

The Role of Ambulatory oxygen to improve skeletal muscle gene expression in Chronic Obstructive Pulmonary Disease in patients with exercise induced hypoxaemia

By

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Doctor of Medicine**

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Abstract

Rationale

Hypoxaemia plays a role in the aetiology of abnormal skeletal muscle function in Chronic obstructive pulmonary disease (COPD) *via* abnormal protein synthesis and mitochondrial function. Patients exhibiting exercise-induced desaturation (EID) have exercise intolerance, perhaps a consequence of muscle hypoxia. Ambulatory oxygen therapy (AOT) is indicated in these patients; however the evidence is derived from single assessment studies. This thesis explores the role of longer term AOT and whether it favourably alters skeletal muscle gene expression in patients with COPD and EID.

Methods

A 12 week randomised controlled trial of AOT against air in 25 patients with COPD and EID was undertaken. Participants underwent skeletal muscle biopsies and exercise assessments. In parallel a systematic review of published literature from 1980-2014 for trials in which AOT was compared to placebo in COPD was completed.

Results

The systematic review showed that AOT had no statistical effect on improving exercise capacity (6 minute walk or endurance shuttle walk tests); $p=0.44$ and $p=0.29$ respectively.

Gene set enrichment analysis show the KEGG pathways of oxidative phosphorylation, PPAR signalling and fatty acid metabolism to be up-regulated following AOT ($q<2\%$) in the clinical trial of AOT versus Air.

Conclusion

AOT has limited long term benefit in improving functional exercise capacity. It may however favourably alter gene expression in patients with COPD and EID.

Dedication

This thesis is dedicated to my parents; for their constant love and support.

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Contents

Chapter 1: Introduction Chronic Obstructive Pulmonary Disease	Page
1.1 Diagnosis and Clinical features	1
1.1.0 Pulmonary Physiology in COPD	4
1.1.1 Heterogeneity in COPD	5
1.2 Pathogenesis	7
1.2.0 Inflammation	7
1.2.1 Protease anti Protease Imbalance	9
1.2.2 Oxidative Stress	10
1.3 Management	12
1.3.0 Non Pharmacological	12
1.3.1 Pharmacological	12
1.3.2 Interventional	14
1.4 Oxygen in COPD	15
1.4.0 Long Term Oxygen Therapy (LTOT)	16
1.4.1 Ambulatory Oxygen Therapy (AOT)	18
Exercise Induced Desaturation	
AOT assessment	
Field Tests	
1.4.2 Short Burst oxygen therapy (SBOT)	23
1.5 Muscle Disease in COPD	24
1.5.0 Skeletal muscle structure	25
1.5.1 Clinical observations	26
Skeletal muscle strength and endurance	

1.5.2	Laboratory Observations	28
	Muscle Mass	
	Skeletal Muscle dysfunction (SKMD) Pathological changes	
1.6	Aetiology of SKMD in COPD	31
1.6.0	Inflammation	31
1.6.1	Hypoxia	32
1.6.2	Oxidative stress	33
1.6.3	Disuse	34
1.7	Aims	35
Chapter 2 Methods		
2.1	Systematic Review	39
2.1.0	Objective 1 Clinical effectiveness of ambulatory oxygen	40
2.1.1	Objective 2 Clinical effectiveness of methods of delivering ambulatory oxygen	41
2.1.2	Objective 3 Cost-effectiveness of ambulatory O ₂	42
2.1.3	Search strategy	43
2.1.4	Study selection, data extraction and risk of bias	44
2.1.5	Statistical methods	45
2.2	Clinical trial Methods	46
2.2.0	Ethics and Governance	50
2.2.1	Participant Selection	50
2.2.2	Demographic data	52
2.2.3	Spirometry and arterial blood gas sampling	52
2.2.4	Muscle biopsy	52
2.2.5	Blood sampling	53

2.2.6	HRQoL and activity monitoring	54
2.2.7	6MWT	54
2.2.8	Blinding, Treatment allocation and statistical analysis	55
2.2.9	Trial monitoring and safety	56
2.3	Clinical Trial Laboratory Methods	56
2.3.0	Enzyme linked immunosorbent assay	56
2.3.1	RNA extraction	59
2.3.2	RNA amplification	62
2.4	Microarrays	63
2.4.0	Normalisation	64
Chapter 3 Systematic Review		
3.1	Results	69
3.1.0	Included studies	69
3.1.1	Risk of bias	71
3.2	Clinical effectiveness of ambulatory oxygen	79
3.2.0	Exercise capacity	79
3.2.1	Quality of life	81
3.3	Impact of device on ambulatory oxygen delivery	84
3.3.0	Exercise capacity	84
3.3.1	Oxygen utilisation	85
3.3.2	Other outcomes	85
3.4	Cost Effectiveness	86
3.5	Discussion	86
Chapter 4 Clinical Outcomes		

4.1 Patient Characteristics	97
4.2 Cylinder usage	98
4.3 Exercise capacity and home activity	99
4.4 Health related quality of life	101
4.5 Systemic inflammation	103
4.6 Discussion	104
Chapter 5 Gene expression response to ambulatory oxygen and air	
5.1 Demographic data	112
5.2 Microarray Quality Assessment	113
5.3 Gene expression	115
5.3.0 Gene set enrichment analysis (GSEA)	117
5.3.1 GSEA results	119
5.4 Principle component analysis	125
5.5 Discussion	134
Chapter 6 Discussion Summary	
6.1 Summary of results	139
6.2 Clinical efficacy	139
6.3 Device efficacy	141
6.4 Changes in Skeletal muscle gene expression	142
6.5 Future work	145
6.5.0 Ambulatory oxygen efficacy	145
6.5.1 Inflammatory response to AOT	145
6.5.2 Gene Expression	146

List of Tables

Table 1.1 Severity of airflow obstruction by FEV1%	3
Table 1.2 Protease and antiproteases involved in the pathogenesis of COPD.	9
Table 1.3 Borg Dyspnoea Scale	21
Table 1.4 Muscle fibre classification	25
Table 3.1 Clinical effectiveness of Ambulatory oxygen studies.	72
Table 3.2 Impact of device on Ambulatory oxygen delivery studies	75
Table 3.3 Risk of bias in included studies	76
Table 4.1 Baseline demographic data for trial participants	98
Table 4.2 Change from baseline in 6MWT distance following interventions and home activity (VMU/min) at the end of each intervention period.	100
Table 4.3 Change from baseline scores CAT and HAD	102
Table 4.4: Systemic cytokine response to oxygen and air during clinical trial	103
Table 5.1 Demographic data for patients included in gene expression data analysis	113
Table 5.2a Enriched KEGG pathways during ambulatory oxygen treatment	119
Table 5.2b Enriched KEGG pathways during air treatment.	120
Table 5.3a: Clinical outcome data for trial patients whom underwent muscle biopsies	131
Table 5.3b Correlation of OXPHOS pathway and patient clinical data.	132

List of Figures

Figure 1.0: Severity Matrix COPD	3
Figure 1.1 and 1.2: CT thorax cross sectional images of emphysema.	6
Figure 1.3 Inflammatory cells and cytokines involved in COPD pathogenesis.	9
Figure 1.4 Nice guidelines for COPD management.	14
Figure 1.5 Kaplan Meier survival curves for LTOT trial.	17
Figure 1.6 The muscle motor unit	26
Figure 1.7 Hypertrophy and atrophy signalling pathways in muscle.	29
Figure 2.1 Trial flow diagram	47
Figure 2.2 Equipment used for muscle biopsy sampling.	53
Figure 2.3 Bergstrom's Procedure	53
Figure 2.4 Standard Elisa lab well	57
Figure 2.5 Sandwich Elisa process.	58
Figure 2.6 RNA extraction process.	61
Figure 2.7 Schematic diagram detailing the microarray process.	65
Figure 2.8 MA plots a) before and b) after normalisation	67
Figure 3.1 Prisma flow diagram	70
Figure 3.2 Forest plots showing the effect of ambulatory oxygen therapy on exercise capacity.	80
Figure 3.3 Forest plot showing the effect of ambulatory oxygen on end test exercise induced dyspnoea	81
Figure 3.4 Forest plots showing the results of ambulatory oxygen on HRQoL in the Pulmonary Rehab studies.	82

Figure 3.5 Forest plots showing results of ambulatory oxygen on HRQoL in domiciliary studies.	83
Figure 3.6 Forest plot showing the effect of OCDs on exercise capacity and exercise saturations	85
Figure 4.0 Study design diagram	92
Figure 4.1 Trial flow diagram	97
Figure 4.2 Correlation of baseline 6MWT and FEV1% predicted	99
Figure 4.3a-c) Correlations of home activity with 6MWT at baseline	101
Figure 4.4: HADs score during treatment arms	102
Figure 4.5 Plasma IL-6 during treatment arms	104
Figure 5.1 Box plot of individual normalised microarray data	114
Figure 5.2 False heat map of distance between microarrays	115
Figure 5.3a Enrichment plot for KEGG ribosome pathway	121
Figure 5.3b Enrichment plot for KEGG oxidative phosphorylation	122
Figure 5.3c Enrichment plot for the only KEGG pathway up-regulated during air	123
Figure 5.4 Functional grouping for KEGG pathways during oxygen treatment.	124
Figure 5.5 Plot of principal components against variance	126
Figure 5.6: Biplot of OXPHOS gene and principal components	127
Figure 5.7 Heatmap of genes enriched in OXPHOS pathway	129
Figure 5.8 Interaction between KEGG pathways, genes and clinical data	133

Appendices

Appendix 1 Search Strategy for systematic review.	148
Appendix 2 Clinical trial documents	
Appendix 2.1 Good clinical practice certificate	151
Appendix 2.2 IRAS application	152
Appendix 2.3 MHRA acceptance letter	162
Appendix 2.4 Local Research and development letter approval	164
Appendix 2.5 NRES approval letter	165
Appendix 2.6 Patient information sheet	168
Appendix 2.7 Ambulatory oxygen in COPD Prescription	175
Appendix 2.8 Case report form	176
Appendix 3 Top 400 genes from pre-ranked list.	178

References

181

Abbreviations

COPD Chronic Obstructive Pulmonary Disease

FEV1 Forced expiratory volume

FVC Forced Vital Capacity

TLC Total lung capacity

RV Residual volume

FRC Functional residual capacity

AATD Alpha One antitrypsin deficiency

NF- κ B Nuclear factor Kappa B

FFM Fat free mass

MMP Matrix metalloproteinase

AAT Alpha One antitrypsin

NE Neutrophil Elastase

TIMPs Tissue inhibitors of matrix metalloproteinases

ROS Reactive Oxygen species

HRQoL Health related quality of life

PR Pulmonary Rehab

SABA short acting beta 2 agonist

SAMA short acting muscarinic antagonist

LAMA Long acting muscarinic antagonist

LABA Long acting beta 2 agonist

LABA/ICS Long acting beta 2 agonist with inhaled corticosteroid

ICS Inhaled corticosteroid

LVRS Lung volume reduction surgery

LTOT Long term oxygen therapy

EID Exercise induced desaturation

6MWT 6 minute walk test

ESWT Endurance shuttle walk test

ISWT Incremental shuttle walk test

DODs Demand oxygen devices

PRC Pendant reservoir cannulae

CFNC Continuous flow nasal cannula

SBOT Short Burst Oxygen therapy

SKMD Skeletal muscle dysfunction

AMPK AMP-activated protein kinase

DNA Deoxyribonucleic acid

RNA Ribonucleic acid

LTB4 Leukotriene B4

PPAR Peroxisome-proliferator activated receptors

MHRA Medicines and Healthcare products regulatory agency

RNS Reactive nitrogen species

SNOSE Sequentially numbered, opaque sealed envelopes

CRF Case Report form

MRC Medical research council

CAT COPD assessment test

GCP Good clinical practice

TNF Tumour necrosis factor

IL Interleukin

HIF- α Hypoxia inducible factor alpha

KEGG Kyoto Encyclopedia of Genes and Genomes

HRG Healthcare resource group

OXPPOS Oxidative phosphorylation

GSEA Gene set enrichment analysis

Chapter 1

Introduction

Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is the 4th leading cause of death worldwide and predicted to be the 3rd by 2020 with the incidence of the disease increasing due to persistent exposure to risk factors and an ageing population(1). In the UK 900,000 people are diagnosed with COPD with another 2 million remaining undiagnosed(2). Estimated costs to the NHS are in the region of £800 million in direct healthcare costs(2), highlighting the need to study the disease to develop effective treatments.

1.1 Diagnosis and Clinical Features

COPD is characterised by airflow limitation that is usually progressive and not fully reversible(3). The airflow limitation is a consequence of chronic inflammation which affects both small airways and lung parenchyma resulting in narrowed small airways and loss of elastic recoil. Tobacco smoking is recognised as an important aetiological risk factor(in the Western world) however, only 15-20% of smokers develop clinically significant disease(4). From this it can be inferred that development of the disease is due to the interplay between genetic susceptibility and inhalation of noxious particles, most commonly from cigarette smoke but also environmental pollutants.

Diagnosis is based on physiological testing using post bronchodilator spirometry where the ratio between forced expiratory volume in the first second (FEV1) and forced vital

capacity (FVC) ratio is <70% in the presence of symptoms in individuals exposed to COPD risk factors(3). The predominant symptoms are:

- Dyspnoea which is generally progressive, persistent and worse with activity or exercise.
- Cough-with or without sputum production.
- Wheeze

The use of fixed FEV1/FVC ratio of <70% has been suggested and used by the Global initiative for chronic obstructive lung disease (GOLD)(3) due to its relative simplicity; however this remains a controversial issue. This is largely due to prevalence studies demonstrating disparity between the fixed ratio and lower limit of normal (the lower 5th percentile for reference population) when used for diagnosis. The use of the fixed ratio may result in misclassification at extremes of age; an overestimation and underestimation of disease in older and young older patients respectively(5, 6). The use of the lower limit of normal is less likely to cause this and in contrast to GOLD and National institute for clinical excellence (NICE)(7) is used by the ATS (American Thoracic Society) and ERS (European Respiratory Society).

The classification of severity airflow obstruction is based on the reduction in FEV1 compared to that predicted by age, gender and height. The FEV1 % predicted (table 1) is standardised, as such classification of severity in the UK-NICE guideline (7) is the same as that across America and Europe; ATS/ERS guidelines(8).

Although airflow obstruction has been used in several international guidelines to define severity, its use alone to define symptoms and management is outdated has been replaced by multicomponent system(3).

GOLD	Severity	FEV1%
1	Mild	>80%
2	Moderate	50-79%
3	Severe	30-49%
4	Very Severe	<30%

Table 1.1: Severity of airflow obstruction by FEV1%

This multicomponent system incorporates; exacerbation history, quality of life and level of dyspnoea. Quality of life and dyspnoea scores are quantified by the COPD assessment tool (CAT) and modified Medical research council (mMRC) respectively. The former is scored out of 40 and the mMRC out of 4 with higher scores indicating greater symptoms. Patients are categorised by these features (figure 1) into groups; A to D which indicate both their symptom burden and exacerbation history. Group A low symptoms and exacerbation burden, group B high symptoms and low exacerbation burden, group C low symptoms and high exacerbation burden and group D high symptom and exacerbation burden.

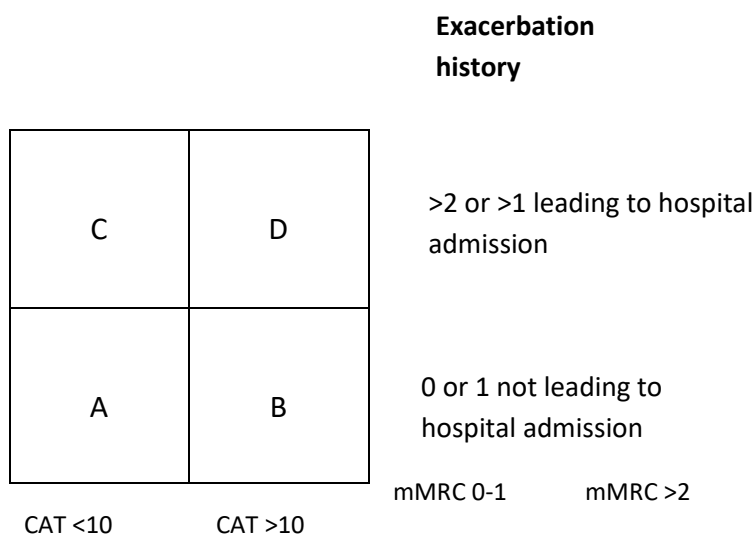


Figure 1.0: Severity Matrix COPD

1.1.0 Pulmonary Physiology in COPD

Physiological testing is the cornerstone of COPD diagnosis. FEV1 has been well characterised in patients with COPD. A lower FEV1% predicted is associated with worse health related quality of life (HRQoL) (9), symptom burden (10), a higher frequency of exacerbations(11) and mortality(12). The transfer factor for carbon monoxide (DLCO/TLCO) measures gas exchange within the lungs. Although less well characterised than FEV1, impairment of DLCO may indicate patients with reduced exercise capacity and those more likely to be affected by exercise induced desaturation (13). KCO (transfer factor per alveolar volume) is a measure of DLCO corrected for alveolar volume and normally indicates parenchymal involvement, which in the context of COPD suggests emphysema (section 1.1.1). The measurement of lung volumes FRC (functional residual capacity), TLC (Total lung capacity) and RV (residual volume) can be made using body plethysmography or inert gas dilution. In COPD these often reveal evidence of gas trapping suggested by raised RV:TLC ratio, raised RV and reduced inspiratory capacity.

Physiological testing via lung function can also indicate bronchodilator reversibility or small airways disease. Bronchodilator reversibility: an increase of 12 % and at least 200ml in FEV1 is common in COPD and more likely when the FEV1 is low(3). It may also suggest coexisting asthma which is likely to have implications for long term management. Small airway disease, measured physiologically by mid expiratory flow (FEF25-75%) is of interest in COPD as changes in their function may be the earliest signs of disease. Cross sectional studies have previously shown that even if pathology was present in the small airways FEV1 remained normal however as pathology progressed FEV1 subsequently declined(14).

1.1.1 Heterogeneity in COPD

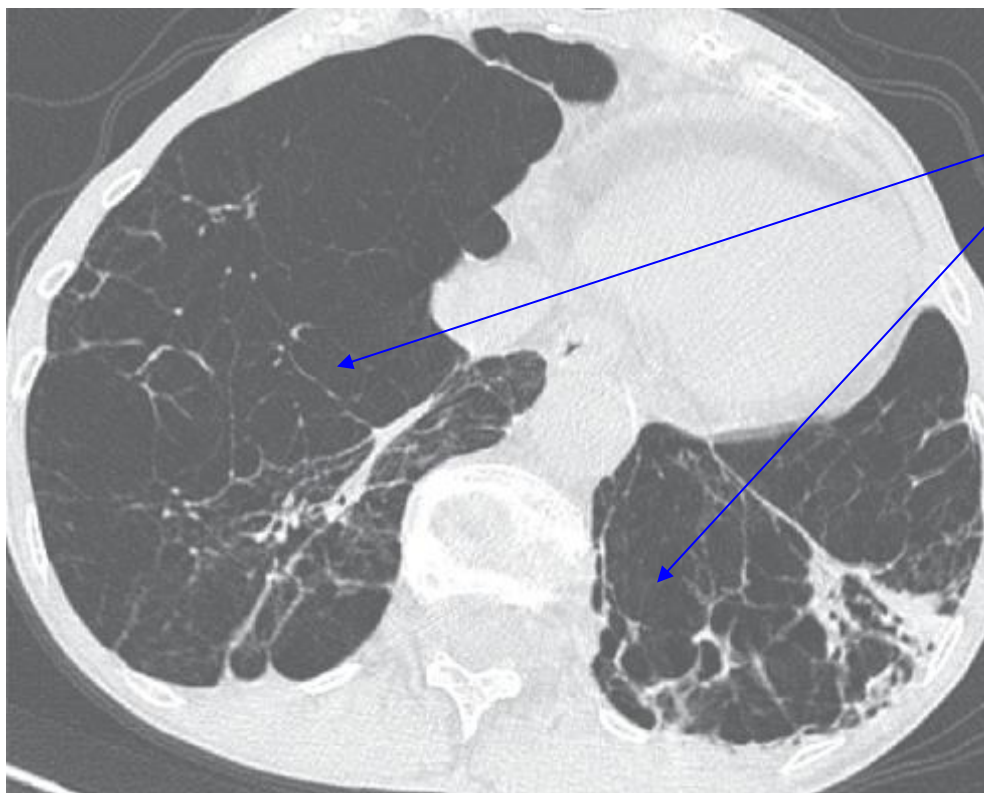
Despite its spirometric definition COPD is a heterogeneous disease that encompasses a spectrum of clinical presentations which may co-exist. Typical clinical presentations include chronic bronchitis and emphysema.

Chronic bronchitis is classically defined as chronic cough with sputum production for at least 3 months of 2 consecutive years(15). It is caused by mucus overproduction and hypersecretion by goblet cells. This contributes to airflow limitation by causing luminal obstruction and altering airway surface tension increasing the tendency for them to collapse(16). Those affected are more likely to have a more rapid decline in lung function(17) and higher exacerbation(18) frequency than those without chronic bronchitis.

Emphysema is the destruction of alveolar walls with permanent abnormal enlargement of airspaces distal to terminal bronchioles(19). The use of computed tomography (CT) has enabled this diagnosis to be made *in vivo* from radiology in addition to its widely accepted histological diagnosis. Emphysema may be subdivided in its distribution on high resolution computer tomography (HRCT) both in terms of its relative geographic position within the lung as a whole (upper vs lower) or position relative to the secondary pulmonary lobule (centrilobular vs panlobular)-figure1.1/1.2.



Centrilobular emphysema



Panlobular emphysema

Figure 1.1 and 1.2: CT thorax cross sectional images of emphysema.

Emphysema in patients with COPD is typically upper lobe predominant and centrilobular in its distribution. However in Alpha one anti trypsin deficiency, a condition where there is a genetic predisposition to emphysema, it is typically lower zone predominant and panlobular(20). The distribution of emphysema within the lung may represent distinct clinical phenotypes. For example, lung volume reduction surgery a procedure which has been shown to improve functional and quality of life outcomes(21) is only effective for upper lobe predominant emphysema. It is important to note that both chronic bronchitis and emphysema; quantified by computer tomography can occur in the absence of airflow obstruction(16, 22).

1.2 Pathogenesis

The pathogenesis of COPD is complex and not completely understood. It involves several inflammatory cell types, molecular pathways and cytokines. Over the years a number of overarching concepts contributing to COPD pathogenesis have emerged; *inflammation*, *protease anti- protease imbalance* and *oxidative stress*.

1.2.0 Inflammation

The concept of inflammation is supported by studies demonstrating increased inflammatory cell infiltrate in the sputum, bronchoalveolar (BAL) fluid and lung biopsies of patients with COPD compared to healthy controls (23). There is some evidence to suggest that the intensity of the inflammatory cell infiltrates correlates with the degree of disease progression(24-27). The key inflammatory cells implicated are macrophages, neutrophils and cytotoxic T cells (CD8+)(28, 29).

Cigarette smoke activates macrophages to release pro-inflammatory mediators including interleukin 8 (IL8), tumour necrosis alpha (TNF- α) as well as chemoattractants such as leukotriene B4 (LTB4)(28, 29).

TNF- α enhances inflammation by activating nuclear factor Kb (NF-kB) which switches on the transcription of inflammatory genes(28). Serum levels of TNF- α have been shown to be higher in patients with COPD than controls and are also associated with the loss of fat free mass (FFM) in patients with COPD(30).

Macrophages also secrete protease enzymes; matrix metalloproteinases (MMP), cathepsins and neutrophil elastase (largely taken up from neutrophils) which degrade the extracellular matrix within the lung. Furthermore, macrophages from patients with COPD have greater elastolytic activity at baseline than smokers without COPD which is accentuated by cigarette smoke(31, 32). Neutrophils migrate to the lungs and respiratory tract under the chemotactic influence of IL8 and LTB4. They secrete oxidants as well as serine proteases most commonly neutrophil elastase which contributes to alveolar destruction. The chemotaxis of neutrophils in COPD has been shown to be aberrant (33) which may potentiate the amount of destruction that occurs during migration in the lung. CD8+ T cells are found in increased numbers of the lung parenchyma of patients with COPD(34, 35). Although their role in COPD pathogenesis is not fully understood they have the ability to induce alveolar cell apoptosis by activating FAS-FAS ligand pathways(36). Majo et al demonstrated association between CD8+ cells and apoptosis of alveolar cells in patients with emphysema(36). The interaction between inflammatory cells and cytokines in the pathogenesis of COPD is summarised in figure 1.3.

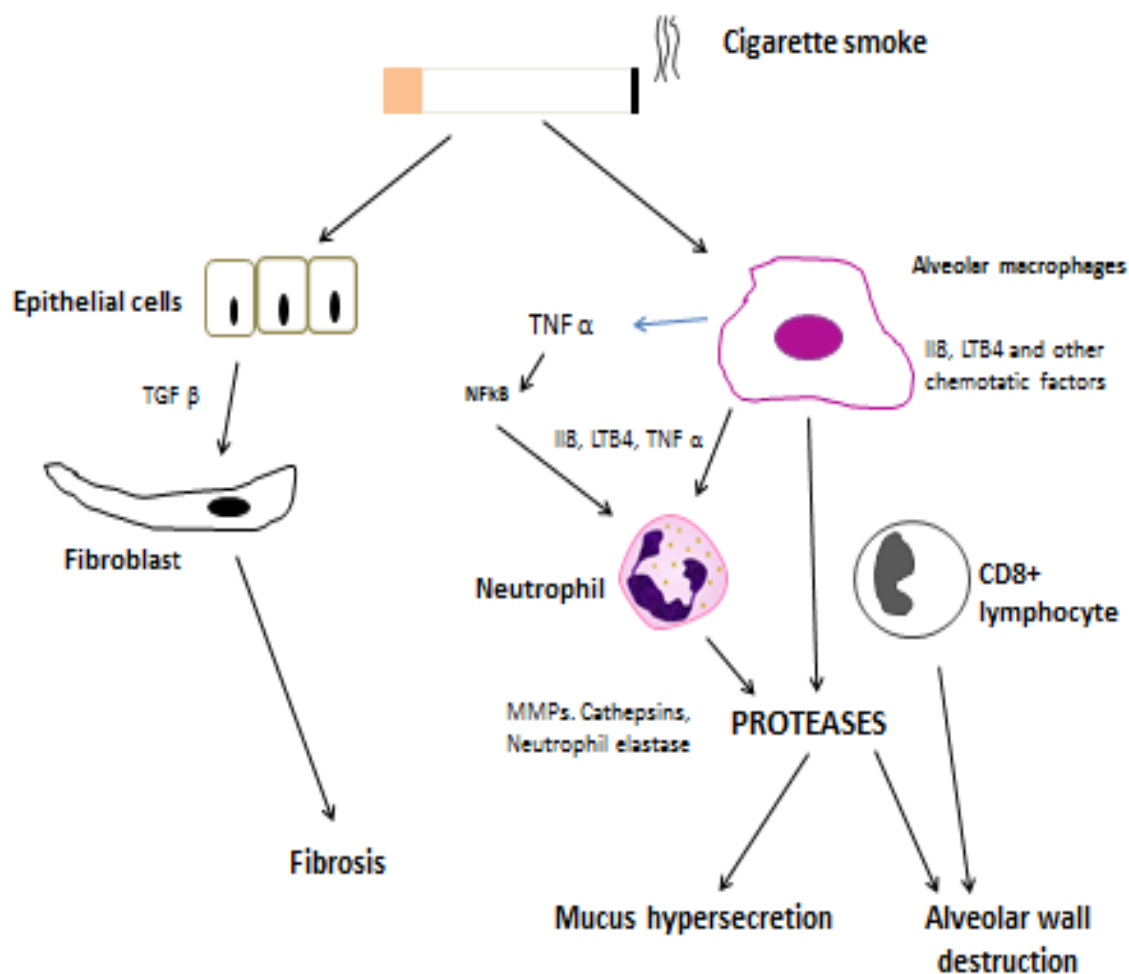


Figure 1.3: Inflammatory cells and cytokines involved in COPD pathogenesis.

1.2.1 Protease-Anti protease imbalance

The concept of protease-anti protease balance contributing to pathogenesis in COPD arose from early studies of alpha one antitrypsin (AAT) deficient patients developing early onset emphysema(37). AAT is an anti-protease which predominantly acts to block the action of neutrophil elastase (NE). Proteases break down the extracellular matrix within

the lung parenchyma. One of the key components is elastin and its destruction reduces the elasticity of the lung parenchyma and development of emphysema. There are two main types of proteases; serine (NE and proteinase 3) and cysteine (cathepsins and MMPs). Each protease is inhibited by one or more anti-proteases (table 1.2).

Protease	Antiprotease
Neutrophil elastase Proteinase 3	α 1 antitrypsin
Cathepsins	Secretory leukoprotease inhibitor
MMPs	Tissue inhibitors of MMPs

Table 1.2: Protease and antiproteases involved in the pathogenesis of COPD.

MMPs are increasingly recognised to play a role in COPD pathogenesis(38). They predominantly break down collagen and gelatin; other components of the extracellular lung matrix and are inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs) (39, 40). Previous studies have demonstrated increased activity of MMP 9 in the lung parenchyma of patients with COPD (40). Animal models support the role of MMPs in pathogenesis as mice lacking MMP9 are protected from small airway fibrosis(41) and those lacking MMP12 are protected from cigarette smoke induced emphysema(42).

1.2.2 Oxidative Stress

There is considerable evidence for the role of oxidative stress in patients with COPD (43-45). Oxidative stress occurs when reactive oxygen species (ROS) such as O_2^- and hydrogen peroxide are produced in excess or overwhelm that of natural antioxidant defence mechanisms. This has deleterious effects and results in damage to tissue by mechanisms including lipid peroxidation, tissue remodelling and DNA damage. Cigarette smoke is a

potent source of oxidants, which can directly damage elastin and collagen(46). Furthermore, the interaction between cigarette smoke, epithelial cells and inflammatory cells increases ROS formation(47, 48). In addition to the direct damage of elastin and collagen, oxidants detrimental effects are also mediated by inactivating anti-proteases but activating certain MMPs(49, 50).

Studies have shown several markers of oxidative stress, for example, hydrogen peroxide, myeloperoxidase (51)and markers of oxidative tissue damage, such as 8-isoprostane 1(52)to be elevated in exhaled breath or exhaled breath condensate from patients with COPD. The major antioxidants in the respiratory tract lining fluid include reduced glutathione, uric acid, vitamin E and ascorbic acid(53). Reduced glutathione production is upregulated in response to oxidative stress in healthy subjects(54). Interestingly studies have shown that glutathione is elevated in BAL fluid from chronic smokers(43, 55) which may suggest that up-regulation of antioxidants occur in an attempt to restore the oxidant anti-oxidant imbalance.

1.3 Management

The treatment of COPD is multifaceted; involving patient education, exercise programs and pharmacological therapy. The main aims are to improve patient's health status, exercise tolerance and reduce patient's symptoms and exacerbations. Patient education of the disease process and recognition of changing symptoms are important. Acute exacerbations are defined as a sustained increase in cough, dyspnoea or sputum production beyond normal day to day variability and have deleterious impacts on patient health related quality of life (HRQoL) and lung function(56, 57). Thus patient recognition

of changes in their symptoms can allow prompt treatment to mitigate these effects. The roles of both pharmacological and non-pharmacological therapies are discussed below.

1.3.0 Non Pharmacological

Pulmonary rehabilitation (PR) is an individualised exercise programme with an educational component and it has established itself as a key non-pharmacological treatment in COPD. In the UK PR programmes are of between 6-12 weeks duration and usually include 2 supervised sessions per week. PR is undertaken in stable patients and recent evidence suggests benefit in those who have had recent exacerbations(58). The principal training methods are endurance (continuous or interval) and resistance training as the benefit in muscle strength from a combination of training modalities seem to be greater than endurance alone(59). The educational components of PR are targeted towards smoking cessation, general information about COPD, self-management and decision making during exacerbations(3).

1.3.1 Pharmacological

The spectrum of pharmacological therapy within COPD is widening(60); however the mainstay of treatment encompasses bronchodilators, inhaled corticosteroids and oxygen therapy. Bronchodilators are broadly subdivided into Beta 2 agonists and anti-muscarinic agents. Their action reduces dynamic hyperinflation by reducing air trapping and facilitates emptying of the lungs. This improves inspiratory lung volumes which reduces breathlessness and improves exercise capacity. Short and long acting bronchodilators are used, with long acting bronchodilators having the advantage of once to twice daily administration which has been suggested to aid compliance. Inhaled corticosteroids (ICS)

target airway inflammation and although this is thought to be predominantly neutrophilic in COPD(61) inhaled corticosteroids have been shown to reduce acute exacerbations and improve HRQoL when combined with long acting bronchodilators when the FEV1 % predicted is <60%.(62). These therapeutic agents are introduced in a stepwise manner either as symptoms or airflow limitation progresses(7) (figure 1.4).

The advent of dual bronchodilators LABA/LAMA is likely to change current guidance. They have been shown to be superior to combination LABA/ICS in improving breathlessness and to reduce exacerbations in landmark clinical trial (63). Notwithstanding current guidance, the concept of defining a patient's "treatable traits" has developed over the last few years(64). This management strategy guides treatment based on a phenotypic presentation or understanding of the causal biological pathway. As such patients with chronic bronchitis defined as persistent productive cough for more than 3 months over 2 consecutive years benefit from the addition of anti-inflammatory agents such as Roflumilast to their treatment regimen whilst COPD patients without this phenotype do not. Roflumilast has subsequently been licensed by NICE as add on to triple therapy (LABA/ICS and LAMA) in this group. Review of previous clinical trials have shown that LABA/ICS treatment is likely to be more effective in reducing exacerbations and slowing lung function decline in COPD patients with eosinophilic inflammation(65) (a peripheral blood eosinophil count of >2%); a hypothesis that has been tested in recent clinical trials(63, 66). Here, the higher blood eosinophil count acts as a biomarker to identify patient who would derive greater benefit particularly from the inhaled corticosteroid in the LABA/ICS combination. Thus, the use of guidelines in concert with

the concept of treatable traits is likely to offer greater precision in the choice of therapy for patients and will likely translate to improved outcomes.

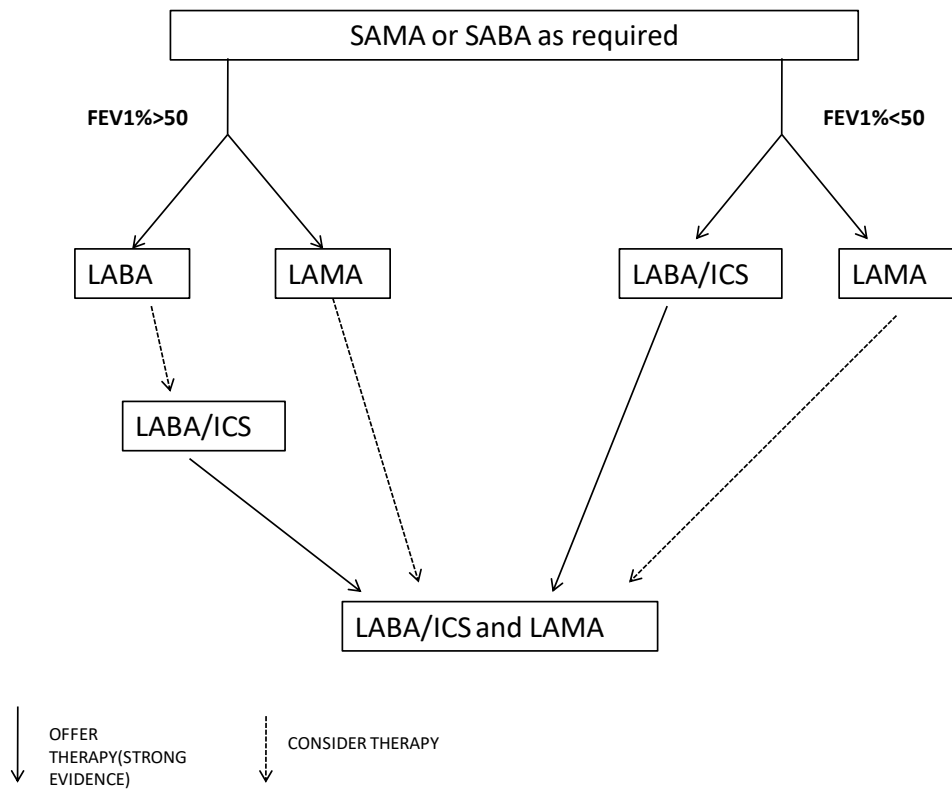


Figure 1.4: NICE guidelines for COPD management. SAMA-short acting muscarinic antagonist, SABA-short acting beta 2 agonist, LABA-long acting beta 2 agonist, LAMA-long acting muscarinic antagonist.

1.3.2 Interventional

Interventional treatments are used as an adjunct to both pharmacological and non-pharmacological treatments or in advanced disease in certain patients. Lung volume reduction surgery (LVRS) is a procedure whereby parts of the lung are resected in an attempt to reduce hyperinflation. It has been shown to improve exercise capacity and

survival in patients with upper zone predominant emphysema and low baseline exercise capacity(67). Other selection criteria for LVRS include FEV₁>20, DLCO>20% and PaCO₂<7.3kPa(21). Lung volume reduction using similar selection criteria can also be undertaken bronchoscopically; a recent meta-analysis (68) concluding it is a safe and effective treatment for patients with emphysema. Finally, lung transplantation in carefully selected patients with very severe COPD transplantation has been shown to improve functional capacity and quality of life outcomes (69).

1.4 Oxygen in COPD

Hypoxaemia is a hallmark feature of COPD as in many individuals gaseous exchange becomes more impaired as the disease progresses, leading to hypoxemia at rest and in some individuals on exercise alone. The mechanisms underlying this are multifactorial and include diffusion limitation, ventilation/perfusion mismatches and shunting. Despite current advances in medical therapy oxygen therapy in the form of LTOT (long term oxygen therapy) is the only treatment in COPD which has demonstrated a mortality benefit in hypoxaemic individuals with COPD(70, 71). Oxygen therapy however, is also used outside of this setting to improve exercise capacity or ameliorate dyspnoea. Use of oxygen therapy during exercise has been shown to reduce dynamic hyperinflation and thus reduce dyspnoea(72). Other postulated mechanisms by which supplemental oxygen exerts beneficial effects are reductions in anaerobic respiration of peripheral muscles; indicated by reduced blood lactate levels(73) and possibly reduced peripheral chemoreceptor activity(74).

1.4.0 Long Term Oxygen Therapy (LTOT)

The indications to use oxygen therapy in hypoxaemic individuals with COPD are derived from two landmark trials (NOTT, MRC) published in the 1980's(70, 71). The MRC was a randomised controlled trial (RCT) designed to assess whether the use of supplementary oxygen for 15hrs/day compared to no supplemental oxygen conferred a survival advantage over 3 years. 87 participants with severe COPD, hypoxaemia ($\text{PaO}_2 < 8\text{kPa}$) and hypercapnia ($\text{PaCO}_2 > 7.4$) were enrolled in which oxygen was administered at 2L/min or higher (to achieve $\text{PaO}_2 > 8\text{kPa}$). Results showed improved survival in those randomised to oxygen vs controls ($p < 0.05$). The NOTT trial provided further evidence for the use of supplementary oxygen in resting hypoxaemia in COPD. This RCT assessed whether continuous supplemental oxygen improved survival compared to nocturnal oxygen alone. Enrolled participants had resting hypoxaemia ($\text{PaO}_2 < 7.3$) or $\text{PaO}_2 < 8\text{kPa}$ in the presence of polycythaemia or electrocardiographic evidence of right heart strain. Results demonstrated a survival advantage with continuous oxygen compared to nocturnal ($P = 0.01$). Of the important secondary outcomes there was significant reduction in polycythaemia and pulmonary vascular resistance(75). Kaplan Meier survival curves for the LTOT trials are shown in figure 1.5.

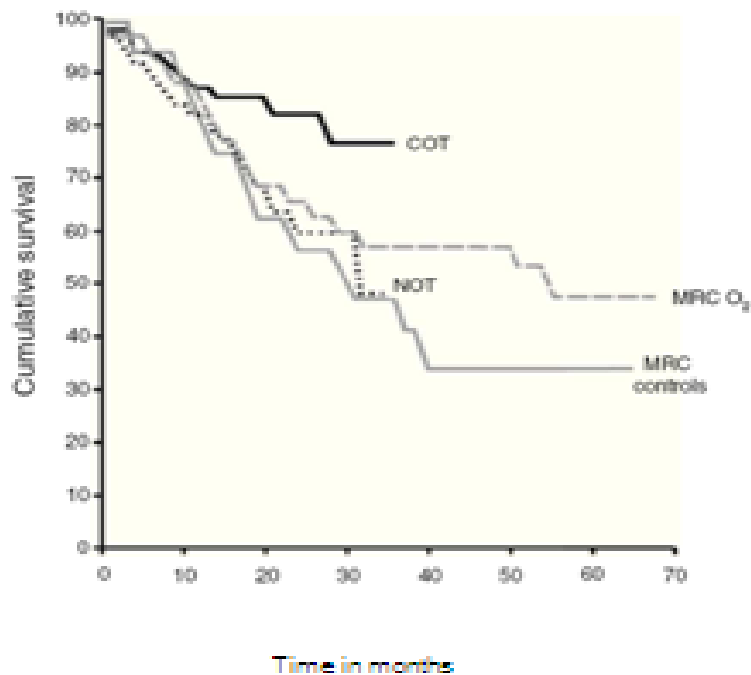


Figure 1.5: Kaplan Meier survival curves for LTOT trial. COT-continuous oxygen therapy, NOT-nocturnal oxygen therapy COT-Continuous oxygen, NOT-Nocturnal oxygen MRC (Medical research council)

Long term oxygen therapy (LTOT) is indicated in stable hypoxaemic COPD when:

- PaO₂ ≤ 7.3kPa
- PaO₂ 7.3-8 kPa in the presence of pulmonary hypertension, nocturnal hypoxaemia or raised haematocrit.

In contrast to the above studies supplemental oxygen therapy has not been shown to improve survival in moderately hypoxaemic individuals with COPD(76) (77). The study by Albert et al(77) which initially recruited patients with stable resting desaturation was re-designed to include those with exercise induced desaturation. It did not demonstrate any benefit of supplementary oxygen with regards to improved survival, reduction in hospital admissions or exercise capacity.

1.4.1 Ambulatory Oxygen Therapy (AOT)

Ambulatory oxygen therapy, defined as the use of supplementary oxygen during activities of daily living is used in patients who meet LTOT criteria(78). It is also used in non-hypoxaemic patients whom exhibit exercise induced desaturation if there is a demonstrable improvement in exercise capacity or breathlessness scores(78). Theoretically the use of ambulatory oxygen therapy (AOT) to mitigate desaturation on exertion may improve oxygen utilisation in peripheral muscles, reduce dynamic hyperinflation (79) and reduce dyspnoea.

Exercise Induced desaturation

Patients with COPD experience exercise induced desaturation (EID) in an unpredictable fashion, exhibiting variability both in the magnitude and temporal onset of EID with mobilisation(80). There is no uniform definition of EID however in the UK it is defined as fall in oxygen saturations by $\geq 4\%$ (nadir $\leq 90\%$)(81). In the United States the nadir of $< 88\%$ is used and forms the basis for the remuneration policy employed by Medicare. Evidence from two studies(82, 83) suggests that the prevalence of EID increases with increasing severity of airflow obstruction and is associated with worse HRQoL, higher mortality and is likely to contribute to exercise limitation in COPD(82). A number of studies(80, 84) have attempted to propose predictors of EID in COPD without consistent consensus. Nevertheless, reduced pulmonary physiology (DLCO $< 50\%$ and FEV1 $< 45\%$), baseline arterial PaO₂ $< 10\text{kPa}$ and female gender seem to be the strongest predictors of EID(80).

The precise mechanism leading to arterial hypoxaemia and desaturation is thought to be due to altered pulmonary physiology. During exercise both ventilatory demand and ventilatory workload are increased. Ventilation and perfusion within the lung are crucial

for normal arterial oxygen haemostasis. This is deleteriously affected in patients with COPD either due to airflow limitation or parenchymal destruction; in those with co-existing emphysema. Low ventilation/perfusion ratio areas within the lung in addition to worsened alveolar deadspace significantly contribute to hypoxaemia in COPD especially during exercise(85, 86). This is potentiated by dynamic hyperinflation which occurs during exercise and which worsens alveolar deadspace and thus hypoxia. Adverse pulmonary mechanics; raised inspiratory capacity and dynamic hyperinflation may contribute to sensation of breathlessness and exercise limitation(87). The impact of exercising muscle is also important. A number of studies have demonstrated higher lactic acid generation at lower workloads in patients with COPD(88). Theoretically arterial hypoxaemia may exacerbate peripheral muscle oxygen utilisation, increase lactic acid production and cause peripheral muscle fatigue. This is supported in one study in which the degree of quadriceps fatigue correlated with peak oxygen utilisation(89). Interestingly muscle afferents in COPD have been shown to be abnormal and may mediate enhanced respiratory effort and perception of dyspnoea(90). Since AOT has some effect on dynamic hyperinflation and will improve arterial oxygenation in areas of V/Q mismatch and potential muscle oxygenation it may provide a means to improve exercise intolerance in selected patients. Increasing arterial oxygenation will also directly inhibit carotid body stimulation reducing the sensation of dyspnoea which has been shown to limit exercise capacity.

AOT assessment

In the assessment of ambulatory oxygen therapy exercise capacity is assessed using specific field tests. The three main types of field tests are: 6 minute walk test (6MWT),

endurance shuttle walk test (ESWT) and incremental shuttle walk test (ISWT). Importantly cycle ergometry should not be used in the assessment of ambulatory oxygen(81). The magnitude of desaturation is greater in walking field test compared to cycling. The study by Mahler et al demonstrated that enhanced alveolar ventilation and subsequent higher partial pressure of alveolar oxygen during cycle ergometry minimised the decrease in arterial oxygen and thus had a “protective” effect against desaturation(91).

Field Tests

The 6MWT is a practical and simple test which has been shown to be a predictor of mortality and morbidity in COPD(92). The test is self-paced in which participants are instructed to walk up and down a 20-50metre corridor for 6 minutes.

“The object of this test is to walk as far as possible for 6 minutes. You will walk back and forth in this hallway. Six minutes is a long time to walk, so you will be exerting yourself. You will probably get out of breath or become exhausted. You are permitted to slow down, to stop, and to rest as necessary. You may lean against the wall while resting, but resume walking as soon as you are able.”(93)

The distance walked, number of stops, Borg dyspnoea score (table 1.3) and oxygen saturations (baseline and nadir) are noted. There is some debate regarding whether a practice walk is needed as in many individuals there is a learning effect with an increase 6MWD on the subsequent by as much as 17% of the original distance (93). Evidence suggest that the 6MWT is less sensitive to change with interventions such as bronchodilators(94, 95)than other exercise tests. Nevertheless it provides robust information of functional status and relates well to activities of daily living (96). As stated

although there has been debate about the use of practice walks updated guidance suggest that they should be undertaken if the 6MWT is being used to measure change over time(97).

Borg Score	
0	Nothing at all
0.5	Very, very slight (just noticeable)
1	Very slight
2	Slight (light)
3	Moderate
4	Somewhat severe
5	Severe(heavy)
6	
7	Very Severe
8	
9	
10	Very, very severe (maximal)

Table 1.3 Borg Dyspnoea Scale

The ISWT uses a 10m course and the walking speed is externally paced by signals from an audio cassette. Incremental increases in walking speed occur each minute, to a point at which the test is terminated because of; breathlessness, the inability to sustain the walking speed or achievement of 85% of maximal heart rate. The ISWT has been shown to produce maximal response in patients with COPD(98)and is responsive to interventions of exercise training. The ESWT is also externally paced whereby participants walk for as long as possible performed at a speed 85% of their maximal capacity (calculated from ISWT). The test enables participants to achieve standardisation of exercise intensity

which is sustained until limited by symptoms. The ESWT has been shown to be repeatable(99) and responsive to interventions of exercise training and bronchodilators.

During ambulatory oxygen assessments participants will perform one of these tests on air and is then repeated with oxygen therapy. Patients are deemed to qualify for AOT if there is a 10% increase in exercise capacity or reduction in Borg score of 1(81) .

The mode of delivery of ambulatory oxygen therapy is variable but can broadly be divided into liquid oxygen against compressed cylinder oxygen delivered via continuous flow devices or oxygen conserving devices. Oxygen conserving devices can be further subdivided to Demand oxygen devices (DODs) or Pendant reservoir cannula (PRC). DODs deliver oxygen during inspiration only whilst PRC are nasal cannulae with a storage reservoir which increase the proportion of oxygen delivered during inspiration theoretically allowing lower flow rates to be used. Oxygen conserving devices deliver oxygen only during inspiration whereas continuous flow systems deliver oxygen throughout the respiratory cycle. Cylinder types can also be further subdivided into lightweight systems some of which are refillable. During the test the participant should carry, push or pull the ambulatory oxygen system alone without help from the assessor.

Ambulatory oxygen does have an acute effect in improving exercise capacity. The 2005 Cochrane review demonstrated clear benefit of short term ambulatory oxygen in improving exercise capacity in moderate to severe COPD(100). However, the review encompassed mainly single assessment studies whereby patients underwent a modality of exercise testing with ambulatory oxygen and compressed air generally over 1 day. Whether the acute improvement in exercise capacity is sustained has not been proven. Interestingly in a subsequent Cochrane review in which ambulatory oxygen was

evaluated in the context of pulmonary rehabilitation programs no benefit was demonstrated(101). Given that most pulmonary rehabilitation programs run longer than 6 weeks it is unclear whether longer term use of ambulatory oxygen still provides continued benefit in improving functional exercise capacity.

1.4.2 Short Burst oxygen Therapy (SBOT)

SBOT is used for the symptomatic relief of dyspnoea in patients who do not meet LTOT or AOT therapy criteria. It is commonly used prior to or following short periods of exertion in patients with COPD. The use of SBOT is controversial and studies investigating its effects have been limited by inconsistency of exercises performed, outcome measures and heterogeneous patient selection. Nevertheless early studies by Woodcock et al(102) and Evans et al(103) have shown some benefit of SBOT by improving submaximal treadmill distance and shortened dyspnoea recover time post exercise respectively. Interestingly however, the study by Evan et al(103) specifically tested the reproducibility of SBOT's ability to improve dyspnoea recovery time. Despite testing the subjects who responded to SBOT initially reproducibility was poor. Subsequent studies(104-106) have shown no benefit in SBOT's ability to improve dyspnoea, exercise tolerance (6MWT) or HRQoL. The largest of these (106) in which 78 patients recently discharged following an exacerbation were randomised to SBOT (2l/min) or cylinder air for 6 months to be used to relieve distressing dyspnoea. SBOT did not improve HRQoL, hospital readmission or healthcare utilisation.

1.5 Muscle disease in COPD

Although characterised by airflow obstruction many patients with COPD exhibit systemic manifestations such as muscle dysfunction, loss of fat free mass, cardiovascular disease(107), diabetes(108), depression and osteoporosis(109). Skeletal muscle dysfunction is characterised by reduced muscle strength and or endurance(3) and is an important systemic manifestation in COPD which contributes to the morbidity of the disease. Although SKMD can occur in upper limbs and to some degree respiratory muscles this thesis concentrates on skeletal lower limb muscle in patients with COPD. One of the key observations is that SKMD is likely to have a detrimental impact on exercise capacity. Exertional dyspnoea and reduction in exercise capacity are common features of COPD, however studies of patient self-reported symptoms suggest that perceived leg effort or fatigue were the predominant limitations to their exercise ability (110, 111). Interestingly in lung transplant recipient patients exercise capacity remains abnormally low, by as much as 50% predicted workload for age and gender despite normalisation of lung function(112). Also pulmonary rehabilitation provides significant improvement in exercise capacity without significant change in lung function (59). These observations taken together suggest that SKMD plays an important role in exercise performance. Furthermore SKMD is recognised to contribute independently of lung function to mortality (113). Reduced mid-thigh cross sectional area and quadriceps strength have been shown to be strong predictors of mortality after adjusting for FEV1, age and sex(113-115). Finally SKMD is likely to contribute to poor health status and healthcare utilisation (116, 117).

1.5.0 Skeletal Muscle Structure

Skeletal muscle is composed of a motor unit (figure 1.6); the muscle fibre and its innervating motor neuron. The functional units of muscle are called sarcomeres. Within each sarcomere are the myofibrillar proteins myosin and actin. The myosin heavy chain in the head region contains an adenosine triphosphate (ATP) binding site for the conversion of ATP into ADP (adenosine diphosphate) and phosphate. The heavy chains contain the myosin heads that form cross bridges with actin which facilitate muscle contraction.

Muscle fibres are classified as fast twitch or slow twitch depending on their contractile speed. Fast twitch fibres are further subdivided to fast twitch fatigue resistant, fast twitch intermediate and fast twitch fatigable. This classification corresponds broadly to muscle fibre identification by myosin heavy chain (MHC) immunoreactivity and myofibrillar ATPase staining (table 1.4).

Fibre	ATPase	MHC
Slow twitch	I	MHCI
Fast twitch fatigue Resistant	IIa	MHCIIa
Fast twitch Fatigable	IIb	MHCIIx/d
Fast twitch Intermediate	IIx	

Table 1.4: Muscle fibre classification

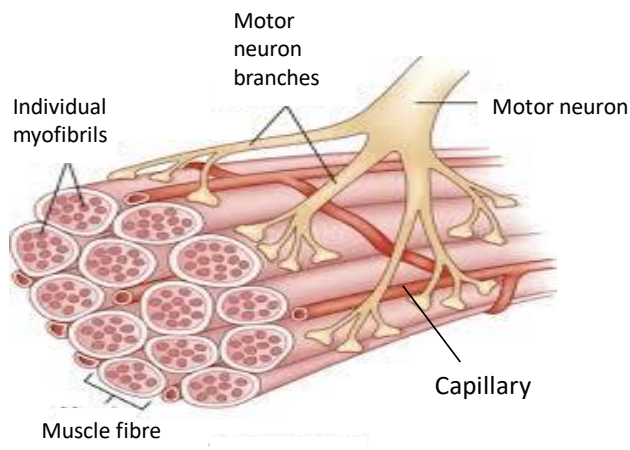


Figure 1.6: The muscle motor unit

1.5.1 Clinical Observations

Skeletal Muscle Strength and Endurance

Skeletal muscle strength is defined as the capacity of the muscle to generate force. This is mediated by three factors; “cross sectional area of the muscle fibres within the motor unit, the number of muscle fibres innervated by the motor neuron and the specific force of the muscle fibre” (118). In general fast twitch intermediate and fatigable generate more force per unit area than slow twitch or fast twitch fatigue resistant (118). The force generated by each fibre depends on intracellular calcium levels, which are important for sarcoplasmic reticulum excitation/contraction and also neuronal activation. The contribution of neuronal activation to muscle strength is important as in the early stages of muscle training gains in strength are mediated by neural adaptation rather than by muscle hypertrophy(119). In COPD especially in severe disease quadriceps muscle strength may be reduced by as much as 30%(120, 121). This difference however

disappears when quadriceps strength is normalised to mid-thigh cross sectional area, implying therefore that muscle atrophy is important in determining muscle strength.

Endurance relates to the ability of muscle to maintain a certain force over time; normally a percentage of maximal force or exercise capacity. In health, oxygen delivery and utilisation rather than ventilatory function or compromised cardiac function limits endurance. This is a complex interplay between muscle enzyme concentration, capillarity, mitochondrial volume density and bioenergetics(118). The energy for skeletal muscle contraction is derived from the de-phosphorylation of ATP. Low intracellular stores of ATP mean that skeletal muscle can only sustain contraction for a few seconds before this is replenished or further ATP generated. One mechanism is the breakdown of Phosphocreatine to phosphate and creatine mediated by the enzyme creatine kinase. Other more effective mechanisms are that of oxidative phosphorylation, Krebs Cycle and to a lesser degree glycolysis. Skeletal muscle endurance is also perturbed in COPD. A number of studies have reported a reduction in quadriceps endurance in patients with COPD(122, 123). Interestingly in one of these studies(122) an artificial stimulation protocol was used thus negating the impact of central fatigue.

Fatigue is another determinant of endurance, of which two types are recognised; central and peripheral. Central fatigue indicates contractile reserve within the muscle, in that additional force can be generated by nerve stimulation in the event of voluntary “task failure”. Peripheral fatigue implies that there is no contractile reserve within the muscle which may be due to transmission at the neuromuscular junction, muscle membrane or muscle microfilaments. There is a complex interplay between central and peripheral fatigue which is not completely understood. In health several studies(124, 125) have

shown that arterial oxygen concentration affects central fatigue and peripheral fatigue; both deleteriously affected by hypoxia. The study Amann et al suggest that central fatigue is likely to be the dominant of the two(126). The study used varying oxygen concentrations; normoxia to hypoxic (15% Oxygen) to show that exercise performance switches from a predominantly peripheral origin during normoxia to central origin during increasing hypoxia. It is highly likely that lowered arterial oxygen concentration may play a role in limiting exercise tolerance in patients with COPD in this manner.

1.5.2 Laboratory observations

Muscle mass

Muscle mass homeostasis is a dynamic process involving a balance between protein synthesis and protein degradation. Protein synthesis is mediated by hypertrophic signalling and protein degradation by atrophic signalling and autophagy. Anabolic hormones, namely Insulin and Insulin like growth factor 1 (IGF-1) stimulate the hypertrophic signalling pathway. They act on the phosphoinositide 3 kinase (PI3 K)/AKT (protein kinase B) pathway(127). AKT in its phosphorylated form stimulates mTOR (mammalian target of rapamycin) which can promote protein synthesis by activation of the 70-kD ribosomal S6 protein kinase (p70S6K) and inhibition of 4E-BP1 (eukaryotic initiation factor 4E binding protein respectively. AKT also directly inhibits glycogen synthase kinase-3 (GSK-3), which represses protein synthesis(127). Atrophy signalling is mediated by the ubiquitin proteasome pathway. Proteins identified for degradation are attached to ubiquitin by E3 (ubiquitin ligating) enzymes of which two have been identified; atrogin 1 (muscle atrophy F-Box) and MuRF1 (muscle ring finger-1). MuRF1 is stimulated upstream by pro-inflammatory cytokines largely mediated by NF- κ B. Atrogin

1 is stimulated upstream by p38 MAPK (mitogen activated kinase), for which oxidative stress and inflammation are potent stimuli. There is a degree of cross talk between these pathways. AKT can block protein degradation by down regulating atrogen 1 and MuRF-1. This is mediated by phosphorylation of FoxO (forkhead box O) transcription factor. The atrophy and hypertrophy pathways are summarised in figure 1.7.

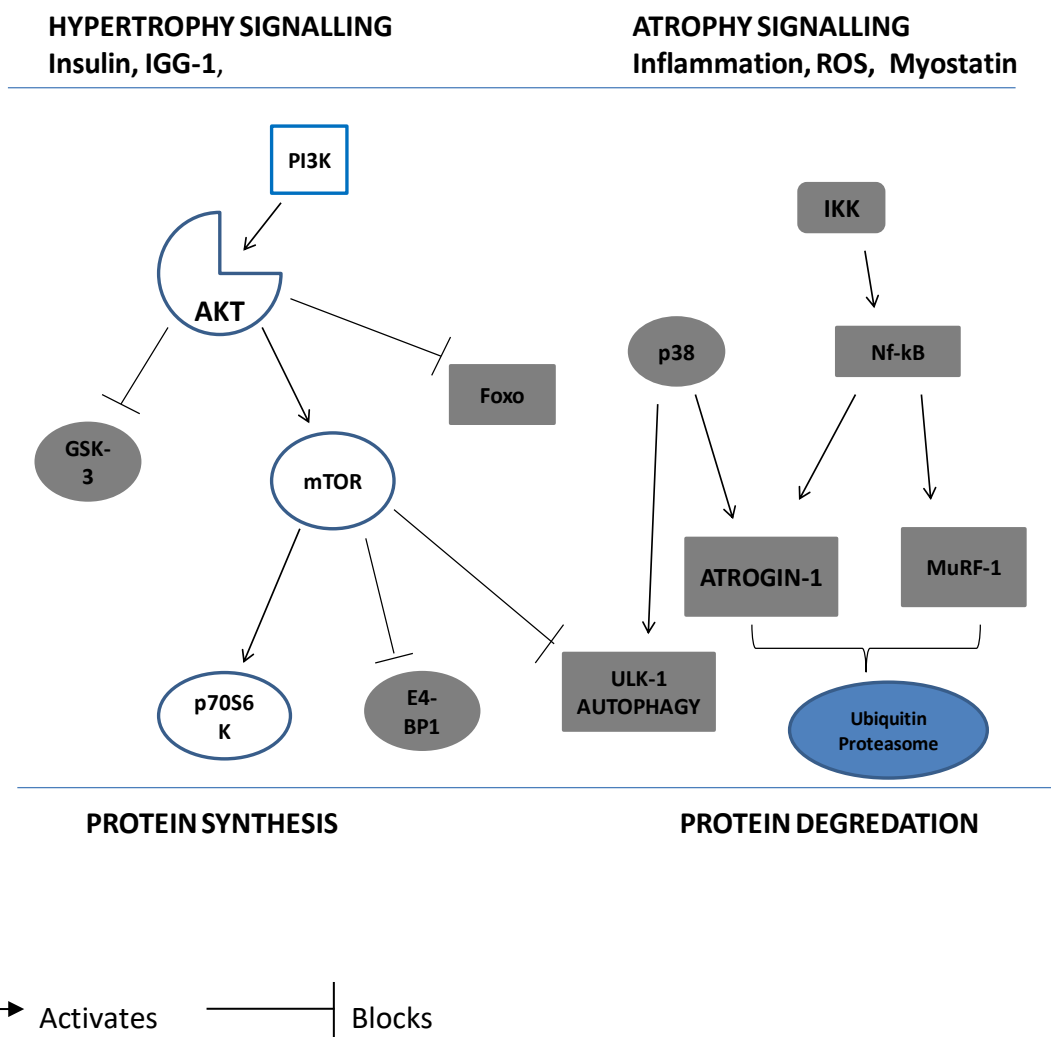


Fig 1.7: Hypertrophy and atrophy signalling pathways in muscle.

Autophagy is a constitutively active process in limb skeletal muscle(118) and is the process whereby proteins are degraded in autophagosomes. Autophagosomes are then delivered to the lysosomes for degradation of their contents. Autophagy is initiated by the ULK1 complex which is under the control of AMP-activated protein kinase (AMPK) and mTOR pathways(128). mTOR phosphorylates and inactivates ULK1 thus inhibiting autophagy whilst AMPK is able to inactivate mTOR. Although the role of autophagy in skeletal muscle has not fully been elucidated, it has been shown to significantly induced in limb muscles following denervation(129).

SKMD-pathological changes

One of the key structural alterations in skeletal muscle of patients with COPD is that of skeletal muscle atrophy and fibre type shift. Fat free mass (FFM) a surrogate for skeletal muscle mass is reduced in COPD (130). The degree of thigh muscle mass lost is relatively greater to that of the whole body weight indicating a preferential loss of muscle tissue over body tissue in COPD.(121). Cross sectional studies have demonstrated increased mRNA levels of atrogin-1 and MuRF-1 (131, 132) in the quadriceps of COPD patients compared to controls indicating upregulation of atrophic pathways. This however bore no relationship to quadriceps function and no difference was found between COPD patients with and without preserved muscle mass(127). COPD patients have a reduction in proportions of type 1 accompanied by increased proportions of type IIx fibres, which is contrary to what occurs in normal ageing(118). The reduction in type 1 fibres mirrors disease severity. Gosker et al(133) showed that as FEV1 declined the proportion of type 1 fibres also decreased. Type I fibres are advantageous as they confer greater oxidative capacity. Thus the switch to type IIx fibres may explain the increased fatigability and

reduced endurance in COPD limb muscle. Although there is atrophy of all fibre types there is some evidence (134) to suggest this occurs preferentially in the type IIx fibre.

Capillary density is important in skeletal muscle as oxidative metabolism is dependent on the capillary supply and mitochondrial volume density. Capillary density has been shown to be reduced in COPD muscles(135), although this is not a consistent observation(136). This is may be due to the heterogeneity between studies with some carried out in the context of exercise training(136) which is known to improve capillarity.

Finally mitochondrial bioenergetics is altered in COPD with reduced oxidative enzyme capacity. Key enzymes involved in oxidative phosphorylation such as citrate synthase and succinate dehydrogenase have been shown to be reduced(137, 138). In line with these observations expression of PGC-1 α /PPAR (peroxisome-proliferator activated receptors) a regulator of cellular oxidative phosphorylation is reduced in the musculature of COPD patients(139).

1.6 Aetiology of SKMD in COPD

1.6.0 Inflammation

Inflammation is a key concept in COPD and its role in the pathogenesis of the disease has been discussed. It may play a role in SKMD. Whether this is a consequence of systemic and or local inflammation is not completely clear, however a number of studies lend support that pro-inflammatory cytokines can adversely affect skeletal muscle growth. A number of pro-inflammatory cytokines have been implicated in pathogenesis; TNF α , IL-6

and IL-8. Spruit et al demonstrated an inverse relationship between quadriceps muscle strength and serum IL-8 levels(140) albeit during exacerbations, whilst elevated IL-6 levels are associated with radiological evidence of quadriceps wasting in COPD(141). In vitro studies have shown that TNF α induces apoptosis in skeletal muscle cells and myoblasts(142).

The predominant system whereby inflammation is thought to exert its role is by increasing activity of the ubiquitin proteasome pathway via Nf κ B activation. A significant increase in Nf κ B nuclear binding has been observed in the limb muscles of patients with COPD(143). Furthermore Nf κ B has been shown to directly inhibit MyoD a myogenic regulatory factor involved in satellite cell differentiation (144).

1.6.1 Hypoxia

In health, under hypoxic conditions such as at altitude functional, structural and morphological changes in skeletal muscle occurs which bear close resemblance to those found in COPD(145). Skeletal muscle endurance, strength (145, 146) and muscle mass are reduced. Furthermore, evidence suggests that hypoxia may have a deleterious impact on the force of muscle contraction by altering the strength of actin myosin cross bridges(147). Interestingly in this study(147) this contractile dysfunction was reversed following re-oxygenation. Hypoxia has been shown to impair the mTOR pathway(148) and thus may contribute to muscle wasting. Further to this, in vitro studies have shown that hypoxia inhibits myoblast differentiation by degradation of MyoD(149). In addition, tissue hypoxia could also play a part in determining systemic inflammation as there is a relationship between extent of arterial hypoxaemia and circulating levels of TNF α , and its receptors, in COPD patients(150). It may also potentiate oxidative stress (118, 147).

During hypoxia the transcription factor hypoxia inducible factor-1 α (HIF-1 α) is increased which may alter bioenergetic pathways by down and up regulating oxidative and glycolytic enzymes respectively(151). This also mirrors the reduced proportion of type I fibres observed in hypoxaemic patients with COPD compared to non-hypoxaemic patients(152). Finally studies of limb muscle transcriptomic have shown this to be altered in COPD. Turan et al(153) used a systems biology approach and found a failure of COPD limb muscles to co-ordinate several tissue remodelling and bioenergetics pathways. This study suggested that this abnormality is linked to an altered expression of histone modifiers which correlate with peak oxygen uptake, suggesting that cell hypoxia may play a role in SKMD through epigenetic mechanisms.

1.6.2 Oxidative Stress

Mitochondria are the main sources of RNS (reactive nitrogen species); derived from nitric oxide and ROS production especially during skeletal muscle contraction. In normal skeletal muscle ROS and RNS are produced at relatively low levels and play a positive role in normal physiological functions(154). However, in COPD the production of these species is at a higher rate and overcomes the antioxidant ability; this is particularly true during acute episodes of exercise or following exacerbations(118). ROS and RNS may particularly target key contractile proteins and enzymes within muscle fibres(154); implicated in myosin heavy chain carbonylation and reduced creatine kinase enzymes. Further evidence for the role of oxidative stress is provided by the identification of oxidation of proteins, DNA and lipids. COPD patients exhibit higher levels of peroxidation, oxidized glutathione, and protein carbonylation in the blood and limb muscles(137, 155-157). The level of oxidative stress may have functional implications.

Koechlin et al(158) reported that use of n-acetylcysteine (an anti-oxidant) improved quadriceps endurance time in patients with COPD. Oxidative stress may also relate to protein degradation and hence muscle loss. ROS species are able to trigger the ubiquitin proteasome and p38 MAPK pathways (118, 154).

1.6.3 Disuse

Physical activity in patients with COPD is generally lower than the average population with more having a sedentary lifestyle (159). This is thought to be a consequence of deconditioning heralded by the dyspnoea spiral in which patients do not exert themselves to avoid dyspnoea which in turn causes a decline in fitness. Physical inactivity leads to a number of adaptive changes in skeletal muscles including altered mitochondrial biogenesis, reduced capillary density and reduced proportions of type I muscle fibres(139, 160) which all negatively affect strength and endurance. The preferential loss of thigh muscle mass relative to body weight may suggest that muscles that are least used, in this case the lower limbs are more susceptible. This is supported in some respect as other muscles such as the diaphragm shows an increase in oxidative capacity and increase in type I fibres in severe COPD(161).

1.7 Aims

This thesis sets out to explore 3 aims

- 1) Systematic review of ambulatory oxygen in COPD
- 2) To assess the biological response in muscle to oxygen therapy
- 3) To assess the clinical effectiveness of ambulatory oxygen therapy in patients with exercise induced desaturation.

Aim 1

Chapter 2 of this thesis will set out the methodology to explore aim 1. Ambulatory oxygen therapy (AOT) is widely used currently in patients with COPD who exhibit exercise induced desaturation, if they show an improvement in exercise capacity or breathlessness scores. The evidence base for this has come largely from single assessment studies whereby AOT was evaluated generally over one day by a given exercise modality(100). In general the majority of trials to date have shown that use of ambulatory oxygen in this setting is likely to be of benefit, in terms of duration of exercise, dyspnoea on exertion and quality of life. The caveat to this is that many of the trials in the review had small sample sizes with no generation of power calculations with reviewers describing methodological quality as low(100). A subsequent review evaluated AOT use in the context of pulmonary rehabilitation and found no clear benefit(101). Since most pulmonary rehabilitation sessions last longer than 6 weeks the longer term benefit is doubtful and has not been clinically proven. Despite this, continued prescriptions for AOT occur on the basis of eligibility tests demonstrating benefit but without knowledge of its longer term efficacy. Furthermore, it remains less clear

whether domiciliary ambulatory oxygen use is beneficial and whether there are long term gains with regard to exercise capacity or quality of life in the patients who use it in this way. Finally, neither of the reviews explored the economic impact of AOT which in the current climate of assessing cost effectiveness of interventions is an important factor. Thus, there are several unanswered questions; does the acute effect of ambulatory oxygen on exercise capacity persist? Does use of AOT provide clinical benefit in a domiciliary setting and is AOT cost effective? Using 3 linked systematic reviews specific objectives were to explore the:

1. The clinical effectiveness of long term ambulatory oxygen therapy (defined by more than 6 weeks duration) in patients with COPD exhibiting EID or exertional dyspnoea who do not meet the criteria for LTOT.
2. The relative clinical effectiveness of different methods of delivering ambulatory oxygen in COPD patients and the optimum mode of delivery of the intervention;
3. The cost-effectiveness of ambulatory oxygen in COPD patients not on LTOT compared to usual care and the relative cost-effectiveness of different methods of delivering ambulatory oxygen.

Chapter 3 details the results of the systematic review; whether AOT has any effect on exercise or quality of life outcomes. It also discusses if the effects of AOT on exercise are field test specific.

Aims 2 and 3

Chapter 2 of this thesis also sets out the methodology and statistical methods used to investigate the skeletal muscle biological response and clinical effectiveness of AOT in patients with exercise induced desaturation (EID). EID is defined as a nadir $<88\%$ (81) on

ambulation is common in patients with chronic obstructive pulmonary disease (COPD). It affects patients with advanced and less advanced disease with evidence suggesting that they have worse health related quality of life, mortality and exercise tolerance(83) than non-hypoxaemic patients who do not exhibit this phenomenon(82). The reason for observed exercise limitation is unclear and hypotheses have been discussed in section 1.4.1 but is thought to reflect muscle hypoxia exacerbated by exertion. It is well established that hypoxia has a role in perturbing muscle function, e.g. impairing protein synthesis and myogenesis by inhibiting the mTOR pathway(148) and contributes to muscle wasting *via* degradation of MyoD(149). Hypoxia has also been linked to mitochondrial dysfunction(151) and a reduction in the proportion of Type I muscle fibres(152), suggesting a reduced endurance exercise capacity, which may be exacerbated by the oxidative stress that is increased in skeletal muscle mitochondria on exposure to acute hypoxaemia(162). Inflammation is a key concept in COPD with a number of cytokines (discussed in 1.6.0) implicated in underlying aetiology of skeletal muscle abnormalities. Whilst numerous studies have assessed the impact of AOT on exercise performance(163), none have assessed the longer term effect of AOT on skeletal muscle gene expression, systemic inflammation or physical activity. This is explored via a 12-week randomised controlled cross over trial with 2 week washout for which the methods and rationale of trial design are outlined in Chapter 2.

Chapter 4 documents the clinical results from the randomised controlled trial and discusses the impact of AOT on functional exercise capacity, physical activity and the inflammatory cytokine response to AOT.

Chapter 5 documents the skeletal muscle response to oxygen which has never been investigated previously. The muscle biopsies were subject to microarray analysis to explore differentially expressed genes or gene sets between oxygen and air. This enabled the identification of genes or gene sets involved in important skeletal muscle transcription. A hypothetical network interaction has been created between the clinical data set including exercise capacity, systemic inflammation and quality of life and the transcriptional changes seen in skeletal muscle.

Chapter 6 concludes the thesis.

Chapter 2

General Methods

2.1 Systematic Review

The review had 3 objectives, the methods for which had a degree of overlap but will be described separately here. As with all systematic reviews achievement of the objectives has been limited by the type and quality of the available published evidence. The objectives to assess were as follows:

Objective 1 - The clinical effectiveness of ambulatory O₂ in COPD patients not on LTOT compared to usual care.

This was to determine the benefit of the addition of ambulatory O₂ to treatment regimens.

Objective 2- The relative clinical effectiveness of different methods of delivering ambulatory O₂ in COPD patients not on LTOT.

This was to determine the optimum mode of delivery of the intervention.

Objective 3- The cost-effectiveness of ambulatory O₂ in COPD patients

This included the cost-effectiveness of ambulatory oxygen in those not on LTOT compared to usual care, and the relative cost-effectiveness of different methods of delivering ambulatory O₂.

For each objective the methods below describe the types of study included in the review, the population, intervention, comparator and outcomes assessed. This is the standard format for systematic review questions according to Cochrane guidance

2.1.0 Objective 1 Clinical Effectiveness of Ambulatory Oxygen compared to Usual Care

Study Design

Randomised Control trials (RCTs), non RCTs and Cross over studies have been included. All studies had a follow up duration of more than 6 weeks. Previous reviews(100, 101) have either been of less than 6 weeks duration or single assessment studies limiting their ability to demonstrate longer term benefit. Therefore any study with duration of less than 6 weeks was excluded.

Population

The population for this objective included adult (18 years or over) patients suffering from stable chronic COPD but not on LTOT. The definition of COPD chosen was broad; any study description of patients defined as having COPD by spirometry (NICE)(7), radiological emphysema or chronic bronchitis, were included. 'Stable' was defined as not currently undergoing, or within 6 weeks of, an exacerbation of their disease. This definition was chosen because the majority of patients with exacerbations demonstrate recovery of symptom scores and lung function within this time frame(18, 164).

Interventions

Ambulatory oxygen therapy was defined as the use of supplemental oxygen during exercise and activities of daily living, consistent with the definition used in BTS guidelines (81).

Comparators

There were several suitable comparators, all of which were equivalent to medical air. Thus compressed air or gaseous mix equivalent to air were suitable, provided they were administered via a similar route to the intervention. Only studies in which the treatment difference between study arms was purely due to use of ambulatory oxygen were considered.

Outcomes

The primary outcomes were exercise capacity as measured but not limited to 6MWT distance, shuttle test distance and maximal power tests. Health related quality of life (HRQoL), rates of exacerbations, hospitalisation and/or health service utilization were also considered.

2.1.1 Objective 2 Clinical Effectiveness of methods of delivering ambulatory Oxygen.

The criteria for population, intervention and outcomes remained the same as objective 1 with the inclusion of:

- Patients who met LTOT criteria
- Studies with follow up of any duration included.

The rationale for this is as follows. The use of ambulatory oxygen delivery devices has largely been restricted to patients using LTOT given the evidence base supporting it, or other forms of supplementary oxygen. A number of studies(165, 166) have demonstrated that a proportion of LTOT patients also experience EID. The sole focus of this objective was to address the efficacy of the delivery device; thus included studies would compare

device against device and not ambulatory oxygen against placebo the above patient group and study duration were included.

Comparator

Any alternative method of delivering ambulatory oxygen to that of the intervention arm was considered a suitable comparator. Only studies where the difference between study arms was purely due to variation in mode of ambulatory oxygen delivery were included. Such variation could be due to the equipment (portable cylinders, liquid canisters, oxygen conserving devices or concentrators) or the way the oxygen is inhaled i.e. interface, (mask or nasal cannulae).

2.1.2 Objective 3-Cost-effectiveness of ambulatory O₂ in COPD patients not on LTOT compared to usual care and the relative cost-effectiveness of different methods of delivering ambulatory O₂

The criterion for Population, Intervention and Comparator were the same as for aim 2-3 combined. Study design and Outcome criteria were:

Study Design

Cost-analysis, cost-effectiveness, cost–utility and cost–benefit studies were targeted.

Outcomes

Assessment of impact on Quality of life and also cost judged by; incremental cost-effectiveness ratios

Criteria for Exclusion from this Review

Studies which included patients on any other form of oxygen therapy (such as LTOT or short burst) apart from ambulatory oxygen therapy were excluded except if stated for

that objective. In addition studies that involved a mixed population were excluded, unless data on those patients relevant to the objective was presented and evaluated separately.

2.1.3 Search strategy

In order to maximise efficiency, two search strategies were undertaken. The literature search included the following databases; MEDLINE, MEDLINE in Process, EMBASE, Cochrane-(CENTRAL), Science Citation Index, NHS Economic evaluation database, PEDro, Health technology assessment database, ClinicalTrials.gov, CINAHL, and the Cochrane Airways specialised register. Following this the references of each included study as well as any review articles found were searched for additional articles that may contain further studies.

COPD treatment has changed considerably over the last 20 years(3, 8)consequently it may be that very old studies, particularly those that occurred prior to the introduction of LTOT, may not be relevant to current patients. For this reason, searches were started in 1980, just prior to the publication of the LTOT trials(167, 168), as these were a landmark point in the therapy of COPD, whose results were widely accepted worldwide shortly after publication. No language restrictions were applied and both published and unpublished studies were sought (via contacting authors directly via email). The search strategy is outlined in Appendix 1.

2.1.4 Study selection, data extraction and risk of bias

The search results were entered in to reference management software (Endnote X5, Philadelphia USA) and duplicate entries electronically and manually removed. The remaining records were assessed for relevance to each objective in a two stage process. Stage 1 involved myself and another reviewer (Dr AM Turner) independently screening titles and where available abstracts against the inclusion/exclusion criteria to identify relevant studies. The aim of this stage was to remove irrelevant studies and thus those records on which a decision on relevance could not be made due to insufficient/ambiguous information. Full copies of all articles passing stage 1 were obtained. In stage 2, two reviewers (myself and Dr AM Turner) independently applied the inclusion/exclusion criteria to these articles. Any disagreements between the other reviewer and I were resolved by consensus or through consultation with a third reviewer (Dr Chirag Dave). A record of the studies excluded was kept along with the reason(s) for exclusion.

One reviewer independently extracted and recorded essential information derived from each included study using a piloted data extraction form); a second reviewer checked all numerical data. For Objective 1 and 2 for each study, the data required on (but not limited to) the following was recorded: Design, completeness of follow up length of follow up; country of origin; study design; sample size; patient inclusion and exclusion criteria; patient characteristics; intervention/comparator, adherence/compliance, outcome measures; statistical methods employed; findings, effect sizes and associated uncertainty.

For Objective 3 data on the following were sought, type of design/analysis and features relevant to this, patient characteristics; intervention/comparator and outcomes were quality of life, costs and incremental cost-effectiveness ratios.

The Methodological quality of the included RCTs was appraised by using the Cochrane risk of bias framework(169); for the comparative studies assessing the adequacy of sequence generation, allocation concealment and blinding for non-RCTs such as the observational studies (prospective and retrospective cohort study) were appraised using appropriate domains of the same tool. The Jadad Scale(170) was also used to assess methodological quality.

2.1.5 Statistical Methods

All trial data was combined using Review Manager 5.2 (Cochrane Collaboration). For objective 1 and 2, meta-analysis was undertaken for studies comparing the same interventions for key outcomes where clinical and methodological details between studies were considered to be sufficiently homogeneous. Thus, only results from the same exercise test protocols were analysed together (i.e. all constant power exercise tests, all functional exercise tests, all maximal exercise tests and all shuttle walk tests). Continuous data was synthesised as mean differences where possible as described below. Statistical heterogeneity was presented and interpreted using the I^2 statistic.

Data was pooled with the generic inverse variance method using change from baseline with the appropriate means and standard deviations for each study if provided. This method ensures larger studies with smaller standard errors are given greater weight than smaller studies with larger standard errors. For the studies that reported the pre and post


training means and respective standard deviations, in order to use the change in baseline outcome measure:

- 1) The pre training means were subtracted from the post training means:
- 2) The standard deviations (SD) for pre and post training were pooled.

Pre and post-test exercise outcomes reported for both intervention groups were from tests performed on compressed air.

A fixed effect was used in all analyses unless heterogeneity defined as ($I^2 > 30\%$) was present in which case a random effect was used. The meta-analyses are presented using forest plots from Review Manager 5.2. Data which was not in a format suitable for meta-analysis have been reported in narrative form.

2.2 Clinical Trial General Methods

The OM-COPD (oxygen for muscles in COPD) trial was a single centre double blind randomized crossover trial comparing 12 weeks of an oxygen/nitrogen mix (equivalent in oxygen concentration inspired by the patient to air) to 12 weeks of oxygen, used when performing activity, in the patients described below. 





Patients were enrolled between September 2012 and April 2014. The trial design diagram is shown in figure 2.1. A mix equivalent to medical air, rather than medical air itself, was required to ensure that colouring of the cylinders could be done in a manner

which allowed complete blinding. Gases were provided at a flow rate of 2l/min and were not to be used for any more than 4 hours of activity per day.

Subjects were randomized using the sequentially numbered, opaque sealed envelopes SNOSE technique(171). A crossover design was chosen to minimise differences due to inter-patient variability, which was likely to be greater than intra-patient variability in this group over the time frame proposed(172).

There was a washout of 2 weeks between interventions. The following were examined; HRQoL, activity levels, 6MWT distance, capillary blood gas, blood tests (for inflammatory cytokines) and spirometry pre and post intervention and muscle biopsy pre and post intervention. This resulted in 6 invasive procedures (3 biopsies, 3 ear lobe capillary blood gases, 3 blood tests) per patient – before the trial, after the first and after the second intervention. Activity monitoring and HRQoL were assessed at the mid-point and end of each intervention, together with a basic clinical assessment of general and respiratory health.

Of the outcomes studied the primary outcome was change in gene expression in skeletal muscle. The biological changes in muscle in response to exercise in patients with COPD have been investigated in several previous studies(153, 173). Many of these have used training programmes akin to pulmonary rehabilitation; at least over 8 weeks looking specifically at target genes or proteins in relation to mitochondrial bioenergetics or muscle hypertrophy, atrophic pathways. Eight weeks was sufficient to induce measurable changes between trained and untrained in patients with COPD. The length of time trial participants were exposed to each intervention in the clinical trial is based around this training timeframe. As it is accepted that training is more likely to be a more

potent stimulus to skeletal muscle change than standalone domiciliary activity a longer 12 week rather 8 week period was used. The hypotheses were that either oxygen upregulate genes/gene sets involved in mitochondrial biogenesis or skeletal muscle hypertrophy. This based on either oxygen having a direct action on skeletal muscle, i.e. mitigating the desaturation which occurred on exertion or that use of AOT would encourage participants to be more active and thus have a beneficial effect on skeletal muscle. As such a 2 week wash out period was used as this would give sufficient time to revert to “normal” activity.

Whilst a key aim of this trial was to examine the systemic response to oxygen, and in particular how this systemic response might influence the muscle, it was not possible to power the microarray analyses hence these were deemed exploratory; they are described further in chapter 5. Secondary outcomes were limited to exercise capacity, physical activity, quality of life and cytokine response.

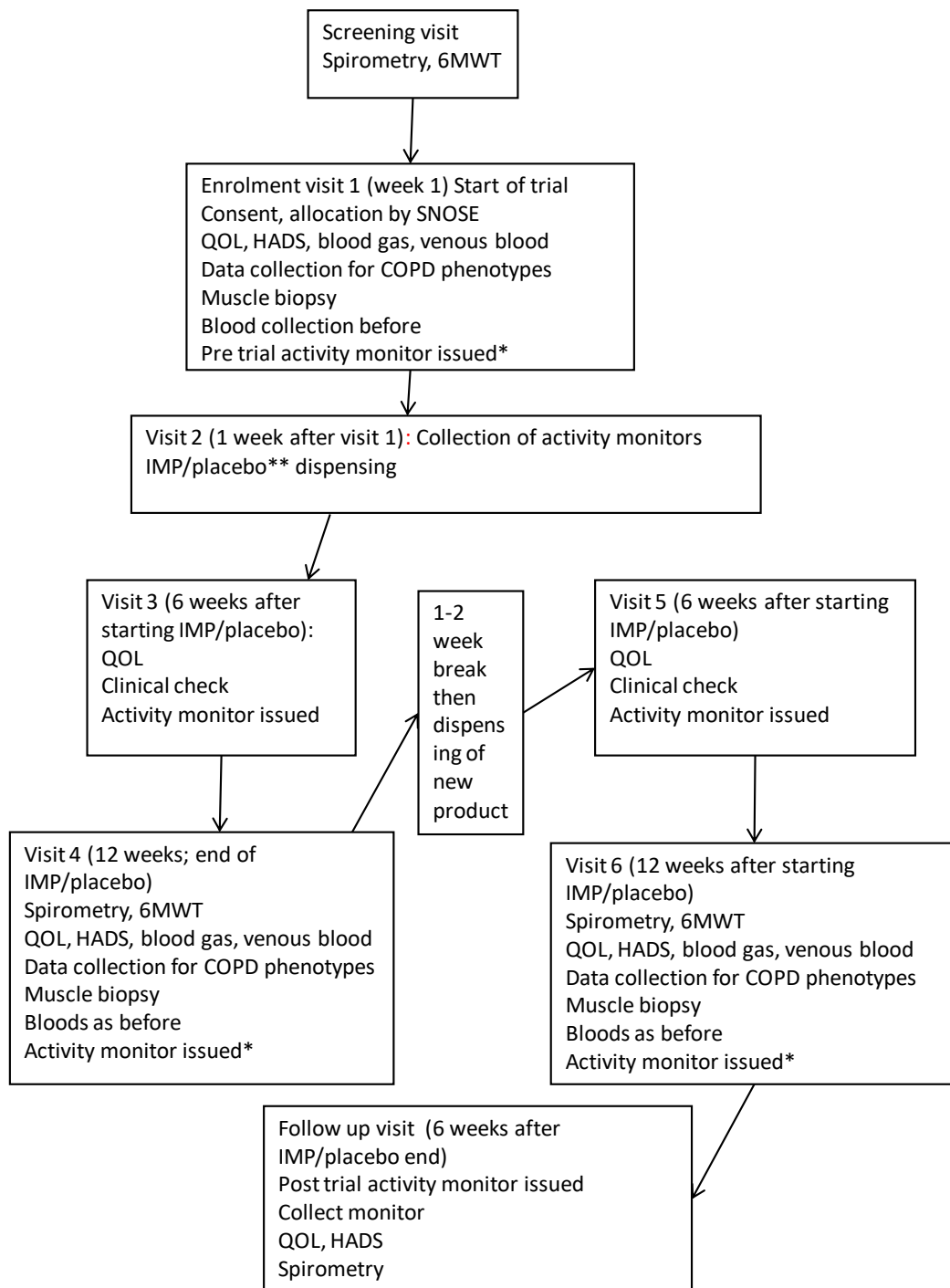


Figure2.1: Trial design diagram.

2.2.0 Ethics and governance

The trial was conducted in compliance with the principles of the Declaration of Helsinki (1996) and the principles of Good Clinical Practice (GCP). Prior to the commencement of the clinical trial I completed GCP in clinical trial management. The trial protocol and initial ethical approval was undertaken by the PI in conjunction with me. Subsequent trial management including but not limited to organising patient visits, data collection, completing adverse event forms, performing muscle biopsies, submitting substantial amendments and maintaining the trial master file was undertaken by me. Development safety update reports (section 2.2.9) along with summary of product characteristics for medical oxygen were completed and sent to Medicines and Healthcare products regulatory agency (MHRA) on a yearly basis. During the clinical trial a number of new standard operating procedures (SOPS) were introduced by the sponsor. Internal trial procedures were modified where possible to bring them in line with the new SOPS e.g. the delegation of adverse event reporting to trial PI/investigators by sponsor. Adverse events were reported to the MHRA as part of the development safety update. Suspected unexpected serious adverse reactions were reported to the sponsor.

2.2.1 Participant selection

Subjects were recruited from adult secondary care respiratory clinics [REDACTED]. Informed written consent was obtained from all participants prior to enrolment. The clinical trial has ethical approval from the regional ethics committee [REDACTED]; key trial documents are shown in the appendix 2

25 patients with COPD were recruited. Enrolled patients had radiological emphysema or spirometrically defined COPD, according to British Thoracic Society standards (i.e. FEV1/FVC <0.7 and FEV1<80% predicted), and no co-morbidities that limited their mobility. All patients had oxygen saturations greater than or equal to 94% at rest and exhibited EID to less than 88% on exertion, as documented during a formal 6MWT. All participants were clinically stable, defined as being more than 6 weeks since their most recent exacerbation.

Exclusion criteria were as follows:

- Age – less than 18
- Immobile due to other medical conditions
- On LTOT
- Unable to understand or retain information
- Uncontrolled angina symptoms
- Evidence of clinically significant harm from oxygen therapy (decompensated type 2 respiratory failure).

All participants underwent the procedures described below as part of the clinical trial, which are summarized in the flowchart (Figure 2.1).

2.2.2 Demographic data

Basic demographic data (gender and date of birth) was collected using a Case Report Form (CRF)-appendix 2. The CRF included lung function at enrolment or that within 6 months of enrolment, the participant clinical phenotypes; delineated by bronchitis, emphysema or bronchiectasis and also medical research council (MRC) dyspnoea score.

2.2.3 Spirometry and Arterial blood gas sampling

All spirometry was performed by ARTP (association for respiratory physiology and technology) accredited respiratory physiologists within the lung function department [REDACTED]. Values obtained were compared to predicted values derived from standard reference equations for Caucasian adults (European Coal and Steel reference values). Blood gas sampling was obtained using the earlobe blood gas sample technique; a well validated alternative to arterial blood gases(174). If any subject had elevated carbon dioxide (>6kPa) on their baseline blood gas this was repeated after giving oxygen at 2l/min for 20 minutes in order to ensure that the subject was unlikely to come to any harm from using oxygen during the study

2.2.4 Muscle biopsy

Percutaneous needle biopsy offers a less invasive alternative to conventional open biopsy with satisfactory muscle yield (up to 300mg).

Muscle biopsy was performed percutaneously from the lateral vastus lateralis under local anaesthesia (2% lignocaine) and strict asepsis using the Bergstroms technique(175) shown

in diagram (figure 2.2 and 2.3) . Samples were flash frozen using a dry ice acetone bath and subsequently stored at -80C. Samples were subsequently used for RNA extraction and microarrays, as detailed in 2.3.3 and 2.4.

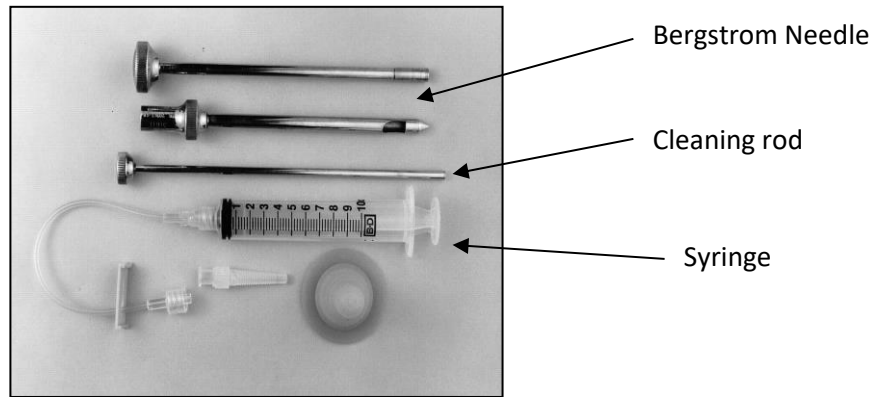


Figure 2.2. Equipment used for muscle biopsy sampling.

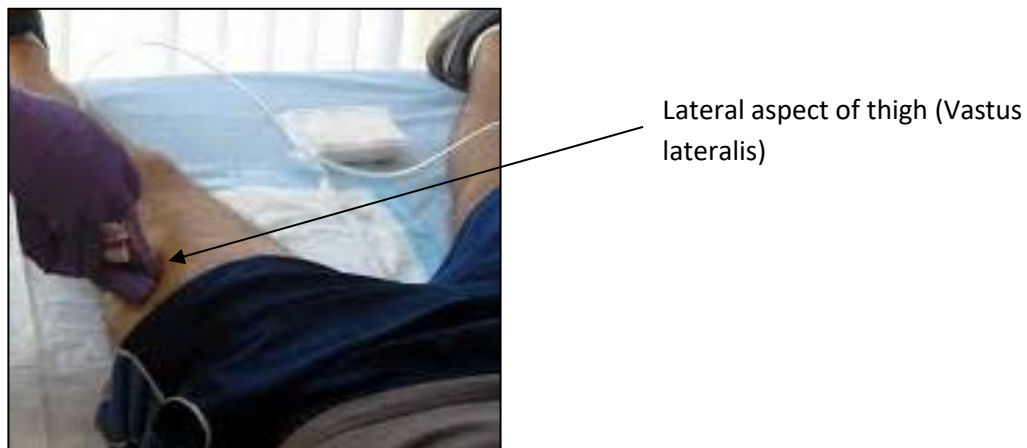


Figure 2.3. Bergstrom's Procedure

2.2.5 Blood Sampling

Plasma was prepared from venous blood which was collected in EDTA blood tubes. The venous blood was centrifuged at 1000g for 10mins with the resultant supernatant (plasma) stored in 0.5ml aliquots at -80°C. Plasma levels of TNF- α , interleukin 6 (IL-6), interleukin 8

(IL-8) and interferon gamma (IFN- γ) were analysed using enzyme linked immunosorbent assays (Ebioscience).

2.2.6 HRQoL and Activity Monitoring

HRQoL was assessed using the COPD assessment test (CAT) accessed from www.catestonline.co.uk and the Hamilton Anxiety and Depression Scale(176). Both of these patient reported questionnaires are well validated(177) and were chosen due to their ease and speed of completion.

Activity monitoring was performed using Actigraph Tri-axial accelerometers. Participants wore the activity monitor around their waist for five days only removing it during sleep and bathing/showering. Five days of home monitoring has been shown previously to adequately overcome day to day variation in activity levels at all stages in COPD(178) and provides a quantitative, objective measure of exercise capacity and energy expenditure in a real-life setting. Data was validated and scored using Actilife version 6 using the Freedson Adult cut off points(179) and expressed as vector magnitude units/min. This accelerometer has been validated by the PROactive consortium to give an accurate indication of physical activity in patients with COPD(180).

2.2.7 6MWT

Participants underwent 6MWT in a 25metre corridor in accordance with ATS guidelines(93). A practice 6MWT was not completed. Patients were encouraged every minute of the 6MWT using two phrases: “Keep up the good work” or “You are doing

well". Patients were allowed to stop and rest during the test, but were instructed to resume walking as soon as they felt able to do so.

2.2.8 Blinding, Treatment allocation and Statistical analysis

There is a universal colour code for medical gas supply cylinders in the United Kingdom. Medical air cylinders are white and black whilst oxygen cylinders are white, therefore if standard cylinders were used blinding could not be achieved. For the purpose of the clinical trial all cylinders were white in colour. A nitrogen/oxygen mix equivalent to air was used in place of air cylinders. All cylinders were prepared by BOC Group PLC and delivered to the clinical trial pharmacy where they were labeled A and B. The code break was held by the clinical trial pharmacist alone and was not revealed to trial investigators until the completion of the trial at the point when which results analysis was undertaken.

Statistical analysis was carried out using SPSS version 22 Armonik, New York, IBM Corp. Normality of data was assessed using Spiro Wilk tests. Throughout the thesis normally distributed data is shown as mean (standard deviation) and non-normally distributed data as median (interquartile range). All statistical analyses were completed prior to the un-blinding of treatment allocation by coding periods as Treatment A and B. For cross-over trial data analysis clinical variables were firstly tested for evidence of carry over effects, i.e. the sum of values over both treatment periods were compared between those who received ambulatory oxygen first or air first and assessing whether there was significant difference between them. Given that no carry-over effects were observed for clinical variables the order of treatment was not accounted for. Therefore change from

baseline for each treatment period was evaluated using paired statistical tests for primary and secondary clinical outcome data; T-tests and Wilcoxon tests were used for parametric and non-parametric data respectively.

2.2.9 Trial monitoring and safety

Trial monitoring was undertaken on more than 2 separate occasions by the trial sponsor. Monitoring visits were used to facilitate trial management and also to ensure procedure were in line with GCP. All monitoring queries were discussed with the PI and co-investigators with action points from monitoring queries dealt with by me.

Adverse events (AEs) defined as any untoward medical occurrence in an enrolled subject, including occurrences which are not necessarily caused by or related to oxygen were recorded at follow up visits by myself and another co-investigator.

2.3 Clinical Trial Laboratory Methods

2.3.0 Enzyme linked immunosorbent assay

This section describes the general technique used to measure plasma levels of IL-6, IL-8, interferon gamma and Tnf- α . The specific details for each assay were followed from the manufacturers protocol.

A quantitative sandwich ELISA was used in which the sample for analysis is quantified against the concentration of known standards; samples of predetermined concentration used to set up a standard curve. The final fluorescence/colour generating step is specific to each ELISA is performed prior to reading the plate in standard microplate reader. All samples were run in duplicate on a 96 well plate as shown in figure 2.4. The intra assay

coefficient of variation for samples was less than 10%. If this was not the case samples were re-analysed.

The sandwich ELISA process is demonstrated in figure 2.5.

A	Std1	Std1	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	Std2	Std2	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	Std3	Std3	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	Std4	Std4	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	Std5	Std5	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	Std5	Std6	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	Std6	Std6	S7	S7	S15	S15	S23	S23	S31	S31	Blank	Blank
H	Std7	Std7	S8	S8	S16	S16	S24	S24	S32	S32		

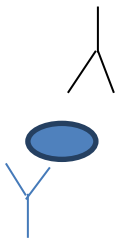
Figure 2.4 Lab well Std-standards of known concentration, S-samples (duplicated per well)



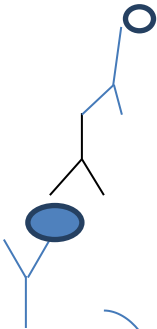
The plate is coated with a capture antibody



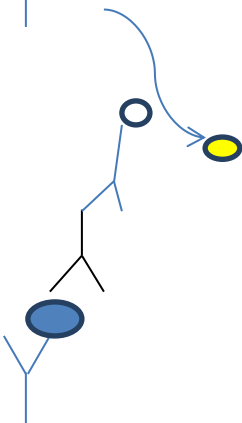
The sample is added and any antigen present binds to the capture antibody.



A detection antibody is added which also binds to the antigen



An enzyme linked antibody is added which binds to the detection antibody



A substrate is added and is converted by the enzyme to its detectable form

Fig 2.5 Sandwich Elisa process.

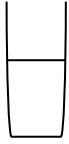
2.3.1 RNA Extraction

RNA was extracted from muscle biopsy samples using the QIAGEN RNeasy kit (Manchester, UK) following the manufacturer's protocol detailed below. This kit was chosen because it provides a reliable, relatively quick and consistent method of producing high quality RNA which can be used for downstream processes such as microarray analysis.

1. "The muscle sample was homogenized by adding 700µl of QIAzol lysis reagent.
2. The homogenate was incubated at room temperature (15-25°C) for 5 minutes.
3. 140µl of chloroform was added to the homogenate and shaken for 15 seconds
4. The mixture from step 3 was incubated at room temperature for 2-3 minutes before being centrifuged at 12,000g at 4°C.
5. The upper aqueous phase was transferred to a new collecting tube using a Pasteur pipette where it was then mixed with 525µl of 100% ethanol.
6. 700 µl of the sample including any precipitate was pipetted, into an RNeasy® Mini column in a 2 ml collection tube. This was then centrifuged at $\geq 8000 \times g$ for 15 s at room temperature. Any follow through was discarded.
7. The above step was repeated with the remaining sample.
8. 700 µl of Buffer RWT was added to the RNeasy Mini column and centrifuged for 15 s at $\geq 8000 \times g$. Any follow through was discarded.
9. 500 µl of Buffer RPE was pipetted onto the RNeasy Mini column and centrifuged for 15 s at $\geq 8000 \times g$. Any follow through was discarded.
10. 500 µl of Buffer RPE was added to the RNeasy Mini column and centrifuged for 2 min at $\geq 8000 \times g$.

11. The RNeasy Mini column was transferred to a new 1.5 ml collection tube. 30–50 μ l of RNase-free water was pipetted directly onto the RNeasy Mini column membrane and centrifuged for 1 min at $\geq 8000 \times g$ before eluting.”

Muscle tissue



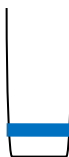
Muscle tissue lyzed with QIAzol.
Ethanol added to the muscle tissue
homogenate and sample
centrifuged



Sample added to RNA column with
addition of buffer and sample then
centrifuged.



Binding of RNA



Sample washed and eluted to yield
total RNA.

Fig. 2.6: RNA extraction process.

2.3.2 RNA amplification

This section describes the process of RNA amplification used for microarray analysis. This was required as following RNA quality assessment (using absorbance) some of the extracted RNA was found to be degraded. RNA amplification of intact and degraded was achieved using the Whole Transcriptome Amplification kit Sigma Aldrich (Gillingham, Dorset, UK). This method has been validated(181) to effectively amplify degraded RNA maintaining transcripts levels representative of intact unamplified .

Protocol for RNA amplification

Library Synthesis Reaction

The Library synthesis buffer and solution were thawed and mixed thoroughly. Any precipitate was dissolved by briefly heating the mixture to 37°C.

1. 100ng of RNA was added to 2.5µL of the Library Synthesis Solution.
2. Nuclease-free water was added to bring the total to 16.6 µL.
3. The resultant solution was mixed and incubated at 70°C for 5 minutes then 18°C
4. 2.5 µL Library synthesis solution, 3.9 µL Water and 2 µL of library synthesis enzyme were added.
5. Incubated in a thermal cycler using the following plan:
 - 18°C for 10 minutes
 - 25°C for 10 minutes
 - 37°C for 30 minutes
 - 42°C for 10 minutes
 - 70°C for 20 minutes
 - 4°C to hold

6. The 25 μ L samples were divided into 5 X 5 μ L aliquots on a fresh plate

Amplification reaction

The amplification mix and 10nM dNTP were thawed.

1. A master mix was prepared by adding 301 μ L of nuclease-free water, 37.5 μ L amplification mix, 7.5 μ L WTA dNTP mix, and 3.75 μ L amplification enzyme.
2. 70 μ L of master mix was added to 5 μ L of the sample from step 6 of the library synthesis reaction
3. Incubated in a thermal cycler:
 - a. 94 $^{\circ}$ C for 2 minutes
 - b. 17 cycles of 94 $^{\circ}$ C for 30 seconds and 70 $^{\circ}$ C for 5 minutes
 - c. End cycle
4. The resulting WTA DNA was stored at -20 $^{\circ}$ C.

2.4 Microarrays

The genetic expression microarray analysis was outsourced to the Biosciences department at the University of Birmingham at a cost of £4900. This included dye labelling, hybridisation, array scanning and feature extraction. The microarray experimental design was undertaken by myself.

Two channel microarray microarrays were performed in a matched pair design i.e. each patient's baseline muscle sample was hybridised along with their corresponding muscle sample following each intervention (baseline muscle sample with air treatment sample, baseline with oxygen treatment sample). The two channel microarray design in this setting was significantly cheaper than a single channel analysis as the same information

could be gleaned using half the arrays. Therefore, each array represented the log ratio between baseline condition and ambulatory oxygen/control and subsequent analysis comparing Air treatment vs Oxygen.

Six hundred nanograms of cDNA from each sample were converted to labelled cDNA coupled to a fluorescent dye (Cy3 for baseline samples and Cy5 for samples following interventions) using Agilent SureTag labelling kit (Agilent, 5190-3400, Cheshire UK). These were competitively hybridised to 8x60k microarrays (Agilent Technologies G4851; Cheshire UK), washed and scanned, then data extracted from the scanned image using Feature Extraction (version 10.7.3, Agilent Technologies). A schematic diagram explaining the microarray experiment procedure is shown in figure 2.7

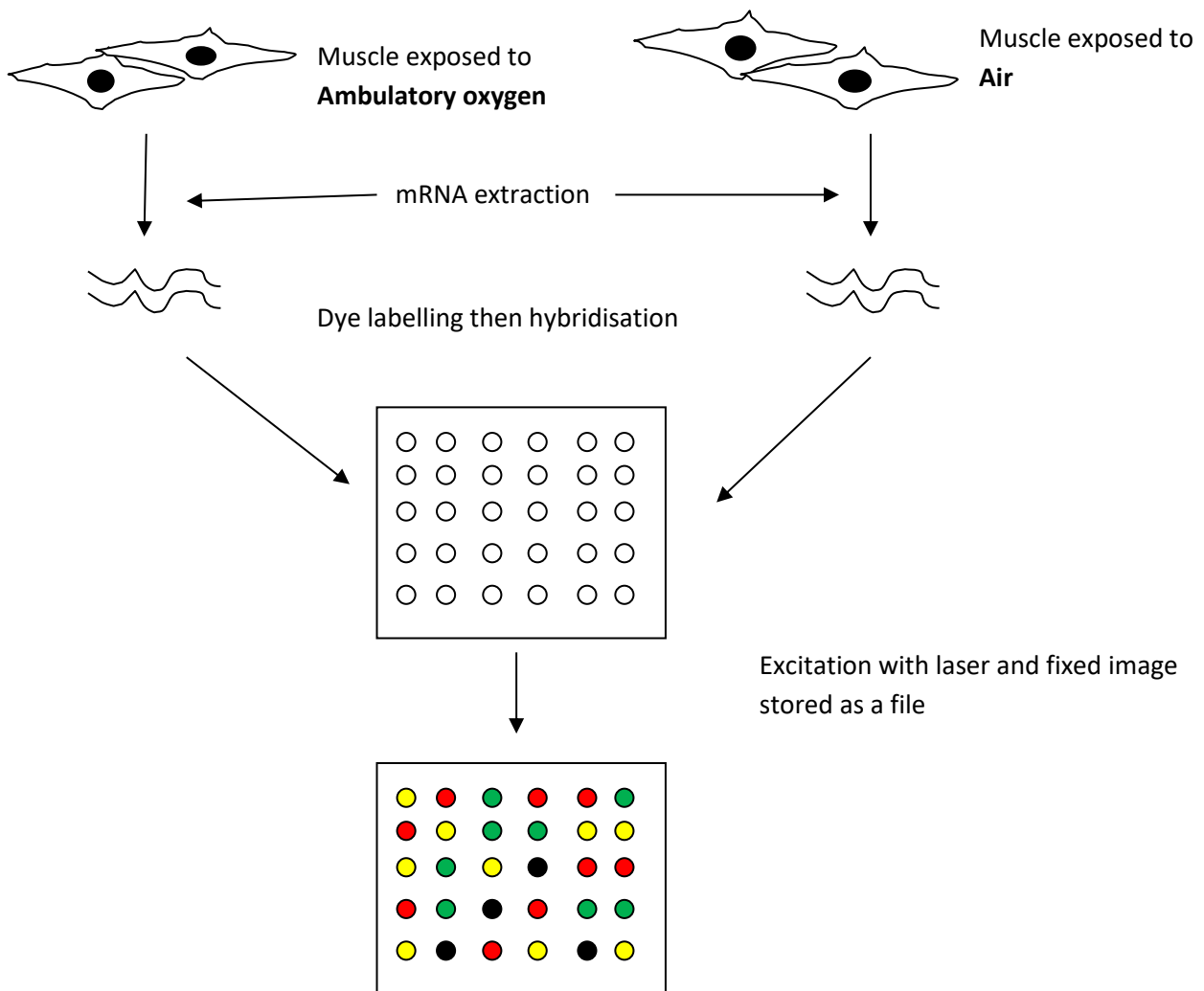


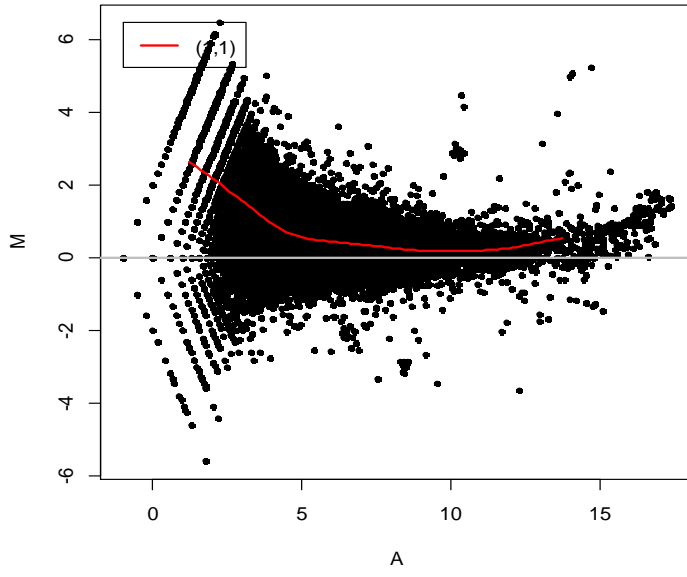
Fig 2.7: Schematic diagram detailing the microarray process.

mRNA is extracted from the muscle cells and labelled with two dyes (Cy3-green and Cy5-red) during the synthesis of cDNA by reverse transcriptase. The cDNA is hybridised to the microarray slide where each cDNA molecule representing a gene binds to the complementary DNA sequence. Following this the microarray slide is then excited with a laser a various wavelengths to detect the red and green dyes. Green spots indicate hybridisation with control samples only (muscle exposed to air), red hybridisation with test targets (muscle exposed to ambulatory oxygen), yellow hybridisation with both samples and black no hybridisation.

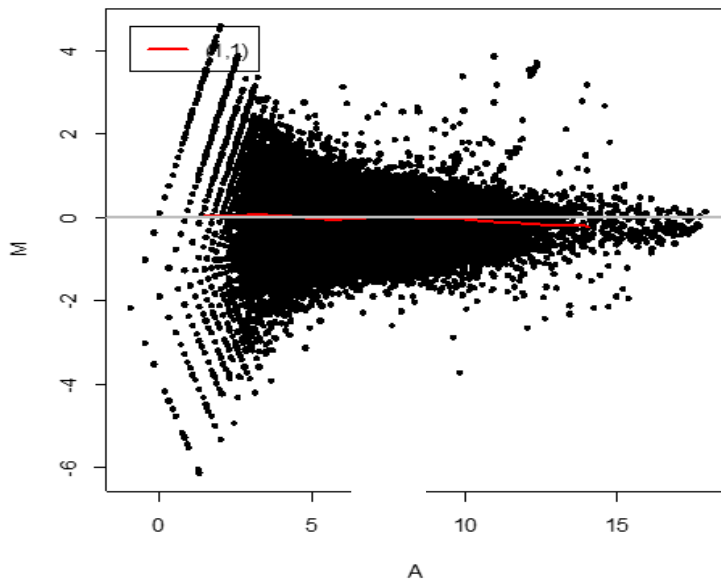
2.4.0 Normalisation

Normalisation and subsequent microarray analysis was undertaken by myself in the program Bioconductor *R*.

The package “marray” was used for microarray normalisation. Normalisation is the process which eliminates sources of variation which affect microarray experiments. These can arise from differing quantities of initial mRNA between samples or differential dye labelling (dye bias). The process of normalisation is based on the general principle that in a microarray experiment only a small proportion of genes will show changes in expression between two different conditions. An MA plot is modified scatterplot used to visualise the red-green intensities from an array. A , is the average log intensity of the two channels and M is the relative expression between the two channels also known as the log-ratio. In an MA plot the points should be localised around the zero line in a situation where there are no technical artifacts. Deviations from this zero line are due to non-biological reasons and are therefore removed in the normalisation process. The “loess” method is a within array normalisation method which was used to normalise the microarray data. Figure 2.8 shows the same array MA plot before and after normalisation.



a)



b)

Figure 2.8: MA plots a) before and b) after normalisation

The significance of microarray package (SAM) was used to perform the microarray analysis of differential gene expression between ambulatory oxygen and air. Exploratory analyses of microarray using gene set enrichment and principal component analysis are further explained in analysis of microarrays chapter 5.

Chapter 3.

Systematic Review

This review aims to clarify the longer term efficacy of AOT in those with EID and exertional dyspnoea when judged by outcomes measures of exercise capacity and quality of life. It also evaluates whether the use of oxygen conserving devices is warranted. Previous reviews have been limited by their short duration (<6weeks) therefore, whether acute eligibility tests for AOT translate into longer term benefit is unclear. In addition, no previous review has evaluated the devices by which AOT is delivered. As discussed in the methods (section 2.1.5), only trials of similar exercise outcomes have been compared i.e. ISWT vs ISWT. Pulmonary rehabilitation (PR) studies have been analysed separately to domiciliary as they are not directly comparable. Although the desired and hypothesised effect of AOT use in both PR and domiciliary studies was to improve exercise capacity analysing them together would conflate the effects of exercise and oxygen.

Some of the results and text from this chapter have been published in the peer reviewed Journal of the COPD foundation: *Chronic Obstr Pulm Dis* (Miami). 2016; 3(1): 419-434.

3.1 Results

Twenty-three studies (620 patients) were included in the systematic review: nine studies evaluated the clinical effectiveness of ambulatory oxygen and thirteen studies evaluated the impact of the delivery device. Generally the studies were small with primary outcomes focused on exercise capacity, Borg dyspnoea scores and exercise saturations. Figure 3.1 summarises the trial flow in the PRISMA format for reporting systematic reviews.

PRISMAFlow Diagram

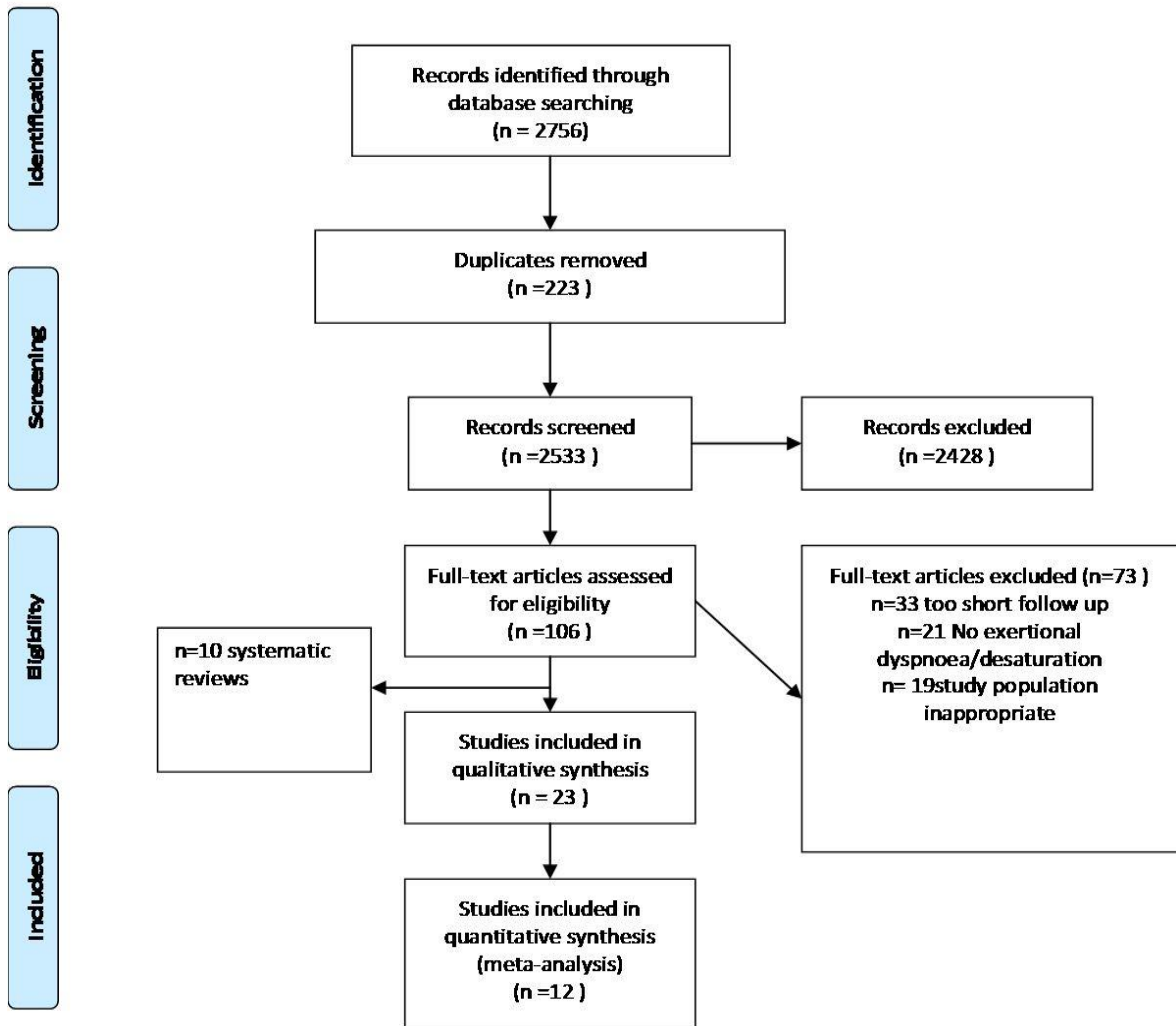


Figure 3.1 Prisma flow diagram

3.1.0 Included Studies

The included studies have been summarised in table 3.1 (clinical effectiveness) and table 3.2 (delivery devices).

3.1.1 Risk of Bias

Allocation concealment, adequate sequence generation, blinding and Jadad score were used to assess this (table 3.3). The Jadad score was poor in 12 of the 23 studies, good in 9 and excellent in 2 of the studies. 9 of the studies had adequate randomisation process; mostly undertaken by computer random number generation or Latin square sequencing. Allocation concealment was adequate in 7 studies; undertaken by a mixture of central randomisation and the SNOSE technique. Blinding was adequate in 6 studies; undertaken by blinding the assessor in the trial or use of identical cylinders arranged by the cylinder suppliers. In a number of the studies especially the older (pre 1995) sequence generation, allocation concealment and /or blinding was unclear or high risk. This information was not available in the individual manuscripts or after contacting the authors.

Author	Inclusion	Exclusion	Study type	Intervention	Comparator	Sample Size	Methods	Outcomes	Results Summary
McDonald et al(182)	a)COPD b)PaO ₂ >60mmHg/8kPa c)clinically stable d)exertional dyspnoea sufficient to interfere with ADLs e)current non smokers	a)symptomatic cardiac dysfunction, angina pectoris and locomotor disability.	Crossover double blinded	Oxygen 4l/min	Air 4l/min	26	Unsupervised 12 week domiciliary oxygen/air use	a)6MWT b)Step test c)Borg Scores d)CRQ	Small significant improvement in 6MWT on home O ₂ . Improvement in all parameters of CRQ on home O ₂ compared to baseline. P<0.02
Rooyackers et al(183)	a) COPD b) SpO ₂ < 90% at maximal exercise c) > 15mmHg / 2.00kPa increase in alveolar-arterial difference in PO ₂ from rest to maximal exercise	a) PaO ₂ < 64mmHg / 8.53kPa b) mean nocturnal SaO ₂ < 90% c) mean pulmonary artery pressure > 25mmHg d) neuromuscular or cardiovascular disease	Parallel un-blinded	Oxygen 4l/min	Air	12 Air group, 12 O ₂ group	10 week supervised PR program. 5 sessions/week	a)PFTs b)Maximal cycle ergometry (watts) c)Endurance cycling time d)6MWT e)CRQ f)Borg Scores	Improvement in peak WR in air group only p<0.01. Improvement in 6MWT and total CRQ scores compared to baseline in both groups p<0.01
Eaton et al (184)	a)COPD b)<88% SpO ₂ on exertion c)resting PaO ₂ >7.3kPa d)clinically stable	a)limiting angina or significant musculoskeletal disability	Crossover-double blinded	Oxygen 4l/min	Air 4l/min	41	Unsupervised 12 week domiciliary oxygen/air use	a)CRQ b)HADS c)SF-36 d)6MWT e)Borg Scores	No significant difference between groups in 6MWT p=0.4 Improvement in total CRQ scores for home O ₂ period compared to home air p=0.002
Emtner et al(185)	a)COPD; FEV ₁ < 50% predicted b) 45-80 years old	a) Symptomatic cardiovascular co-morbidities or other disease	Parallel double blinded	Oxygen 3l/min	Air 3l/min	14 O ₂ group, 15 Air group	Supervised 7 week exercise program with 3 sessions/week	a)Maximal cycle ergometry (watts) b)Constant power cycle ergometry	Improvement in peak exercise tolerance test in both groups p<0.05. Endurance in constant

	c) clinically stable d) PaO ₂ > 55mmHg / 7.33kPa at rest e) SpO ₂ > 88% during constant WR test	that might contribute to exercise limitation b) regular participation in formal exercise program c) participation within a formal rehab program in last 2 years						c) CRQ d) SF-36 e) Borg Scores	WR improved significantly in oxygen group compared to air group p < 0.05.
Nonoyama et al (186)	a) COPD b) < 88% SpO ₂ on 6MWT c) Dyspnoea interfering with ADLs	a) LTOT	N of 1 RCTs	Oxygen 2l/min	Air 2l/min	27	Unsupervised 6 weeks domiciliary oxygen/air use	a) CRQ b) SGRQ c) 5MWT d) Borg Scores	Improvement in 5MWT and Borg dyspnoea scores for home O ₂ vs home air p = 0.04. No significant difference in HRQoL between home O ₂ or home air
Janaudis-Ferreira et al (187)	a) COPD FEV ₁ 70% b) < 92% SpO ₂ on 6MWT c) PaO ₂ > 8kPa at rest d) clinically stable d) not smoking 6 months prior to study	a) severe cardiac, orthopaedic or neurological problems that would influence exercise performance b) LTOT	Parallel single blinded	Oxygen 5l/min	Air 5l/min	10 Air group, 10 O ₂ group	Supervised 8 week training program	a) 6MWT b) time < 90% saturations c) Borg Score	Improvement in 6MWT in both groups compared to baseline p < 0.008 Air group and p < 0.005 O ₂ group. Greater proportion of patients achieve MCID for 6MWT in air group p = 0.01
Moore et al (188)	a) COPD b) PaO ₂ 7.3kPa c) MRC dyspnoea > 3	a) Domiciliary oxygen use b) Participation in PR c) Locomotor	Parallel double blinded	Oxygen 6l/min	Air 6l/min	75 cylinder air, 68 oxygen	Unsupervised 12 weeks domiciliary oxygen/air use	a) 6MWT b) CRQ c) BDI/TDI d) HADs	No significant difference between CRQ dyspnoea score, 6MWT or BDI/TDI scores between groups.

		difficulties							
Dyer et al(189)	a)COPD b)desaturation>4% and below 90% on ESWT c)>10% improvement in ESWT with AO.	a)any form of Home Oxygen	Parallel single blinded	Oxygen 2-6L/min	Air	23 Air group, 24 O2 group	Supervised 7 week exercise program-Twice weekly sessions	a)ESWT b)CRQ c)HADS	Improvement in ESWT in both groups compared to baseline p<0.0001. Improvement in ESWT in O2 group vs air group p<0.001
Ringbaek et al(190)	a)COPD; FEV1<80% predicted b)MRC 3-5 c)desaturation>4% and below 90% on ISWT/ESWT d)SpO2>90% at rest	a)LTOT b)Significant musculoskeletal, cardiac or cognitive problems	Parallel un-blinded	Oxygen 2l/min	Air	22 O2 group, 23 air group	20 week training program supervised for the first 7 weeks	a)ESWT b)SGRQ	Improvement in ESWT (time and distance) in both groups p<0.001

Table 3.1: Clinical effectiveness of Ambulatory oxygen studies.

5MWT5 minute walk test, 6MWT 6 minute walk test, CRQ chronic respiratory disease questionnaire, HADS Hamilton Depression and Anxiety Scale, WR work rate, ESWT endurance shuttle walk test, PFTs Pulmonary Function Test, SGRQ Saint George's Respiratory Questionnaire, BDI/TDI baseline/transitional dyspnoea scores, MCID minimal clinically important difference.

Author	Inclusion	Exclusion	Study type	Intervention	Comparator	Sample Size	Methods	Outcome	Results Summary
M Soffer et al(191)	COPD or restrictive ventilatory disease. SpO2<90%	NR	Non-RCT Cross over	DODS	CFNC	20 (13 COPD)	Treadmill exercise	a)O2 conservation ratio at 90% SpO2 b)% O2 savings	DODs requires lower flow rate to achieve 90% saturations compared to CFNC. P<0.05 (data from COPD patients alone)
R Carter et al (192)	COPD Exercise desaturation Resting SpO2 ≤90%	NR	Cross over	DODs	CFNC	10	Treadmill exercise test (1-1.5mpH)	SpO2 on exercise	DODs achieves statistically higher SpO2 on exercise compared to CFNC p<0.001
B Tiep et al(193)	COPD	No cardiovascular or orthopaedic limitations to exercise	Non-RCT Cross over	DODs	CFNC	9	Treadmill exercise test	O2 flow setting required to achieve SpO2>90%	DODs requires lower flow rate to achieve 90% saturations p<0.0001
Bower et al(194)	Chronic lung disease Clinically stable	NR	Cross over	DODS	CFNC	5 COPD 1 COPD/scoliosis	Treadmill exercise test	a)Oxygen usage percentage b)exercise SpO2	No significant difference in exercise SpO2. DODS mean O2 utilisation is 44% that of CFNC O2 utilisation
S. Braun et al (195)	COPD: FEV1/FVC ratio <60%, LTOT	NR	Cross over	DODS (5 devices)	CFNC	10	12minute walk test	Average and lowest SpO2 at rest and exertion	No significant difference between the mean of the lowest SpO2 during exercise between intervention and comparator
C Roberts et al (196)	COPD Exercise desaturation <90% Clinically stable	NR	Cross over	DODs	CFNC	15	6MWT	a)6MWT b)Time spent <90% SpO2	CFNC significantly improved 6MWD and reduced Time <90% SpO2 (compared to baseline) whereas DODs only improved 6MWT

A.Cuvelier et al(197)	COPD-ATS criteria Clinically stable FEV1/FVC<65% FEV1<55% PaO2≤60mmHg at rest/PaO2>60mmHg with evidence of exercise desaturation	NR	Cross over	Refilled O2 cylinders – HomeFill system	Standard commercial O2 cylinders	10	Successive 6MWT carrying standard O2 and refilled O2 cylinders	a)6MWD b)mean SpO2 at end of 6MWT	No significant difference between 6MWD orSpO2 between cylinder types.
B Tjep et al (198)	Chronic lung disease Desaturation on exercise Clinically stable All patients using supplementary O2	NR	Non-RCT Crossover	DODS	CFNC	10 (9 COPD)	Treadmill exercise test. O2 flow required to maintain saturations between 92 and 94%	O2 flow setting required to achieve SpO2>90%	No significant difference in SpO2 at higher DODs setting. DODs confers 4.3 fold O2 saving on exercise
W.Chatila et al (199)	COPD-GOLD criteria Clinically stable the preceding 3 months	Cardiovascular disease	Non-RCT crossover	Vapotherm (High flow oxygen 20l/min)	Low flow oxygen (2.5-6l/min)	10 (5 completed exercise test)	Cycling-unloaded bicycle	a)V _T b)V _E c)Work of Breathing d)Inspiratory time fraction d)RR/V _T	No significant difference in outcome measure (a,b or c) between Vapotherm or Low flow oxygen
C Fuhrman et al (200)	COPD FEV1<50 %, PaO2<65mmHg. Current or former smoker No exacerbations in preceding 3months	Symptomatic cardiovascular disease. Any condition with contraindication to exercise testing	Cross over	DODs (4 devices)	CFNC	13	6MWT	a)SpO2 at rest and exercise b)6MWT	No significant difference in 6MWD with any DODS device compared with CFNC

S. Strickland et al (201)	COPD-GOLD Stage 4 SpO ₂ <90% LTOT Clinically stable preceding 6 weeks	Symptomatic Cardiovascular disease Physical limitations precluding walking	Cross over	a)Liquid O ₂ system b)HomeFill compressed O ₂ system c)Portable O ₂ concentrator	Standard commercial O ₂ cylinders	39	6MWT	SpO ₂ on exercise	No significant difference between SpO ₂ between interventions or comparator
A Palwai et al (202)	Obstructive lung disease, SpO ₂ <90% at rest or on exertion. Hypoxaemia warranting use of supplementary O ₂ .	NR	Cross over	DODS (4 devices)	CFNC	13	Treadmill exercise test Modified Naughton protocol	a)Work stages completed b)SpO ₂ c)V _E	Significantly higher METs achieved with CFNC p=0.006
Casaburi et al (203)	COPD FEV ₁ <60% PaO ₂ <8kPa Clinically stable preceding month	Uncontrolled heart failure. Orthopedic, neurological or cognitive limitations to exercise. Current smokers	RCT Parallel	Lightweight aluminium cylinders	E cylinders	22	Unsupervised domestic activity	Oxygen utilisation Home activity	No significant difference in oxygen utilisation or patterns of activity between lightweight and standard cylinder
S Marti et al (204)	COPD and ILD Exercise desaturation<88%	Active smoker Use of AO therapy prior to study Recent exacerbation	Crossover	DODS/PRC	CFNC	28 COPD 31 ILD	6MWT using intervention /comparator	a)% of patients with correction of exercise desaturation b)6MWD c)Borg scores	Exercise desaturation corrected in 79% of patients with DODs and CFNC and in 86% with PRC (data for COPD patients alone). No significant difference between devices

Table 3.2 Impact of device on Ambulatory oxygen delivery studies.DODs Demand oxygen devices, PRC Pendant reservoir cannulae, NR not reported, ATS American Thoracic Society, METS Metabolic equivalents

Study	Adequate Sequence generation	Allocation Concealment	Blinding	Jadad Score
Soffer 1985	Unclear	Unclear	No-high risk	2
Carter 1986	Unclear	Unclear	No-high risk	2
Bower 1988	Unclear	Unclear	No-high risk	2
Braun 1992	Unclear	Unclear	No-high risk	2
McDonald 1995	Unclear	Unclear	Yes-low risk	4
Roberts 1996	Yes-low risk	Yes-low risk	No-high risk	3
Rooyackers 1997	Yes-low risk	Yes-low risk	Unclear	2
Tiep 1997	No-high risk	No-high risk	No-high risk	1
Cuvelier 2002	Unclear	Unclear	No-high risk	2
Eaton 2002	Unclear	Unclear	Yes-low risk	4
Tiep 2002	No-high risk	No-high risk	No-high risk	1
Emtner 2003	Yes-low risk	Unclear	Yes-low risk	5
Chatila 2004	No-high risk	No-high risk	No-high risk	1
Fuhrman 2004	Unclear	Unclear	No-high risk	2
Nonoyama 2007	Unclear	Unclear	Yes-low risk	4
Janaudis-Ferreira 2009	Yes-low risk	Unclear	Unclear	3
Strickland 2009	Unclear	Unclear	No-high risk	2
Palwai 2010	Unclear	Unclear	Yes-low risk	4
Moore 2011	Yes-low risk	Yes-low risk	Yes-low risk	5
Casaburi 2012	Yes-low risk	Yes-low risk	No-high risk	3
Dyer 2012	Yes-low risk	Yes-low risk	No-high risk	3
Marti 2013	Yes-low risk	Yes-low risk	Unclear	2
Ringbaek 2013	Yes-low risk	Yes-low risk	Unclear	3

Table 3.3: Risk of bias in the included studies.

3.2 Clinical effectiveness of ambulatory oxygen

3.2.0 Exercise Capacity

4 RCTs (182, 183, 187, 188) used 6MWT distance in assessing exercise capacity. In the PR (pulmonary rehabilitation) studies (183, 187) the common effect (weighted mean difference) was 21.97 metres in favour of the control group. This result was mirrored in the meta-analysis of the domiciliary studies (182, 188); common effect 0.56 metres in favour of control. However, neither result was statistically significant (95% CI -77.25 to 33.32) and (95% CI -26.96 to 28.08) respectively, Figure 3.2. 2 PR RCTs (189, 190) used ESWT; they differed substantially in their results and therefore the pooled figure may be less meaningful. It was not statistically significant, common effect 150.02 seconds in favour of oxygen (95% CI -124.74 to 427.77 seconds, Figure 3.2). 2 PR RCTs (183, 185) used incremental work rate exercise testing via cycle ergometry to assess exercise capacity and found no difference with oxygen; 1.45 watts in favour of control (95% CI -13.54 to 10.64 watts), Figure 3.2). 2 PR RCTs (183, 185) measured constant power exercise tests using cycle ergometry. There was a strong trend toward longer exercise time with oxygen (2.76 minutes longer (95% CI -0.07 to 5.58 minutes, Figure 3.2).

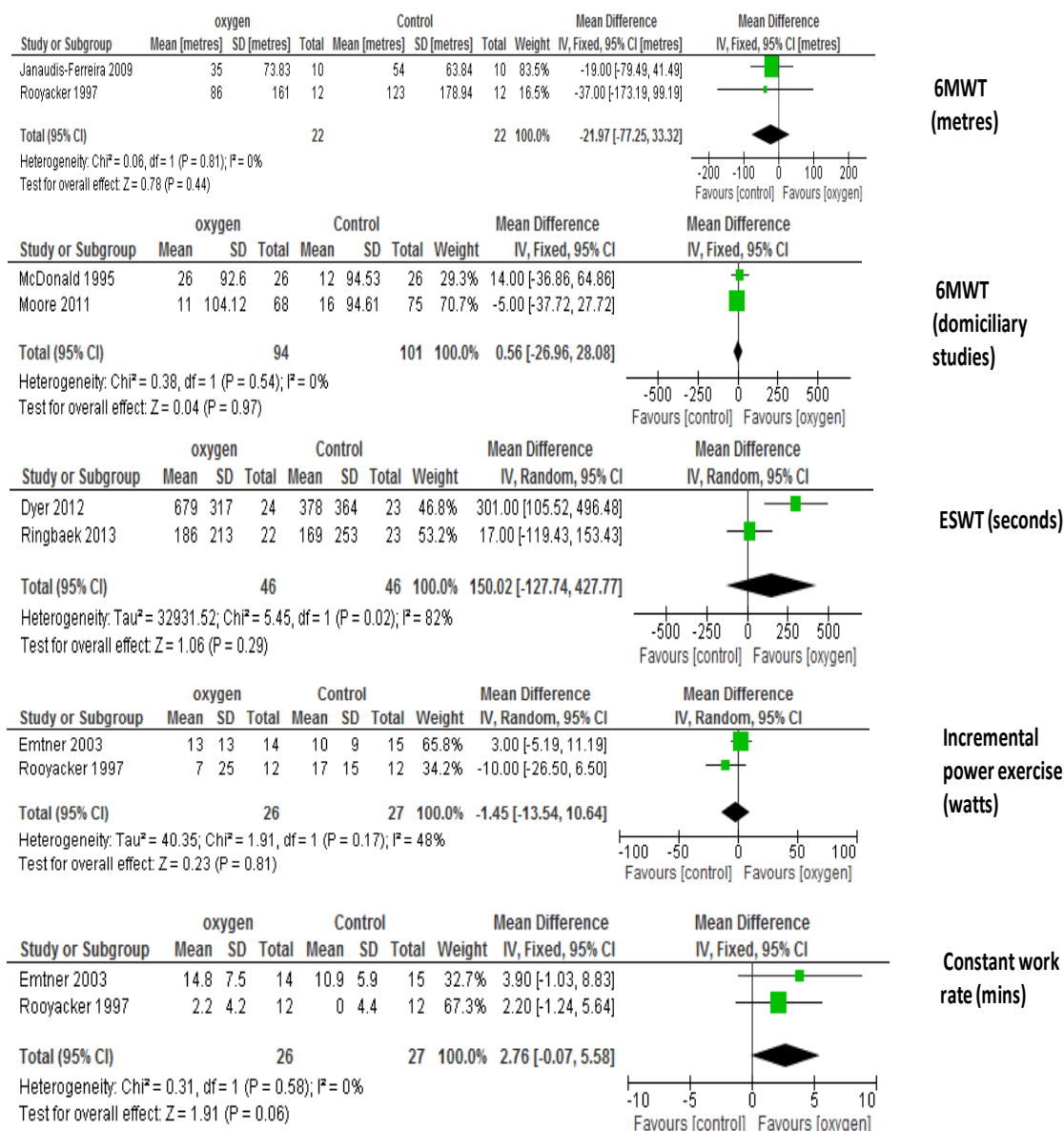


Figure 3.2 Forest plots showing the effect of ambulatory oxygen therapy on exercise capacity.

Two studies (both domiciliary) (184, 186) could not be used in the meta-analysis as one study provided p values only(184) and the other was the only one to use 5MWT(186).

Neither showed any statistical benefit of long term ambulatory oxygen on 6MWT distance (p=0.9) or 5MWT distance for individual participants.

Borg dyspnoea Scores

End of test Borg score were measured in 2 of the meta-analysed PR studies that used 6MWT(182, 183, 187) and 2 RCTs using incremental work exercise testing(183, 185). Heterogeneity between the studies was not significant ($I^2=0\%$). Neither were statistically significant; effect 0.16(-0.61 to 0.93) and 0.25(-0.81 to 1.31) respectively; Figure 3.3).

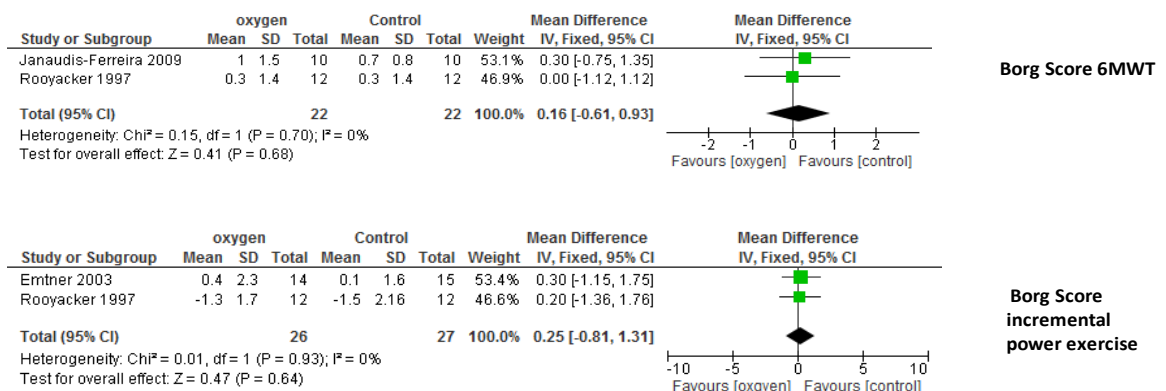


Figure 3.3 Forest plot showing the effect of ambulatory oxygen on end test exercise induced dyspnoea.

3.2.1 Quality of life

This was measured by CRQ (chronic respiratory questionnaire) in 7 studies(182-186, 188, 189) 3 of which were PR studies. In the PR studies there was no statistical difference between oxygen and control in any domain, however there was a strong trend to

statistical significance in CRQ emotion and fatigue favouring oxygen; $p=0.06$ and $p=0.09$ respectively (Figure 3.4). In the domiciliary studies the effect in all domains was in favour of oxygen treatment, significant only in dyspnoea ($p=0.002$) and fatigue ($p=0.01$) (Figure 3.5).

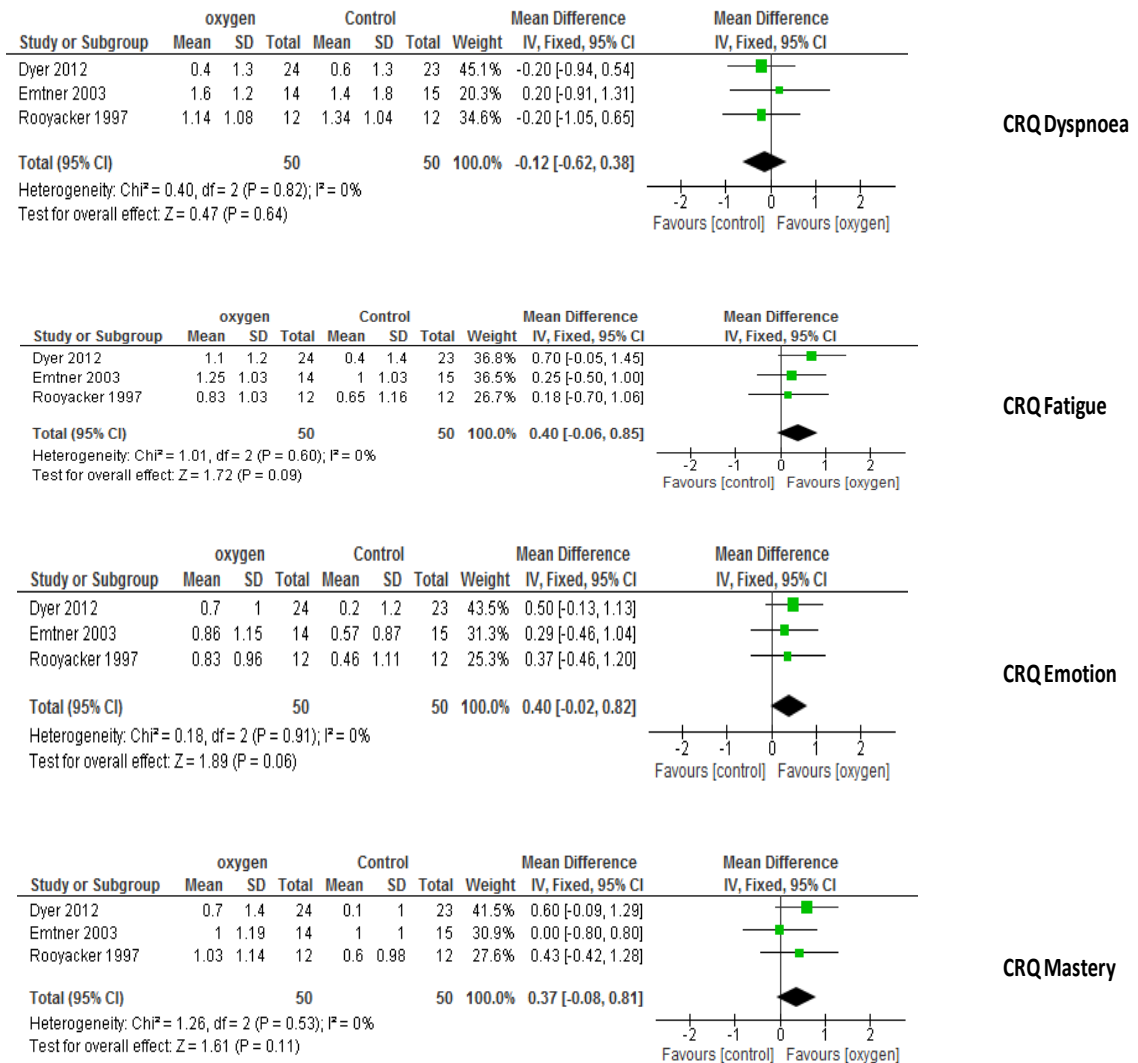


Figure 3.4 Forest plots showing the results of ambulatory oxygen on HRQoL in the Pulmonary Rehab studies.

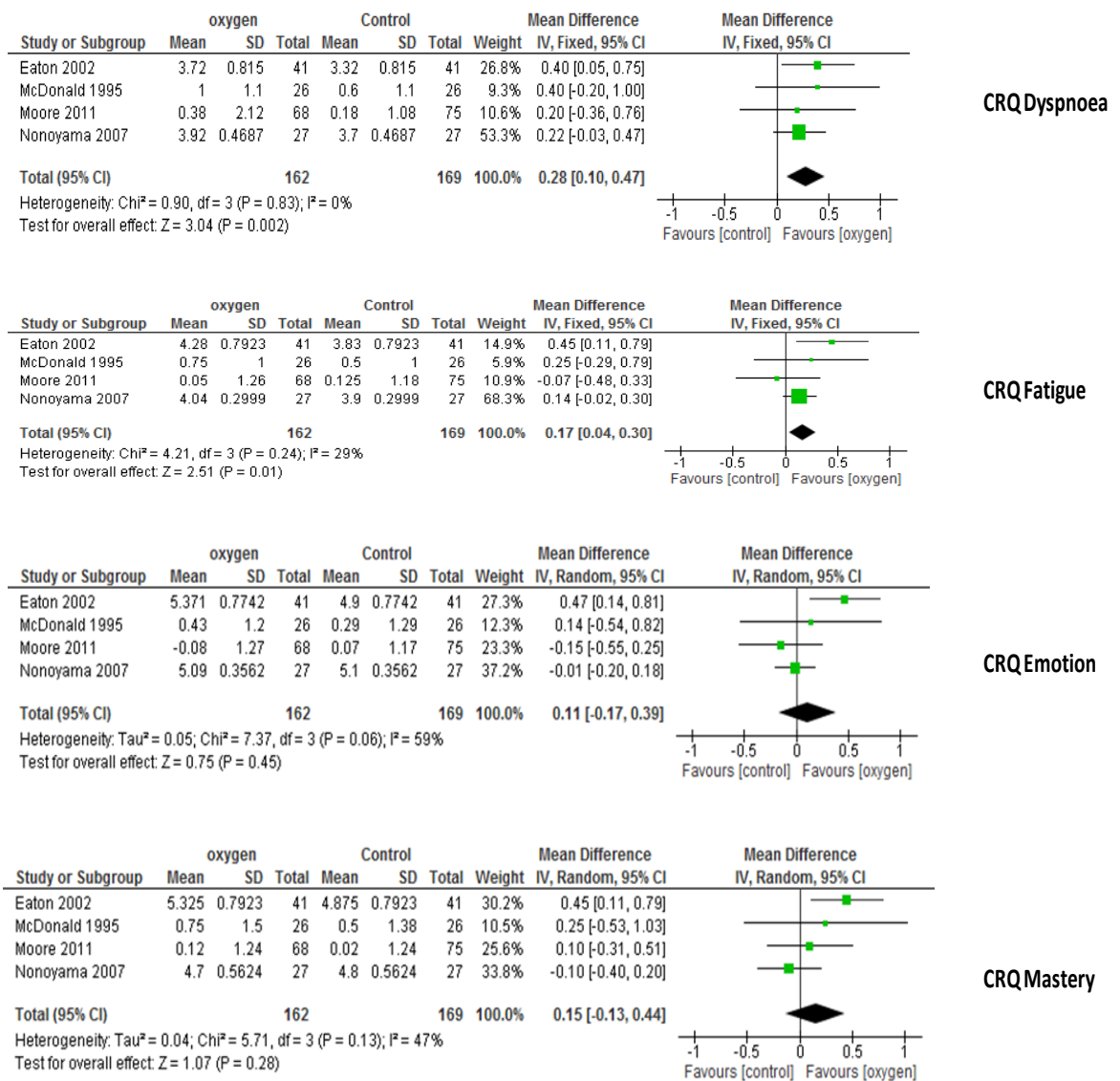


Figure 3.5 Forest plots showing results of ambulatory oxygen on HRQoL in domiciliary studies.

3.3 Impact of device on ambulatory oxygen delivery

14 studies evaluated a variety of oxygen conserving devices (OCDs) and cylinder types(191-204) (table 3). Modes of delivery included DODs (demand oxygen devices), PRC (Pendant reserve cannulae) and continuous flow nasal cannulae (CFNC). In some studies multiple OCDs were compared. Where this was the case, the most effective OCD was used in the meta-analysis comparing it to standard CFNC.

5 OCDs studies(192, 194, 196, 200, 204)were used in two separate meta-analyses. Tests for heterogeneity were not significant (both $I^2=0$).

3.3.0 Exercise capacity

3 of the 5 meta-analysed studies compared DODS against standard CFNC using 6MWT as an outcome measure(196, 200, 204). DODS and CFNC did not differ (CI-10.28 to 1.60), Figure 3.6).

Exercise oxygen saturations

Oxygen saturations were used in 2 of the 5 studies(192, 194) with methods of delivery being PRC and DODs respectively. Oxygen saturations were 2.03% higher with OCDs compared to CFNC, (95% CI 0.09 to 3.98, $p=0.04$, Figure3.6). In contrast, Roberts et al demonstrated that DODs caused a significantly longer period of desaturation <90% than CFNC $p<0.001$.

3.3.1 Oxygen utilisation

Three studies(191, 193, 198) evaluated the ability of OCDs versus standard CFNC to conserve oxygen in patients during exercise by calculating an oxygen conservation ratio at 90% SpO₂ (i.e. the relative difference in oxygen flow required to maintain oxygen

saturation with both devices at 90% SpO₂). 2 studies used DODs(193, 198) and 1 PRC(191). Although a higher DODS setting was required to maintain equivalent oxygen saturations, DODs still conferred 2.9-7.6 fold oxygen saving which was significant when compared to standard CFNC $p < 0.001$. A similar result was demonstrated with PRC; oxygen utilisation was significantly lower than CFNC $p < 0.05$ (191).

3.3.2 Other outcomes

Individual studies demonstrated no significant difference in exercise capacity with either refilled cylinders or portable oxygen concentrators when compared to standard commercial cylinders(197, 201). Patterns of either home activity or oxygen utilisation were not significantly different when lightweight cylinders were used compared to heavier E cylinders.(203)

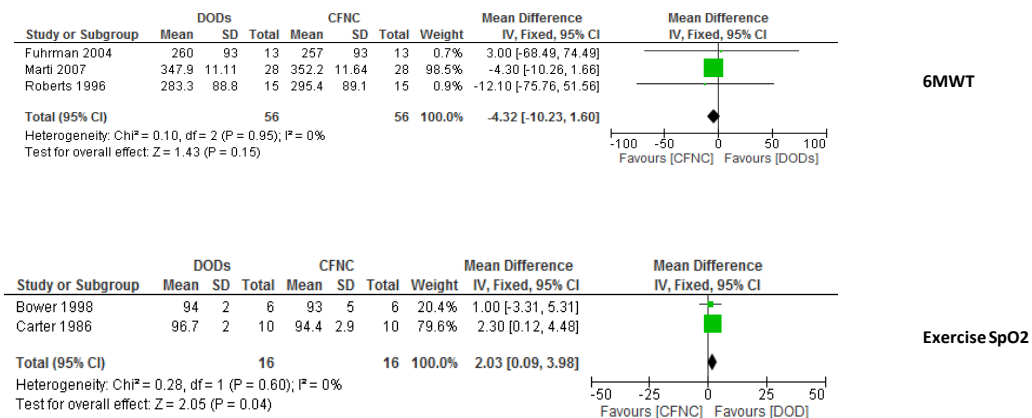


Figure 3.6 Forest plot showing the effect of OCDs on exercise capacity and exercise saturations

3.4 Cost effectiveness

There were no published studies found from the searches which assessed cost incremental ratios or cost effectiveness for ambulatory oxygen in addition to usual care.

Since there were no studies that assessed this, and clinical effectiveness could not be proven in any domain, it was not possible to answer the question of whether ambulatory oxygen is cost-effective. However it may be relevant to consider what the potential cost of ambulatory oxygen would be if used widely in the UK. The prevalence of patients with COPD on 3 medicinal preparations (e.g. LABA/ICS,SABA and LAMA) and likely severe disease is 8.7%(2). Studies indicate the prevalence of EID in severe COPD at approximately 30%(80, 82). There are approximately 820,000(2) people with COPD in the UK and thus a conservative estimate of patients with EID in the UK is approximately 21,000. Previous studies have indicated variable ambulatory oxygen usage from 40-50 minutes daily(182, 188) to 3.5 hrs(186). Using tariff data from Hertel et al(205) (daily unit cost £14.70) estimated annual costs would be £3.85 million if all patients were treated for 40-50 minutes per day. This is a considerable burden to the health system, particularly if effectiveness is not well established. The results from the included studies imply that continuing to prescribe based on acute response which is reassessed in those who have completed pulmonary rehabilitation may be a way of limiting cost, and possibly treating those likely to benefit most at home.

3.5 Discussion

This review evaluated the use of ambulatory oxygen in three separate ways: efficacy against placebo, optimum mode of delivery and cost. Firstly, we have shown that

ambulatory oxygen is unlikely to be effective at improving functional exercise capacity or symptoms of breathlessness in unselected patients with EID, although there may be some patients who respond well. Secondly we have shown that use of OCDs is appropriate, in that outcomes are similar or better to CFNC but at lower oxygen consumption. Since the review showed no significant overall effect of oxygen, and there were no published cost-effectiveness studies we were unable to calculate an incremental cost effectiveness ratio (ICER). Each area of the results will be discussed here in turn.

Clinical effectiveness of ambulatory oxygen

The prescription of ambulatory oxygen therapy in COPD is governed by an improvement in exercise capacity or Borg dyspnoea scores, wherein a 10% improvement in distance walked or reduction ≥ 1 in Borg Score indicates it should be used in patients with EID > 4% (78). The majority of our results suggest that there is not a benefit of this magnitude (Figures 3.2 and 3.3). It is important to highlight that in the included studies the definition of EID was not uniform, ranging from mild (<92%) to more severe desaturation (<88%). Since results for some outcomes were heterogeneous it is important to consider how patient selection influenced results. Two studies (182, 188) used exertional dyspnoea as their main inclusion criterion and were analysed separately, although 1/3 of patients in the Moore et al (188) study also exhibited EID. Few studies specified “acute responders” (i.e. those with >10% improvement in walking distance); The only study (189) which did use ESWT as the outcome measure and showed significant improvement in those randomised to ambulatory oxygen compared to compressed air, implying that appropriate patient selection is important in optimising clinical outcome. Interestingly however, 7 studies (182-186, 188, 190) included a single assessment acute oxygen test for all participants as part of their protocol. All but one (190) demonstrated a significant

mean improvement in exercise capacity for participants with acute oxygen therapy compared to compressed air which is in line with a previous Cochrane review(100). Whether the acute effects of oxygen persist in the long term in trained or untrained participants was addressed in 2 of the included studies(183, 184) as they all included a repeat acute oxygen test at the end of the study period. Rooyackers et al(183) and Eaton et al(184) both demonstrated that this acute effect was no longer statistically significant when using 6MWT as the outcome measure. It would therefore seem that any acute improvement in walking distance observed in a single assessment study is not maintained. This has implications for clinical practice as prescriptions for ambulatory oxygen based on improvements in exercise capacity observed in single assessment studies as suggested by guidelines(78) are not sustained or are minimal in the longer term. However, what currently determines the longer term efficacy of AOT is the subjective assessment of whether patients feel it has helped them increase their own physical activity. Where patients feel there has been no benefit; AOT is removed.

The way in which exercise capacity was measured may also be relevant to the interpretation of results. Largely it was measured using field tests (ESWT or 6MWT) which particularly in the case of the 6MWT have been suggested to relate more to activities of daily living(96, 206). The meta-analysis of exercise capacity with respect to 6MWT slightly favoured the control group and showed no benefit of ambulatory oxygen, implying that in normal daily life it would not be expected to help patients be more active. In the ESWT meta-analysis, although the result was not statistically significant in favour of oxygen therapy it may be clinically significant. The MCID for the ESWT is estimated at 45-85 seconds (207). The common effect of 150 seconds exceeded this although this must be approached with caution because the heterogeneity between the

two studies(189, 190)was considerable. Both enrolled patients exhibiting EID>4% and predominantly severe COPD. During the ESWT in the Dyer et al (189)study patients had their oxygen cylinders carried by an assistant whereas this was not the case in Ringbaek et al(190). It is recognised that any improvement in exercise capacity can be negated by the weight of the ambulatory oxygen system if carried by patients alone(196, 208), thus it is possible the Dyer study overinflated the benefits of treatment in real life by providing assistance. The meta-analysis of endurance cycle time strongly favoured oxygen treatment and was close to approaching statistical significance. Furthermore this is likely to be an underestimate of the effect of ambulatory oxygen treatment as 16 endurance tests in the oxygen group were terminated at 30mins compared to 6 endurance tests in the control group(185). The responsiveness of constant work rate test such as the ESWT and cycle endurance to intervention is far better than 6MWT(209) which may explain the discrepancy between these different exercise outcome results. However at present no MCID for cycle ergometry has been established and its relationship to activities of daily living is unclear. Thus far no study of ambulatory oxygen therapy has used home activity or activities of daily living as an outcome measure. Real world performance of ambulatory oxygen has been largely gauged by 6MWT results. It is now possible to measure home activity with a number of validated accelerometers(180). This was part of the rationale for including home activity as an outcome measure in the Ambulatory oxygen study for muscles in COPD trial (NCT01722370) which forms chapter 4 of this thesis. Given that 6MWT is also an outcome measure we will be able to explore the relationship between them.

Some studies(183, 185, 187, 189, 190) were carried out as part of pulmonary rehabilitation. Although no statistical benefit of long term ambulatory oxygen was demonstrated, exercise capacity (particularly 6MWT) in the pulmonary rehabilitation studies exceeded that of the domiciliary studies(182, 188)whether patients were randomised to ambulatory oxygen or placebo, figure 2.1 and 2.2. What is also clear is the improvement in 6MWT distance gained by pulmonary rehabilitation far exceeds that gained by ambulatory oxygen(183). This supports current guidelines(78) in that any assessment of ambulatory oxygen therapy should be made following pulmonary rehabilitation.

Finally, it is important to consider the meta-analyses possible using CRQ domains. In general there was a benefit of ambulatory oxygen on QoL, which was most pronounced in the dyspnoea and fatigue domains; the others were not statistically significant but showed a strong trend in the same direction. However, the effect size was only 0.28 and 0.17 in the statistically significant domains. Although the MCID for PR adjuncts has not been established it is less than the proposed 0.5 points which is considered to represent the MCID for an individual CRQ domain(210). This explains the apparent discrepancy between Borg score and CRQ dyspnoea score meta-analyses; neither were clinically significant, thus they concur, despite the fact that statistically the CRQ score was better with oxygen. The effect size is also considerably less than the effect on CRQ of pulmonary rehabilitation (0.62 points) (211) confirming that assessment for ambulatory oxygen should only be done after rehabilitation.

Impact of device on ambulatory oxygen delivery

The mode of delivery of ambulatory oxygen therapy largely encompasses the cylinder type and the interface used. Oxygen conserving devices (DODS/PRC) have been used for

many years and the theory supporting their use has been discussed elsewhere (193). In short “classic” DODs devices deliver oxygen during the initial phase of inspiration alone whilst PRC stores oxygen during exhalation to be delivered at the next inspiratory phase. The role of OCDs is to conserve oxygen however in doing so must perform equivalently to standard CFNC in ameliorating oxygen desaturation. Some studies(192, 194, 196, 200, 204) included in the meta-analysis indicate that oxygen conserving devices perform equivalently to standard CFNC at maintaining exercise oxygen saturation and 6MWT. Importantly however, it is likely that oxygen conserving devices achieves these outcomes at a much lower oxygen utilisation than CFNC. This is likely to have implications in terms of oxygen cylinder usage and subsequent cost. Although the primary role of OCDs is not to improve exercise performance over CFNC, patients use these devices on the premise that they are ambulatory. Thus it is reassuring that functional performance is not negated by the weight of the system.

It is important to note that there was considerable variation between OCDs and their performance and in the meta-analyses the best performing OCD was used. Notably in the study by Palwai et al (202) one of the DODs systems performed no better than ambulation on room air. Also there may be some patients that do not tolerate OCDs and their use may cause further desaturation. This is supported by the study by Marti et al (204) in which use of DODs failed to correct EID in approximately a fifth of participants. This has been explained by a change in respiratory pattern on more strenuous exertion where by the predominance of mouth breathing rather than nasal a fails to trigger the flow sensor(196).

Oxygen cylinders have historically been the main method of delivering portable oxygen, however newer devices such as portable concentrators are now available. These devices have variable weight with cylinders weighing between 3.4-8kg and portable concentrators between 3-4kg. As mentioned previously the weight of the ambulatory oxygen system (cylinder in this case) is an important factor as the portable oxygen system should be carried by the patient during their ambulatory oxygen assessment and following this in their ambulatory activity. The weight of the portable oxygen systems has previously been cited as one of the reasons why patients with COPD have poor concordance with ambulatory oxygen prescriptions(212). An interesting observation from one of the studies (203) is that ambulatory cylinder weight had no impact on home activity. One would hypothesize that a lower weight would facilitate ambulation. This was not the case despite prior efforts of focused education on increasing oxygen treatment understanding and ambulation.

The comparison of standard cylinders (197) to refillable (Homefill), portable concentrators and lightweight cylinders demonstrated no difference in exercise capacity (6MWT), oxygen saturations or time used. From this one can conclude that most portable oxygen systems perform equivalently and when used should be coupled with oxygen conserving devices in the first instance. Currently when ordering ambulatory oxygen, the HOOOF (Home oxygen order form) system tends to select OCD devices for those who are most active which is in line with current evidence(81).

There are a number of limitations to the meta-analysed data in this chapter. The quality of data from the published papers was poor to average limited by small sample sizes and high risk of selection and detection bias especially in the older studies. In addition,

patient selection and heterogeneity of outcomes may limit the generalisability of the results. There were no published studies on cost effectiveness of AOT. Whilst cost effectiveness has been evaluated to some extent in inhaled therapies in COPD the lack of data in this area with respect to AOT may be due to the difficulty analysing specific outcomes. Inhaled therapies have been shown to reduce exacerbations rates; mild to severe and consequently in many cases healthcare utilisation. These outcomes can be defined by cost, especially in the context of hospital admissions where which exacerbations are grouped under a Healthcare Resource Group (HRG) with an indicated cost(205). AOT main effect is to increase functional exercise capacity and negate desaturation. Whilst AOT will have an effect on QoL and exercise capacity it is difficult to define the economical impact of these benefits. Finally as alluded to in this section, outcomes in this chapter did not include other clinically relevant endpoints such as exacerbations or mortality largely due to paucity of information.

Conclusion

Although limited by patient selection and heterogeneity in outcomes this review has shown that in unselected patients with EID AOT is unlikely to provide lasting clinically meaningful improvements in exercise capacity, when judged by the 6mwt or in BORG scores. However, some patients may respond well and derive benefit either clinically or subjectively. Pulmonary rehabilitation should be undertaken before AOT assessment as the improvement in exercise capacity from PR far exceeds any improvement when standalone oxygen is given to patients in a domiciliary setting. When AOT is prescribed, O₂CDs should be use in preference to CFNC as they are likely to achieve similar oxygen saturations and mitigate oxygen desaturation at lower total oxygen usage.

Several studies in this review were in the setting of pulmonary rehabilitation, which have been shown to improve exercise outcomes(213) with the resistance component improving markers of muscle myogenesis(214). Domiciliary studies whether supported by AOT have never used skeletal muscle myogenesis or gene expression as an outcome measure. Since there is no exercise component in these studies; benefits in exercise or skeletal muscle outcomes must either be derived from a direct action of oxygen or improvements in physical activity; one of the assumptions made when AOT is prescribed. Moreover, no study included in this review measured physical activity directly by use of activity monitors or using patient reported questionnaires of activity during the study period. Therefore, it is unclear what affect AOT would have on patient physical activity and whether this translates to improved exercise or skeletal muscle outcomes. This is the subject of investigation in chapter 4.

Chapter 4

Clinical Outcomes

This chapter outlines the clinical results from the Ambulatory oxygen for muscles in COPD trial (NCT01722370); the methods for which are discussed in chapter 2.2. This study investigated the role of AOT on skeletal muscle gene expression, systemic inflammation, physical activity, exercise capacity and quality of life.

Patients were recruited between September 2012 and August 2014 and randomised to 12 weeks of AOT or placebo air in a cross over fashion. Skeletal muscle biopsies were taken at trial enrolment (baseline sample) and after each intervention. Markers of systemic inflammation and exercise capacity were measured at the same timepoints as above. In addition to measuring physical activity at trial enrolment and after each intervention, physical activity was also measured at the midpoint of each intervention, i.e. at 6 weeks. The study design diagram is shown in figure 4.0

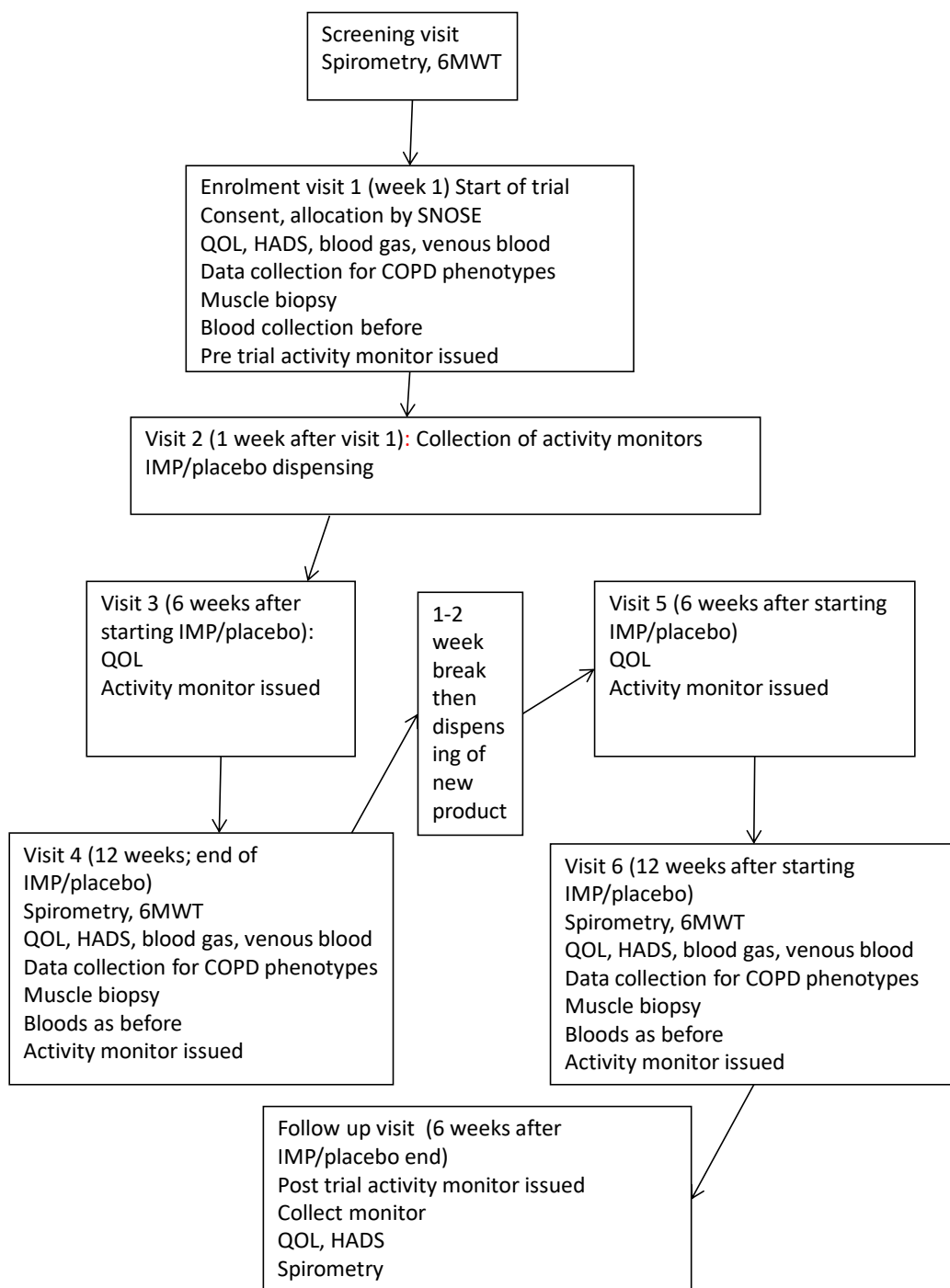


Figure 4.0: Study design Diagram

4.1 Patient characteristics

Twenty five participants were recruited to the clinical trial following the initial screening of thirty two patients. All participants had completed pulmonary rehabilitation within 2 years prior to trial enrolment and demonstrated exercise induced desaturation to a nadir <88%. All but one patient had obstructive lung disease with a FEV1/FVC ratio of <70% with the remaining patient having radiological upper zone predominant emphysema.

Seventeen patients completed the trial. The reasons for non-completion were that 1 patient required LTOT, 3 were lost to follow up and 4 withdrew consent. The trial study plan is shown in figure 4.1.

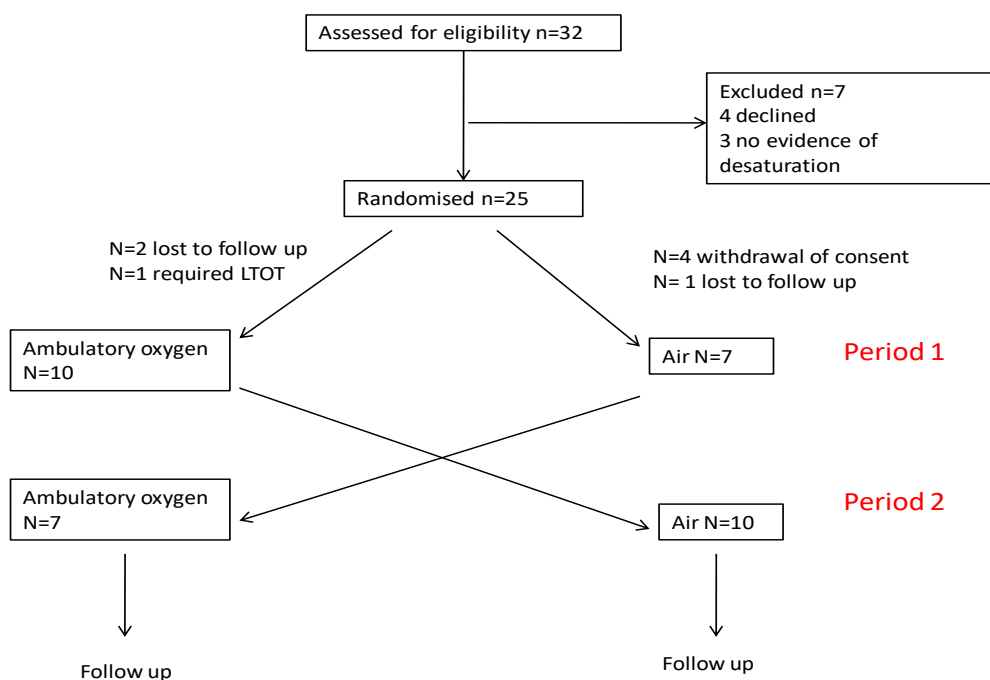


Figure 4.1: Trial flow diagram

In general the recruited patients had spirometrically severe COPD, were very symptomatic and had anxiety/depression scores indicative of clinically significant psychological co-morbidity. There was considerable variability in baseline exercise capacity, as demonstrated by the 6 minute walk test distance and there was a wide range of body mass indices (BMI). Table 4.1 shows the baseline demographic and clinical data.

Age (years)	67.35(7.45)
Gender (M:F)	7:10
FEV1 absolute (litres)	1.18(0.7)
FEV1% predicted	50(23.6)
FVC absolute (litres)	2.27(0.72)
FVC % predicted	80.5(18.87)
Desaturation nadir %	86(85-88)
BMI (kg/m ²)	28.77(19.2-30.07)
Baseline 6MWT distance (metres)	223(143.9)
Baseline CAT score	27.94(6.45)
Baseline HAD Score	17.17(5.97)
Baseline home activity (VMU/min)	251.3(157.3-430.07)

Table 4.1: Baseline demographic data for trial participants. Data expressed as mean (Standard deviation), Median(25th-75th Quartile)

4.2 Cylinder usage

Cylinders weighed approximately 4kg. Oxygen flow rates were set at 2l/min and patients were instructed to use this flow rate when performing routine physical activity. The mean number of cylinders used during oxygen treatment was 5 (1.62) compared to 4.82(2.21)

during the air treatment phase ($p=0.78$). The cylinders provided by BOC had a run time of approximately 5hrs at 2l/min. Given the mean cylinder usage, this would equate to less than one hour; approximately 30mins air/oxygen use daily.

4.3 Exercise capacity and Home Activity

At baseline 6MWT distance correlated significantly with baseline lung function (FEV1%) ($r=0.63$, $p=0.007$, figure 4.3) but no other clinical variable. 6MWT distance was not significantly different in patients with high ($BMI>25$) or low ($BMI<25$) $p=0.399$. The extent of exertional desaturation did not correlate with 6MWT distance during air or oxygen treatment (Spearman's $R=-0.001$, $p=0.97$ and Spearman's $R=0.11$, $p=0.68$) respectively.

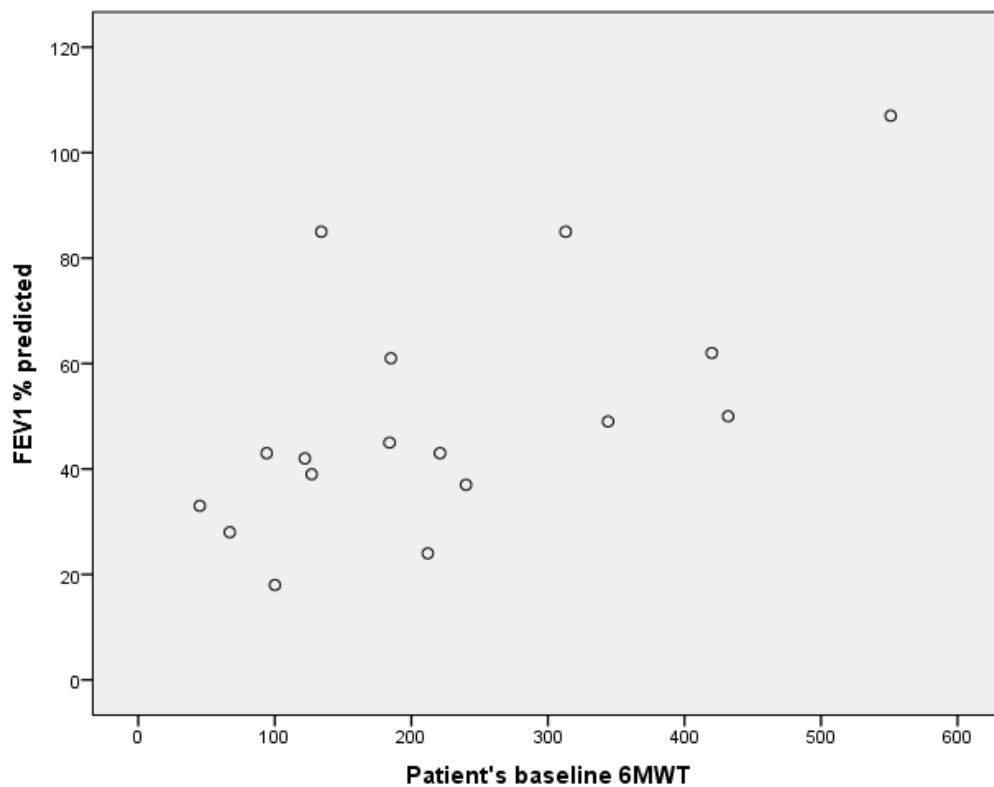


Figure 4.2: Correlation of baseline 6MWT and FEV1% predicted $r=0.63$ $p=0.007$

Exercise capacity (6MWT) increased from baseline in both arms of treatment. The magnitude of improvement was greater during ambulatory oxygen treatment (34.06metres compared to 16.25 metres). However, neither of these improvements from baseline was statistically significant $p=0.279$ and $p=0.51$ for ambulatory oxygen and air treatments respectively. Use of the tri-axial accelerometer showed that patients were generally inactive with no patient spending more than 10% of the time in moderate to vigorous physical activity at any point during the trial. Home activity improved from baseline during AOT both at trial midpoint and at trial end. Neither of these changes were statistically significant. Midpoint home activity was not significantly different to home activity at the end of the treatment phase during AOT or air treatment; $p=0.91$ and $p=0.86$ respectively. Table 4.2 summarises these results.

	Air	Oxygen	Air vs Baseline	Oxygen vs Baseline	Air vs Oxygen
6MWT	16.25m (99.25)	34.06m (125.33)	$P=0.51$	$P=0.279$	$P=0.65$
Home Activity At Trial midpoint	241.75(169.7-376.8)	279(207.3-415.86)	$P=0.99$	$P=0.37$	$P=0.41$
Home activity Trial end (VMU/min)	238.3(171.3-374.38)	278.9 (207.4-413.43)	$P=0.875$	$P=0.308$	$P=0.32$

Table 4.2: Table shows the change from baseline in 6MWT distance following interventions and home activity (VMU/min) at the end of each intervention period.

Interestingly home activity following oxygen treatment correlated significantly with 6MWT following ambulatory oxygen treatment (Spearman's $R=0.751$, $p=0.005$). This

relationship however was not present at baseline (Spearman's $R=0.399$, $p=0.199$) or following air treatment (Spearman's $R=0.441$, $p=0.152$, figure 4.3a-c).

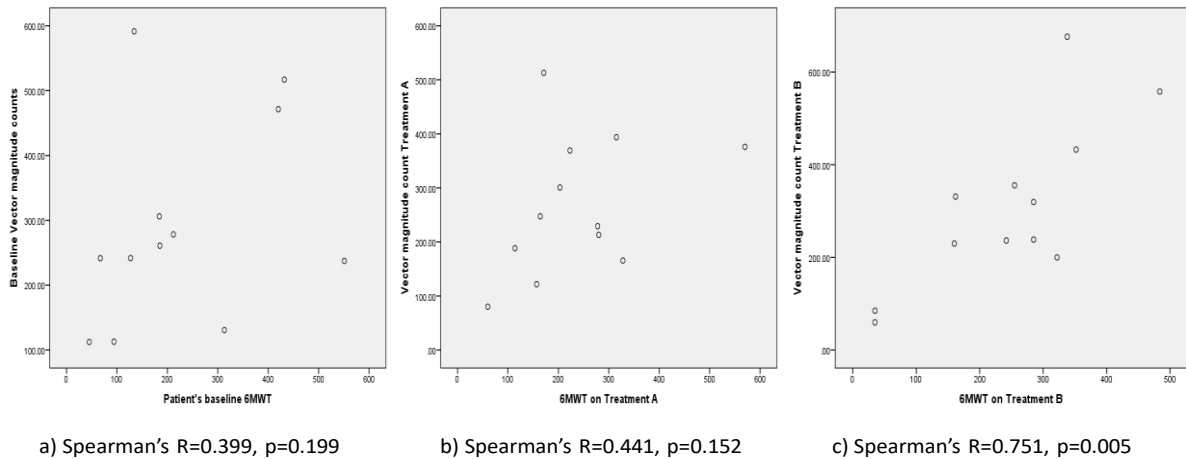


Figure 4.3 a-c) Correlation of home activity with 6MWT distance at baseline, following air (Treatment A) and ambulatory oxygen (Treatment B)

4.4 Health related quality of life and Anxiety and Depression

All recruited patients had baseline CAT scores of more than 20 indicating that COPD had a clinically significant impact on their quality of life. As with exercise capacity the response to treatment was heterogeneous. Following oxygen treatment 11/17 patients had reduced (improved symptom) scores compared to 8/17 following air treatment. The mean change from baseline was $-1.59(6.86)$ following oxygen treatment and $0.64(4.24)$ following air. Neither were statistically significant ($p=0.354$ and $p=0.538$ respectively).

All but one patient at baseline had evidence of clinically significant anxiety and depression. Following both interventions there was a reduction (improved symptoms) in

HADs score; -2.47(4.45) with oxygen treatment and -1.82(4.57). Table 4.3 summarises these results.

	Air	Oxygen	Air vs Baseline	Oxygen vs Baseline	Air vs Oxygen
CAT	0.64(4.24)	-1.59(6.86)	P=0.35	P=0.54	P=0.26
HADs	-2.47(4.45)	-1.82(4.57)	P=0.13	P=0.04	P=0.79

Table 4.3: Change from baseline scores for HADs and CAT.

Only the improvement in HADs from baseline following oxygen treatment reached statistical significance; $p=0.04$ compared to $p=0.13$ following air treatment, however there was no significant difference between AOT and air treatment. Figure 4.4 shows the individual HADs patient scores during treatment phases.

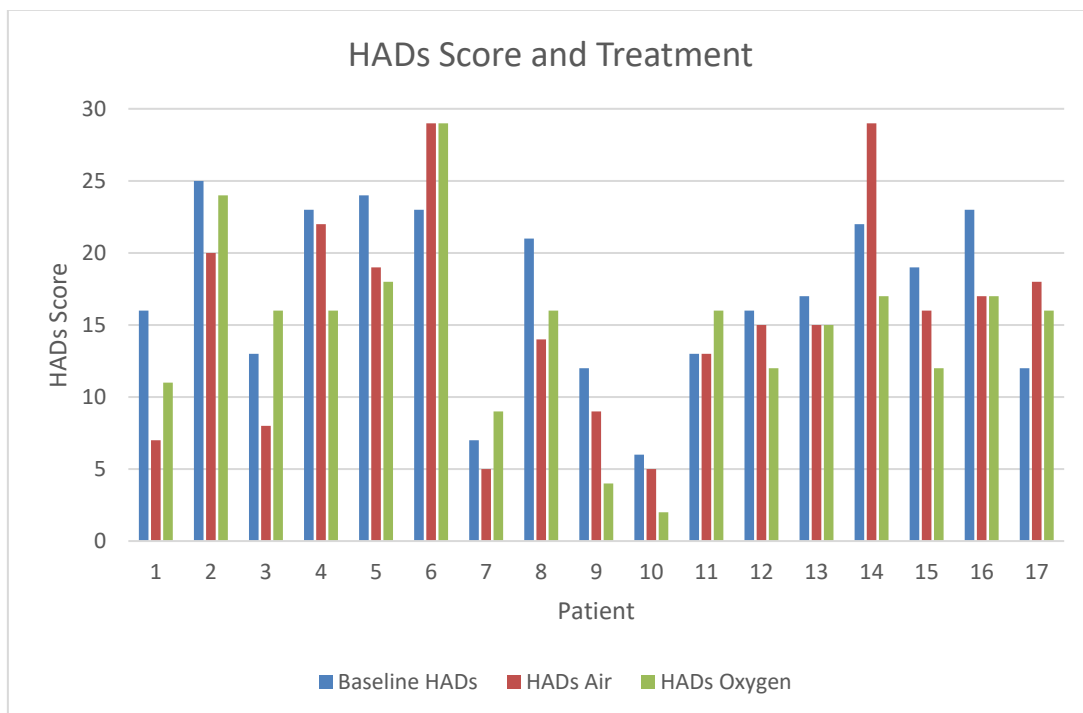


Figure 4.4: HADs score during treatment arms

4.5 Systemic Inflammation

The results of the plasma cytokine levels in response to oxygen and air treatment are shown in table 4.4. TNF alpha levels were generally low and did not show significant change following either intervention.

	Baseline	Air	Oxygen	Air vs Baseline	Oxygen vs Baseline	Air vs Oxygen
Il-6 pg/ml	19.01(10.88-63.47)	28.61(10.52-62.72)	12.17(7.82-41.91)	P=0.94	P=0.04	P=0.35
Il-8 pg/ml	5.29(4.35-7.33)	6.33(5.08-8.03)	5.57(4.44-6.64)	P=0.48	P=0.58	P=0.89
IFN-g pg/ml	181.25(99.73-273.84)	215.06(132.37-372.07)	267.92(123.73-285.87)	P=0.31	P=0.35	P=0.76
TNF- α pg/ml	0.57(0.45-0.81)	0.54(0.48-1.13)	0.48(0.4-1.05)	P=0.53	P=0.37	P=0.92

Table 4.4: Systemic cytokine response to oxygen and air during clinical trial.

Plasma interferon gamma levels rose following both treatments but this change was not significantly different from baseline. The only significant change from baseline during the clinical study was a reduction in plasma IL-6 levels following oxygen treatment although there was between group difference was not significant. Individual patient responses of IL-6 and treatment are shown in figure 4.5

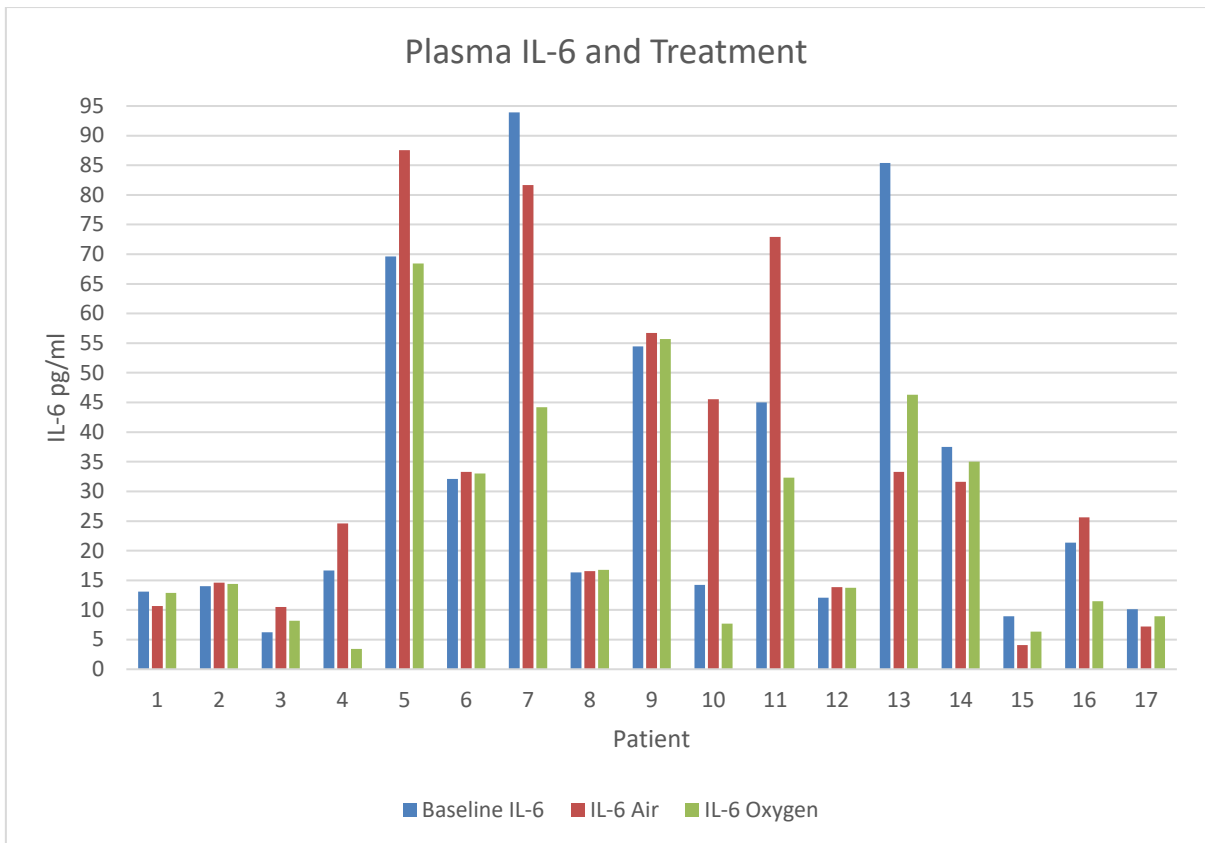


Figure 4.5: Plasma IL-6 during treatment arms

4.6 Discussion

The secondary aims of this study were to determine whether ambulatory oxygen therapy had any long term impact on functional exercise capacity, quality of life or systemic inflammation. The results from the systematic review analysis (chapter 3) suggest that it would have little impact on these clinical outcomes save for quality of life. The benefits of this study in comparison to others presented in chapter 3 are that the duration of the study is far longer than any previous study which assessed ambulatory oxygen in a domiciliary setting. It also investigated, systemic inflammatory response and home activity alongside functional exercise capacity, as measured by the 6MWT. The hypothesised mechanisms by which AOT may mitigate EID and improve exercise capacity

have been discussed in 1.4.1 and 1.5.1. Briefly they include, improved V/Q ratios and arterial oxygen concentration, reduction in peripheral chemoreceptor activity, reduction in peripheral/central fatigue and reduction in skeletal muscle lactic acid production. Collectively these mechanisms are likely to result in either improved arterial oxygenation or reduction in the sensation of dyspnoea which may both beneficially effect exercise capacity or activity. Central to this therein is the use of AOT. During the trial cylinder usage both for AOT and air was low; roughly equating to less than 30mins daily use. It is unlikely therefore that usage was sufficient to mediate either direct action of oxygen or that AOT encouraged participants in the trial to be more active. The effects of AOT on functional exercise capacity and home activity are discussed in turn.

Functional exercise capacity has been shown to be reduced in COPD patients(215) with a number of factors determining poor 6MWT performance (defined as a 6MWT distance <350metres)(216). Determinants of 6MWT distance are complex, however airflow limitation and the presence of depressive symptoms are two variables which have been shown to influence it(216). The significant positive correlation between FEV1 % predicted and 6MWT in the data figure 4.3 supports this although at best only explains 39% of the variance in baseline 6MWT in the cohort of patients enrolled. Low BMI has been also linked to poor 6MWT performance(217). However in this cohort 6MWT distance at baseline did not differ when the cohort was dichotomised too high and low BMI. This in part may be due to the small numbers of patients in the study. Also, it is acknowledged that BMI is a poor surrogate for fat free mass which is linked to sarcopenia and reduced exercise capacity(218). The improvement in 6MWT distance compared to baseline following AOT was almost double that of air and greater than that shown in other

domiciliary studies (182, 188). This improvement although not statistically significant it is important to explore if it has any clinical relevance. Evidence from the ECLIPSE (Evaluation of COPD Longitudinally to Identify Predictive Surrogate End Points) cohort suggests that the MCID (minimum clinically important difference) for 6MWT is 30metres and represents increased risk of death from exacerbations(219). Since some patients enrolled in the study improved by more than the MCID and the study population was smaller than intended at the outset, thus could be underpowered, it would be worthwhile to conduct a larger study to determine if the improvement is genuine or not.

Domestic activity is also reduced in patients with COPD. The results from the clinical study suggest that baseline activity in the enrolled participants was relatively low (median 238.3 VMU/min) with very little time spent doing moderate or vigorous activity. This is consistent with prior observational work in our local population(220) gathered as preliminary data prior to this trial. This study showed the patients with COPD spent a greater proportion of time sedentary and far less doing moderate to vigorous physical activity. A VMU of 290/min roughly equates to a walking speed on a treadmill of 1mph(221), which is significantly slower than in health (~4 mph). The reduced activity levels in the enrolled patients are consistent with a number of other studies (203, 220). The study by Casburi et al (203) demonstrated physical activity was low VMU 108.9 (48.6) albeit in patients receiving LTOT therapy.

Domestic activity improved following oxygen treatment although this was not statistically significant. The trend to improved domestic activity with oxygen therapy was also demonstrated in a study by Sandland et al (166) which included heterogeneous COPD population of patients with EID (nadir \leq 90%) and those on LTOT. Interestingly there was

not a significant change in home activity at the trial midpoints i.e. when participants were using either intervention. This again indicates that AOT usage did not encourage increased activity in this cohort of participants. At present no MCID for data derived from accelerometers has been established so the clinical relevance of the improved activity with oxygen is not clear. Interestingly however the correlation between home activity and 6MWT during oxygen treatment was strongly significant (Spearman's Rho 0.751 $p=0.005$); a relationship that was not present at baseline nor following air treatment. This indicates firstly that the two variables correlate well, which one of the cited benefits of the 6MWT and secondly that the improvement in 6MWT with ambulatory oxygen is likely to be relevant as it may also have a clinically meaningful translation to home day to day activity.

In the future the measurement of physical activity in patients with COPD is likely to involve the use of structured questionnaires. Whilst accelerometers such as Actigraph have been well validated in this group of patients the ability of these devices to capture patients' experience/perceptions of activity is doubtful. Patients often limit physical activity to avoid symptoms(222) and may find themselves modifying their lifestyle because of this. This important information would not be gleaned from an accelerometer alone and has led to the formation of two questionnaires; daily proactive physical activity in COPD and clinical proactive physical activity in COPD which have shown promise in terms of their validity and reliability.

Health related quality of life (HRQoL) and anxiety and depression were assessed using the CAT and HADs scores. The CAT and HADs are measured out of 40 and 28 respectively with higher scores indicating worse symptoms. There was a trend to improved scores

following ambulatory oxygen therapy, significant only for HADs. The CAT assessment has not been previously used in studies of domiciliary ambulatory oxygen therapy. Previous studies used CRQ(182, 184, 188) which encompass 4 domains; fatigue, dyspnoea, emotion and mastery. The results from the systematic review in chapter 3 show use of ambulatory oxygen in a domiciliary setting significantly improved dyspnoea and fatigue alone; $p=0.002$ and $p=0.01$ respectively. Although these were significant improvements neither were clinically relevant; both effect sizes falling below the MCID. The trend to improvement in CAT score in this study concurs with the above and not likely to be clinically relevant given the MCID estimated for CAT is 2 points(223).

This study suggests that ambulatory oxygen therapy has a greater effect on anxiety and depression compared to the functional impact of COPD symptoms. Anxiety and depression are common co-morbidities in COPD with prevalence ranging between 13-46%(224). Both are likely to adversely affect quality of life and treatment adherence(225) and as alluded to earlier depressive symptoms also detrimentally affect functional performance i.e. 6MWT. The management is multifaceted and involves use of antidepressants, cognitive behavioural therapy and pulmonary rehabilitation. Here we show use of ambulatory oxygen influences anxiety and depression; significantly improved HADs score following treatment. Although there is no direct comparison, CRQ mastery and emotion scores which cover similar themes to HADs have shown a trend to improvement with oxygen use in studies of ambulatory oxygen therapy(182, 184) which supports this. Of note the HADs score also improved following air treatment. It is well recognised that many patient enrolled into clinical trials have improved quality of life even if randomised to placebo. This is in part due to the regular review visits patients undertake during a

clinical trial; in this case 6. However the role of “placebo” air is likely be important. The patient perception of oxygen in COPD is complex. Many patients see the use of oxygen therapy as a milestone of disease progression and are keen to avoid its use(226). Contrary to this the perception of oxygen therapy for some is that of a treatment which will improve dyspnoea, fatigue or help them do more(227). The perception that dyspnoea and hypoxia are interlinked and remedied solely by oxygen therapy seems to be a common view for some patients with COPD. From experience there is an intrinsic attachment to oxygen therapy when it is prescribed and there is some degree of anxiety if patients are told they no longer need it. It is highly plausible that placebo air would be more prone to this placebo effect given the above.

Systemic inflammation is thought to be a key component in COPD and implicated in the aetiology of SKMD as discussed in chapter 1. The cytokines measured in the study showed a differential response to ambulatory oxygen treatment. Plasma IL-6 and to some degree TNF α were reduced compared to baseline following oxygen treatment, with the reduction in plasma IL-6 reaching statistical significance although there was not a between group difference. Both TNF α and IL-6 are pro-inflammatory cytokines, recognised to be altered in COPD(228). TNF α signalling is mediated through two cell surface receptors (TNFR1 and TNFR2) whilst IL-6 classically via the IL-6 receptor(229-231). Both have been shown to activate the Nf-kB (nuclear factor Kappa B), which under normal conditions is present within the cytoplasm in an inactive state, bound to an inhibitory protein. Stimulation via either TNF α or IL-6 leads to an intracellular signalling cascade resulting IKK (inhibitor of kB kinase) phosphorylation of the Nf-kB/inhibitor complex which releases Nf-kB to the nucleus of the cell. Here, Nf-kB co-ordinates the

transcription of a number of chemokines, cytokines and ubiquitin proteasome pathway (UbP) leading to muscle atrophy(229). TNF α and IL-6 have been shown to cause muscle atrophy and impair myogenic differentiation in murine and in vitro studies(232, 233). Elevated levels of IL-6 have also been associated with radiological evidence of quadriceps wasting in COPD patients(141).

In contrast plasma IL-8 and IFN-g levels rose compared to baseline following oxygen treatment. The role of IFN-g in skeletal muscle signalling is not completely understood. Some studies have demonstrated that IFN-g is required for efficient skeletal muscle regeneration (234, 235). However in a murine model over expression of IFN-g at the neuromuscular junction caused a necrotizing myopathy (236). Muscle repair and differentiation occurs following a number of stimuli including exercise and is likely to have occurred during the trial period following episodes of exertion in the participant cohort. One would hypothesize that the elevated levels from baseline in both treatments (although not significant) represents this. IL-8 levels have been inversely correlated with quadriceps muscle strength albeit during exacerbations of COPD (140) and may also activate the NF κ B pathway in the same way as TNF α and IL-6. Although plasma levels IL8 rose during oxygen treatment, the rise was not significant.

There are a number of limitations to these results. Practice walks were not undertaken during the 6MWT. The standards for performing the 6MWT were published after patients were enrolled into the clinical trial. It is recognised that there is a learning effect when undertaking the 6MWT and this may have over inflated the benefits of oxygen therapy. Cylinder usage was low although mean usage however between the two treatments did not differ. This is consistent with previous data from local COPD patients showing that

activity levels were low(220), such that we feel the low usage of cylinders represents a true reflection of activity levels. The clinical trial was double blinded with cylinder flow rates were regulated at 2l/min. We accept that this may not have ameliorate EID in all participants. The cytokines measured were from the systemic circulation; changes in the systemic circulation may not directly represent those which occur at the muscle level and there is some evidence to suggest that this may be negligible (237). It is also likely the study was underpowered to detect differences in the clinical outcomes measured. Assessment of quadriceps strength is a notable omission from the study outcomes. This would have aided in the phenotyping of patients with skeletal muscle dysfunction and not just those with reduced exercise capacity. However we were limited in this by lack of the equipment to do this at our hospital enrolment and clinical site.

Conclusion

Ambulatory oxygen therapy use in patients with exertional hypoxaemia did not significantly alter functional exercise capacity, home physical activity which is low in this population of patients or quality of life. The reduction in plasma IL-6 from baseline would need to be investigated further however in this cohort of patients AOT did not reduce systemic inflammation. The low cylinder usage during the trial is likely a key mediating factor in all of these outcomes.

Chapter 5

Gene expression response to Ambulatory Oxygen and Air

This chapter outlines the gene expression results for the Ambulatory oxygen for muscles in COPD trial; the general and statistical methods of which are discussed in chapter 2. The results from chapter 4 suggest that AOT may have little effect on skeletal muscle gene expression given the lack of effect on clinical outcomes. Skeletal muscle biopsies were taken at trial enrolment (trial diagram, page 96) and at the end of each intervention with muscle biopsies undergoing transcriptomic analysis to explore differentially expressed genes during AOT and air treatment. Microarrays rather than target gene or protein analysis were used as it lends itself more to exploratory analysis when evaluating high throughput gene expression changes. Differential gene expression was undertaken using the R Bioconductor platform. The microarrays experimental design was two channel meaning that each baseline sample of muscle was hybridised with an intervention sample i.e. baseline sample with sample following AOT. Using this design enabled a direct comparison of air vs AOT.

5.1 Demographic Data

Eight patients underwent muscle biopsy at all three time points; at baseline and following air or oxygen interventions. The remainder of the patients refused further biopsies stating pain or difficulty with ambulation as the main reasons. They were therefore excluded from the microarray data analyses.

The demographic data for the patients included in the gene expression analyses is shown in table 5.1

Age (years)	67.13 (8.74)
Sex (M:F)	5:3
FEV1%	48.88 (26.2)
Baseline 6MWT (metres)	226.88(158.2)
BMI (kg/m²)	29.72(18.54-31.10)
O2 saturations at baseline (%)	93(1.4)
Nadir O2 saturations during 6MWT	84.6(4.0)
pH	7.4(0.01)
pCO2 (kPa)	5.5(0.45)
pO2 (kPa)	8.36(0.89)
HCO3 (mmol)	25.3(1.57)
CAT	29.5 (5.5)
HAD	18.75(5)
Activity (VMU/min)	226.18(65.4)
TNFα pg/ml	0.55(0.17)
IL-6 pg/ml	29.93(22.1)
IL-8 pg/ml	6.25(3.7)
IFGγ pg/ml	187.5(77.8)

Mean (SD), median (25th -75th Centile)

Table 5.1: Demographic data for patients included in gene expression data analysis.

5.2 Microarray Quality Assessment

Array data quality can be affected during the experimental process. When analysing arrays together the quality of each individual array needs to be assessed. The quality of microarray data was assessed using the “arrayQualityMetrics”(238) in Bioconductor

R(239). This package enables each individual microarray to be evaluated separately and in conjunction with the other microarrays to detect arrays where which the data quality may have been subject to errors during the experimental process, e.g. hybridisation or dye labelling. This can take the form of boxplots (representing summaries of the signal intensity distributions of the arrays, figure 5.1) or a heatmap (representing the distance between arrays, figure 5.2). The detected arrays are outliers and if used may compromise the accuracy of final output gene expression data.

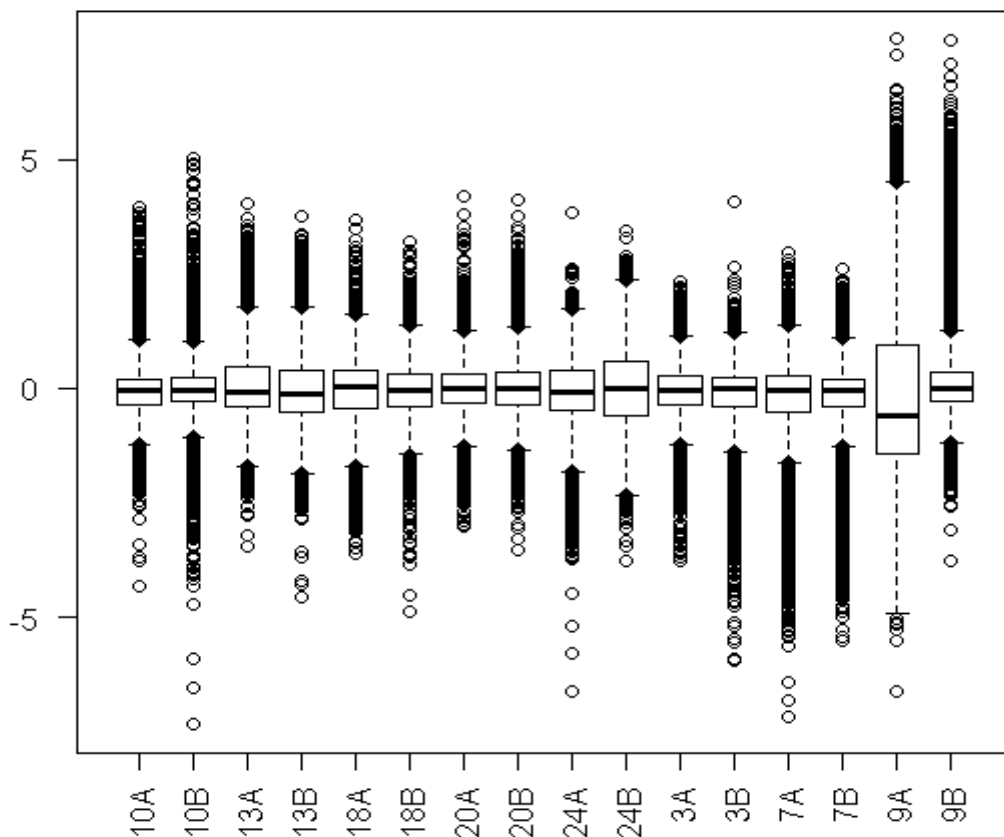


Figure 5.1 Box plot of individual normalised microarray data (log₂ Ratio of red and Green channels). The microarray for 9BaselineTreatmentA was identified as an outlier.

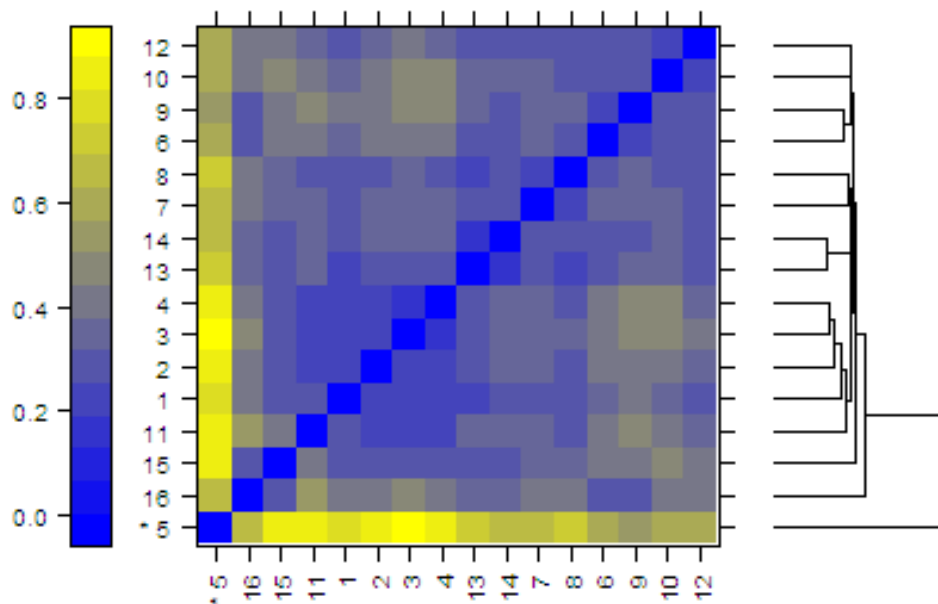


Figure 5.2. False heat map of distance between microarrays. Outlier detection was performed by looking for arrays for which the sum of the distances to all other arrays was exceptionally large. In this analysis array 5 (corresponding to patient 9 Baseline treatment (A) was identified as an outlier.

Patient 9's microarray data was therefore excluded from analysis as it failed both quality metrics.

5.3 Gene Expression

Microarray data pre-processing (discussed in chapter 2.4 methods) was performed prior to differential gene expression. Differential gene expression during ambulatory oxygen and air treatments was explored using the Significance Analysis of Microarrays (SAM)(240) in R. The SAM package allocates a score to each gene on the basis of change

in gene expression relative to the standard deviation of repeated measurements. In cases where genes with scores greater than an adjustable threshold occur SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance (false discovery rate, q value). A two-class paired analysis (i.e. comparing oxygen vs. treatment in the same patients) was undertaken.

At a single gene level differential expression between ambulatory oxygen and air was not detectable.

Since differential expression at a single gene level was not detectable, for the purpose of gene set enrichment analyses (GSEA) all genes that were up or down regulated during each intervention period were extracted. A pre-ranked gene list based on the SAM score was then used for GSEA in the web based program BROAD(241, 242). The SAM score was chosen over fold change for the formation of the pre-ranked list. Although fold change analysis may yield more reproducible gene expression data(243, 244) it does not take into account the variance of the expression values measured. The SAM score compares the difference in mean expression levels between two groups also taking into account the variability of the data not only from the gene tested, but also from other genes exhibiting a similar degree of change(245). This results in more accurate inference of gene expression change when the number of replicates is small(246).

11,479 genes were identified (gene list of top 400 genes in appendix 3 for GSEA. Positive values correspond to genes up-regulated during Treatment period A (Air) whilst negative values correspond to up-regulated genes during Treatment Period B (Ambulatory oxygen).

5.3.0 Gene Set Enrichment Analysis (GSEA)

GSEA was performed using the web based program BROAD Institute(241, 242). GSEA is a computation method that determines whether a priori set of genes show significant differences between two biological or phenotypic states. Genes are ranked based on their correlation between their expression and in this case the biological states or air and AOT. The aim of GSEA is to determine whether the genes are randomly distributed or are dichotomised to the top or bottom of the list. The latter occurs in genes which demonstrate biological or phenotypic distinction. GSEA has 3 core processes which are used to derive its results; enrichment score calculation, estimating the significance of enrichment scores and adjustment for multiple hypothesis testing(241, 242).

Enrichment Score

“The enrichment score (ES) determines the degree to which a gene set is represented at the extremes of a ranked list”. The calculation is made by walking down the list increasing a running sum statistic (magnitude depending on correlation between gene and phenotype) when the gene is encountered in the set or decreasing it when it not encountered (241, 242). Thus scores which are highly negative or positive are likely to demonstrate highly represented gene sets.

Significance of Enrichment Score

The statistical significance of enrichment score is based on an empirical phenotype based permutation test. A null distribution for the enrichment score is generated by permuting data for the phenotypes i.e. oxygen versus air and computing the enrichment score of the gene sets for this data. “The statistical significance of the observed score is then calculated relative to the null distribution”(241, 242).

Adjustment for multiple testing.

The enrichment score for each gene set is normalised (NES) to account for the size of the set.

$$\text{NES} = \frac{\text{actual ES}}{\text{Mean (ESs against all permutations of dataset)}}$$

The false discovery rate (FDR) is then calculated for each NES. The FDR is the estimated probability that a gene set with a given NES signifies a false positive finding(241).

The gene sets used in the analyses were derived from the Kyoto Encyclopaedia of genes and genomes (KEGG) which are a manually curated list of genes. These KEGG pathways indicate current knowledge of molecular interactions or networks for various metabolic and cellular processes. The output from BROAD indicate which gene sets for individual KEGG pathways were up or down-regulated for the phenotypes (air versus oxygen) when the pre ranked list of identified genes were used (appendix 3). Disease specific pathways were removed from the KEGG analysis to optimise biological interpretability of the results. To undertake GSEA using Broad, KEGG version 5.0 was used as the gene set database, collapse data set option was set to false as the up/down regulated probes had already been annotated with gene symbols as part of the pre-processing (chapter 2.4) and the weighted scoring system for enrichment was applied.

5.3.1 GSEA Results

The results of the GSEA analysis using KEGG pathways are shown in table 5.2a and 5.2b.

KEGG pathway	Size of Gene Set	ES	NES	FDR value	q
RIBOSOME	82	-0.75	-3.62	0	
OXIDATIVE_PHOSPHORYLATION	117	-0.58	-2.96	0	
PROTEASOME	42	-0.67	-2.78	0	
SPLICEOSOME	104	-0.51	-2.57	0	
PROPANOATE_METABOLISM	28	-0.66	-2.49	0	
AMINOACYL_TRNA_BIOSYNTHESIS	27	-0.64	-2.42	0	
FATTY_ACID_METABOLISM	34	-0.62	-2.40	0	
RNA_DEGRADATION	41	-0.58	-2.36	0	
UBIQUITIN_MEDIATED_PROTEOLYSIS	96	-0.48	-2.36	0	
VASOPRESSIN_REGULATED_WATER_REABSORPTION	32	-0.59	-2.31	1.62E-04	
FOCAL_ADHESION	160	-0.41	-2.19	1.52E-04	
PYRUVATE_METABOLISM	34	-0.56	-2.18	2.58E-04	
ADHERENS_JUNCTION	57	-0.48	-2.17	2.44E-04	
NEUROTROPHIN_SIGNALING_PATHWAY	100	-0.42	-2.10	6.80E-04	
ANTIGEN_PROCESSING_AND_PRESENTATION	64	-0.46	-2.10	6.46E-04	
VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION	37	-0.52	-2.10	6.16E-04	
INSULIN_SIGNALING_PATHWAY	114	-0.40	-2.07	0.001261	
LYSOSOME	90	-0.42	-2.01	0.001882	
CITRATE_CYCLE_TCA_CYCLE	26	-0.55	-2.01	0.001814	
NUCLEOTIDE_EXCISION_REPAIR	36	-0.49	-1.96	0.002849	
GLYCOLYSIS_GLUONEOGENESIS	50	-0.45	-1.93	0.003951	

TGF_BETA_SIGNALING_PATHWAY	60	-0.42	-1.92	0.004215
PURINE_METABOLISM	109	-0.37	-1.89	0.005972
PPAR_SIGNALING_PATHWAY	53	-0.42	-1.85	0.007892
TRYPTOPHAN_METABOLISM	32	-0.48	-1.85	0.007679
REGULATION_OF_ACTIN_CYTOSKELETON	163	-0.34	-1.84	0.008137

Table 5.2a Enriched KEGG pathways up-regulated during ambulatory oxygen treatment

KEGG pathway	Size of Gene Set	ES	NES	FDR q value
OLFACTORY_TRANSDUCTION	125	0.27574	2.04	0.01992

Table 5.2b Enriched KEGG pathway up-regulated during air treatment.

Enrichment plots for the top two KEGG pathways up-regulated during ambulatory oxygen treatment and the KEGG pathway up-regulated during air treatment are shown below (figures 5.3a-5.3c).

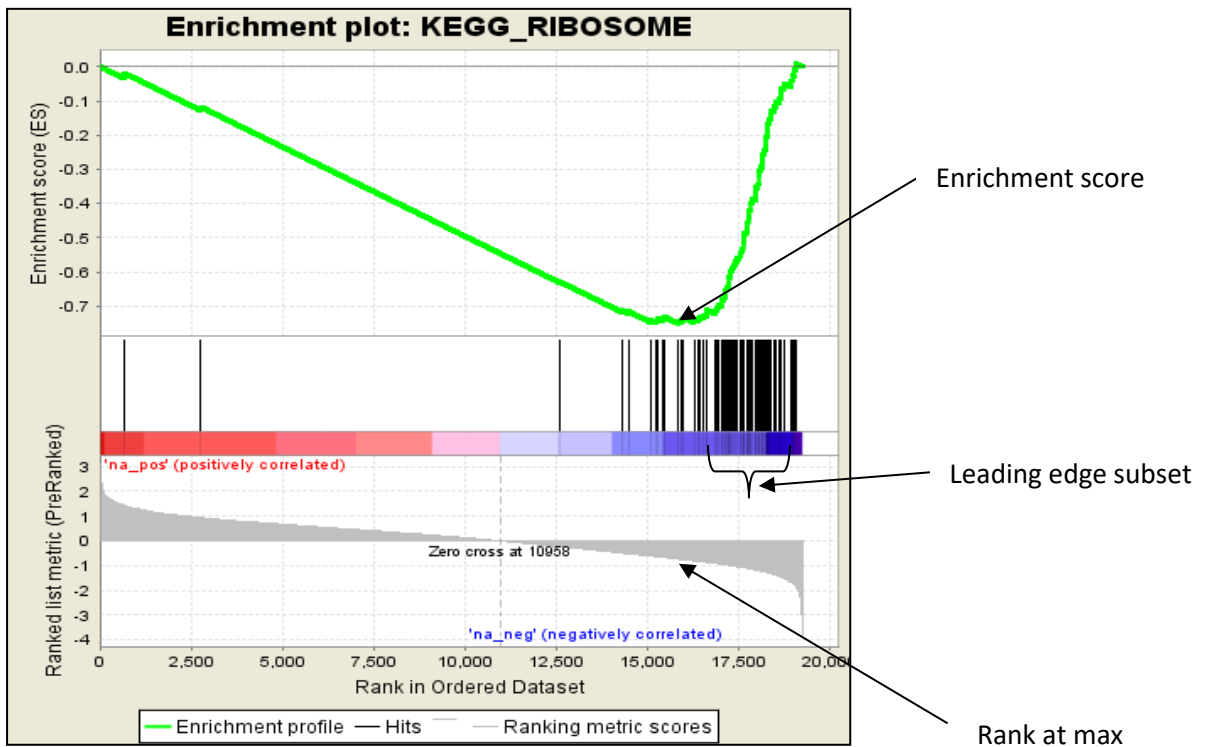


Figure 5.3a. Enrichment plot for KEGG ribosome pathway

The enrichment plot for the ribosome pathway shows the primary genes involved towards the bottom end of the list. The enrichment score is the furthest deviation from zero shown here at the lowest point of the plot; score -0.75 (scale -1 to 1). The “leading edge subset” of a gene set are the genes which contribute most to the enrichment score. The rank at max is a metric which measures a genes correlation with a particular phenotype, in this case AOT and is the position in the ranked list at which the maximum enrichment score was achieved.

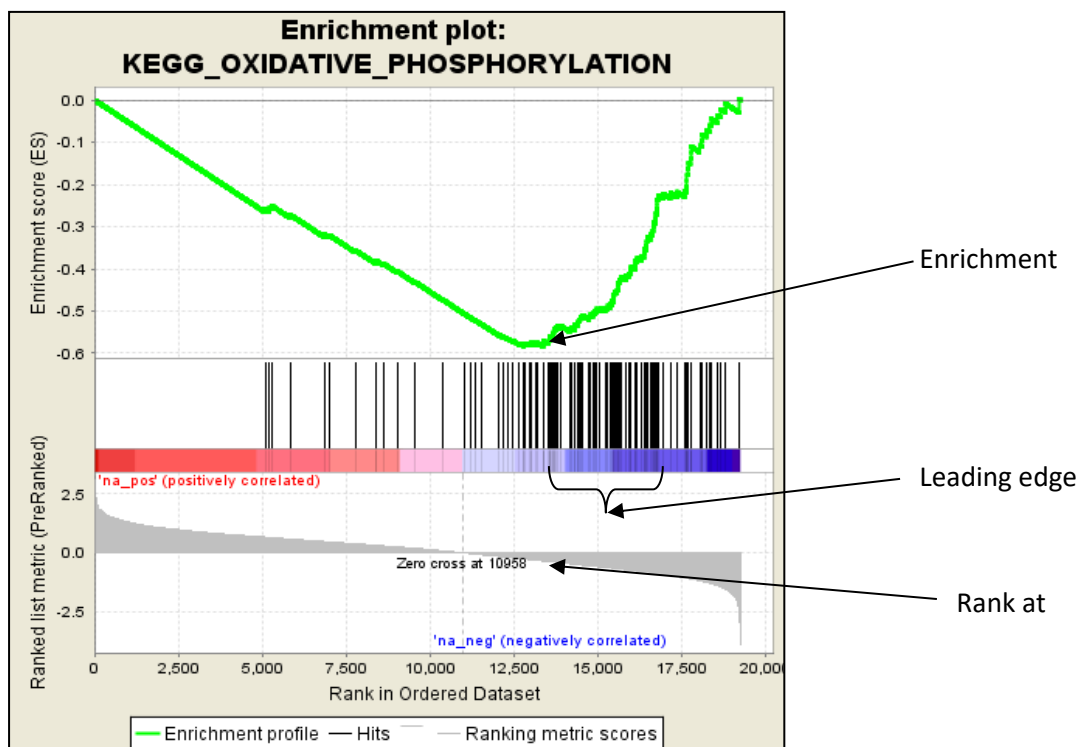


Figure 5.3b Enrichment plot for KEGG oxidative phosphorylation; up-regulated during oxygen treatment.

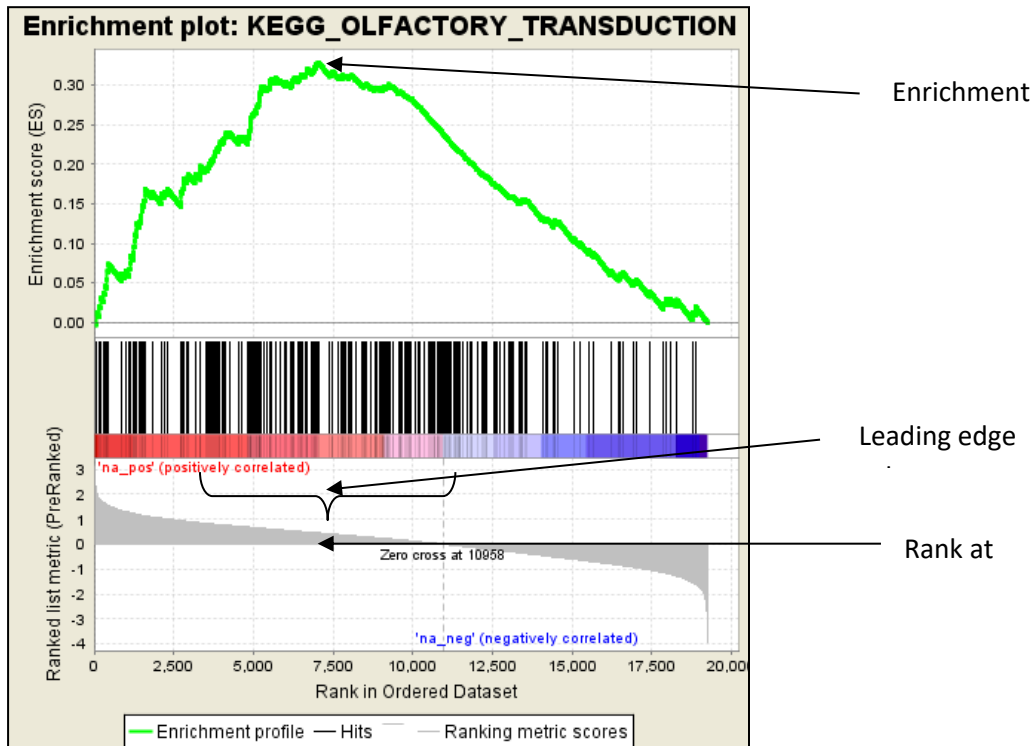


Figure 5.3c Enrichment plot for the only KEGG pathway up-regulated during air

The rank at max in this enrichment plot corresponds to the positive genes in the set (during air treatment). In contrast to the leading edge subsets in figures 5.3a-b the subset in this enrichment plot is broad and not concentrated at either end ranking generally indicating a poorly represented gene set.

The enrichment plots for both Ribosome and Oxidative phosphorylation show gene set correlations towards the bottom of the list with a relatively tightly packed leading edge subset; particularly in the case of KEGG ribosome. Both have relatively high enrichment scores (max -1 to 1). In contrast the KEGG pathway of olfactory transduction; although significant has low enrichment score with its leading edge genes distributed throughout the list.

27 KEGG pathways met the FDR<1% cut off. 26 enriched pathways were up-regulated during oxygen treatment whilst only 1 KEGG pathway was up-regulated during air treatment (table 5.3b). The enriched KEGG pathways up-regulated during ambulatory oxygen treatment could be clustered into three functional groups; mitochondrial bioenergetics, protein turnover and metabolic pathways (figure 5.4).

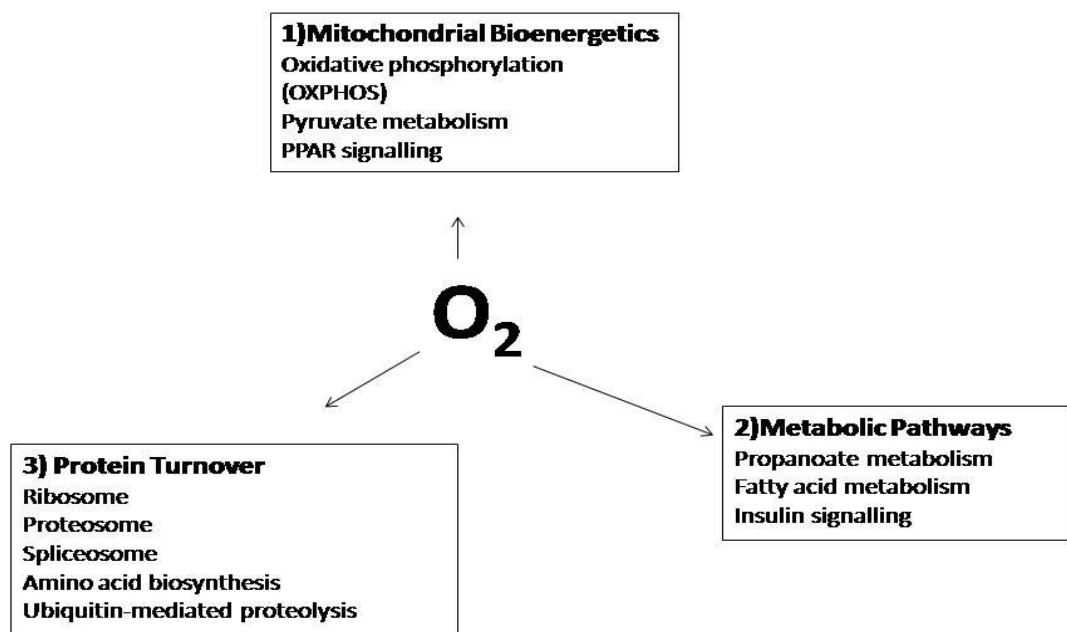


Figure 5.4: Functional grouping for KEGG pathways upregulated during oxygen treatment.

The mitochondrial bioenergetic pathway of oxidative phosphorylation (OXPHOS) was chosen for further examination due to (a) its biological relevance and (b) being in the top two enriched pathways. A correlation based analysis was conducted with the patient clinical outcome data using principal components as described below.

5.4 Principal component analysis

Principal component analysis is a data reduction technique. Using a mathematical algorithm it reduces the dimensionality of a data set whilst retaining as much of the possible variation within it. It accomplishes this by identifying the directions (principal components) in which the variation in the data is maximal. When applied to microarray data it can be used to perform cluster gene analysis, find dominant patterns of gene expression or to summarise transcriptional activity of a “gene set”. Here it has been used to summarise the transcriptional activity of the KEGG oxidative phosphorylation pathway. This was achieved by using the “prcomp” function in R (239) taking the normalised microarray data and genes from the leading edge subset of the OXPHOS pathway to compute the first principal component.

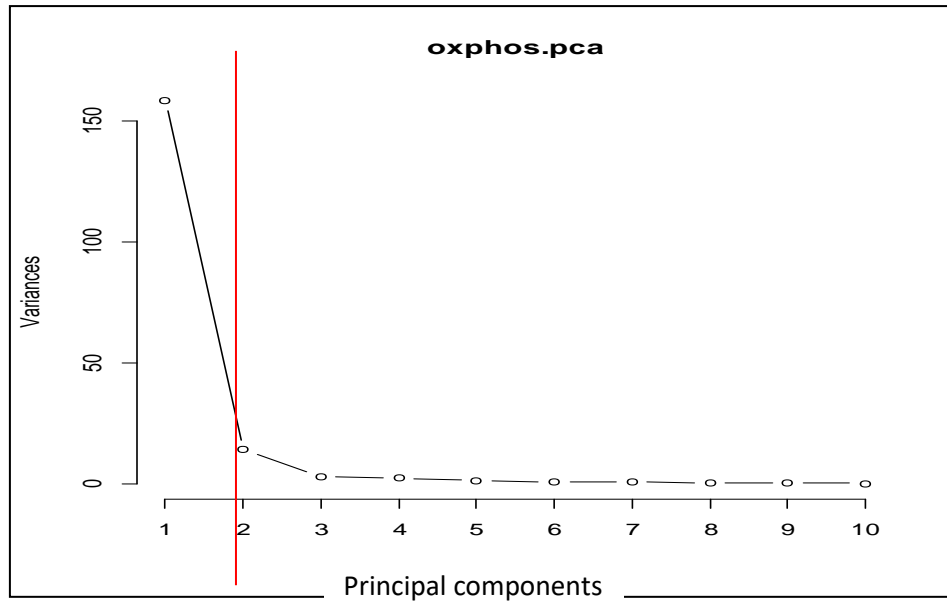


Figure 5.5: Plot of principal components against variance:

The red vertical line shows the cut off for the component that accounts for the most variance; the first principal component retained 86% of the variance. This indicates that the transcriptional state of the involved genes in the OXPHOS pathway can be summarised by this variable. The diagram below is a biplot (fig 5.6); a visual representation of the two principal components, microarray observations and the OXPHOS genes as vectors (red). The first principal component (PC) is in the horizontal axis and second component on the vertical axis. The genes involved are predominantly moving along the horizontal axis (1st PC) highlighting again that the 1st PC account for the majority of the variance.

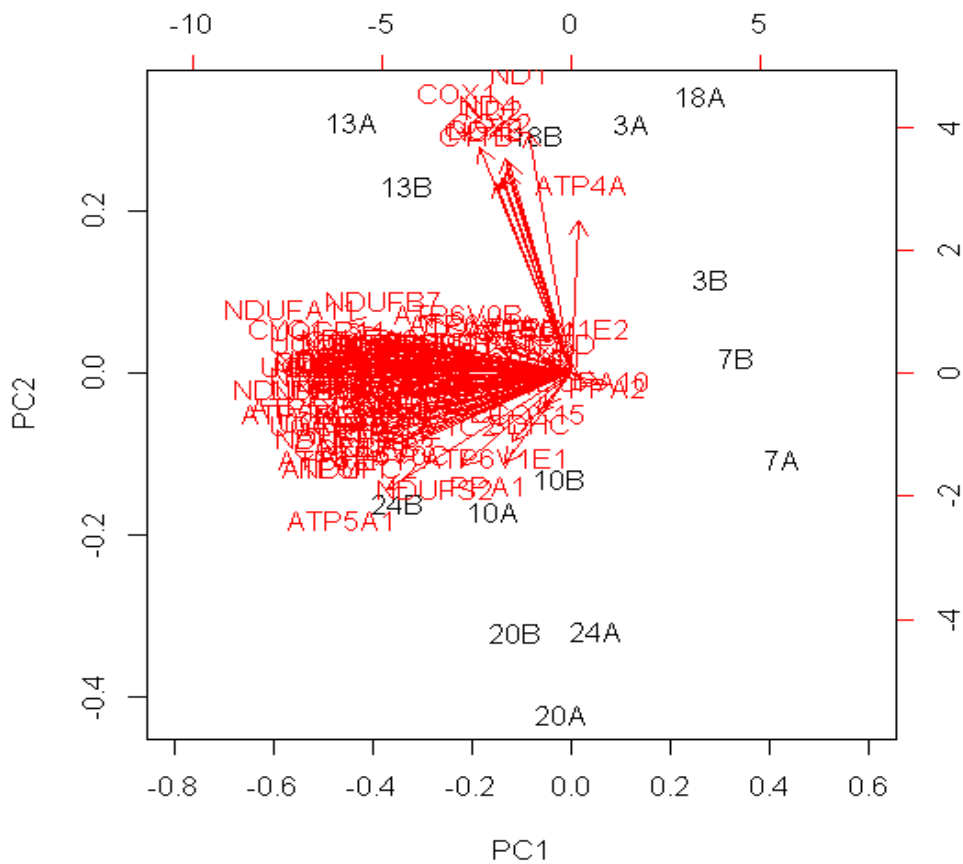


Figure 5.6: Biplot of OXPHOS gene and principal components.

The biplot can also be used to analyse which patients (microarray observations) demonstrated a response to treatment in line with the summarised OXPHOS pathway. The major transcriptional direction of vector (genes) is from right to left. Each patient has two microarray observations on the biplot corresponding to Treatment A (Air) and B (oxygen). The patients where whom their “B” observations lie to the left of the “A” indicate a greater transcriptional change occurred during oxygen treatment. This suggests that there were 4 responders (subjects 7, 18, 20, 24) and 3 non responders (subjects 3, 10 and 13) to ambulatory oxygen therapy. This is also evident from the heatmap of the gene enriched the OXPHOS pathway (figure 5.7). Analysis of baseline

characteristics between these two groups did not demonstrate any significant differences between lung function, age, BMI or systemic inflammation ($p>0.05$).

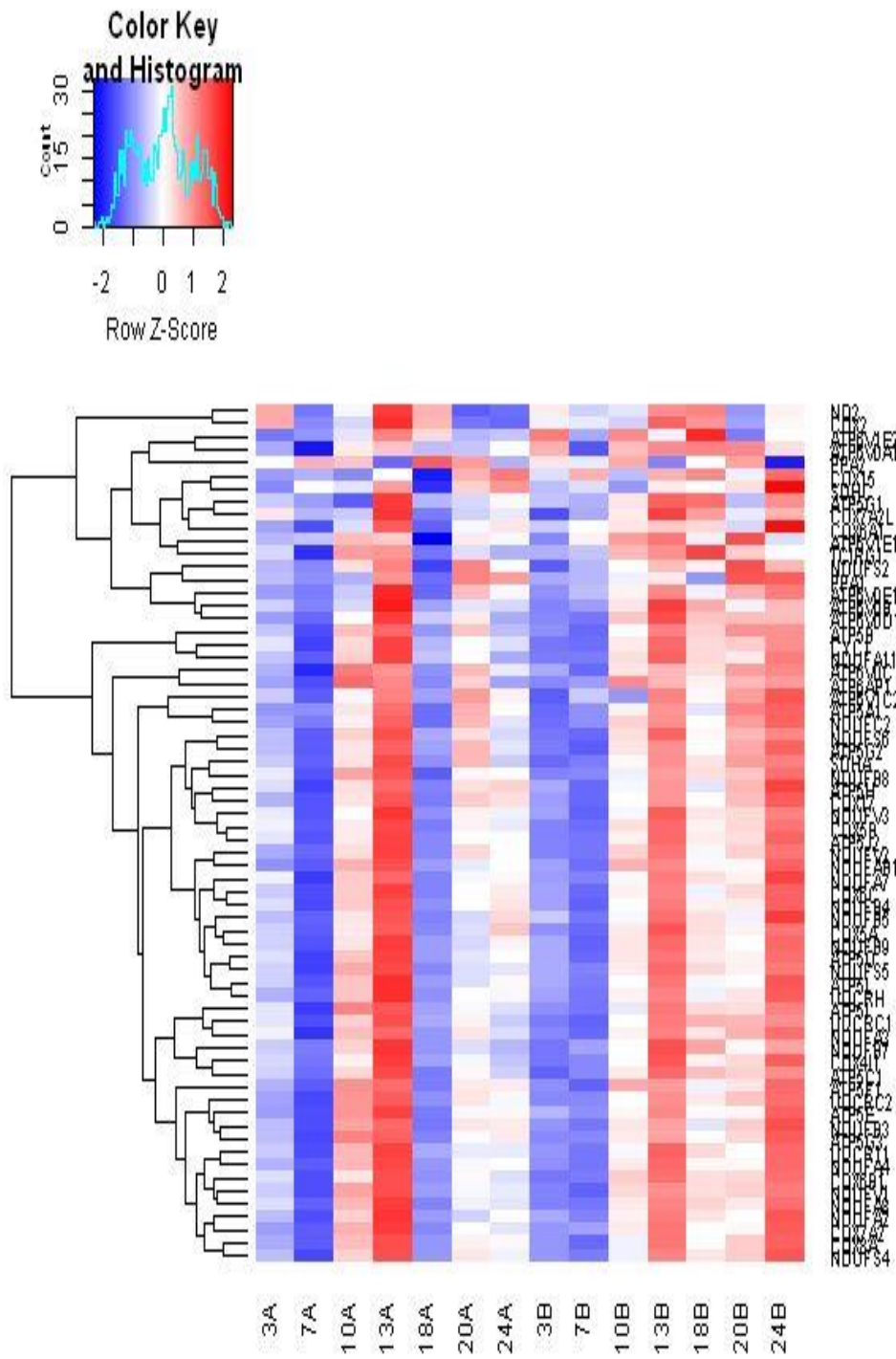


Figure 5.7 Heatmap of genes enriched in OXPHOS pathway: Patient arrays horizontal and genes listed vertically.

Red-indicates increased expression, blue reduced expression and white mean.

A correlation based analysis using Spearman's Rank was undertaken between transcriptional activity of the OXPHOS pathway and patient clinical outcomes (table 5.3a) for those who underwent muscle biopsies. Pearson's correlation test (adjusted for age and FEV1%) was used to assess the strength of the relationship between transcriptional activity and clinical data with one tailed hypothesis significance testing.

The results are shown in table 5.3b below.

Subject	Δ6MWT		ΔCAT		ΔHAD		ΔIL-6pg/ml		ΔIFN- γ pg/ml		ΔIL-8pg/ml		ΔTNF-α pg/ml	
	Air	O ₂	Air	O ₂	Air	O ₂	Air	O ₂	Air	O ₂	Air	O ₂	Air	O ₂
3	38	167	9	16	-9	-5	-2.43	-0.19	-37.35	127.80	2.27	-0.84	-0.07	-0.06
7	0	199	8	0	-1	0	-12.21	-49.69	-38.41	-85.52	3.15	-0.51	0.03	0.01
10	97	93	-4	-4	-5	-6	7.96	-13.23	135.95	-78.45	2.15	0.76	0.23	0.00
13	-9	73	4	0	-3	-8	-5.91	-2.49	18.15	8.28	-0.19	1.27	0.42	0.27
18	-60	-54	1	-1	0	3	27.90	-12.66	198.29	94.76	1.13	1.99	0.02	0.03
20	65	66	7	-2	-1	-4	4.25	1.97	93.32	44.28	3.28	-0.09	-0.09	-0.09
24	39	78	0	-8	-6	-6	4.22	-9.93	-64.36	24.32	-1.14	-0.38	-0.03	-0.17

Table 5.3a: Clinical outcome data for trial patients whom underwent muscle biopsies. Results shown are change from trial enrolment baseline for each clinical variable in each of the subjects.

Clinical outcome	Oxygen		Air	
	r	p value	r	p value
6MWT	0.75	p=0.07	-0.70	p=0.09
CAT	0.50	p=0.20	0.18	p=0.38
HAD	0.60	p=0.14	0.64	p=0.12
IL-6	-0.81	p=0.05	0.42	p=0.24
IL-8	0.15	p=0.41	0.28	p=0.32
IFN γ	-0.36	p=0.27	0.25	p=0.35

Table 5.3b: Correlation of OXPHOS pathway and patient clinical data using Spearman's Rank.

The strongest correlation between clinical variables and transcriptional activity of OXPHOS pathway was IL-6 during ambulatory oxygen treatment; showing a significant inverse relationship, $p=0.05$. 6MWT and OXPHOS activity showed a strong relationship during both treatments; strongly positively correlated during oxygen treatment and a strong inverse relationship during air treatment.

A hypothetical interaction between selected genes contributing to KEGG pathways, KEGGS pathways, cytokine and clinical outcomes is shown in figure 5.8.

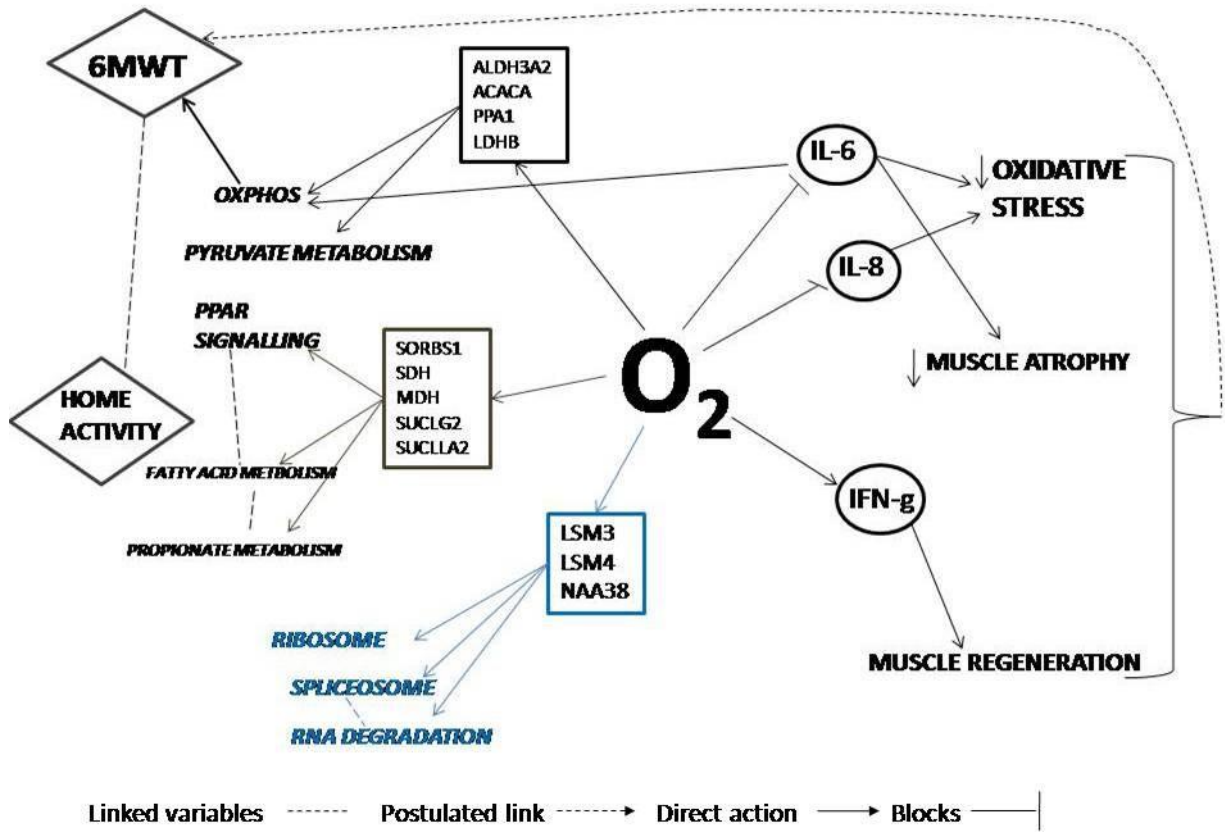


Figure 5.8 Interaction between KEGG pathways, genes and clinical data.

5.5 Discussion

This work has shown that although differential expression at single gene level is absent, GSEA demonstrates important up-regulated pathways involving aspects of mitochondrial function which accompanied ambulatory oxygen treatment. Skeletal muscle mitochondrial function has been shown to be abnormal in COPD, with respect to both reduced mitochondrial density and biogenesis (139, 247). Mitochondria are the main producers of ATP via the Tricarboxylic acid cycle (TCA) and OXPHOS and constitute one of the main producers of reactive oxygen species (ROS), which activate pathways leading to cell apoptosis (118, 248). Thus, defects in mitochondrial function will adversely affect both energy production and cell death. The results from our analysis indicate that hypoxia maybe contributing to the abnormal mitochondrial function in the studied patients. Furthermore a previous study (153) which used a systems biology approach to elucidate the potential drivers for skeletal muscle abnormalities found that at an individual gene level a number of histone deacetylase enzymes could discriminate between healthy and diseased muscle; all of which correlated with oxygen utilization.

Under hypoxemic conditions hypoxia inducible factor alpha (HIF- α) is up-regulated due to reduced protein degradation by the von Hippel Lindau Protein (249). HIF- α has been shown to suppress the peroxisome proliferator activator receptor (PPAR) pathway (250), one the key exponents of mitochondrial oxidative metabolism. Activation of PGC1- α (peroxisome proliferator activator receptor γ cofactor α) up-regulates genes involved in the TCA cycle and OXPHOS via nuclear respiratory factors 1 and 2. (251). Both OXPHOS and TCA are oxygen dependent and were significantly up-regulated during oxygen treatment compared to that of air in the KEGG pathway GSEA analysis. This is in line with

other studies which have used biomarkers of TCA and OXPHOS function; citrate synthase and complex III respectively to show these are reduced during hypoxia (252, 253). The enriched pathways during AOT could be manually separated into three functional groups (figure 5.4) there is a degree of overlap between groups 1 and 2. For example, in beta oxidation a component of fatty acid metabolism generates acetyl-coA and NADPH (nicotinamide adenine dinucleotide phosphate) which enters the TCA cycle and electron transport chain respectively (254). Evidence from both human and murine studies suggests that there is a tendency for beta oxidation to be reduced following hypoxic stimuli and as a consequence reducing mitochondrial energy production (253, 255). The last functional group of protein turnover indicate that both pathways of protein synthesis and protein degradation were significantly up-regulated during oxygen treatment. Whilst protein synthesis is important in gaining muscle mass it is interesting that the ubiquitin proteasome pathway (UbP) is also up-regulated. This is the key proteolytic pathway which underpins muscle atrophy. Cross sectional studies have shown markers of the UbP, specifically MuRF1 (muscle ring finger protein) and Atrogin 1, to be elevated in COPD patients with low muscle mass compared to controls (127). The up-regulation of both pathways during oxygen treatment suggests that muscle mass homeostasis is tightly regulated and the genes involved in this process are differentially affected by hypoxia. Alternatively, it is possible that fibre type-specific changes—atrophy in Type II and maintenance in Type I – produce simultaneous up-regulation in both protein synthesis and degradation genes. The only KEGG pathway up-regulated during air treatment to the FDR threshold of <1% was olfactory transduction. The reasons for this pathway being up-regulated are not clear. It has no relationship to muscle function or mitochondrial energy production.

The correlations between OXPHOS transcriptional activity and clinical data were generally strongest during AOT. The significant inverse relationship between IL-6 and OXPHOS is interesting. This suggests that the cellular process of OXPHOS is deleteriously affected by increasing levels of IL-6. Given that, IL-6 was also significantly reduced compared to baseline during AOT, the relationship between inflammation (especially this cytokine) and impaired cellular mitochondrial process seem highly plausible. A previous study also demonstrated a significant relationship between systemic inflammation and the expression of energy metabolism genes in skeletal muscle from COPD patients, where CXCL10 and CXCL9 were significantly inversely correlated to the OXPHOS and TCA pathways(256). Van den Burst et al(257) previously demonstrated a relationship between loss of muscle oxidative phenotype and reduced endurance in patients with COPD. This is supported by the correlations we observed between functional exercise capacity and OXPHOS transcriptional activity during both treatments. During the air treatment phase i.e. “control arm” an inverse relationship was noted suggesting that continued hypoxia during exertion had a negative impact on OXPHOS. The reverse was true during AOT.

There are a number of limitations to these results. Primarily, the sample size used for the microarray data analysis was small. Only 8 of the 17 patients underwent muscle biopsies that could be used in the microarray data analysis at all-time points, due to the unavoidable factor of patient refusal. Overall recruitment to the trial was extended to account for dropouts, but due to time and funding constraints no additional amendments to extend recruitment and account for these refusals could be justified, given that the number who might be needed was not known. This is because no formal power

calculations were undertaken, as the exploratory endpoint of change in gene expression had never been measured previously with respect to treatment with oxygen therapy. Nevertheless previous studies using gene expression in muscle as an endpoint have demonstrated differences with a few as 10 patients (173), hence why a pilot of this size was justifiable. Analysis of the participants who completed muscle biopsy compared to those who continued in the trial with secondary outcome data show that they do not differ significantly with respect to age $p=0.91$, FEV1% $p=0.85$, baseline 6MWT $p=0.92$, oxygen saturations $p=0.2$, desaturation nadir $p=0.34$ or baseline BMI $p=0.88$. Therefore, although the dropout rate from was higher than expected the sample included was representative of all trial participants. The correlation gene expression analyses were single tailed which can lead to inaccurate or biased results. Nevertheless, the associations demonstrated have been supported by previous literature(256, 257) and thus warrant further investigation.

Gene set enrichment analysis was performed on the resulting microarray gene results as, after correcting for multiple hypotheses testing, no individual gene met the threshold for statistical significance. GSEA is useful in this context as small changes in a number of genes encoding the same pathway may have more biological relevance to a cellular process than a single gene(241) especially if the changes in the studied biological system are subtle. GSEA is a robust and commonly used tool to further explore microarray data in the presence or absence of identified genes.

Conclusion

Ambulatory oxygen treatment did not result in detectable statistically differentially expressed genes after correction for multiple hypotheses testing. Although differential expression was undetectable GSEA analysis revealed important pathways of mitochondrial bioenergetics that were up-regulated in muscle following ambulatory oxygen treatment suggesting that intermittent hypoxia is likely to be contributing to abnormal mitochondrial function in these patients. In addition use of computational analysis reveal of a significant inverse relationship between levels of inflammation and oxidative phosphorylation suggesting a potential link between systemic inflammation and aberrant mitochondrial function. Analyses were limited by the size of the population studied and their relative inactivity, such that periods of hypoxia due to activity may have been infrequent and short in duration.

Chapter 6 Discussion Summary

6.1 Summary of Results

This thesis concerns the role of ambulatory oxygen in patients with COPD and exercise induced desaturation. It specifically assesses its clinical efficacy with regard to exercise capacity, quality of life, clinical device efficacy and effect on skeletal muscle gene expression via the randomised clinical trial: detailed in chapter 4.

6.2 Clinical efficacy

Ten studies including OM COPD evaluated the role of AOT to improve exercise capacity. The modality of the exercise test and the context in which the test were carried out varied. Five of the studies were supervised; associated with a structured exercise program, whereas the rest were domiciliary with instructions given to patients when to use AOT. It is therefore important to understand that due to the heterogeneity of the studies included firm conclusions with regard to AOT clinical efficacy may be limited. Functional exercise capacity was measured by 6MWT in the majority of studies which did not use endurance tests. In both supervised PR and domiciliary studies AOT had limited benefit of improving 6MWT with all studies bar the OM COPD favouring control. The latter study showed a non-significant improvement in 6MWT with AOT, which may have clinical relevance in patients with COPD and EID in relation to mortality from hospitalisation. The magnitude of improvement in functional exercise capacity was far greater in the supervised studies than domiciliary. Clearly the impact of structured physical exercise programs and the subsequent physiological adaptation gained from

exercise is superior to any benefit gained from AOT. All of the endurance test studies or constant work rate tests were supervised. Collectively they demonstrate that AOT is likely to have benefit in improving endurance/time exercised if judged by this modality with all studies favouring the AOT mirrored also in the meta-analyses (chapter 3). These observations taken together suggest either there are different mechanism by which AOT affects these different tests or endurance tests are more sensitive to therapies such as AOT or both. The main postulated mechanisms for AOT efficacy are to reduce carotid body activation and cause a subsequent reduction in respiratory rate. Since dyspnoea is a limitation to exercise tolerance in patients with COPD, a reduction in respiratory rate and sensation of dyspnoea is likely to improve exercise capacity. A reduction in dynamic inflation has also been observed in one study(72). A number of studies have shown that endurance test are more responsive to interventions than the 6MWT when bronchodilators have been used(258). Given that bronchodilators reduce hyperinflation in a similar mechanistic fashion to AOT it seems highly plausible that endurance test should respond better to AOT than the 6MWT(258). The design of the exercise tests are also very different. The 6MWT is self paced while endurance tests such as ESWT are externally paced (calculated from an externally paced incremental shuttle walk). Evidence suggests that the cardiorespiratory response in patients with COPD differ when either test is undertaken. During endurance tests heart rate, minute ventilation and respiratory rate increase close to maximal levels whereas these three indices are sub-maximal during a 6MWT. One could hypothesize that any treatment which reduces dynamic hyperinflation is unlikely to demonstrate benefit if the test used causes a sub-maximal response especially in respiratory rate and minute ventilation and therefore unlikely to evoke a “hyperinflation” physiological threshold.

The Chronic respiratory disease questionnaire (CRQ), HADs and CAT examined the impact of AOT on HRQoL. In both supervised PR and domiciliary studies AOT demonstrated statistically significant benefit over control when the CRQ was used. The CRQ covers themes such as anxiety, physical function and breathlessness. The OM COPD trial described in chapter 4 used the HAD and CAT which when combined cover similar areas to the CRQ. Whilst a statistically significant improvement in HADs score was noted after AOT occurred, a marked improvement in the control arm also occurred, a phenomenon often seen in the placebo arm of clinical trials. Despite the statistical improvement in both CRQ and HAD, the clinical relevance is doubtful as all the improvements in CRQ fall below the MCID and no MCID for HADs has been established. What is clear from the results is that AOT seems to give patients the perception that they are less breathless and more in control of their disease. The perception of dyspnoea in COPD is complex, with a number of bio-psychological factors involved. The apparent benefit in HRQoL may come from a number of altered perceptions of safety, symptom relief, possible placebo effect and motivational factors.

6.3 Device Efficacy

The results from chapter 3 indicate that whilst there is considerable variation in OCDs devices their performance is superior to CFNC in maintaining exercise saturations and 6MWT. The OCDs achieve these outcomes at much lower oxygen utilization than CFNC. This is likely to have implications in terms of oxygen cylinder usage and subsequent cost as although initial outlay for OCDs is higher the longer term benefit of lower oxygen utilisation will more than recoup this. It is important that the choice of OCDs is tailored

to the individual taking into account whether they are predominately mouth or nasal breathers as the effort of ambulation or exercise increases. Since most of the OCDs are triggered by nasal inspiratory flow a change to mouth breathing during increased effort would render these OCDs less useful and cause desaturation to the user.

6.4 Changes in Skeletal Muscle gene expression

There is a wealth of data to suggest that skeletal muscle in patients with COPD differs to controls with respect to reduction in proportion of type 1 fibres(152), abnormal mitochondrial density(133), increased oxidative stress and loss of oxidative phosphorylation phenotype(257). The difficulty has been in trying to determine the main driver to these alterations. Hypoxia is implicated in its pathogenesis. The pilot clinical trial (chapter 4) investigated the role of oxygen supplementation during activity in patients with COPD and exercise induced desaturation. The hypothesis was that peripheral muscle hypoxia is increased specifically in these patients on exertion especially in those with low activity levels. Administration of AOT would either encourage participants to be more active and via activity have a favourable effect on gene expression or have a direct effect on muscle in its own right. Unfortunately at a single gene level there was no detectable difference in gene expression between AOT or air treatment. The study aimed to recruit 25 participants however there was a high dropout rate due to many participants not being able to tolerate serial muscle biopsies. Although it is unlikely given the results from chapter 4&5 that AOT directly impacts on muscle results may have been more favourable had it been the total number recruited were involved in the gene expression analysis. In addition, participant activity was low and did not change significantly during the trial indicating that AOT did not encourage

participants to become more active. Furthermore, linked to low activity was low cylinder usage during the trial period; equating to roughly 30mins per day. Given both the low activity and cylinder usage it is not surprising that no gene or cytokine changes were demonstrated. Participants were given clear instructions on when and how to use cylinders, however a more detailed discussion prior to trial enrolment on the merits and rationale for usage should have been undertaken. Other reasons for absence of detectable change in gene expression have been discussed in section 4.2.5.

The results of the GSEA demonstrate upregulation of multiple mitochondrial and cell homeostasis pathways during AOT. Given that these pathways were not upregulated during air treatment the attenuation of hypoxia during exertion would seem to be contributing to these changes. The study by Turan et al(153) also suggested hypoxia as the cause for the abnormal response in COPD muscles with an underlying epigenetic mechanism. They were able to show that the expression of a number of oxygen correlated histone modifiers could discriminate between healthy and COPD muscle; sirtuins and histone deacetylases. Hypoxia can lead to an altered redox state which has been shown to be abnormal in COPD (154, 259). Sirtuins are NAD^+ dependent deacetylases and their activity especially SIRT1 and SIRT3 are sensitive to changes in NAD^+ to NAD^+/NADH ratio which is adversely affected by hypoxia. Enhanced SIRT activity, namely SIRT3 is associated with improved mitochondrial function and exercise performance. Murine studies of mice lacking SIRT3 show abnormalities of a number of mitochondrial proteins associated with impaired electron transport chain activity and fatty acid metabolism(260). Indeed the transcriptional correlation analysis showed strongly positive correlation between OXPHOS and 6MWT during AOT almost reaching

statistical significance with the converse was true during air treatment. Collectively one could speculate would suggest that hypoxia is implicated in the altered muscle gene expression and regulation causing abnormal mitochondrial function indicated by the results of the GSEA.

The results from chapter 4 showed that IL-6 was the only cytokine which demonstrated significant change during the clinical trial with reduction in plasma IL-6 during AOT compared to baseline values albeit with no between group differences. The transcriptional correlation analysis also showed a significant inverse relationship between plasma IL-6 and the mitochondrial OXPHOS pathway suggesting that lower IL-6 levels confer enhanced OXPHOS activity. The role of IL-6 on muscle biology is complex with a few studies showing both pro-myogenic and atrophic effects of the cytokine(261). Exercise especially endurance or eccentric causes acute inflammation within skeletal muscle and studies have shown that IL-6, IL-6 mRNA rise dramatically during acute exercise(262). The adaptations noted following regular exercise lead to reduction of oxidative damage and improved mitochondrial biogenesis (largely mediated by PGC-1 α). Thus to adapt appropriately episode of acute inflammation during exercise are required. Regular exercise may reduce basal IL-6 levels and also the magnitude of response to IL-6 and the efficiency of the bioenergetic changes(262). Whilst acute changes seem to be beneficial, murine studies have shown that IL-6 over expression can cause mitochondrial dysfunction (263) a process which can be rectified with physical activity. A number of epidemiological studies have also shown a negative relationship between physical activity and IL-6 levels. Therefore it is possible that the use of AOT ameliorates basal IL-6 levels akin to exercise in the cohort of patients in the study.

6.5 Future Work

6.5.0 Ambulatory oxygen Efficacy

The clinical efficacy of AOT in improving exercise capacity is largely dependent on the exercise test modality chosen. The evidence suggests that AOT use does result in statistically significant improvements in endurance or constant work rate exercise tests. The results from the systematic review would have been useful to inform the exercise modality chosen in the clinical trial (chapter 4). Although this thesis via the clinical trial adds to the evidence that AOT has limited role in statistically improving 6MWT, the effect of AOT would have been better assessed by an endurance shuttle walk test which would have been achievable at the trial site. If the 6MWT was to be used in future to assess the longer term benefits of AOT any future trial should only enrol acute responders; i.e. participants with 10% improvement in 6MWT following AOT use.

6.5.1 Inflammatory response to AOT

Inflammation is one of the hypothesized aetiological factors in abnormal muscle function predominantly via NfκB activation. The inflammatory response can either be systemic or local. It is highly plausible that changes in systemic inflammation may not reflect those at the muscle level and may indicate a different mechanism. The OM COPD trial did demonstrate a significant reduction in plasma IL-6. It is unclear whether muscle IL-6 mRNA would show a similar pattern. It would be important to assess both local and systemic inflammation in future studies. Systemic inflammation could be assessed as

described in chapter 2 whilst local inflammation assessed by extracting RNA from muscle subsequently using the appropriate probes and primers to quantify this.

6.5.2 Gene Expression

The pilot study of AOT effect on skeletal muscle gene expression has given some insight to how its use may perturb some of the deleterious changes observed in COPD patients. Since this study was small and recruitment falling short of target the number of participant included in a future similar would need to be significantly larger. Data from previous studies(173, 264) indicate that between 200 and 1100 genes maybe differentially expressed in exercise in COPD patients or those with reduced fat free mass. Assuming approximately 18000 are not differentially expressed genes, 0.9 power to detect a fold change of 2 between treatments and an anticipated standard deviation of 0.6 (matched pair design) (265) a sample size of 19 in each arm would be required. This would firstly help confirm the results from the clinical trial (chapter 4) and may yield individual differentially expressed genes. This information would be used in concert with cytokine data to further explore the relationship between hypoxia, inflammatory response and changes in gene expression. The clinical trial work tentatively suggests that mitochondrial functions such as oxidative phosphorylation are important in the aetiology of muscle dysfunction. As mentioned above one of the key problems with the OM COPD trial was recruitment. This in part may have been due to the number of skeletal muscle biopsies participants had to undertake; 3. An alternative approach would use a randomised double blind parallel trial of AOT vs Air over a similar time period as the OM COPD trial, with muscle again as the primary end point. However, one would analyse target OXPHOS proteins e.g. (complexes 1-4) and also genes of the OXPHOS pathways

such as PGC-1 alpha, PPAR from skeletal muscle at baseline and at the end of the trial. The analysis of target proteins may prove more fruitful as one would expect any change from AOT usage to be over a period of weeks/month. In contrast changes in relative expression of genes involved in mitochondrial or muscle hypertrophy occur over hours to a day following sufficient stimuli e.g. exercise.

Appendix 1

Search Strategy –Clinical and DeviceEfficacy

1. chronic obstructive pulmonary disease/
2. copd.mp.
3. smoking-related lung disease\$.mp.
4. lung emphysema/
5. exp bronchitis/
6. (chronic respiratory adj (disease or disorder)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
7. (chronic obstructive lung adj (disease or disorder)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
8. (chronic obstructive pulmonary adj (disease or disorder)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
9. (chronic obstructive airway adj (disease or disorder)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
10. (chronic obstructive respiratory adj (disease or disorder)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
11. chronic ventilatory failure.mp.
12. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11
13. oxygen therapy/
14. exp ambulatory care/
15. 13 and 14
16. ((ambulat\$ or portable or supplemental or O2) adj2 (oxygen or therap\$)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]

17. 15 or 16

18. 12 and 17

Search Strategy –Cost effectiveness

1 chronic obstructive lung disease/ (67861)

2 copd.mp. (37001)

3 smoking-related lung disease\$.mp. (97)

4 lung emphysema/ (14765)

5 exp bronchitis/ (45660)

6 (chronic respiratory adj (disease or disorder)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword] (1569)

7 (chronic obstructive pulmonary adj (disease or disorder)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword] (34872)

8 (chronic obstructive lung adj (disease or disorder)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword] (68620)

9 (chronic obstructive airway adj (disease or disorder)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword] (337)

10 (chronic obstructive respiratory adj (disease or disorder)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword] (106)

11 chronic ventilatory failure.mp. (92)

12 or/1-11 (130339)

13 oxygen therapy/ (19975)

14 exp ambulatory care/ (38150)

15 13 and 14 (123)

16 ((ambulat\$ or portable or supplemental or O2) adj2 (oxygen or therap\$)).mp. (9268)

17 15 or 16 (9324)

18 12 and 17 (932)

19 cost benefit analysis/ (65629)

20 cost effectiveness analysis/ (90660)

21 cost minimization analysis/ (2303)

22 cost utility analysis/ (4841)

23 economic evaluation/ (8349)

24 (cost or costs or costed or costly or costing).tw. (403593)

25 (economic\$ or pharmaco-economic\$ or price\$ or pricing).tw. (210580)

26 (technology adj assessment\$).tw. (4263)

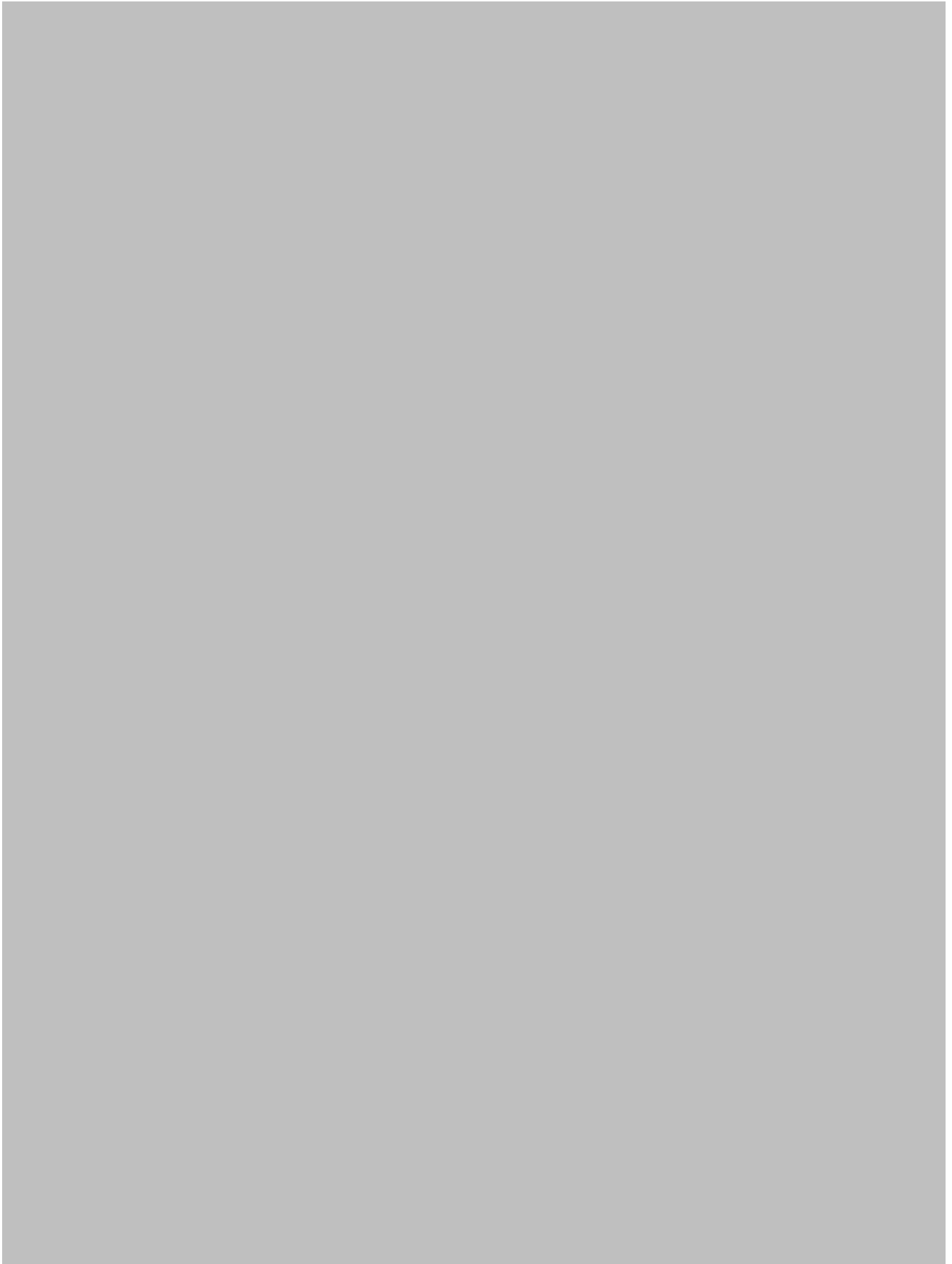
27 or/19-26 (614168)

28 18 and 27 (95)

Appendix 2

Appendix 2.1

Appendix 2.2

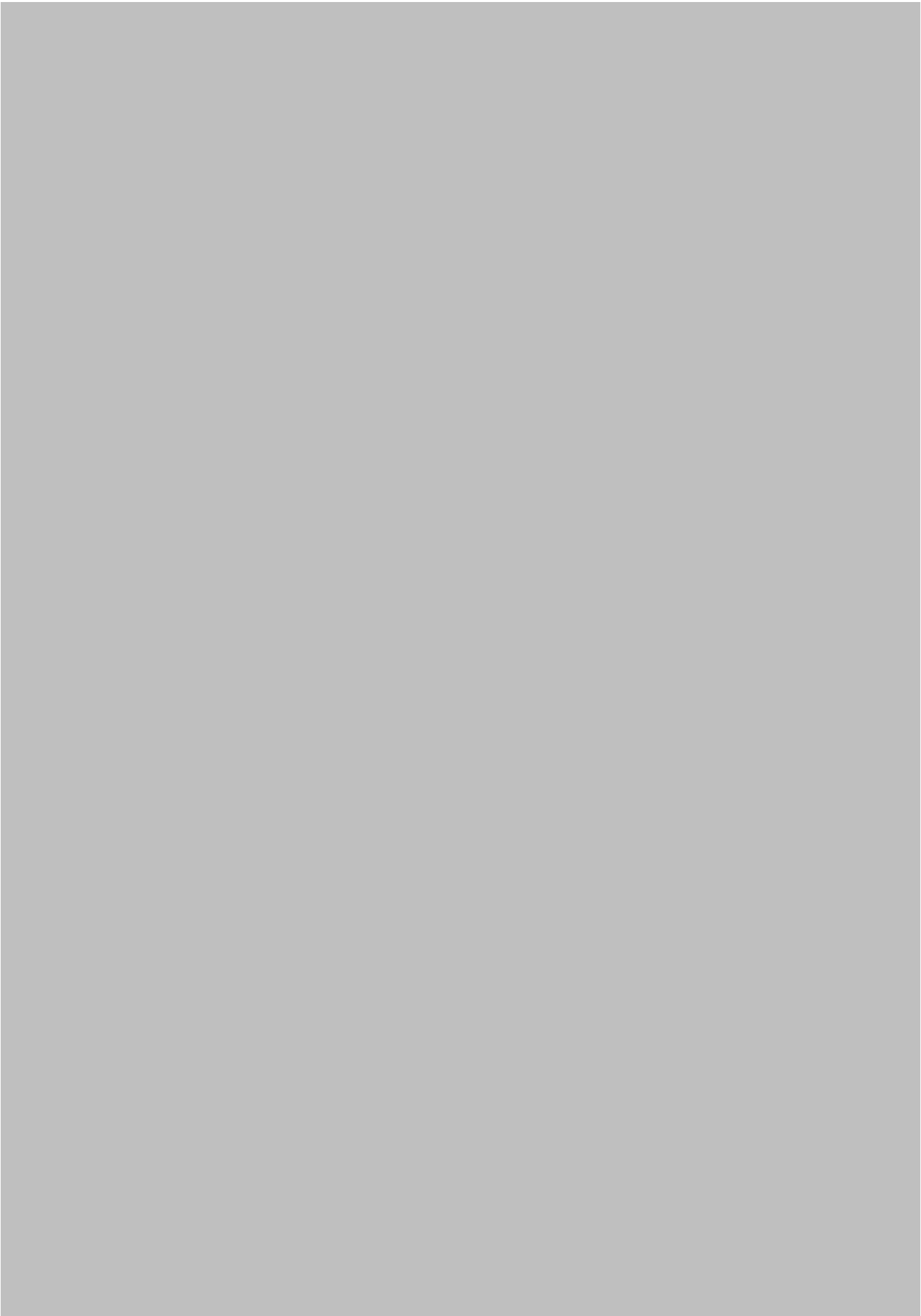






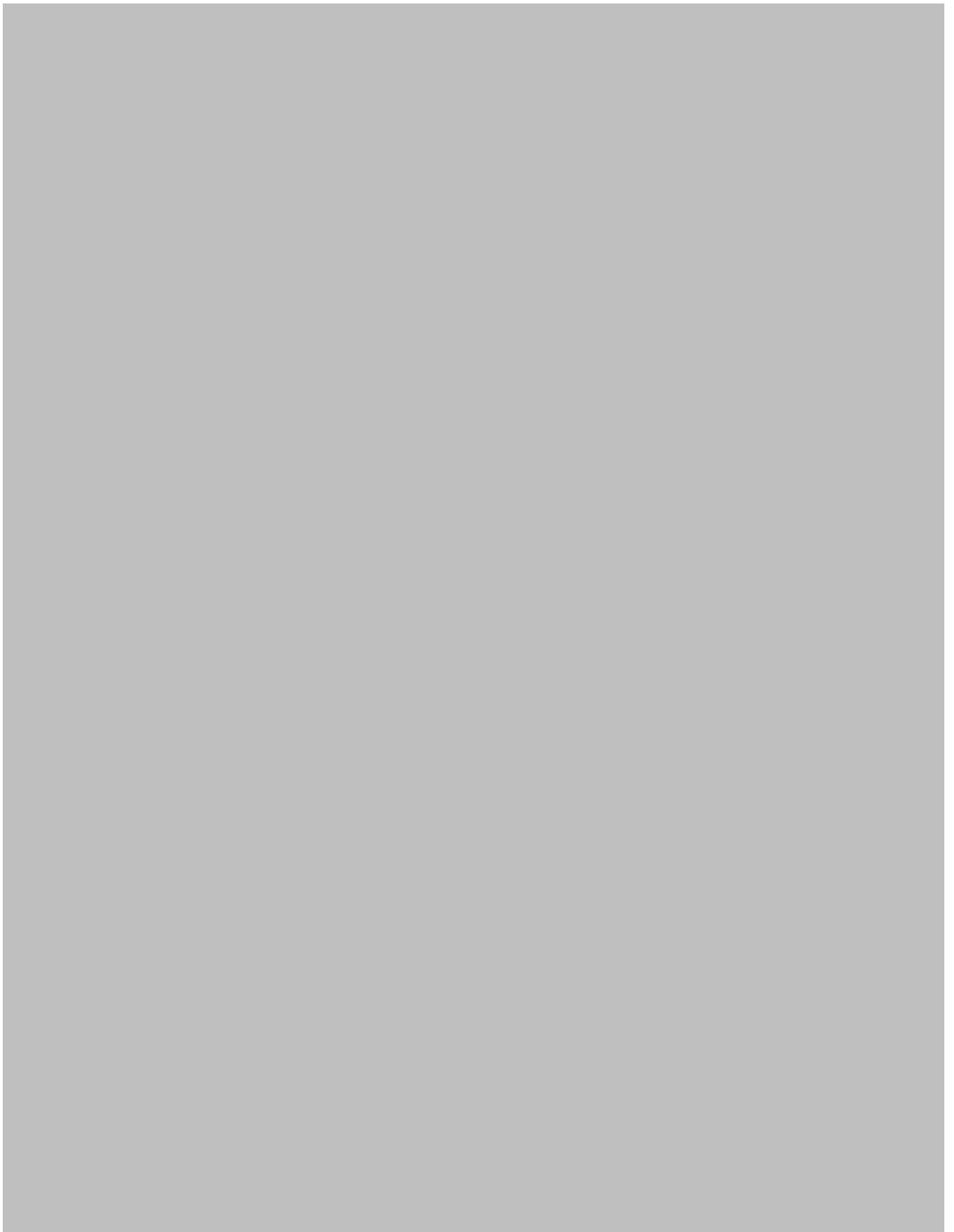




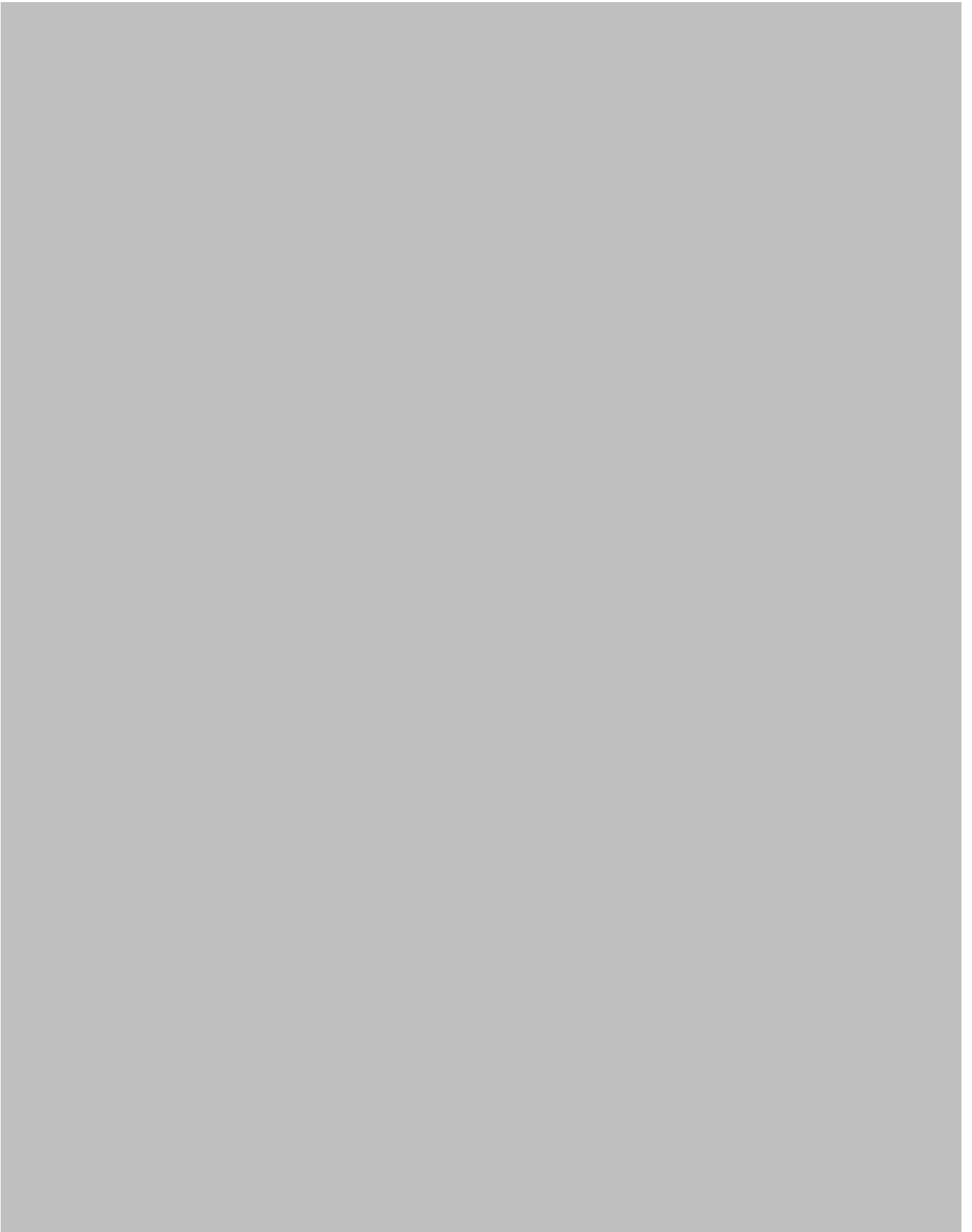


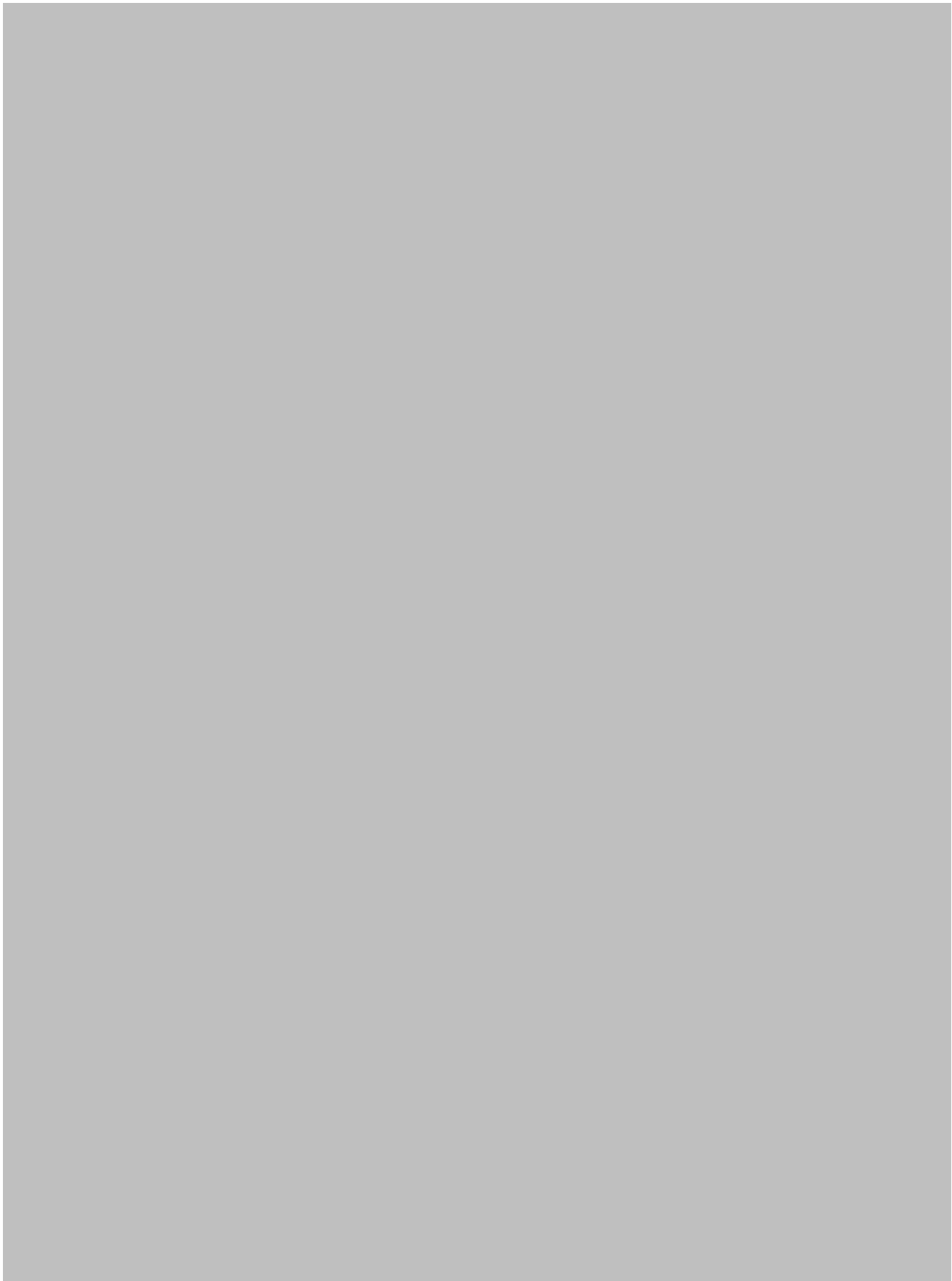


Appendix 2.3



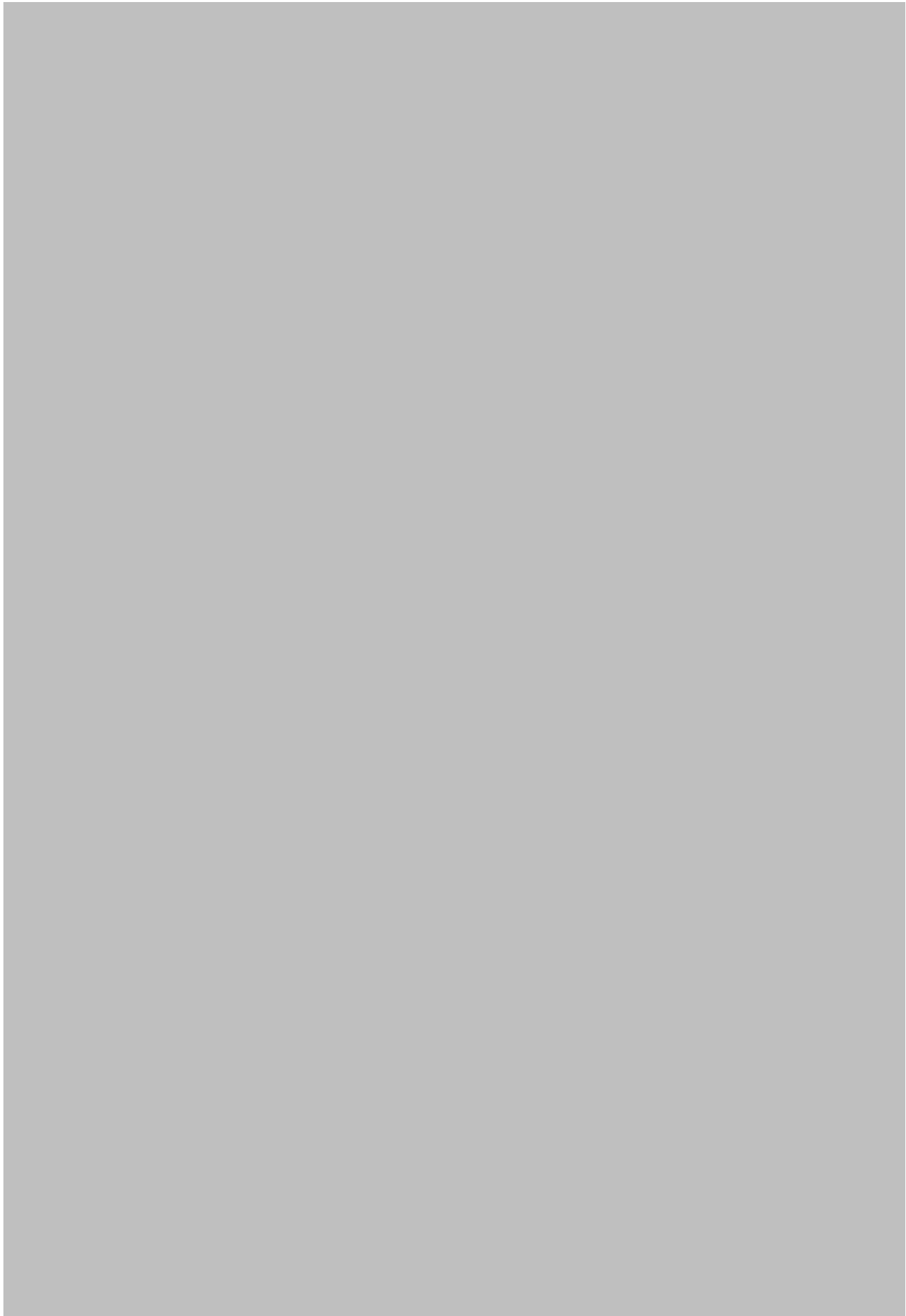
Appendix 2.4



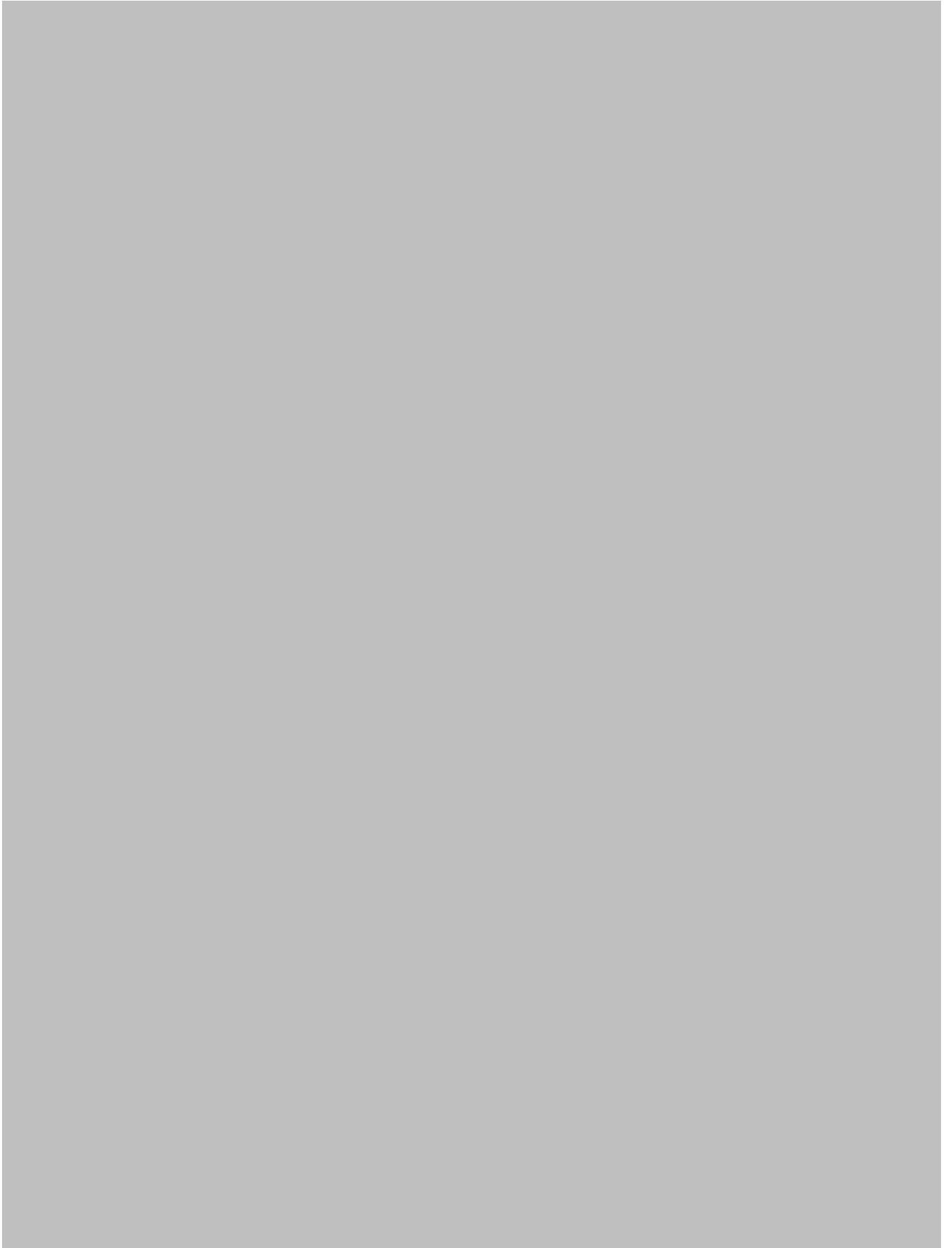


Appendix 2.5





Appendix 2.6









CONSENT FORM

Study Number: ERN-11-1670

Patient Identification Number for this trial:

Title of Project:

Name of Researcher:

Please initial box

I confirm that I have read and understand the information sheet dated..... version.....for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	
I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from the University of Birmingham, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
I agree to my GP being informed of my participation in the study.	
I agree to take part in the above study.	

Name of Patient Date.....

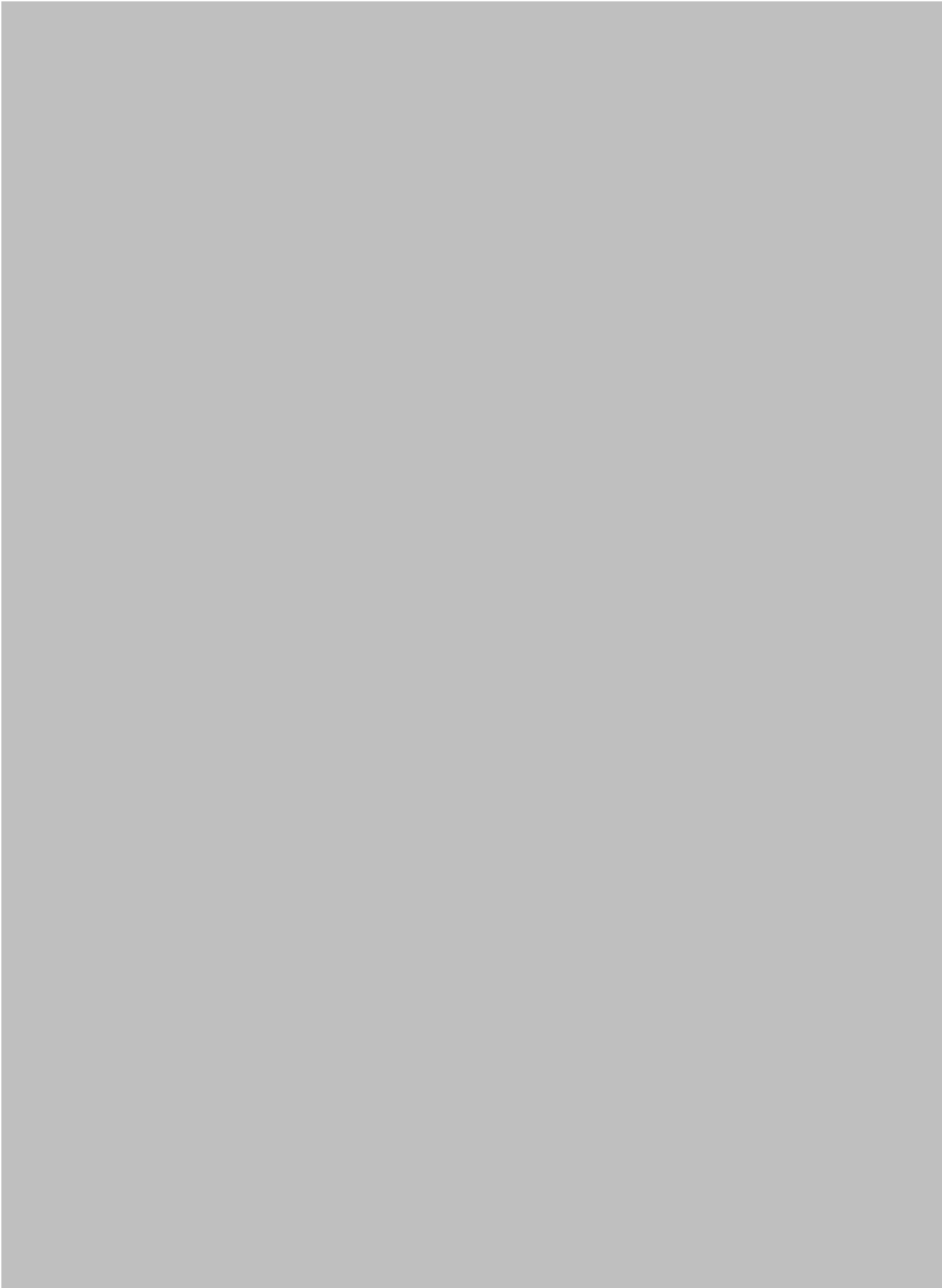
Signature.....

Name of Person taking consent Date.....

Signature.....

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

Appendix 2.7



Appendix 2.8

Can muscle dysfunction in COPD be altered by oxygenation in patients with intermittent hypoxia on exertion?

Proforma Visit 0

Patient ID _____ D.O.B _____

Age _____

Sex Male Female

O2 sats _____

ABG_(0.21) _____

	Measured	Predicted	%Predicted	Lower Limit
FEV1				
FVC				

Chronic bronchitis

Emphysema

Bronchiectasis

Common comorbidities: IHD DM Heart failure (R or L) Osteoporosis GORD

MRC dyspnoea score _____

CAT score _____

HAD score _____

Anxiety Component	/21
Depression Component	/21

6MWT Distance _____

Samples collected

Serum sample Results

Activity monitor data

Activity Level	Time Spent per Day Hours:mins					
	Day 1	2	3	4	5	Ave
Sedentary						
Light						
Moderate						
Vigorous						
Total						

Proforma for clinic visits 14, 28, 34 and 35 would be broadly similar

Extra information collected at those time points:

ADVERSE EVENTS

Recent exacerbation requiring hospital admission Y N

Recent exacerbation treated in community Y N

Adverse event

Clinic visit wk8 and 22 would just include (HAD, CAT and extra info collected as above).

Appendix 3

KIAA0355	TOMM20	RASSF1	AKT2
ING1	JDP2	ACAD9	DOK1
WARS	PSMD6	DYNC1LI2	LMBRD1
USP1	KLHDC2	XLOC_010470	DROSHA
SAC3D1	IGSF8	TPPP	ARFIP1
LZTS2	BST2	CELF5	ADIPOR1
PNKD	USP35	RNF130	MXD4
XLOC_014512	MYADM	SURF4	EMC4
TGFB1	CRIP1	DDX49	BNIP3L
RPS6KA2	SLC40A1	C1QA	TMEM221
TRAP1	CRNDE	INTS10	PHYHD1
XLOC_007868	CTNS	MRC2	SNRPF
COL1A2	C11orf83	PDXK	FTL
SPON2	FAM174B	LOC101927712	NOB1
SYT6	AHNAK2	TBC1D9B	GPATCH3
LRRC8A	IRF2BPL	FBXO10	NDUFAF6
LOC102725379	SERPINB1	UBE2H	PREP
RAB31	GOLGA3	PEA15	SETBP1
ARPC5	C20orf24	PLEKHB2	DPCD
LARP6	LOC101928089	CDC123	EHD2
PLOD1	LFNG	COPRS	LIG1
AGMAT	KANSL1	SND1	ZNF667-AS1
GZMA	TKT	C2orf47	TMEM59
SLC25A39	SMIM4	XLOC_013142	TMEM11
BCL2L1	COL4A3BP	RNPEP	MCFD2
CDK11A	FLNA	SSNA1	RPRML
SZRD1	GUCA2B	MRPS11	C1orf174
BCAP31	USP38	LGALS3	XLOC_004260
NSUN5	TTC40	AP1B1	NUPR1
REST	EMC8	TXNRD3	PNPLA2
CEP63	LRRD1	AKR1C3	SUMO2
ATP6V0A1	GPATCH4	DPP7	FAM71E1
SLC14A1	C2orf88	XLOC_008233	TRADD
SMAD4	AVPI1	ARAP1	GPBP1
ZNF189	CDR1	MET	PI16
CTDP1	SSR1	IGFBP5	LINC00943
PMPCA	MMGT1	PHLDB1	RANBP9
UGDH	BCL2	RBM17	XLOC_003147
GPKOW	HIST1H3I	TBX1	FNDC1
C5orf22	TMEM120A	PEAK1	MIR143HG
ALDH3A2	EBLN3	EML4	SARNP
TAPBP	CCDC22	CHRDL1	MRPL54
NUP85	C16orf58	RAI1	RPS17
KEAP1	RAB5C	VSTM4	VMA21
TAOK3	FAM120AOS	PRMT2	CYHR1
FRA10AC1	GADL1	ABCD1	SCPEP1
CYB5A	TMEM237	ADAR	RGCC
WDR61	LOC390705	ZFAS1	ACACB

KIAA0226	TUBA1C	CCDC3	MGLL
FAM213A	IL15	ENDOD1	LMAN2
CHCHD7	ATP2B4	FAM20B	HYI
TOR1AIP2	F8A1	HSP90B1	RAP1GAP2
TOR1AIP1	NUDT1	MTERFD2	RMDN3
MRPS30	NCAM1	SLC39A3	SNCB
BBX	GUCD1	HPS1	ARHGEF11
BCKDHB	NFIL3	REXO2	URGCP
CSF1R	PMVK	SLC43A3	EPB41L4A-AS1
AIMP1	PPP1R8	TNXB	PLIN1
LPL	ATRAID	RAB11B	RAB3GAP1
CROCC	BTBD9	RPL22L1	ITIH5
MRPL10	SPTAN1	PTPN2	TMEM204
GNE	MICU1	EGFR-AS1	RAPGEF1
YIPF6	GPX4	C6orf226	TLE2
NDRG1	TMEM43	PLEKHO2	AKR1C1
FTO	HLA-DPB1	USP22	TMSB4X
C1orf43	TCF25	HTRA1	MRPL36
KANK2	SNORD38A	TRIM24	S100A13
MTDH	SLC25A6	PSEN2	TLN1
MARS	LRRRC8C	LYRM4	DDI2
RNF149	DNPH1	KANK3	KCNS3
STK19	GGT5	LRRRC59	HERC2P4
ZNF32	BTBD1	COL1A1	C10orf71-AS1
TMEM110	DHCR24	C22orf34	SUCO
ZFAND3	ARNT2	PFKFB3	CAV1
ISLR	CMTM3	HTRA2	DAG1
SELENBP1	MRPL45	COQ5	XRCC5
PTPN6	GUK1	LINC00657	GPX1
C12orf4	HAS3	FBXO7	ARHGDI
COBL	LPCAT1	ORMDL2	OR3A3
IFI27	RPA3	LOC440864	MLLT4
HSPG2	YME1L1	HIST1H4I	BARD1
SPDYE4	NPC2	PTPN18	SIDT2
NT5C	TTC33	SKIV2L2	ZNF174
SERPINE1	RPL13A	ADPRHL1	C20orf166-AS1
EIF1AX	FHL2	SRRT	LYPLA2
RPL28	CTTN	XLOC_006689	CNP
IFITM2	XYLT2	KLHL6	AKR7A2
MN1	KIAA1549L	PPP2R2D	SEPP1
MEF2A	KEL	SH3GL1P2	LOC100507144
PITX2	FOXP1	KARS	ECSIT
LSP1	SCAND1	NAA10	CHTF8
CHSY1	CYBA	MEDAG	STX2
NUCB2	ACTR3C	HIST1H2AC	FECH
IFI30	MARCKS	COA3	MUM1
CCDC6	GNG10	SNCA	XLOC_007433
HIGD2A	SEPN1	PBX1	USP4
CAT	MRPS6	LRP1	ZCCHC24
YPEL5	STRADB	AQP7	SLC25A46

CRELD1	TMSB10	CCDC80	COL6A4P2
POLR2C	STT3A	DHDDS	EHMT1

Top 400 genes in ranked list in tables running from top to bottom.

References

1. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med.* 2006;3(11):e442.
2. NICE. National Costing Report:Chronic Obstructive Pulmonary Disease 2011 [Available from: <https://www.nice.org.uk/guidance/cg101/resources/cg101-chronic-obstructive-pulmonary-disease-update-costing-report2>].
3. Global initiative for Chronic Obstructive Lung Disease 2015 [Available from: www.goldcopd.org].
4. Fletcher C, Peto R. The natural history of chronic airflow obstruction. *Br Med J.* 1977;1(6077):1645-8.
5. Roberts SD, Farber MO, Knox KS, Phillips GS, Bhatt NY, Mastronarde JG, et al. FEV1/FVC ratio of 70% misclassifies patients with obstruction at the extremes of age. *Chest.* 2006;130(1):200-6.
6. Hardie JA, Buist AS, Vollmer WM, Ellingsen I, Bakke PS, Morkve O. Risk of over-diagnosis of COPD in asymptomatic elderly never-smokers. *Eur Respir J.* 2002;20(5):1117-22.
7. CG101 N. Chronic obstructive pulmonary disease: Management of chronic obstructive pulmonary disease in adults in primary and secondary care 2010 [Available from: <https://www.nice.org.uk/guidance/cg101>].
8. American Thoracic Society/European Respiratory Society Task Force. Standards for the Diagnosis and Management of Patients with COPD 2004.[Accessed 19th December 2014].
9. Gonzalez E, Herrejon A, Inchaurreaga I, Blanquer R. Determinants of health-related quality of life in patients with pulmonary emphysema. *Respir Med.* 2005;99(5):638-44.
10. Leidy NK, Schmier JK, Jones MK, Lloyd J, Rocchiccioli K. Evaluating symptoms in chronic obstructive pulmonary disease: validation of the Breathlessness, Cough and Sputum Scale. *Respir Med.* 2003;97 Suppl A:S59-70.
11. Fan VS, Ramsey SD, Make BJ, Martinez FJ. Physiologic variables and functional status independently predict COPD hospitalizations and emergency department visits in patients with severe COPD. *COPD.* 2007;4(1):29-39.
12. Celli BR, Cote CG, Marin JM, Casanova C, Montes de Oca M, Mendez RA, et al. The body-mass index, airflow obstruction, dyspnea, and exercise capacity index in chronic obstructive pulmonary disease. *N Engl J Med.* 2004;350(10):1005-12.
13. Panos RJ, Eschenbacher W. Exertional desaturation in patients with chronic obstructive pulmonary disease. *COPD.* 2009;6(6):478-87.
14. Cosio M, Ghezzo H, Hogg JC, Corbin R, Loveland M, Dosman J, et al. The relations between structural changes in small airways and pulmonary-function tests. *N Engl J Med.* 1978;298(23):1277-81.
15. Definition and classification of chronic bronchitis for clinical and epidemiological purposes. A report to the Medical Research Council by their Committee on the Aetiology of Chronic Bronchitis. *Lancet.* 1965;1(7389):775-9.
16. Kim V, Criner GJ. Chronic bronchitis and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2013;187(3):228-37.
17. Vestbo J, Prescott E, Lange P. Association of chronic mucus hypersecretion with FEV1 decline and chronic obstructive pulmonary disease morbidity. Copenhagen City Heart Study Group. *Am J Respir Crit Care Med.* 1996;153(5):1530-5.
18. Seemungal TA, Donaldson GC, Bhowmik A, Jeffries DJ, Wedzicha JA. Time course and recovery of exacerbations in patients with chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine.* 2000;161(5):1608-13.

19. Snider GL, Kleinerman JL, Thurlbeck WM, Bengali ZH. The Definition of Emphysema - Report of a National-Heart-Lung-and-Blood-Institute, Division of Lung-Diseases Workshop. *American Review of Respiratory Disease*. 1985;132(1):182-5.
20. Needham M, Stockley RA. Alpha 1-antitrypsin deficiency. 3: Clinical manifestations and natural history. *Thorax*. 2004;59(5):441-5.
21. Fishman A, Martinez F, Naunheim K, Piantadosi S, Wise R, Ries A, et al. A randomized trial comparing lung-volume-reduction surgery with medical therapy for severe emphysema. *N Engl J Med*. 2003;348(21):2059-73.
22. Reid J, Cockcroft D. Severe centrilobular emphysema in a patient without airflow obstruction. *Chest*. 2002;121(1):307-8.
23. Di Stefano A, Caramori G, Ricciardolo FL, Capelli A, Adcock IM, Donner CF. Cellular and molecular mechanisms in chronic obstructive pulmonary disease: an overview. *Clin Exp Allergy*. 2004;34(8):1156-67.
24. Tetley TD. Inflammatory cells and chronic obstructive pulmonary disease. *Curr Drug Targets Inflamm Allergy*. 2005;4(6):607-18.
25. Domagala-Kulawik J, Maskey-Warzechowska M, Kraszewska I, Chazan R. The cellular composition and macrophage phenotype in induced sputum in smokers and ex-smokers with COPD. *Chest*. 2003;123(4):1054-9.
26. Gadgil A, Zhu X, Scirba FC, Duncan SR. Altered T-cell phenotypes in chronic obstructive pulmonary disease. *Proc Am Thorac Soc*. 2006;3(6):487-8.
27. Sparrow D, Glynn RJ, Cohen M, Weiss ST. The relationship of the peripheral leukocyte count and cigarette smoking to pulmonary function among adult men. *Chest*. 1984;86(3):383-6.
28. Barnes PJ. Mediators of chronic obstructive pulmonary disease. *Pharmacol Rev*. 2004;56(4):515-48.
29. MacNee W. Pathogenesis of chronic obstructive pulmonary disease. *Proc Am Thorac Soc*. 2005;2(4):258-66; discussion 90-1.
30. de Godoy I, Donahoe M, Calhoun WJ, Mancino J, Rogers RM. Elevated TNF-alpha production by peripheral blood monocytes of weight-losing COPD patients. *Am J Respir Crit Care Med*. 1996;153(2):633-7.
31. Lim S, Roche N, Oliver BG, Mattos W, Barnes PJ, Chung KF. Balance of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 from alveolar macrophages in cigarette smokers. Regulation by interleukin-10. *Am J Respir Crit Care Med*. 2000;162(4 Pt 1):1355-60.
32. Capelli A, Di Stefano A, Gnemmi I, Balbo P, Cerutti CG, Balbi B, et al. Increased MCP-1 and MIP-1beta in bronchoalveolar lavage fluid of chronic bronchitics. *Eur Respir J*. 1999;14(1):160-5.
33. Sapey E, Stockley JA, Greenwood H, Ahmad A, Bayley D, Lord JM, et al. Behavioral and structural differences in migrating peripheral neutrophils from patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2011;183(9):1176-86.
34. Finkelstein R, Fraser RS, Ghezzi H, Cosio MG. Alveolar inflammation and its relation to emphysema in smokers. *Am J Respir Crit Care Med*. 1995;152(5 Pt 1):1666-72.
35. Saetta M, Di Stefano A, Turato G, Facchini FM, Corbino L, Mapp CE, et al. CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 1998;157(3 Pt 1):822-6.
36. Majo J, Ghezzi H, Cosio MG. Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema. *Eur Respir J*. 2001;17(5):946-53.
37. Eriksson S. Studies in Alpha1-Antitrypsin Deficiency. *Acta Med Scand*. 1965;S177:5-+.
38. Atkinson JJ, Senior RM. Matrix metalloproteinase-9 in lung remodeling. *Am J Respir Cell Mol Biol*. 2003;28(1):12-24.
39. Betsuyaku T, Nishimura M, Takeyabu K, Tanino M, Venge P, Xu S, et al. Neutrophil granule proteins in bronchoalveolar lavage fluid from subjects with subclinical emphysema. *Am J Respir Crit Care Med*. 1999;159(6):1985-91.

40. Ohnishi K, Takagi M, Kurokawa Y, Satomi S, Konttinen YT. Matrix metalloproteinase-mediated extracellular matrix protein degradation in human pulmonary emphysema. *Lab Invest.* 1998;78(9):1077-87.
41. Lanone S, Zheng T, Zhu Z, Liu W, Lee CG, Ma B, et al. Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and -12 in IL-13-induced inflammation and remodeling. *J Clin Invest.* 2002;110(4):463-74.
42. Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science.* 1997;277(5334):2002-4.
43. Morrison D, Rahman I, Lannan S, MacNee W. Epithelial permeability, inflammation, and oxidant stress in the air spaces of smokers. *Am J Respir Crit Care Med.* 1999;159(2):473-9.
44. Lannan S, Donaldson K, Brown D, MacNee W. Effect of cigarette smoke and its condensates on alveolar epithelial cell injury in vitro. *Am J Physiol.* 1994;266(1 Pt 1):L92-100.
45. Rahman I, Morrison D, Donaldson K, MacNee W. Systemic oxidative stress in asthma, COPD, and smokers. *Am J Respir Crit Care Med.* 1996;154(4 Pt 1):1055-60.
46. Cantin A, Crystal RG. Oxidants, Antioxidants and the Pathogenesis of Emphysema. *European Journal of Respiratory Diseases.* 1985;66:7-17.
47. Li XY, Gilmour PS, Donaldson K, MacNee W. Free radical activity and pro-inflammatory effects of particulate air pollution (PM10) in vivo and in vitro. *Thorax.* 1996;51(12):1216-22.
48. Li XY, Rahman I, Donaldson K, MacNee W. Mechanisms of cigarette smoke induced increased airspace permeability. *Thorax.* 1996;51(5):465-71.
49. Cavarra E, Lucattelli M, Gambelli F, Bartalesi B, Fineschi S, Szarka A, et al. Human SLPI inactivation after cigarette smoke exposure in a new in vivo model of pulmonary oxidative stress. *Am J Physiol Lung Cell Mol Physiol.* 2001;281(2):L412-7.
50. Shapiro SD. Proteolysis in the lung. *Eur Respir J Suppl.* 2003;44:30s-2s.
51. Paredi P, Kharitonov SA, Barnes PJ. Analysis of expired air for oxidation products. *Am J Respir Crit Care Med.* 2002;166(12 Pt 2):S31-7.
52. Paredi P, Kharitonov SA, Leak D, Ward S, Cramer D, Barnes PJ. Exhaled ethane, a marker of lipid peroxidation, is elevated in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2000;162(2 Pt 1):369-73.
53. Cantin AM, Fells GA, Hubbard RC, Crystal RG. Antioxidant macromolecules in the epithelial lining fluid of the normal human lower respiratory tract. *J Clin Invest.* 1990;86(3):962-71.
54. Shi MM, Iwamoto T, Forman HJ. gamma-Glutamylcysteine synthetase and GSH increase in quinone-induced oxidative stress in BPAEC. *Am J Physiol.* 1994;267(4 Pt 1):L414-21.
55. Linden M, Hakansson L, Ohlsson K, Sjodin K, Tegner H, Tunek A, et al. Glutathione in bronchoalveolar lavage fluid from smokers is related to humoral markers of inflammatory cell activity. *Inflammation.* 1989;13(6):651-8.
56. Donaldson GC, Seemungal TA, Bhowmik A, Wedzicha JA. Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease. *Thorax.* 2002;57(10):847-52.
57. Seemungal TA, Donaldson GC, Paul EA, Bestall JC, Jeffries DJ, Wedzicha JA. Effect of exacerbation on quality of life in patients with chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine.* 1998;157(5 Pt 1):1418-22.
58. Puhan MA, Gimeno-Santos E, Scharplatz M, Troosters T, Walters EH, Steurer J. Pulmonary rehabilitation following exacerbations of chronic obstructive pulmonary disease. *Cochrane Database Syst Rev.* 2011(10):CD005305.
59. Bolton CE, Bevan-Smith EF, Blakey JD, Crowe P, Elkin SL, Garrod R, et al. British Thoracic Society guideline on pulmonary rehabilitation in adults. *Thorax.* 2013;68 Suppl 2:ii1-30.
60. Ejiófor S, Turner AM. Pharmacotherapies for COPD. *Clin Med Insights Circ Respir Pulm Med.* 2013;7:17-34.

61. Baraldo S, Turato G, Badin C, Bazzan E, Beghe B, Zuin R, et al. Neutrophilic infiltration within the airway smooth muscle in patients with COPD. *Thorax*. 2004;59(4):308-12.
62. Calverley PM, Anderson JA, Celli B, Ferguson GT, Jenkins C, Jones PW, et al. Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease. *N Engl J Med*. 2007;356(8):775-89.
63. Wedzicha JA, Banerji D, Chapman KR, Vestbo J, Roche N, Ayers RT, et al. Indacaterol-Glycopyrronium versus Salmeterol-Fluticasone for COPD. *N Engl J Med*. 2016;374(23):2222-34.
64. Agusti A, Bel E, Thomas M, Vogelmeier C, Brusselle G, Holgate S, et al. Treatable traits: toward precision medicine of chronic airway diseases. *Eur Respir J*. 2016;47(2):410-9.
65. Barnes NC, Sharma R, Lettis S, Calverley PM. Blood eosinophils as a marker of response to inhaled corticosteroids in COPD. *Eur Respir J*. 2016;47(5):1374-82.
66. Lipson DA, Barnhart F, Brealey N, Brooks J, Criner GJ, Day NC, et al. Once-Daily Single-Inhale Triple versus Dual Therapy in Patients with COPD. *N Engl J Med*. 2018;378(18):1671-80.
67. Naunheim KS, Wood DE, Mohsenifar Z, Sternberg AL, Criner GJ, DeCamp MM, et al. Long-term follow-up of patients receiving lung-volume-reduction surgery versus medical therapy for severe emphysema by the National Emphysema Treatment Trial Research Group. *Ann Thorac Surg*. 2006;82(2):431-43.
68. Choi M, Lee WS, Lee M, Jeon K, Sheen S, Jheon S, et al. Effectiveness of bronchoscopic lung volume reduction using unilateral endobronchial valve: a systematic review and meta-analysis. *Int J Chron Obstruct Pulmon Dis*. 2015;10:703-10.
69. Christie JD, Edwards LB, Kucheryavaya AY, Aurora P, Dobbels F, Kirk R, et al. The Registry of the International Society for Heart and Lung Transplantation: twenty-seventh official adult lung and heart-lung transplant report--2010. *J Heart Lung Transplant*. 2010;29(10):1104-18.
70. Continuous or nocturnal oxygen therapy in hypoxemic chronic obstructive lung disease. A Clinical Trial. *Annals of Internal Medicine*. 1980;93(3):391-8.
71. Long term domiciliary oxygen therapy in chronic hypoxic cor pulmonale complicating chronic bronchitis and emphysema. Report of the Medical Research Council. *Lancet*. 1981;1:681-6.
72. Somfay A, Porszasz J, Lee SM, Casaburi R. Dose-response effect of oxygen on hyperinflation and exercise endurance in nonhypoxaemic COPD patients. *Eur Respir J*. 2001;18(1):77-84.
73. O'Donnell DE, Bain DJ, Webb KA. Factors contributing to relief of exertional breathlessness during hyperoxia in chronic airflow limitation. *Am J Respir Crit Care Med*. 1997;155(2):530-5.
74. Casaburi R, Patessio A, Ioli F, Zanaboni S, Donner CF, Wasserman K. Reductions in exercise lactic acidosis and ventilation as a result of exercise training in patients with obstructive lung disease. *Am Rev Respir Dis*. 1991;143(1):9-18.
75. Stoller JK, Panos RJ, Krachman S, Doherty DE, Make B, Long-term Oxygen Treatment Trial Research G. Oxygen therapy for patients with COPD: current evidence and the long-term oxygen treatment trial. *Chest*. 2010;138(1):179-87.
76. Gorecka D, Gorzelak K, Sliwinski P, Tobiasz M, Zielinski J. Effect of long-term oxygen therapy on survival in patients with chronic obstructive pulmonary disease with moderate hypoxaemia. *Thorax*. 1997;52(8):674-9.
77. Long-Term Oxygen Treatment Trial Research G, Albert RK, Au DH, Blackford AL, Casaburi R, Cooper JA, Jr., et al. A Randomized Trial of Long-Term Oxygen for COPD with Moderate Desaturation. *N Engl J Med*. 2016;375(17):1617-27.
78. British Thoracic Society Clinical component for home oxygen services in England and Wales. 2006:Accessed 19th November 2014.
79. Somfay A, Porszasz J, Lee SM, Casaburi R. Dose-response effect of oxygen on hyperinflation and exercise endurance in nonhypoxaemic COPD patients. *European Respiratory Journal*. 2001;18(1):77-84.

80. van Gestel AJ, Clarenbach CF, Stowhas AC, Teschler S, Russi EW, Teschler H, et al. Prevalence and prediction of exercise-induced oxygen desaturation in patients with chronic obstructive pulmonary disease. *Respiration*. 2012;84(5):353-9.
81. Hardinge M, Annandale J, Bourne S, Cooper B, Evans A, Freeman D, et al. British Thoracic Society guidelines for home oxygen use in adults. *Thorax*. 2015;70 Suppl 1:i1-i43.
82. Stolz D, Boersma W, Blasi F, Louis R, Milenkovic B, Kostikas K, et al. Exertional hypoxemia in stable COPD is common and predicted by circulating proadrenomedullin. *Chest*. 2014;146(2):328-38.
83. Casanova C, Cote C, Marin JM, Pinto-Plata V, de Torres JP, Aguirre-Jaime A, et al. Distance and oxygen desaturation during the 6-min walk test as predictors of long-term mortality in patients with COPD. *Chest*. 2008;134(4):746-52.
84. Andrianopoulos V, Franssen FM, Peeters JP, Ubachs TJ, Bukari H, Groenen M, et al. Exercise-induced oxygen desaturation in COPD patients without resting hypoxemia. *Respir Physiol Neurobiol*. 2014;190:40-6.
85. Vogiatzis I, Zakynthinos G, Andrianopoulos V. Mechanisms of physical activity limitation in chronic lung diseases. *Pulm Med*. 2012;2012:634761.
86. Elbehairy AF, Ciavaglia CE, Webb KA, Guenette JA, Jensen D, Mourad SM, et al. Pulmonary Gas Exchange Abnormalities in Mild Chronic Obstructive Pulmonary Disease. Implications for Dyspnea and Exercise Intolerance. *Am J Respir Crit Care Med*. 2015;191(12):1384-94.
87. O'Donnell DE, Reville SM, Webb KA. Dynamic hyperinflation and exercise intolerance in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2001;164(5):770-7.
88. Maltais F, Simard AA, Simard C, Jobin J, Desgagnes P, LeBlanc P. Oxidative capacity of the skeletal muscle and lactic acid kinetics during exercise in normal subjects and in patients with COPD. *Am J Respir Crit Care Med*. 1996;153(1):288-93.
89. Jeffery Mador M, Kufel TJ, Pineda L. Quadriceps fatigue after cycle exercise in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2000;161(2 Pt 1):447-53.
90. Bruce RM, Turner A, White MJ. Ventilatory responses to muscle metaboreflex activation in chronic obstructive pulmonary disease. *J Physiol*. 2016;594(20):6025-35.
91. Mahler DA, Gifford AH, Waterman LA, Ward J, Machala S, Baird JC. Mechanism of greater oxygen desaturation during walking compared with cycling in patients with COPD. *Chest*. 2011;140(2):351-8.
92. Spruit MA, Polkey MI, Celli B, Edwards LD, Watkins ML, Pinto-Plata V, et al. Predicting outcomes from 6-minute walk distance in chronic obstructive pulmonary disease. *J Am Med Assoc*. 2012;307(3):291-7.
93. ATS statement: guidelines for the six-minute walk test. *Am J Respir Crit Care Med*. 2002;166(1):111-7.
94. Cazzola M, Biscione GL, Pasqua F, Crigna G, Appodia M, Cardaci V, et al. Use of 6-min and 12-min walking test for assessing the efficacy of formoterol in COPD. *Respir Med*. 2008;102(10):1425-30.
95. Boyd G, Morice AH, Pounsford JC, Siebert M, Pelsis N, Crawford C. An evaluation of salmeterol in the treatment of chronic obstructive pulmonary disease (COPD). *Eur Respir J*. 1997;10(4):815-21.
96. Guyatt GH, Thompson PJ, Berman LB, Sullivan MJ, Townsend M, Jones NL, et al. How should we measure function in patients with chronic heart and lung disease? *J Chronic Dis*. 1985;38(6):517-24.
97. Holland AE, Spruit MA, Singh SJ. How to carry out a field walking test in chronic respiratory disease. *Breathe (Sheff)*. 2015;11(2):128-39.
98. Singh SJ, Morgan MD, Hardman AE, Rowe C, Bardsley PA. Comparison of oxygen uptake during a conventional treadmill test and the shuttle walking test in chronic airflow limitation. *Eur Respir J*. 1994;7(11):2016-20.

99. Reville SM, Morgan MD, Singh SJ, Williams J, Hardman AE. The endurance shuttle walk: a new field test for the assessment of endurance capacity in chronic obstructive pulmonary disease. *Thorax*. 1999;54(3):213-22.
100. Bradley JM, O'Neill B. Short term ambulatory oxygen for chronic obstructive pulmonary disease. *Cochrane database of systematic reviews (Online)*. 2005(2):CD004356.
101. Nonoyama ML, Brooks D, Lacasse Y, Guyatt GH, Goldstein RS. Oxygen therapy during exercise training in chronic obstructive pulmonary disease. *Cochrane Database Syst Rev*. 2007(2):CD005372.
102. Woodcock AA, Gross ER, Geddes DM. Oxygen relieves breathlessness in "pink puffers". *Lancet*. 1981;1(8226):907-9.
103. Evans TW, Waterhouse JC, Carter A, Nicholl JF, Howard P. Short burst oxygen treatment for breathlessness in chronic obstructive airways disease. *Thorax*. 1986;41(8):611-5.
104. Nandi K, Smith AA, Crawford A, MacRae KD, Garrod R, Seed WA, et al. Oxygen supplementation before or after submaximal exercise in patients with chronic obstructive pulmonary disease. *Thorax*. 2003;58(8):670-3.
105. Lewis CA, Eaton TE, Young P, Kolbe J. Short-burst oxygen immediately before and after exercise is ineffective in nonhypoxic COPD patients. *Eur Respir J*. 2003;22(4):584-8.
106. Eaton T, Fergusson W, Kolbe J, Lewis CA, West T. Short-burst oxygen therapy for COPD patients: a 6-month randomised, controlled study. *Eur Respir J*. 2006;27(4):697-704.
107. Curkendall SM, DeLuise C, Jones JK, Lanes S, Stang MR, Goehring E, Jr., et al. Cardiovascular disease in patients with chronic obstructive pulmonary disease, Saskatchewan Canada cardiovascular disease in COPD patients. *Ann Epidemiol*. 2006;16(1):63-70.
108. Schmidt MI, Duncan BB, Sharrett AR, Lindberg G, Savage PJ, Offenbacher S, et al. Markers of inflammation and prediction of diabetes mellitus in adults (Atherosclerosis Risk in Communities study): a cohort study. *Lancet*. 1999;353(9165):1649-52.
109. Sin DD, Man JP, Man SF. The risk of osteoporosis in Caucasian men and women with obstructive airways disease. *Am J Med*. 2003;114(1):10-4.
110. Wuyam B, Payen JF, Levy P, Bensaidane H, Reutenauer H, Le Bas JF, et al. Metabolism and aerobic capacity of skeletal muscle in chronic respiratory failure related to chronic obstructive pulmonary disease. *Eur Respir J*. 1992;5(2):157-62.
111. Killian KJ, Leblanc P, Martin DH, Summers E, Jones NL, Campbell EJ. Exercise capacity and ventilatory, circulatory, and symptom limitation in patients with chronic airflow limitation. *Am Rev Respir Dis*. 1992;146(4):935-40.
112. Williams TJ, Patterson GA, McClean PA, Zamel N, Maurer JR. Maximal exercise testing in single and double lung transplant recipients. *Am Rev Respir Dis*. 1992;145(1):101-5.
113. Swallow EB, Reyes D, Hopkinson NS, Man WD, Porcher R, Cetti EJ, et al. Quadriceps strength predicts mortality in patients with moderate to severe chronic obstructive pulmonary disease. *Thorax*. 2007;62(2):115-20.
114. Marquis K, Debigare R, Lacasse Y, LeBlanc P, Jobin J, Carrier G, et al. Midthigh muscle cross-sectional area is a better predictor of mortality than body mass index in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2002;166(6):809-13.
115. Schols AM, Wouters EF, Soeters PB, Westerterp KR. Body composition by bioelectrical-impedance analysis compared with deuterium dilution and skinfold anthropometry in patients with chronic obstructive pulmonary disease. *Am J Clin Nutr*. 1991;53(2):421-4.
116. Simpson K, Killian K, McCartney N, Stubbing DG, Jones NL. Randomised controlled trial of weightlifting exercise in patients with chronic airflow limitation. *Thorax*. 1992;47(2):70-5.
117. Decramer M, Gosselink R, Troosters T, Verschueren M, Evers G. Muscle weakness is related to utilization of health care resources in COPD patients. *Eur Respir J*. 1997;10(2):417-23.
118. Maltais F, Decramer M, Casaburi R, Barreiro E, Burelle Y, Debigare R, et al. An official American Thoracic Society/European Respiratory Society statement: update on limb muscle

- dysfunction in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2014;189(9):e15-62.
119. Gabriel DA, Kamen G, Frost G. Neural adaptations to resistive exercise: mechanisms and recommendations for training practices. *Sports Med*. 2006;36(2):133-49.
 120. Gosselink R, Troosters T, Decramer M. Peripheral muscle weakness contributes to exercise limitation in COPD. *Am J Respir Crit Care Med*. 1996;153(3):976-80.
 121. Bernard S, LeBlanc P, Whittom F, Carrier G, Jobin J, Belleau R, et al. Peripheral muscle weakness in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 1998;158(2):629-34.
 122. Swallow EB, Gosker HR, Ward KA, Moore AJ, Dayer MJ, Hopkinson NS, et al. A novel technique for nonvolitional assessment of quadriceps muscle endurance in humans. *J Appl Physiol* (1985). 2007;103(3):739-46.
 123. Allaire J, Maltais F, Doyon JF, Noel M, LeBlanc P, Carrier G, et al. Peripheral muscle endurance and the oxidative profile of the quadriceps in patients with COPD. *Thorax*. 2004;59(8):673-8.
 124. Amann M, Eldridge MW, Lovering AT, Stickland MK, Pegelow DF, Dempsey JA. Arterial oxygenation influences central motor output and exercise performance via effects on peripheral locomotor muscle fatigue in humans. *J Physiol*. 2006;575(Pt 3):937-52.
 125. Romer LM, Haverkamp HC, Amann M, Lovering AT, Pegelow DF, Dempsey JA. Effect of acute severe hypoxia on peripheral fatigue and endurance capacity in healthy humans. *Am J Physiol Regul Integr Comp Physiol*. 2007;292(1):R598-606.
 126. Amann M, Romer LM, Subudhi AW, Pegelow DF, Dempsey JA. Severity of arterial hypoxaemia affects the relative contributions of peripheral muscle fatigue to exercise performance in healthy humans. *J Physiol*. 2007;581(Pt 1):389-403.
 127. Doucet M, Russell AP, Leger B, Debigare R, Joanisse DR, Caron MA, et al. Muscle atrophy and hypertrophy signaling in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2007;176(3):261-9.
 128. Hussain SN, Sandri M. Role of autophagy in COPD skeletal muscle dysfunction. *J Appl Physiol* (1985). 2013;114(9):1273-81.
 129. Novak I, Kirkin V, McEwan DG, Zhang J, Wild P, Rozenknop A, et al. Nix is a selective autophagy receptor for mitochondrial clearance. *EMBO Rep*. 2010;11(1):45-51.
 130. Engelen MP, Schols AM, Does JD, Wouters EF. Skeletal muscle weakness is associated with wasting of extremity fat-free mass but not with airflow obstruction in patients with chronic obstructive pulmonary disease. *Am J Clin Nutr*. 2000;71(3):733-8.
 131. Plant PJ, Brooks D, Faughnan M, Bayley T, Bain J, Singer L, et al. Cellular markers of muscle atrophy in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol*. 2010;42(4):461-71.
 132. Doucet M, Dube A, Joanisse DR, Debigare R, Michaud A, Pare ME, et al. Atrophy and hypertrophy signalling of the quadriceps and diaphragm in COPD. *Thorax*. 2010;65(11):963-70.
 133. Gosker HR, Zeegers MP, Wouters EF, Schols AM. Muscle fibre type shifting in the vastus lateralis of patients with COPD is associated with disease severity: a systematic review and meta-analysis. *Thorax*. 2007;62(11):944-9.
 134. Gosker HR, Engelen MP, van Mameren H, van Dijk PJ, van der Vusse GJ, Wouters EF, et al. Muscle fiber type IIX atrophy is involved in the loss of fat-free mass in chronic obstructive pulmonary disease. *The American journal of clinical nutrition*. 2002;76(1):113-9.
 135. Jobin J, Maltais F, Doyon JF, LeBlanc P, Simard PM, Simard AA, et al. Chronic obstructive pulmonary disease: capillarity and fiber-type characteristics of skeletal muscle. *J Cardiopulm Rehabil*. 1998;18(6):432-7.
 136. Whittom F, Jobin J, Simard PM, Leblanc P, Simard C, Bernard S, et al. Histochemical and morphological characteristics of the vastus lateralis muscle in patients with chronic obstructive pulmonary disease. *Med Sci Sports Exerc*. 1998;30(10):1467-74.

137. Maltais F, LeBlanc P, Whittom F, Simard C, Marquis K, Belanger M, et al. Oxidative enzyme activities of the vastus lateralis muscle and the functional status in patients with COPD. *Thorax*. 2000;55(10):848-53.
138. Puente-Maestu L, Perez-Parra J, Godoy R, Moreno N, Tejedor A, Gonzalez-Aragoneses F, et al. Abnormal mitochondrial function in locomotor and respiratory muscles of COPD patients. *Eur Respir J*. 2009;33(5):1045-52.
139. Remels AH, Schrauwen P, Broekhuizen R, Willems J, Kersten S, Gosker HR, et al. Peroxisome proliferator-activated receptor expression is reduced in skeletal muscle in COPD. *The European respiratory journal*. 2007;30(2):245-52.
140. Spruit MA, Gosselink R, Troosters T, Kasran A, Gayan-Ramirez G, Bogaerts P, et al. Muscle force during an acute exacerbation in hospitalised patients with COPD and its relationship with CXCL8 and IGF-I. *Thorax*. 2003;58(9):752-6.
141. Debigare R, Marquis K, Cote CH, Tremblay RR, Michaud A, LeBlanc P, et al. Catabolic/anabolic balance and muscle wasting in patients with COPD. *Chest*. 2003;124(1):83-9.
142. Beutler B, Cerami A. Cachectin and tumour necrosis factor as two sides of the same biological coin. *Nature*. 1986;320(6063):584-8.
143. Agusti A, Morla M, Sauleda J, Saus C, Busquets X. NF-kappaB activation and iNOS upregulation in skeletal muscle of patients with COPD and low body weight. *Thorax*. 2004;59(6):483-7.
144. Langen RC, Van Der Velden JL, Schols AM, Kelders MC, Wouters EF, Janssen-Heininger YM. Tumor necrosis factor-alpha inhibits myogenic differentiation through MyoD protein destabilization. *FASEB J*. 2004;18(2):227-37.
145. Caquelard F, Burnet H, Tagliarini F, Cauchy E, Richalet JP, Jammes Y. Effects of prolonged hypobaric hypoxia on human skeletal muscle function and electromyographic events. *Clin Sci (Lond)*. 2000;98(3):329-37.
146. Badier M, Guillot C, Lagier-Tessonier F, Jammes Y. EMG changes in respiratory and skeletal muscles during isometric contraction under normoxic, hypoxemic, or ischemic conditions. *Muscle Nerve*. 1994;17(5):500-8.
147. Ottenheijm CA, Heunks LM, Geraedts MC, Dekhuijzen PN. Hypoxia-induced skeletal muscle fiber dysfunction: role for reactive nitrogen species. *Am J Physiol Lung Cell Mol Physiol*. 2006;290(1):L127-35.
148. Proud CG. The multifaceted role of mTOR in cellular stress responses. *DNA Repair (Amst)*. 2004;3(8-9):927-34.
149. Di Carlo A, De Mori R, Martelli F, Pompilio G, Capogrossi MC, Germani A. Hypoxia inhibits myogenic differentiation through accelerated MyoD degradation. *J Biol Chem*. 2004;279(16):16332-8.
150. Takabatake N, Nakamura H, Abe S, Inoue S, Hino T, Saito H, et al. The relationship between chronic hypoxemia and activation of the tumor necrosis factor-alpha system in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2000;161(4 Pt 1):1179-84.
151. Hoppeler H, Vogt M, Weibel ER, Fluck M. Response of skeletal muscle mitochondria to hypoxia. *Exp Physiol*. 2003;88(1):109-19.
152. Gosker HR, van Mameren H, van Dijk PJ, Engelen MP, van der Vusse GJ, Wouters EF, et al. Skeletal muscle fibre-type shifting and metabolic profile in patients with chronic obstructive pulmonary disease. *Eur Respir J*. 2002;19(4):617-25.
153. Turan N, Kalko S, Stincone A, Clarke K, Sabah A, Howlett K, et al. A systems biology approach identifies molecular networks defining skeletal muscle abnormalities in chronic obstructive pulmonary disease. *PLoS Comput Biol*. 2011;7(9):e1002129.
154. Barreiro E. Protein carbonylation and muscle function in COPD and other conditions. *Mass Spectrom Rev*. 2014;33(3):219-36.

155. Fermoselle C, Rabinovich R, Ausin P, Puig-Vilanova E, Coronell C, Sanchez F, et al. Does oxidative stress modulate limb muscle atrophy in severe COPD patients? *Eur Respir J*. 2012;40(4):851-62.
156. Rodriguez DA, Kalko S, Puig-Vilanova E, Perez-Olabarria M, Falciani F, Gea J, et al. Muscle and blood redox status after exercise training in severe COPD patients. *Free Radic Biol Med*. 2012;52(1):88-94.
157. Barreiro E, Gea J, Matar G, Hussain SN. Expression and carbonylation of creatine kinase in the quadriceps femoris muscles of patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol*. 2005;33(6):636-42.
158. Koechlin C, Couillard A, Simar D, Cristol JP, Bellet H, Hayot M, et al. Does oxidative stress alter quadriceps endurance in chronic obstructive pulmonary disease? *Am J Respir Crit Care Med*. 2004;169(9):1022-7.
159. Pitta F, Troosters T, Spruit MA, Decramer M, Gosselink R. Activity monitoring for assessment of physical activities in daily life in patients with chronic obstructive pulmonary disease. *Arch Phys Med Rehabil*. 2005;86(10):1979-85.
160. Franssen FM, Wouters EF, Schols AM. The contribution of starvation, deconditioning and ageing to the observed alterations in peripheral skeletal muscle in chronic organ diseases. *Clin Nutr*. 2002;21(1):1-14.
161. Levine S, Gregory C, Nguyen T, Shrager J, Kaiser L, Rubinstein N, et al. Bioenergetic adaptation of individual human diaphragmatic myofibers to severe COPD. *J Appl Physiol* (1985). 2002;92(3):1205-13.
162. Magalhaes J, Ascensao A, Soares JM, Ferreira R, Neuparth MJ, Marques F, et al. Acute and severe hypobaric hypoxia increases oxidative stress and impairs mitochondrial function in mouse skeletal muscle. *J Appl Physiol* (1985). 2005;99(4):1247-53.
163. Ejiófor SI BS, Gassamma B, Turner AM. Ambulatory oxygen for exercise-induced desaturation and dyspnea in Chronic Obstructive Pulmonary Disease (COPD): Systematic review and meta-analysis. *Chronic Obstr Pulm Dis* (Miami). 2016;3(1):419-34.
164. Aaron SD, Donaldson GC, Whitmore GA, Hurst JR, Ramsay T, Wedzicha JA. Time course and pattern of COPD exacerbation onset. *Thorax*. 2012;67(3):238-43.
165. Garrod R, Paul EA, Wedzicha JA. Supplemental oxygen during pulmonary rehabilitation in patients with COPD with exercise hypoxaemia. *Thorax*. 2000;55(7):539-43.
166. Sandland CJ, Morgan MD, Singh SJ. Patterns of domestic activity and ambulatory oxygen usage in COPD. *Chest*. 2008;134(4):753-60.
167. Continuous or Nocturnal Oxygen Therapy in Hypoxemic Chronic Obstructive Lung Disease A Clinical Trial. *Annals of Internal Medicine*. 1980;93(3):391-8.
168. Long term domiciliary oxygen therapy in chronic hypoxic cor pulmonale complicating chronic bronchitis and emphysema. Report of the Medical Research Council Working Party. *Lancet*. 1981;1(8222):681-6.
169. collaboration TC. *Cochrane Handbook for Systematic Reviews of Interventions* 2011. Available from: <http://www.cochrane-handbook.org>.
170. Jadad AR, Moore RA, Carroll D, Jenkinson C, Reynolds DJ, Gavaghan DJ, et al. Assessing the quality of reports of randomized clinical trials: is blinding necessary? *Control Clin Trials*. 1996;17(1):1-12.
171. Schulz KF, Grimes DA. Generation of allocation sequences in randomised trials: chance, not choice. *Lancet*. 2002;359(9305):515-9.
172. Maclure M. The case-crossover design: a method for studying transient effects on the risk of acute events. *Am J Epidemiol*. 1991;133(2):144-53.
173. Radom-Aizik S, Kaminski N, Hayek S, Halkin H, Cooper DM, Ben-Dov I. Effects of exercise training on quadriceps muscle gene expression in chronic obstructive pulmonary disease. *J Appl Physiol* (1985). 2007;102(5):1976-84.

174. Wimpres S, Vara DD, Brightling CE. Improving the sampling technique of arterialized capillary samples to obtain more accurate PaO₂ measurements. *Chron Respir Dis*. 2005;2(1):47-50.
175. Bergstrom J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest*. 1975;35(7):609-16.
176. Zigmund AS, Snaith RP. The hospital anxiety and depression scale. *Acta Psychiatr Scand*. 1983;67(6):361-70.
177. Jones PW, Harding G, Berry P, Wiklund I, Chen WH, Kline Leidy N. Development and first validation of the COPD Assessment Test. *Eur Respir J*. 2009;34(3):648-54.
178. Watz H, Waschki B, Meyer T, Magnussen H. Physical activity in patients with COPD. *Eur Respir J*. 2009;33(2):262-72.
179. Freedson PS, Melanson E, Sirard J. Calibration of the Computer Science and Applications, Inc. accelerometer. *Med Sci Sports Exerc*. 1998;30(5):777-81.
180. Rabinovich RA, Louvaris Z, Raste Y, Langer D, Van Remoortel H, Giavedoni S, et al. Validity of physical activity monitors during daily life in patients with COPD. *Eur Respir J*. 2013;42(5):1205-15.
181. TransPlex Complete Whole Transcriptome Amplification Kit: Whole Transcriptome Amplification of Highly-Degraded RNA from FFPE Tissues [Available from: <http://www.sigmaldrich.com/technical-documents/articles/life-science-innovations/transplex-rna-amplification.html>].
182. McDonald CF, Blyth CM, Lazarus MD, Marschner I, Barter CE. Exertional oxygen of limited benefit in patients with chronic obstructive pulmonary disease and mild hypoxemia. *American Journal of Respiratory & Critical Care Medicine*. 1995;152(5 Pt 1):1616-9.
183. Rooyackers JM, Dekhuijzen PN, Van Herwaarden CL, Folgering HT. Training with supplemental oxygen in patients with COPD and hypoxaemia at peak exercise. *European Respiratory Journal*. 1997;10(6):1278-84.
184. Eaton T, Garrett JE, Young P, Fergusson W, Kolbe J, Rudkin S, et al. Ambulatory oxygen improves quality of life of COPD patients: a randomised controlled study. *European Respiratory Journal*. 2002;20(2):306-12.
185. Emtner M, Porszasz J, Burns M, Somfay A, Casaburi R. Benefits of supplemental oxygen in exercise training in nonhypoxemic chronic obstructive pulmonary disease patients. *American Journal of Respiratory & Critical Care Medicine*. 2003;168(9):1034-42.
186. Nonoyama ML, Brooks D, Guyatt GH, Goldstein RS. Effect of oxygen on health quality of life in patients with chronic obstructive pulmonary disease with transient exertional hypoxemia. *American Journal of Respiratory & Critical Care Medicine*. 2007;176(4):343-9.
187. Janaudis-Ferreira TH-LK, Lundgren R, Wadell K. Differences in training effects following training with and without supplemental oxygen in patients with COPD. *Advances in Physiotherapy*. 2009;11:186-92.
188. Moore RP, Berlowitz DJ, Denehy L, Pretto JJ, Brazzale DJ, Sharpe K, et al. A randomised trial of domiciliary, ambulatory oxygen in patients with COPD and dyspnoea but without resting hypoxaemia. *Thorax*. 2011;66(1):32-7.
189. Dyer F, Callaghan J, Cheema K, Bott J. Ambulatory oxygen improves the effectiveness of pulmonary rehabilitation in selected patients with chronic obstructive pulmonary disease. *Chronic Respiratory Disease*. 2012;9(2):83-91.
190. Ringbaek T, Martinez G, Lange P. The long-term effect of ambulatory oxygen in normoxaemic COPD patients: A randomised study. *Chronic Respiratory Disease*. 2013;10(2):77-84.
191. Soffer M, Tashkin DP, Shapiro BJ, Littner M, Harvey E, Farr S. Conservation of oxygen supply using a reservoir nasal cannula in hypoxemic patients at rest and during exercise. *Chest*. 1985;88(5):663-8.

192. Carter R, Williams JS, Berry J, Peavler M, Griner D, Tiep B. Evaluation of the pendant oxygen-conserving nasal cannula during exercise. *Chest*. 1986;89(6):806-10.
193. Tiep BL, Carter R, Nicotra B. Demand oxygen delivery during exercise. *Chest*. 1987;91(1):15-20.
194. Bower JS, Brook CJ, Zimmer K, Davis D. Performance of a demand oxygen saver system during rest, exercise, and sleep in hypoxemic patients. *Chest*. 1988;94(1):77-80.
195. Braun SR, Spratt G, Scott GC, Eilersieck M. Comparison of six oxygen delivery systems for COPD patients at rest and during exercise. *Chest*. 1992;102(3):694-8,(19 ref):694-8.
196. Roberts CM, Bell J, Wedzicha JA. Comparison of the efficacy of a demand oxygen delivery system with continuous low flow oxygen in subjects with stable COPD and severe oxygen desaturation on walking. *Thorax*. 1996;51(8):831-4.
197. Cuvelier A, Nuir JF, Chakroun N, Aboab J, Onea G, Benhamou D. Refillable oxygen cylinders may be an alternative for ambulatory oxygen therapy in COPD. *Chest*. 2002;122(2):451-6.
198. Tiep BL, Barnett J, Schiffman G, Sanchez O, Carter R. Maintaining oxygenation via demand oxygen delivery during rest and exercise. *Respiratory Care*. 2002;47(8):887-92.
199. Chatila W, Nugent T, Vance G, Gaughan J, Criner GJ. The effects of high-flow vs low-flow oxygen on exercise in advanced obstructive airways disease. *Chest*. 2004;126(4):1108-15,(21 ref):1108-15.
200. Fuhrman C, Chouaid C, Herigault R, Housset B, Adnot S. Comparison of four demand oxygen delivery systems at rest and during exercise for chronic obstructive pulmonary disease. *Respiratory Medicine*. 2004;98(10):938f__44.
201. Strickland SL, Hogan TM, Hogan RG, Sohal HS, McKenzie WN, Petroski GF. A randomized multi-arm repeated-measures prospective study of several modalities of portable oxygen delivery during assessment of functional exercise capacity. *Respiratory Care*. 2009;54(3):344-9.
202. Palwai A, Skowronski M, Coreno A, Drummond C, McFadden ER. Critical comparisons of the clinical performance of oxygenf__conserving devices. *American Journal of Respiratory and Critical Care Medicine*. 2010;181(10):1061f__71.
203. Casaburi R, Porszasz J, Hecht A, Tiep B, Albert RK, Anthonisen NR, et al. Influence of lightweight ambulatory oxygen on oxygen use and activity patterns of COPD patients receiving long-term oxygen therapy. *COPD*. 2012;9(1):3-11.
204. Marti S, Pajares V, Morante F, Ramon MA, Lara J, Ferrer J, et al. Are oxygen-conserving devices effective for correcting exercise hypoxemia? *Respir Care*. 2013;58(10):1606-13.
205. Hertel N, Kotchie RW, Samyshkin Y, Radford M, Humphreys S, Jameson K. Cost-effectiveness of available treatment options for patients suffering from severe COPD in the UK: a fully incremental analysis. *Int J Chron Obstruct Pulmon Dis*. 2012;7:183-99.
206. Redelmeier DA, Bayoumi AM, Goldstein RS, Guyatt GH. Interpreting small differences in functional status: the Six Minute Walk test in chronic lung disease patients. *Am J Respir Crit Care Med*. 1997;155(4):1278-82.
207. Pepin V, Laviolette L, Brouillard C, Sewell L, Singh SJ, Revill SM, et al. Significance of changes in endurance shuttle walking performance. *Thorax*. 2011;66(2):115-20.
208. Leggett RJ, Flenley DC. Portable oxygen and exercise tolerance in patients with chronic hypoxic cor pulmonale. *Br Med J*. 1977;2(6079):84-6.
209. Borel B, Provencher S, Saey D, Maltais F. Responsiveness of Various Exercise-Testing Protocols to Therapeutic Interventions in COPD. *Pulm Med*. 2013;2013:410748.
210. Schunemann HJ, Puhan M, Goldstein R, Jaeschke R, Guyatt GH. Measurement properties and interpretability of the Chronic respiratory disease questionnaire (CRQ). *COPD*. 2005;2(1):81-9.
211. Kon SS, Dilaver D, Mittal M, Nolan CM, Clark AL, Canavan JL, et al. The Clinical COPD Questionnaire: response to pulmonary rehabilitation and minimal clinically important difference. *Thorax*. 2014;69(9):793-8.

212. Arnold E, Bruton A, Donovan-Hall M, Fenwick A, Dibb B, Walker E. Ambulatory oxygen: why do COPD patients not use their portable systems as prescribed? A qualitative study. *BMC Pulm Med.* 2011;11:9.
213. McCarthy B, Casey D, Devane D, Murphy K, Murphy E, Lacasse Y. Pulmonary rehabilitation for chronic obstructive pulmonary disease. *Cochrane Database Syst Rev.* 2015(2):CD003793.
214. Constantin D, Menon MK, Houchen-Wolloff L, Morgan MD, Singh SJ, Greenhaff P, et al. Skeletal muscle molecular responses to resistance training and dietary supplementation in COPD. *Thorax.* 2013;68(7):625-33.
215. Aliverti A, Macklem PT. How and why exercise is impaired in COPD. *Respiration.* 2001;68(3):229-39.
216. Spruit MA, Watkins ML, Edwards LD, Vestbo J, Calverley PM, Pinto-Plata V, et al. Determinants of poor 6-min walking distance in patients with COPD: the ECLIPSE cohort. *Respir Med.* 2010;104(6):849-57.
217. Ischaki E, Papatheodorou G, Gaki E, Papa I, Koulouris N, Loukides S. Body mass and fat-free mass indices in COPD: relation with variables expressing disease severity. *Chest.* 2007;132(1):164-9.
218. Jones SE, Maddocks M, Kon SS, Canavan JL, Nolan CM, Clark AL, et al. Sarcopenia in COPD: prevalence, clinical correlates and response to pulmonary rehabilitation. *Thorax.* 2015;70(3):213-8.
219. Polkey MI, Spruit MA, Edwards LD, Watkins ML, Pinto-Plata V, Vestbo J, et al. Six-minute-walk test in chronic obstructive pulmonary disease: minimal clinically important difference for death or hospitalization. *Am J Respir Crit Care Med.* 2013;187(4):382-6.
220. Rogers C, Balanos, G Wood, A. Assessing the validity of a home activity monitor in patients with COPD. *Thorax.* 2011;66(Supplement 4):A85-A6.
221. Hecht A, Ma S, Porszasz J, Casaburi R, Network CCR. Methodology for using long-term accelerometry monitoring to describe daily activity patterns in COPD. *COPD.* 2009;6(2):121-9.
222. Giacomini M, DeJean D, Simeonov D, Smith A. Experiences of living and dying with COPD: a systematic review and synthesis of the qualitative empirical literature. *Ont Health Technol Assess Ser.* 2012;12(13):1-47.
223. Kon SS, Canavan JL, Jones SE, Nolan CM, Clark AL, Dickson MJ, et al. Minimum clinically important difference for the COPD Assessment Test: a prospective analysis. *Lancet Respir Med.* 2014;2(3):195-203.
224. Willgoss TG, Yohannes AM. Anxiety disorders in patients with COPD: a systematic review. *Respir Care.* 2013;58(5):858-66.
225. Yohannes AM, Alexopoulos GS. Depression and anxiety in patients with COPD. *Eur Respir Rev.* 2014;23(133):345-9.
226. Landers A, Wiseman R, Pitama S, Beckert L. Patient perceptions of severe COPD and transitions towards death: a qualitative study identifying milestones and developing key opportunities. *NPJ Prim Care Respir Med.* 2015;25:15043.
227. Giovanna H. Bueno IG, Ilda Godoy. Patients' perception on long-term domiciliary oxygen therapy. *European Respiratory Journal.* 2015;46(suppl 59).
228. Di Francia M, Barbier D, Mege JL, Orehek J. Tumor necrosis factor-alpha levels and weight loss in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 1994;150(5 Pt 1):1453-5.
229. Sedger LM, McDermott MF. TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. *Cytokine Growth Factor Rev.* 2014;25(4):453-72.
230. Bradley JR. TNF-mediated inflammatory disease. *J Pathol.* 2008;214(2):149-60.
231. Brasier AR. The nuclear factor-kappaB-interleukin-6 signalling pathway mediating vascular inflammation. *Cardiovasc Res.* 2010;86(2):211-8.

232. Fong Y, Moldawer LL, Marano M, Wei H, Barber A, Manogue K, et al. Cachectin/TNF or IL-1 alpha induces cachexia with redistribution of body proteins. *Am J Physiol*. 1989;256(3 Pt 2):R659-65.
233. Janssen SP, Gayan-Ramirez G, Van den Bergh A, Herijgers P, Maes K, Verbeken E, et al. Interleukin-6 causes myocardial failure and skeletal muscle atrophy in rats. *Circulation*. 2005;111(8):996-1005.
234. Cheng M, Nguyen MH, Fantuzzi G, Koh TJ. Endogenous interferon-gamma is required for efficient skeletal muscle regeneration. *Am J Physiol Cell Physiol*. 2008;294(5):C1183-91.
235. Foster W, Li Y, Usas A, Somogyi G, Huard J. Gamma interferon as an antifibrosis agent in skeletal muscle. *J Orthop Res*. 2003;21(5):798-804.
236. Shelton GD, Calcutt NA, Garrett RS, Gu D, Sarvetnick N, Campana WM, et al. Necrotizing myopathy induced by overexpression of interferon-gamma in transgenic mice. *Muscle Nerve*. 1999;22(2):156-65.
237. Crul T, Spruit MA, Gayan-Ramirez G, Quarck R, Gosselink R, Troosters T, et al. Markers of inflammation and disuse in vastus lateralis of chronic obstructive pulmonary disease patients. *Eur J Clin Invest*. 2007;37(11):897-904.
238. Kauffmann A, Gentleman R, Huber W. arrayQualityMetrics--a bioconductor package for quality assessment of microarray data. *Bioinformatics*. 2009;25(3):415-6.
239. Huber W, Carey VJ, Gentleman R, Anders S, Carlson M, Carvalho BS, et al. Orchestrating high-throughput genomic analysis with Bioconductor. *Nat Methods*. 2015;12(2):115-21.
240. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A*. 2001;98(9):5116-21.
241. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005;102(43):15545-50.
242. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet*. 2003;34(3):267-73.
243. Guo L, Lobenhofer EK, Wang C, Shippy R, Harris SC, Zhang L, et al. Rat toxicogenomic study reveals analytical consistency across microarray platforms. *Nat Biotechnol*. 2006;24(9):1162-9.
244. Shi L, Tong W, Fang H, Scherf U, Han J, Puri RK, et al. Cross-platform comparability of microarray technology: intra-platform consistency and appropriate data analysis procedures are essential. *BMC Bioinformatics*. 2005;6 Suppl 2:S12.
245. Tarca AL, Romero R, Draghici S. Analysis of microarray experiments of gene expression profiling. *Am J Obstet Gynecol*. 2006;195(2):373-88.
246. Smyth GK, Yang YH, Speed T. Statistical issues in cDNA microarray data analysis. *Methods Mol Biol*. 2003;224:111-36.
247. Gosker HR, Hesselink MK, Duimel H, Ward KA, Schols AM. Reduced mitochondrial density in the vastus lateralis muscle of patients with COPD. *Eur Respir J*. 2007;30(1):73-9.
248. Meyer A, Zoll J, Charles AL, Charloux A, de Blay F, Diemunsch P, et al. Skeletal muscle mitochondrial dysfunction during chronic obstructive pulmonary disease: central actor and therapeutic target. *Exp Physiol*. 2013;98(6):1063-78.
249. Zheng X, Ruas JL, Cao R, Salomons FA, Cao Y, Poellinger L, et al. Cell-type-specific regulation of degradation of hypoxia-inducible factor 1 alpha: role of subcellular compartmentalization. *Mol Cell Biol*. 2006;26(12):4628-41.
250. Narravula S, Colgan SP. Hypoxia-inducible factor 1-mediated inhibition of peroxisome proliferator-activated receptor alpha expression during hypoxia. *J Immunol*. 2001;166(12):7543-8.
251. Ventura-Clapier R, Garnier A, Veksler V. Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha. *Cardiovasc Res*. 2008;79(2):208-17.

252. Levett DZ, Radford EJ, Menassa DA, Graber EF, Morash AJ, Hoppeler H, et al. Acclimatization of skeletal muscle mitochondria to high-altitude hypoxia during an ascent of Everest. *FASEB J.* 2012;26(4):1431-41.
253. Jacobs RA, Siebenmann C, Hug M, Toigo M, Meinild AK, Lundby C. Twenty-eight days at 3454-m altitude diminishes respiratory capacity but enhances efficiency in human skeletal muscle mitochondria. *FASEB J.* 2012;26(12):5192-200.
254. Eaton S, Bartlett K, Pourfarzam M. Mammalian mitochondrial beta-oxidation. *Biochem J.* 1996;320 (Pt 2):345-57.
255. Morash AJ, Kotwica AO, Murray AJ. Tissue-specific changes in fatty acid oxidation in hypoxic heart and skeletal muscle. *Am J Physiol Regul Integr Comp Physiol.* 2013;305(5):R534-41.
256. Davidsen PK, Herbert JM, Antczak P, Clarke K, Ferrer E, Peinado VI, et al. A systems biology approach reveals a link between systemic cytokines and skeletal muscle energy metabolism in a rodent smoking model and human COPD. *Genome Med.* 2014;6(8):59.
257. van den Borst B, Slot IG, Hellwig VA, Vosse BA, Kelders MC, Barreiro E, et al. Loss of quadriceps muscle oxidative phenotype and decreased endurance in patients with mild-to-moderate COPD. *J Appl Physiol (1985).* 2013;114(9):1319-28.
258. Pepin V, Saey D, Whittom F, LeBlanc P, Maltais F. Walking versus cycling: sensitivity to bronchodilation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2005;172(12):1517-22.
259. Couillard A, Koechlin C, Cristol JP, Varray A, Prefaut C. Evidence of local exercise-induced systemic oxidative stress in chronic obstructive pulmonary disease patients. *Eur Respir J.* 2002;20(5):1123-9.
260. Lombard DB, Alt FW, Cheng HL, Bunkenborg J, Streeper RS, Mostoslavsky R, et al. Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation. *Mol Cell Biol.* 2007;27(24):8807-14.
261. Munoz-Canoves P, Scheele C, Pedersen BK, Serrano AL. Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword? *FEBS J.* 2013;280(17):4131-48.
262. Fischer CP. Interleukin-6 in acute exercise and training: what is the biological relevance? *Exerc Immunol Rev.* 2006;12:6-33.
263. White JP, Puppa MJ, Sato S, Gao S, Price RL, Baynes JW, et al. IL-6 regulation on skeletal muscle mitochondrial remodeling during cancer cachexia in the ApcMin/+ mouse. *Skelet Muscle.* 2012;2:14.
264. Rabinovich RA, Drost E, Manning JR, Dunbar DR, Diaz-Ramos M, Lakhdar R, et al. Genome-wide mRNA expression profiling in vastus lateralis of COPD patients with low and normal fat free mass index and healthy controls. *Respir Res.* 2015;16:1.
265. Wei C, Li J, Bumgarner RE. Sample size for detecting differentially expressed genes in microarray experiments. *BMC Genomics.* 2004;5:87.