figure 5.7).**

### 5.3.3 Effects of MitoQ on ozone induced inflammation, mitochondrial membrane potential, mitochondrial and cellular ROS levels in mouse lung tissue

MitoQ treatment resulted in a reduction in ozone induced total BAL cell counts compared with dTPP alone **(Figure 5.8).** This was associated with a restoration in the mitochondrial membrane potential **(Figure 5.9)**, a reduction in ROS levels in isolated mitochondria **(Figure 5.10)**, and a reduction in total cellular ROS levels **(Figure 5.11)**.

### 5.3.4 Effects of MitoQ on mitochondrial membrane potential, mitochondrial and cellular ROS levels in mice muscle tissue

MitoQ treatment had no effect on the mitochondrial membrane potential in ozone exposed mice muscle tissue compared with dTPP alone and there was no difference in membrane potential between the ozone and air exposed mice **(Figure 5.12).** MitoQ treatment had no effect on mitochondrial ROS levels in ozone exposed mice muscle tissue compared with dTPP alone. A difference was observed in mitochondrial ROS levels between the Air/MitoQ and the Ozone/dTPP group **(Figure 5.13).** MitoQ treatment had no effect on cellular ROS levels in ozone exposed mice muscle tissue compared with dTPP alone and no discernible difference in cellular ROS levels was found between the air and ozone groups **(Figure 5.14).**

### 5.3.5 The functional significance of MitoQ - ozone induced airway hyper-responsiveness

Pulmonary airway resistance ($R_L$) was calculated from derived measures of volume (whole-body plethysmography values) and transpulmonary pressure (assessed via an oesophageal catheter, EMMS, Hants, United Kingdom). Mice were ventilated at a standard frequency

every minute with a tidal volume of 250µl. $R_L$ was recorded for three minutes after increasing concentrations of aerosolised acetylcholine (4-256 mg/ml) (Sigma, United Kingdom) were administered. $R_L$ was expressed as a percentage change from baseline measures and the concentration of acetylcholine concentration required to increase $R_L$ by 100% from baseline was calculated ($PC_{100}$). This value was logarithmically transformed (log $PC_{100}$) given the overall lung capacity of the mouse is small at 1ml. Log $PC_{100}$ was taken as a measure of airway hyperresponsiveness to acetylcholine (Li, Wiegman et al. 2013). Airway hyperresponsiveness (AHR) to acetylcholine was greater in the ozone exposed mice at 1 and 6 weeks compared to air groups **(Figure 5.15)**. Treatment with MitoQ resulted in a significant improvement in AHR compared to control treated mice **(Figure 5.16)**.

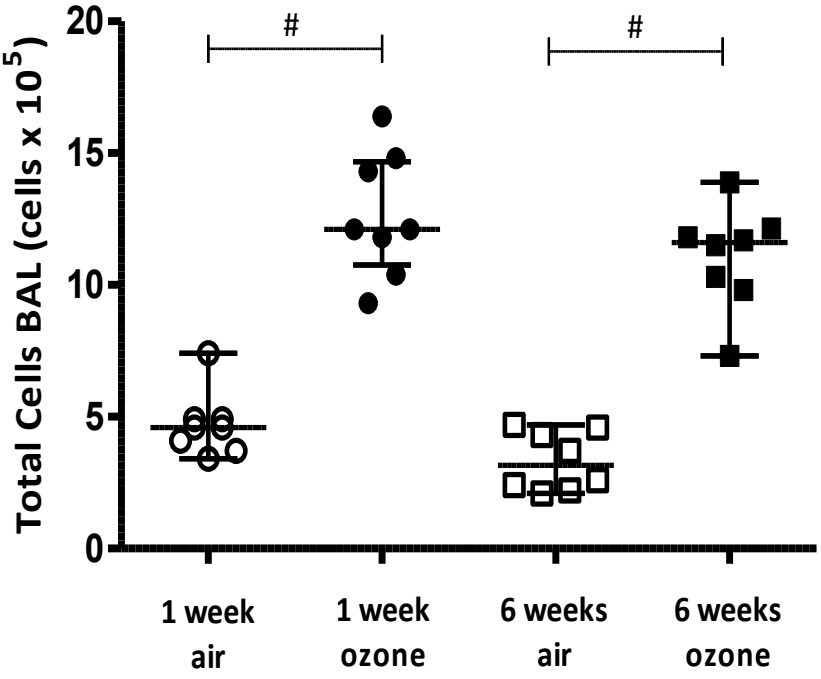
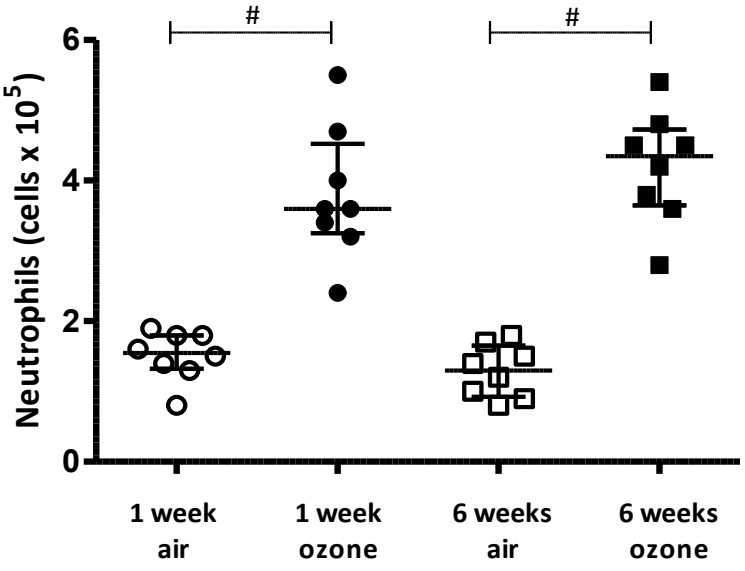



**Figure 5.1 -** Ozone exposed mice showed marked increases in total BAL cell counts compared with those in air exposed control mice after 1 and 6-weeks. $p<0.01$ #

A




B

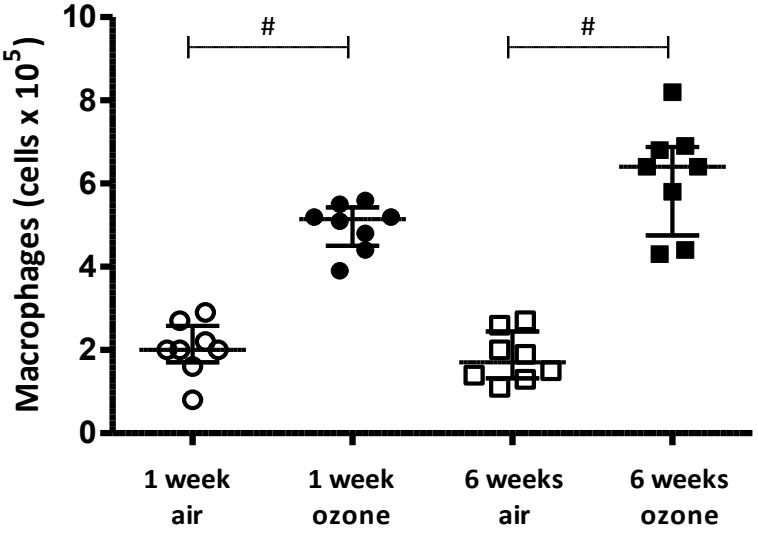
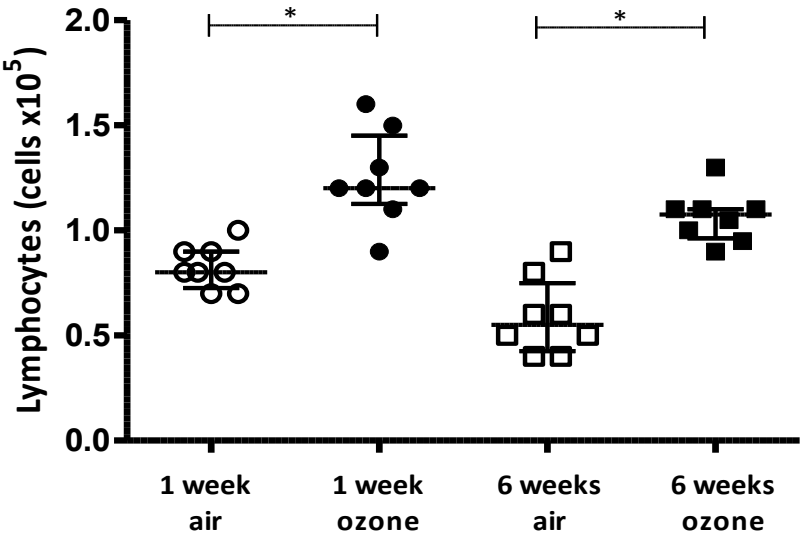



**Figure 5.2 -** Ozone exposed mice have considerably higher cell counts for neutrophils (A) and macrophages (B) when compared to air exposed mice. $p<0.01$ #

A




B

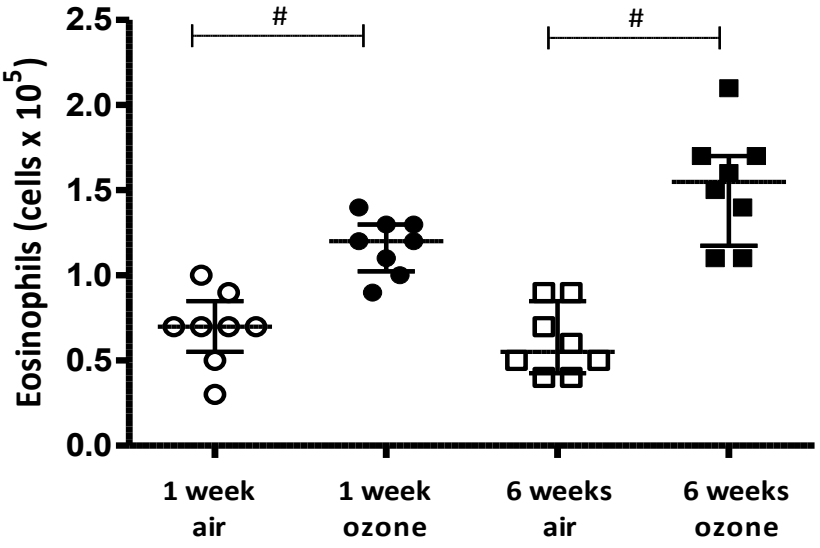



**Figure 5.3 -** Ozone exposed mice have considerably higher cell counts for lymphocytes (A) and eosinophils (B) when compared to air exposed mice. $p<0.05$*, $p<0.01$ #

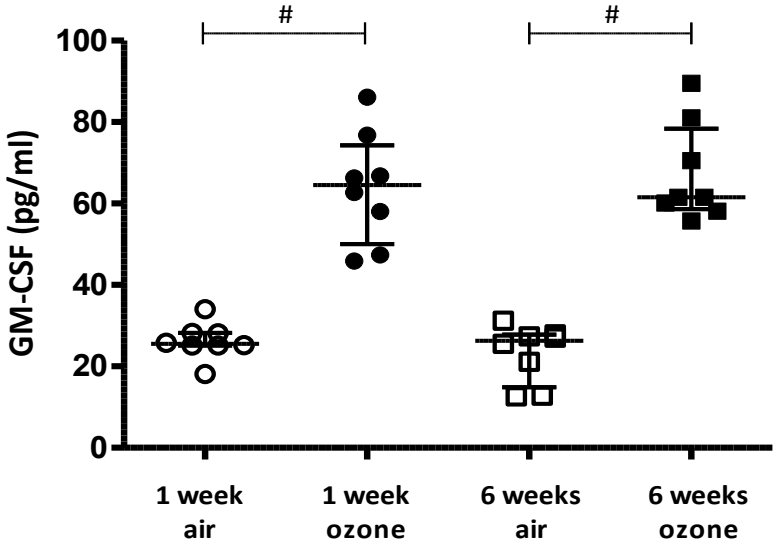



**Figure 5.4 -** 1 and 6-week ozone exposure induces an increase in the pro-inflammatory cytokine GM-CSF. $p<0.01$ #

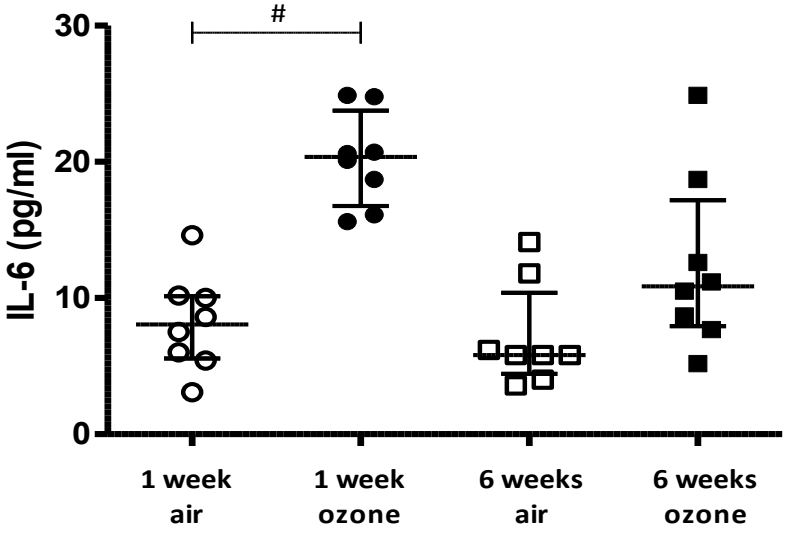



**Figure 5.5 –** 1-week ozone exposure induces an increase in the pro-inflammatory cytokine IL-6. No significant difference was seen in the 6-week ozone exposure group. $p<0.01$ #

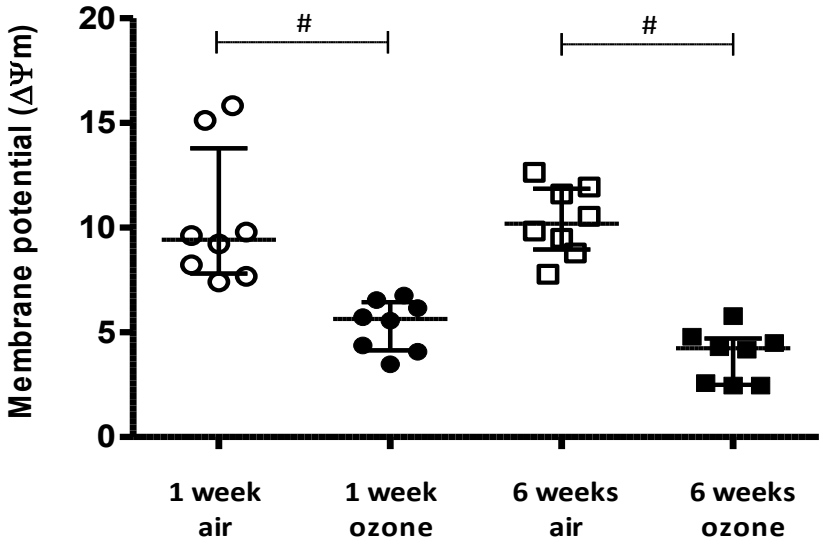



**Figure 5.6 -** Mitochondrial membrane potential tested in mitochondria isolated from mouse lung tissue was reduced in the 1 and 6- week ozone exposed mice. $p<0.01$ #

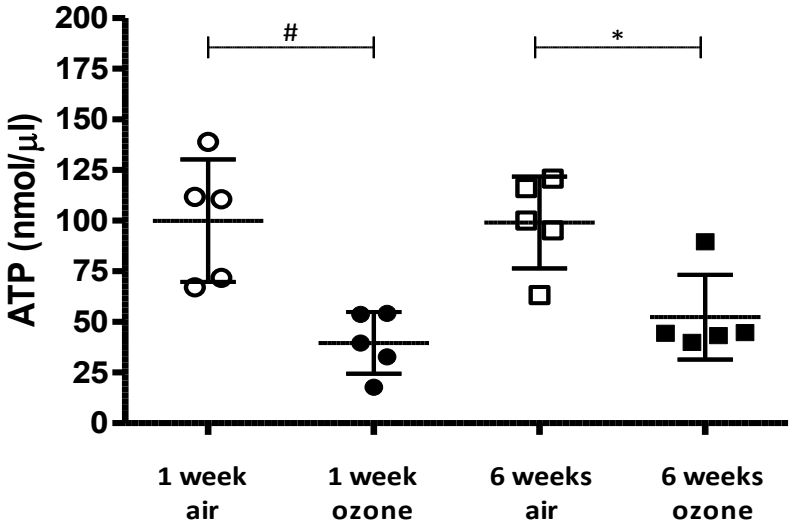



**Figure 5.7 -** ATP production in mouse lung tissue was reduced in both the 1 and 6-week ozone exposed mice when compared to the air exposed control groups. $p<0.05$*, $p<0.01$ #

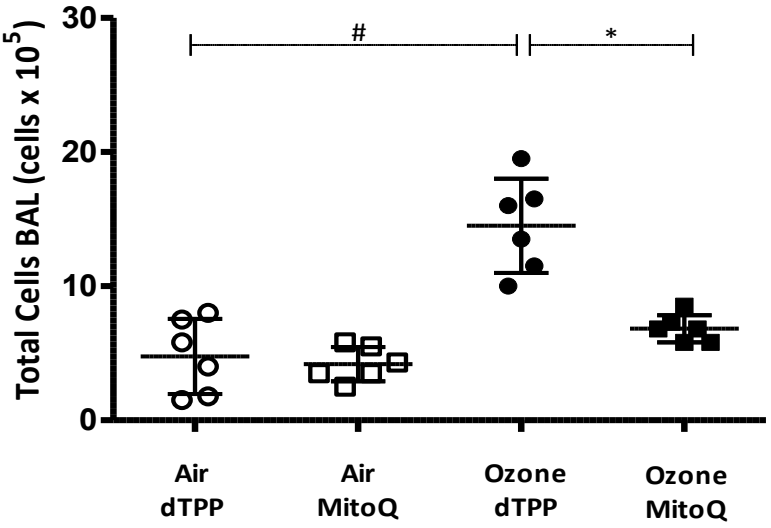



**Figure 5.8 –** MitoQ treatment resulted in a reduction in ozone induced total BAL cell counts compared with dTPP alone. $p<0.05$*, $p<0.01$ #

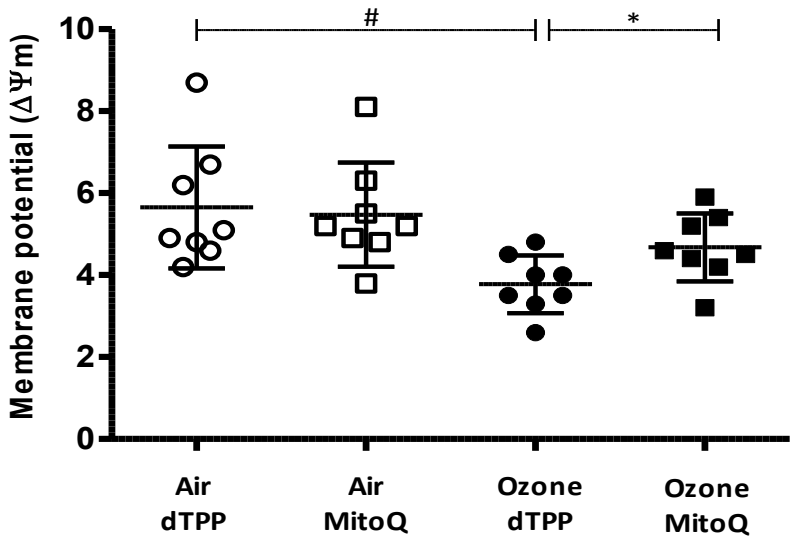



**Figure 5.9 –** MitoQ treatment resulted in restoration of the mitochondrial membrane potential in ozone exposed mice lung tissue compared with dTPP alone. $p<0.05$*, $p<0.01$ #

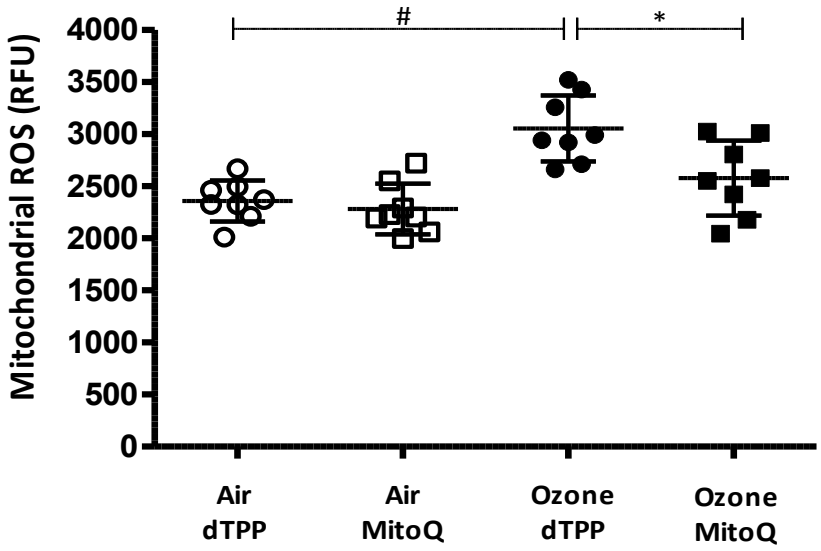



**Figure 5.10 –** MitoQ treatment resulted in a reduction of mitochondrial ROS levels in ozone exposed mice lung tissue compared with dTPP alone. $p<0.05$*, $p<0.01$ #

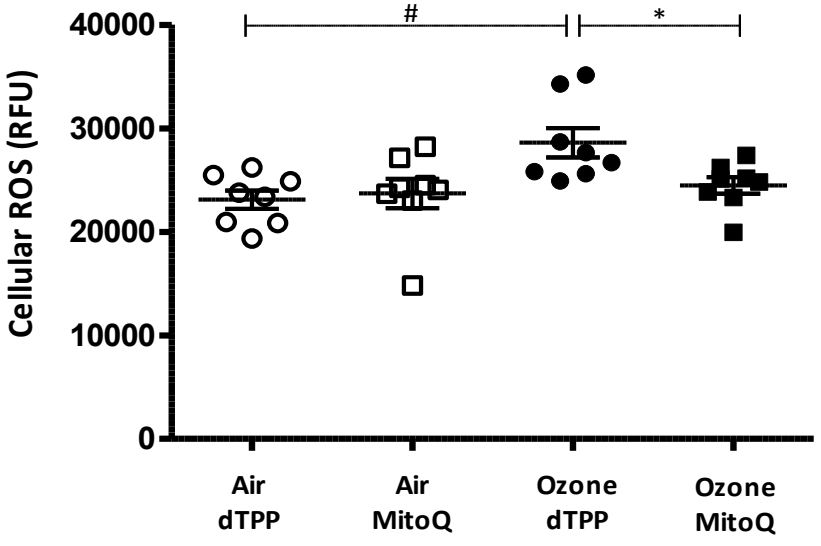



**Figure 5.11 –** MitoQ treatment resulted in a reduction of cellular ROS levels in ozone exposed mice lung tissue compared with dTPP alone. $p<0.05$*, $p<0.01$ #

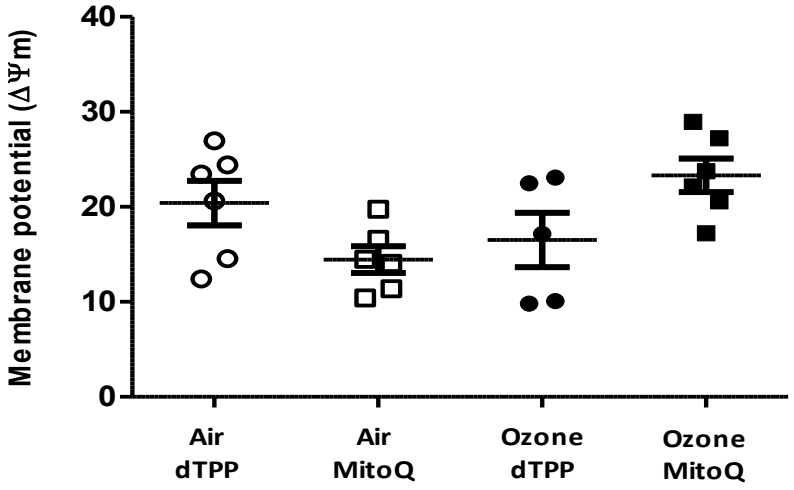



**Figure 5.12 –** MitoQ treatment had no effect on mitochondrial membrane potential in ozone exposed mice muscle tissue compared with dTPP alone

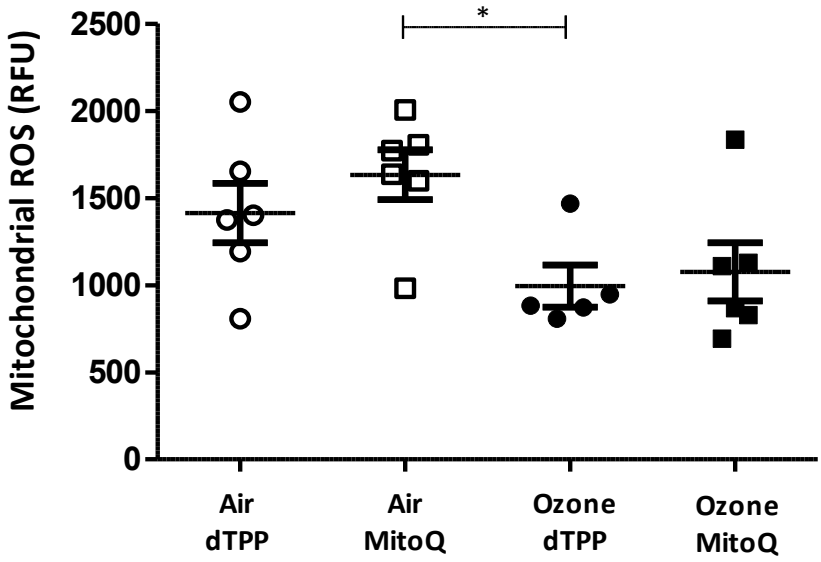



**Figure 5.13 –** MitoQ treatment had no effect on the mitochondrial membrane potential in ozone exposed mice muscle tissue compared with dTPP alone. A difference was observed in mitochondrial ROS levels between the Air/MitoQ and the Ozone/dTPP group. $p<0.05$*, $p<0.01$ #

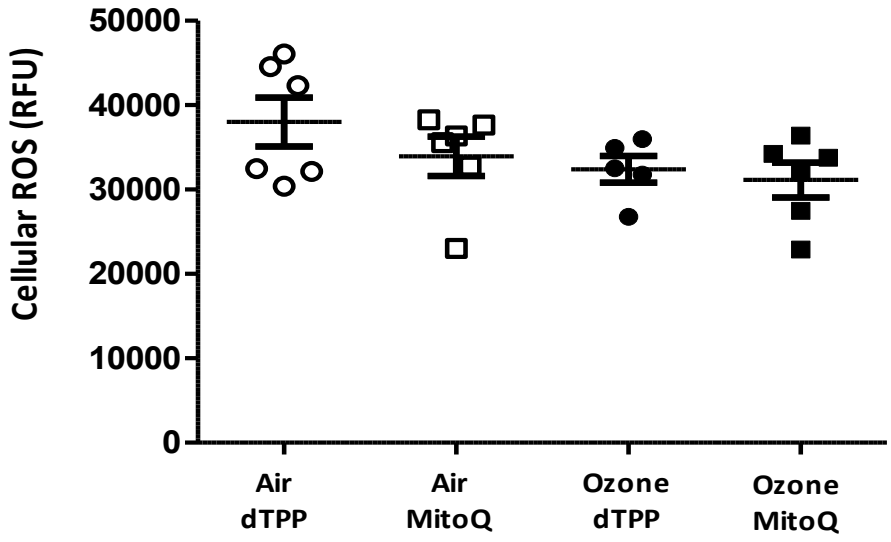



**Figure 5.14 –** MitoQ treatment had no effect on cellular ROS levels in ozone exposed mice muscle tissue compared with dTPP alone.

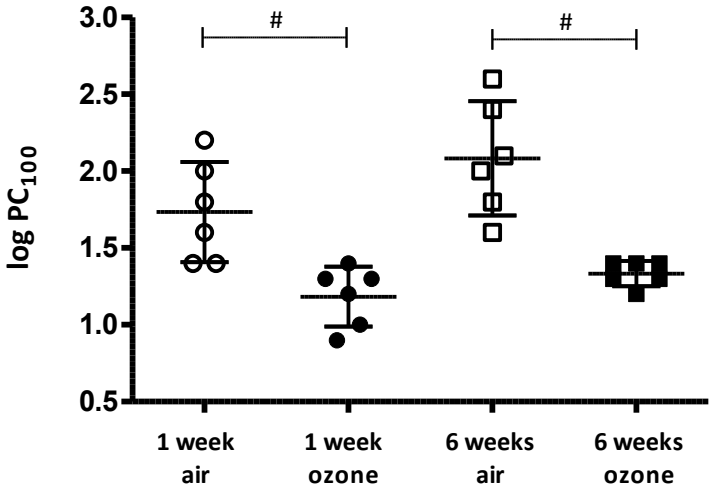



**Figure 5.15 –** Airway hyperresponsiveness (AHR) to acetylcholine was greater in the ozone exposed mice at 1 and 6 weeks compared to air groups with lower concentrations of aerosolized acetylcholine inducing a 100% increase in lung resistance (log $PC_{100}$). $p<0.01$ #

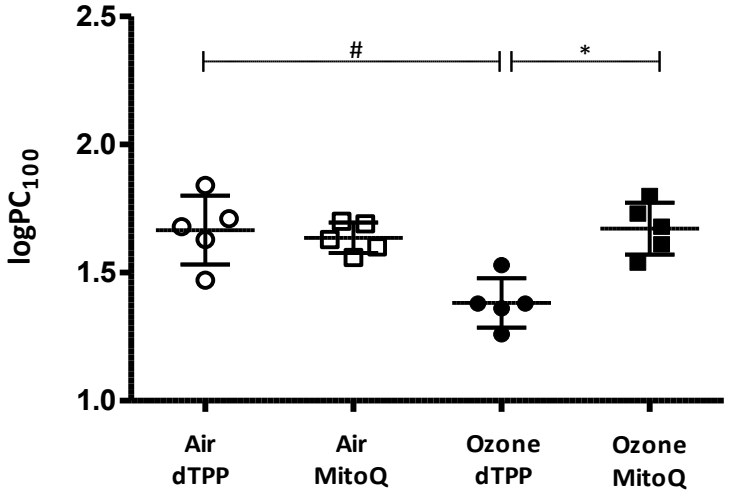



**Figure 5.16 –** Treatment with MitoQ resulted in a significant improvement in AHR compared to control treated mice with higher concentrations of aerosolized acetylcholine required to induce a 100% increase in lung resistance (log $PC_{100}$). $p<0.05$*, $p<0.01$ #

## 5.4 Discussion

### 5.4.1 Summary of results

I observed an increased inflammatory profile in the BAL fluid of mice exposed to ozone when compared to the air exposed groups for all cell types, corroborating previous work from Professor Chung's group in 2013 (LI, Wiegman et al. 2013). I also noted an increase in the BAL pro-inflammatory cytokine profile as evidenced by an increase in IL-6 and GM-CSF levels. Along with this I was able to demonstrate that this model was appropriate to induce mitochondrial dysfunction in lung tissue and that this effect was as a result of excessive oxidative stress. This has been shown as an increase in mitochondrial and cellular ROS levels in the lung tissue of ozone exposed mice, and a corresponding reduction in mitochondrial membrane potential and ATP production. MitoQ partially reversed these processes in the lung, suggesting that mitochondrial targeted antioxidant therapies may have therapeutic benefit in COPD. This data also suggests that specifically targeting mitochondrial derived ROS may be more effective than targeting cellular ROS alone as has been previously trialled with N-acetylcysteine (LI, Wiegman et al. 2013).

In line with what has been observed in the vastus lateralis muscle of human subjects (data presented in Chapter 4) there is no significant difference in mitochondrial membrane potential between air and ozone groups when considering mouse vastus lateralis tissue - as such there were no differences noted with MitoQ therapy. Perhaps unexpectedly there was no significant increase in mitochondrial or cellular ROS levels in the muscle of the ozone exposed mice when compared with the air exposed groups after 6 weeks of exposure.

I have shown alterations in the functional airway phenotype of mice exposed to ozone. This has been demonstrated as an increase in airway $R_L$ at lower concentrations of inhaled acetylcholine. This marker of airway hyperresponsiveness suggests that ozone contributes to airflow limitation in mice. The finding that MitoQ partially alleviates this suggests that its role in improving airflow obstruction is modulated by its effects on mitochondrial function in the murine lung.

### 5.4.2 Critique of methods

Although I have been able to demonstrate mitochondrial dysfunction in lung tissue of ozone exposed mice these results will need to be validated with further experiments. The unexpected finding that ozone does not increase mitochondrial and cellular ROS levels in mouse muscle raises questions. Although we have corroborated previous work that has shown 1 and 6 weeks of ozone exposure was a long enough period to induce lung changes, mouse muscle has not previously been studied. Conceptually this lack of effect within the muscle could arise because the exposure time is inadequate to induce spill-over changes in the skeletal muscle of mice or it may be that the muscle structure of mice is better protected than man against the deleterious effects of oxidative stress. This also raises the question that the spill-over model may be incorrect and that changes in man occur for other reasons then spill-over of inflammatory mediators from the lung.

Lung function assessment in mice is difficult given their small overall size and total lung capacity. Here we measured lung resistance in response to aerosolized acetylcholine and this is therefore a test of AHR. This is a common result that is presented in the ozone model of COPD (Russell, Chung et al. 2016) (Li, Wiegman et al. 2013), however it suggests a

reversible hyper-reactive airway phenotype. This is not something that is typically expected in COPD; again this raises the question whether 6 weeks of ozone exposure is long enough to truly induce typical COPD changes.

The ozone model of COPD has over time become more favoured given that it has been shown to rapidly induce pulmonary manifestations of COPD such as enlarged lung alveoli and emphysematous destruction of lung parenchyma in only 6 weeks, making it an attractive model to test therapeutic targets, compared to approximately 3-12 months with required by cigarette smoking models (Sun, Zhou et al. 2017). The findings presented here raise the question that this model too rapidly induces pulmonary manifestations of COPD without being representative of extra-pulmonary manifestations of COPD such as skeletal muscle defects.

Perhaps the biggest criticism to this work is the timing of administration of the MitoQ. It was delivered twice weekly for the duration of ozone exposures - to more faithfully model human COPD it would be necessary to show that it was able to reverse the process of mitochondrial dysfunction therefore would need to be administered after some or all ozone exposures were complete and disease had been established. The current data demonstrate a prophylactic effect rather than a treatment effect.

### 5.4.3 Significance of findings

Within the limitations of the small cohort of patients studied in this thesis (and in the literature), I have nevertheless been able to demonstrate for the first time that the reduction in mitochondrial membrane potential in human airway tissue of patients with COPD can also be induced in mice exposed to ozone. The finding that mitochondrial and cellular ROS levels in the skeletal muscle of mice are not elevated with this model may suggest that the changes in man are not due to spill-over of inflammatory mediators.

Although we know that anatomical differences exist between the murine pulmonary system and the human system these findings are exciting when considering mitochondrial targeted therapies which will first need to be proven in animal models. Although a long way off, when thinking about MitoQ use in human studies the different dosing strategies between mouse and humans that we have seen in other conditions will need to be considered. In the animal model presented in this chapter, doses of 5mg/kg are routinely used when administered intra peritoneally and this goes as high as 20mg/kg if administered orally. No toxic effects have been noted in mice with these doses (Smith and Murphy 2010). Studies in man have used doses of 0.5-1mg/kg and again have been shown to be safe and tolerable but these dosing regimens have certainly yielded less benefit that animal counterpart studies (Snow, Rolfe et al. 2010), perhaps unsurprisingly given the smaller per kg dose. Thus a current barrier to progress are the magnitude of therapeutic doses in man; safety and tolerability of doses which are probably sub therapeutic have been the focus in previous studies. A current study registered by clinical-trials.gov (NCT02597023) investigating the efficacy of MitoQ at a dose of 0.25mg/kg versus a placebo to improve physiological performance in middle age and elderly patients is due to complete in August 2018. It may

shed more light on the overall benefits of MitoQ at relatively low doses or again has the potential to demonstrate that human doses are sub therapeutic. This trial will be of interest given the endpoints are those which are known to be affected in patients with COPD – arterial stiffness and markers of oxidative stress.

### 5.4.4 Conclusion

I demonstrated for the first time that the ozone-exposed mouse model is a suitable model to investigate pulmonary mitochondrial dysfunction and that the mitochondrial dysfunction is driven by oxidative stress. I have been able to show that although pulmonary changes are apparent using this model it does not appear to mimic all of the systemic pathophysiological features of COPD and therefore at 6 weeks there is limited value to investigating skeletal muscle. The benefit of MitoQ to reverse the pulmonary inflammatory profile and AHR and reduce mitochondrial dysfunction is promising. Going forward we should aim to establish the duration of ozone models that provoke mouse muscle changes and should perhaps study murine skeletal muscle in more detail routinely - to enable a more optimal model of COPD to be developed. For now this model can be used to test pulmonary targeted therapies in COPD but is of limited benefit when thinking of dedicated muscle targeted treatments.

# Chapter 6: Discussion

## 6.1 General discussion

The detrimental pathophysiological cycle of COPD means that skeletal muscle weakness and fatigue exist. This will limit exercise performance due to leg discomfort and mitochondrial anaerobic metabolism which causes lactic acid production. Lactic acid is buffered by bicarbonate and thus carbon dioxide is produced. Patients with COPD also suffer with expiratory flow limitation and cannot therefore clear the excess carbon dioxide by increasing ventilation and as such these patients will experience breathlessness which further discourages exercise and leads to greater physical inactivity and increased skeletal muscle deficits (Polkey and Moxham 2011). The key to treating patients with COPD is to tackle these aspects of the illness, and while COPD is a very heterogeneous condition with a diverse clinical phenotype, these principles need to be borne in mind when looking to personalise treatment strategies and develop novel therapies that are of benefit.

Improving ventilation and ventilatory capacity on exercise is one way to try and break the detrimental pathophysiological cycle that patients with COPD suffer. This has been shown by Davey and colleagues in 2015; a study investigating the effects of endo-bronchial valves in patients with elevated hyperinflation indices and emphysema yielded exciting results. They found that in COPD patients, with heterogeneous emphysema and intact interlobular fissures, insertion of targeted endo-bronchial valves was beneficial; not only to improve respiratory indices but also six minute walk distance and exercise endurance time (Davey, Zoumot et al. 201%). In Chapter 3 I have been able to show that dynamic laryngeal narrowing, during exercise, in patients with COPD is associated with lung function indices of

disease severity, dynamic hyperinflation and exercise performance. The association with dynamic hyperinflation and respiratory duty cycle suggest that the mechanism of action is to increase PEEPi and splint the airways in order to optimise operational lung mechanics. The beneficial effects of this will be to reduce expiratory flow limitation by prolonging the expiratory phase of the respiratory cycle which will serve to abate dynamic hyperinflation and thus more appropriately increase minute ventilation. It may be postulated that these effects will improve exercise performance and this is a hypothesis that should be considered going forward.

In Chapter 4 I have for the first time shown that mitochondrial dysfunction exists in the airways of patients with COPD and that this is associated with the severity of the clinical phenotype; with strong correlations between mitochondrial membrane potential, lung function indices and exercise performance measures. I was not able to investigate the effects of dynamic laryngeal narrowing in patients with airway mitochondrial dysfunction as the overlapping patient groups that enrolled into both studies were too small to meaningfully analyse (seven patients across four groups). However the phenotypic association between airway mitochondrial function and disease severity, in particular $FEV_1$ and $VO_2$ peak, does lead to the supposition that mitochondrial targeted therapies may be of benefit to improve airflow limitation in COPD and therefore may help break the detrimental pathophysiological cycle that ultimately causes poor exercise performance.

The findings in Chapter 5 in which I investigated the effects of MitoQ, a mitochondrial targeted antioxidant, were promising and corroborated the possible benefits of mitochondrial targeted therapy in COPD. I was able to show that MitoQ partially restored mitochondrial function within the lungs of mice that had been exposed to ozone as an

oxidative stressor, and that this led to improvements in murine lung function. Although the limitations of these studies have been discussed in the relevant critique of the methodology sections (Section 4.4 and 5.4), they do suggest that clinical trials of MitoQ in COPD patients may be warranted, particularly as MitoQ has been shown as a safe and tolerable therapy for human administration (Smith and Murphy 2010) (Snow, Rolfe et al. 2010) (Gane, Weilert et al. 2010).

The findings from this thesis that mitochondrial function in the quadriceps muscle of patients with COPD was no different to control subjects was not altogether surprising and is most likely due to changes in muscle fibre type and size. The demonstration of definitive mitochondrial density change (as opposed to functional change) and increased ROS levels in skeletal muscle has been reported previously (Meyer, Zoll et al. 2013), as has a reduction in proportion of type I (mitochondria enriched) muscle fibres (Natanek, Gosker et al. 2013). The functional significance of this is early lactic acidosis and fatigue which contributes to greater inactivity which in turn perpetuates the poor skeletal muscle phenotype (Maltais, Simard et al. 1996) (Polkey and Moxham 2011).

Perhaps the most novel aspect of this study is the simultaneous study of airway and skeletal muscle from the same COPD patients. What is of interest from this is that the oxidative stress burden in COPD patients' skeletal muscle was elevated to a similar degree as their airway oxidative stress burden suggesting that the detrimental changes in muscle are driven by more than the spill-over of mediators from the lung. While I have been unable to prove or disprove the spill-over theory this finding potentially suggests that COPD should be considered a systemic condition with the pulmonary and skeletal muscle systems being two of the affected organs. Previous clinical phenotypic findings and associated mortality data

from other studies add weight to this alternative theory particularly as we know that skeletal muscle weakness occurs very early in the disease process before respiratory symptoms manifest (Seymour, Spruit et al. 2010), and that skeletal muscle dysfunction is a predictor of mortality in COPD irrespective of lung function (Patel, Natanek et al. 2014).

When considering strategies to develop pharmacological treatments for disease, appropriate animal models are of importance. The novel finding that pulmonary system mitochondrial dysfunction can be induced using ozone in a murine model of COPD, which shares similar pathological processes to that seen in the airways and lungs of patients with COPD (Triantataphyllopoulos, Hussain et al. 2011) is of interest and confirms that this is an appropriate model to trial lung targeted mitochondrial therapies. However, this is the first time the peripheral muscles have been studied in this murine model and given the lack of change that I found in mitochondrial and cellular ROS levels in the vastus lateralis of mice this model may be of limited benefit when considering the study of muscle specific therapies. It suggests that the time frame of the model may be too short to induce skeletal muscle changes or that once again the muscle changes of COPD are due to more than the spill-over of inflammatory mediators and ROS from the lung.

## 6.2 Study limitations

There are various limitations to the studies presented in this thesis. They are critiqued in greater detail in the relevant chapters. Overall however when addressing the study rationale from section 1.11 the question linking the human studies has not been answered given the small number of patients that underwent both dynamic laryngeal assessment during exercise and subsequent endobronchial biopsy and muscle biopsy. Overall with only 7 common patients between 4 different groups no meaningful analysis could be undertaken. A more definitive power calculation incorporating all the variables assessed would have been a way to ensure that this did not happen. Overall therefore these observations provide appropriate feasibility data for which future studies can be based.

### 6.3 Future work

Building upon work presented in this thesis by reconciling the findings with what has already been presented in the literature is an important area for future work. The finding that the upper airway exerts a functional effect in patients with more significant airflow obstruction is in keeping with previous work by Higenbottam and Payne in 1982 which observed expiratory narrowing of the glottis at rest in patients with COPD (Higenbottam and Payne 1982). Here I have been able to demonstrate the phenomenon of dynamic laryngeal narrowing, present at rest and on exercise, correlates more strongly with the respiratory duty cycle and the dynamic hyperinflation index. This is of interest and suggests that a study of phenotypically well characterised COPD subjects with similar hyperinflation indices and lung function would be of merit to ascertain the exact benefits of expiratory laryngeal narrowing on exercise performance measures. It perhaps demonstrates the importance of all aspects of the airway in patients with COPD - potentially targeting the upper airway, as well as the middle and lower airways, could have therapeutic potential to optimise operational lung mechanics.

Further work to establish a possible causative effect of mitochondrial dysfunction triggering airway inflammation could potentially be another avenue of study. In particular the links with airway TGF-β, which has previously been shown to regulate superoxide dismutase (Michaeloudes, Sukhar et al. 2011) a potent antioxidant within the mitochondria but more importantly as an inducer of IL-6 release which triggers airway smooth muscle proliferation through inflammation (Michaeloudes, Sukhar et al. 2011). The hypothesis that mitochondrial dysfunction is the cause of inflammation and a higher oxidative stress burden has not been tested here, rather an association has been found. If further work looks to

establish a possible role for mitochondrial targeted airway therapy in patients with COPD greater mechanistic associations to the aforementioned pathways will first be required. Following the results of a current study (due to complete in August 2018), registered by clinical-trials.gov (NCT02597023); investigating the efficacy of MitoQ at a dose of 0.25mg/kg versus a placebo to improve physiological performance in elderly patients, will be of interest given that COPD shares key patho-biological features with aging (Ito and Barnes 2009) and the endpoints are those which are known to be affected in patients with COPD – arterial stiffness and markers of oxidative stress.

Whilst this thesis has not demonstrated a role for defective mitochondria in the skeletal muscle of COPD patients it has demonstrated that there is a comparable oxidative stress burden in the skeletal muscle compartment to that which is found in the airway. This suggests that the complexity of the muscle biology that is observed is likely to be different to that occurring in the pulmonary system. The study of muscle targeted therapies remains an exciting area of work which may potentially provide global therapeutic benefits to COPD patients.

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## Appendix: Publications arising from this thesis

### First author and co-first author publications

**Haji GS**, Polkey MI, Hull JH. Dynamic laryngeal narrowing in COPD may have effects on the trachea. Thorax. 2015;70(7):692.

**Haji GS*,** Baz M*, Menzies-Gow A, Tanner RJ, Hopkinson NS, Polkey MI, Hull JH. Dynamic laryngeal narrowing during exercise: a mechanism for generating intrinsic PEEP in COPD? Thorax. 2015;70(3):251-257

Wiegman CH*, Michaeloudes C*,**Haji G*,** Narang P, Clarke CJ, Russell KE, Bao W, Pavlidis S, Barnes PJ, Kanerva J, Bittner A, Rao N, Murphy MP, Kirkham PA, Chung KF, Adcock IM; COPDMAP. Oxidative stress-induced mitochondrial dysfunction drives inflammation and airway smooth muscle remodelling in patients with chronic obstructive pulmonary disease. J Allergy Clin Immunol. 2015;136(3):769-80.

### Other Publications

Micaheloudes C, Kuo CH, **Haji G,** Finch DK, Halayako AJ, Kirkham P, Chung KF*, Adcock IM*. Metabolic repatterning in COPD airway smooth muscle cells. Eur Respir J. 2017;50(5).

Curtis KJ, Meyrick VM, Mehta B, **Haji GS**, Li K, Montgomery H, Man WD, Polkey MI, Hopkinson NS. Angiotensin-Converting Enzyme Inhibition as an Adjunct to Pulmonary Rehabilitation in Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med. 2016 Dec 1;194(11):1349-1357.

Patel MS, Donaldson AV, Lewis A, Natanek SA, Lee JY, Andersson YM, **Haji G**, Jackson SG, Bolognese BJ, Foley JP, Podolin PL, Bruijnzeel PL, Hart N, Hopkinson NS, Man WD, Kemp PR, Polkey MI. Klotho and smoking--An interplay influencing the skeletal muscle function deficits that occur in COPD. Respir Med. 2016;113:50-56