Skeletal Muscle, Exercise and Activity in Pulmonary Hypertension

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Thesis Submitted to Newcastle University for

Doctor of Medicine (M.D)

August 2017

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Declaration

The research thesis submitted is entirely all my own work except for certain contributions by specific people that are mentioned in the acknowledgement section and in the relevant chapters. The works of others that are quoted are referenced accordingly at the end of the paragraph or sentence and full references are given in the bibliography.

Abstract

Pulmonary Arterial hypertension (PAH) is a rare and progressive condition presenting with exercise intolerance, leading to right ventricle (RV) failure and death. There has been significant progress in understanding the basic pathophysiology leading to the development of a number of targeted therapies, resulting in improved prognosis. Despite this, patients remain limited in performing exertional activities with a poorer quality of life. Recent research has focused on PAH being a multi-systemic disease with skeletal muscle dysfunction contributing to exercise intolerance. There needs to be greater understanding of the physiological and behavioural mechanisms that limit daily functional capabilities in PAH patients.

The aims of the thesis were to study the role of skeletal muscle mitochondrial function, the limitations in central and peripheral haemodynamics on maximum exercise, and develop a greater understanding of whether habitual daily physical activity levels are improved by current pharmaceutical treatments.

Using ^{31}Phosphorous-magnetic resonance spectroscopy (^{31}P-MRS), oxygen delivery as opposed to impaired mitochondrial function would explain the abnormal skeletal muscle bioenergetics observed. This is further supported by analysing skeletal muscle biopsy samples demonstrating that mitochondrial protein expression and function was normal, therefore not contributing to impaired exercise capacity. Using continuous non-invasive cardiac output, chronotropic incompetence and reduced peripheral oxygen extraction are the predominant mechanisms leading to impaired peak oxygen consumption. Finally, in a pilot study targeted-therapies failed to change habitual daily physical activity and fatigue levels in PAH patients despite a significant observed change in submaximal exercise capacity.

In conclusion, a number of physiological mechanisms that impair exercise capacity and habitual physical activity in PAH are beyond the currently available targeted therapies. Further research is needed into how best to improve exercise capacity, fatigue and activity levels that will directly lead to improvement in quality of life for PAH patients.

Acknowledgements

I am most grateful and thankful for my supervisors, Professors Paul Corris and Michael Trenell. Professor Corris gave me the opportunity to gain clinical and research experience within his National Pulmonary Hypertension Service (Newcastle). This gave me an excellent chance to gain not only clinical experience in pulmonary hypertension (PH) but also in all aspects of respiratory medicine by working in highly specialised tertiary service at the Freeman Hospital. Furthermore, Professor Corris provided wonderful opportunities to present my work at national and international conferences. However, the greatest benefit was by working closely with him directly in the clinical setting. I gained and learnt immeasurable amount regarding not only the management of PH patients, but patients with other respiratory conditions. Professor Trenell was incredibly supportive throughout the research period and appreciate the guidance he has provided. I am grateful for his time, critical appraisal and review of the thesis.

Although not able to be acknowledged formally as co-supervisors, Drs Grainne Gorman and Djordje Jakovljevic provided significant support and teaching of research methodology. I am most grateful in their review and feedback of two ethics committee applications for research approval of the projects. Grainne taught me how to perform muscle biopsies independently for which I am very grateful. Djordje spent time in teaching me how to perform non-invasive haemodynamics during exercise, use of healthy control data in chapter 5 and provided guidance in the completion of the exercise project.

I am grateful Jehill Parikh and Kieren Hollingsworth for analysing the spectroscopy data presented in chapter 3 to the standard required. Gavin Falkous taught me how to use the cryostat to cut muscle sections, perform immunohistochemistry and to analyse these under the microscope. Mariana Rocha and Karolina Rygiel who taught me laboratory techniques and computer skills to perform quadruple immunofluorescence. John Grady provided final statistical analyses support within the laboratory. I am thankful to Professors Doug Turnbull and Robert Taylor for allowing me to work at the Wellcome Trust Mitochondrial Laboratory Service (Newcastle). Sarah Chapman taught me to how to use the equipment in performing physical activity monitoring and provided her own time inputting raw physical activity data through r-program coded by Vincent van Hees to generate numerical values for further analyses.

I am very thankful to have worked with the pulmonary hypertension specialist nurses at Freeman Hospital: Margaret Day, Julia De Soyza and Rachel Crackett. Their constant support during the research period and assistance in the recruitment of patients was invaluable. They are brilliant nurses, who are very attentive to the needs of their patients and without whom the PH service would not be possible. Furthermore, I have learnt much and appreciated the support from Professor Andrew Fisher, Professor John Simpson, Dr Guy MacGowan and Dr Jim Lordan. Special mention must be made of Logan Thirugnanasothy, who was my desk partner during the research period, who provided much needed support and feedback.

I am thankful to my parents for their continued support and undivided attention. Finally, I am most grateful for my wife, Aranee throughout the research period, in particular for looking after our daughter, Laavanya whilst finishing the thesis.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviations are defined at first in the theses, but some of the more common ones are listed below.

AVO_2diff – Arterio – Venous Oxygen difference

CO – Cardiac Output

CI – Cardiac Index

CPEX – Cardiopulmonary Exercise Testing

6MWD – 6 minutes-walk distance

IPAH – Idiopathic Pulmonary Arterial Hypertension

MVPA – Moderate – Vigorous Physical Activity

MET – Metabolic Equivalent

OXPHOS – Oxidative Phosphorylation

PH – Pulmonary Hypertension

PAH – Pulmonary Arterial Hypertension

$PCRt_{1/2}$ – Phosphocreatine recovery half-time

SV – Stroke Volume

WHO- FC – World Health Organization Functional Class

Chapter 1 Introduction

1.1 Pulmonary Hypertension

1.1.1 Definition

Pulmonary hypertension (PH) is a disorder that can affect cardiac and respiratory diseases, afflicting the pulmonary vessels, leading to impaired right ventricle (RV) function and early death without appropriate treatment. (Humbert et al., 2014) Patients suffer from insidious onset of breathlessness, fatigue, syncope and sudden death. The underlying aetiology leading to the development of PH guides the treatment and prognosis. Since the 1990s a number of successful therapies that have been developed for the treatment of specific types of PH, with the availability of surgical options including lung or heart-lung transplantation as a final treatment strategy. (Simonneau et al., 2016) Although the survival of patients with PH has improved, the disease remains incurable with patients living with a poor quality of life.

1.1.2 Causes and Classification

Since the first World Symposium on Pulmonary Hypertension (WSPH) in 1973 in Geneva, Switzerland, the classification system has been in place to facilitate a common diagnostic and treatment approach. This system continued to undergo modifications at subsequent WSPH symposiums held every five years, with the current classification being in place from the meeting held in Nice 2013 (Figure 1). (Simonneau et al., 2013)

Pulmonary Arterial Hypertension (PAH) makes up group 1 of the classification system and so far, has been the group which has shown significant beneficial outcomes from targeted therapies.

Figure 1: Updated Classification System of Pulmonary Hypertension

1. Pulmonary Arterial Hypertension
- 1.1 Idiopathic
- 1.2 Heritable
  - 1.2.1 BMPR2
  - 1.2.2 ALK-1, ENG, SMAD9, CAV1, KCNK3
  - 1.2.3 Unknown
- 1.3 Drug and toxin induced
- 1.4 Associated with
  - 1.4.1 Connective tissue disease
  - 1.4.2 HIV infection
  - 1.4.3 Portal Hypertension
  - 1.4.4 Congenital heart diseases
  - 1.4.5 Schistosomiasis

1' Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis

1'' Persistent pulmonary hypertension of the newborn (PPHN)

2. Pulmonary hypertension due to left heart disease
- 2.1 Left ventricular systolic dysfunction
- 2.2 Left ventricular diastolic dysfunction
- 2.3 Valvular disease
- 2.4 Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies

3. Pulmonary hypertension due to lung diseases and/or hypoxia
- 3.1 Chronic obstructive pulmonary disease
- 3.2 Interstitial lung disease
- 3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern
- 3.4 Sleep-disordered breathing
- 3.5 Alveolar hypoventilation disorders
- 3.6 Chronic exposure to high altitude

4. Chronic thromboembolic pulmonary hypertension (CTEPH)

5. Pulmonary hypertension with unclear multifactorial mechanisms
- 5.1 Hematologic disorders: chronic haemolytic anaemia, myeloproliferative disorders, splenectomy
- 5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis
- 5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
- 5.4 Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure, segmental PH

Reproduced from (Simonneau et al., 2013)

1.2 Pulmonary Arterial Hypertension

1.2.1 Epidemiology

Pulmonary arterial hypertension (PAH) is rare disease with a low incidence and prevalence. The major national published registries report an incidence per million of PAH between 2.0-7.6 cases with prevalence of 10.6-26 cases per million. (Hoeper et al., 2013a) The mean age of diagnosis has moved away from average of 36 ± 15years in 1987, to between 50 ± 14 and 65 ± 15 years in the last 10 years. (Rich et al., 1987, Badesch et al., 2010, Hoeper et al., 2013b) Females make up 65-80% of patients. (Humbert et al., 2006, Benza et al., 2010)

1.2.2 Prognosis

Prognosis can be determined by clinical characteristics including underlying aetiology, age, World Health Organization (WHO) functional class, cardiopulmonary haemodynamics and exercise capacity. (McLaughlin et al., 2004) More pertinently, how the right ventricle responds to the increased afterload at rest and on physical exertion has gained significant importance in determining prognosis in recent years. (Naeije and Manes, 2014) In the early 1990s, median reported survival of PAH patients was 2.8 years, and a 5- year survival rate of 34%. (D'Alonzo et al., 1991) The advent of increased number of PAH-targeted therapies, has led to improved survival with a reported 1, 3 and 5-year survival rate are approximately 84%, 67% and 58%, respectively. (Thenappan et al., 2010)

1.2.3 Pathophysiology

PAH pathophysiology understanding has increased considerably in the last 30 years. The pulmonary vessels are normally under low-pressure with approximately one-tenth of the resistance to blood flow when compared to the systemic circulation. (McLaughlin and McGoon, 2006) There are number of different molecular and cellular mechanisms that play a vital role in progressive pulmonary vasculopathy leading to vasoconstriction, cell proliferation, and in-situ thrombosis. (Humbert et al., 2014)

There are three main cellular pathways identified, leading to the development of targeted therapies. The first is prostacyclin which has vasodilating and antiproliferative actions in pulmonary vessels. (Jones et al., 1989) In PAH patients, prostacyclin synthetase activity is reduced therefore prostacyclin (PGI_2) levels are reduced. (Tuder et al., 1999) Endothelin -1

levels are elevated in PH. (Giaid et al., 1993) The second is endothelin-1(ET-1) which has both vasoconstrictive and proliferative function, by acting on two receptors, ET_A and ET_B. Prognosis of PAH patients has been correlated with ET-1 levels. (Rubens et al., 2001) The third is nitric oxide (NO) which leads to vasodilation. In PAH there is decreased NO synthase expression leading to vasoconstriction. (Giaid and Saleh, 1995) There are other mechanisms involved, including mitochondrial dysfunction, role of voltage-dependent potassium channels, activation of the coagulation cascade, platelet and endothelial cell dysfunction. (Archer et al., 2008)

The general understanding of the pathophysiology of PAH has advanced most recently as the ability to sequence genes has improved. Mutations in bone morphogenetic protein receptor II (BMPRII) leads to lack of apoptosis and uncontrolled cellular proliferation in pulmonary vessels. This is the most common mutation causing familial PAH, responsible for 60-80% cases. (Newman et al., 2001) The mutation is also found in 20% of patients with sporadic idiopathic PAH. Other genetic, rarer alterations have been discovered including ALK-1, SMAD9, KCNK3, endoglin and caveolin-1. (Tuder et al., 2013).

The concept of "multiple-hits" hypothesis has been proposed whereby a genetic substrate with a mutation or polymorphism being present with an environmental stimulus of unknown origin leads to the development of PAH. (McLaughlin and McGoon, 2006, Machado et al., 2005, Yuan and Rubin, 2005)

1.2.4 Diagnosis

The diagnostic process in any patient suspected of PAH consists of the basics including careful history and examination, and then followed by screening transthoracic echocardiography. The gold-standard of haemodynamic measurement is by right heart catheterisation, carried out for accurate diagnosis, assess the severity and then decide on appropriate treatment. (McLaughlin and McGoon, 2006) An overview of the clinical diagnosis process will now be briefly described.

1.2.4.1 History and examination

Patients typically report shortness of breath, fatigue, syncopal episodes and peripheral oedema. History should be focused on finding and excluding potential risk factors for PAH.

Clinical examination to find features of the underlying cause of PH, including those of portal hypertension,human, immunodeficiency virus (HIV) and connective tissue disease. There may be clinical signs of right ventricle failure including peripheral oedema, abdominal distension and raised jugular venous pressure. (Galie et al., 2016)

1.2.4.2 Clinical Investigations

Baseline pathology tests are undertaken to assess haematology and biochemistry functions. At the same time, testing of thyroid function, autoantibodies, blood-borne viruses including HIV, hepatitis B and C are carried out. The severity of right heart dysfunction can be assessed by measuring N-terminal prohormone brain naturietic peptide (NT-proBNP) and is an independent predictor of outcome. (Galie et al., 2016). Pulmonary function tests and arterial blood gases allow for further assessment of any underlying airway or interstitial lung disease. (Trip et al., 2013, Sun et al., 2003)

Electrocardiogram features in PAH may show right-axis deviation, right ventricular hypertrophy, with ST- and T- abnormality with inversion to suggest right ventricular strain. (Henkens et al., 2008, McLaughlin and McGoon, 2006)

Transthoracic echocardiogram (TTE) is a commonly used tool to evaluate patients for the potential diagnosis of PH by assessing the effect on RV function and estimating pulmonary arterial pressure (PAP) by doppler measurements. There is a strong correlation between right ventricular systolic pressure estimated from the TTE to the mean pulmonary arterial pressure on invasive right catheter measurements. (Currie et al., 1985) Furthermore, TTE allows for assessment of the left ventricle size and function, including estimating the size of the left atrium to determine whether left heart disease is the cause of PH. (Howard et al., 2012)

Apart from chest radiography, more detailed imaging is undertaken to determine the underlying aetiology of PH. This will include high resolution computer tomography or contrast-enhanced CT scan to look for features of interstitial lung disease, or alternative causes of PAH including the presence of oesophageal dilation in systemic sclerosis or vascular abnormalities due to congenital cardiac defects. (Rajaram et al., 2015)

Computer tomography pulmonary angiogram and ventilation-perfusion (V/Q)scans are carried out to determine the presence of chronic thromboembolic disease, with V/Q having

higher sensitivity. Even without chronic thromboembolic disease, in PAH V/Q scan can demonstrate peripheral unmatched or non-segmental defects in perfusion. (Tunariu et al., 2007, McLaughlin and McGoon, 2006)

1.2.4.3 Right heart catheterisation

The gold-standard method of diagnosing PAH is by right heart catheterisation (RHC) and allows for the assessment of the severity of cardiopulmonary haemodynamics. (Galie et al., 2016) By international consensus, the haemdynamic definition of PAH is a mean pulmonary arterial pressure (mPAP) of ≥ 25mmHg, pulmonary capillary wedge pressure (PCWP) of ≤15mmHg and pulmonary vascular resistance (PVR) of > 3 Wood units. (Hoeper et al., 2013a) Furthermore, RHC can measure baseline resting cardiac output, thereby determining the impact of obstruction to blood flow on how well the right ventricle is functioning.(Hoeper et al., 1999) Vasoreactivity testing is undertaken in selected patients by using nitric oxide or prostacyclin analogues to determine if these patients drop their respective mPAP in response. If they do, these select patients are treated with calcium channel blockers as the first step. (Figure 2) (Benza et al., 2015)

1.2.4.4 Exercise tests

Exercise capacity can be determined by a number of different methods including the six-minutes' walk distance (6MWD) and symptom-limited cardiopulmonary exercise testing (CPEX) most commonly using a cycle ergometer. The absolute 6MWD achieved is affected by age, gender, co-morbidities, learning and motivation.(Galie et al., 2016) Baseline and follow-up absolute 6MWD has prognostic implications, with patients achieving less than 150m having poorer outcome and those with values more than 380m have improved survival. (Sitbon et al., 2002, Barst et al., 1996, Miyamoto et al., 2000) The percentage change or absolute change in 6MWD has no prognostic implications. (Farber, 2012)

CPEX as opposed to 6MWD, is performed to maximal exercise, allowing assessment of exercise capacity, gas exchange and cardiac function. CPEX testing has been shown to correlate to functional class but less well with baseline haemodynamics.(Sun et al., 2001) Maximal oxygen consumption of greater than > 10.1ml.kg^{-1}.min^{-1} and peak systolic blood pressure > 120mmHg associated with improved survival. (Sun et al., 2001)

Exercise testing allows the clinicians to assess the functional impact of the disease on the individual basis. By following objective, standardised protocol the tests can be repeated when following up patients to determine the effect of treatment.

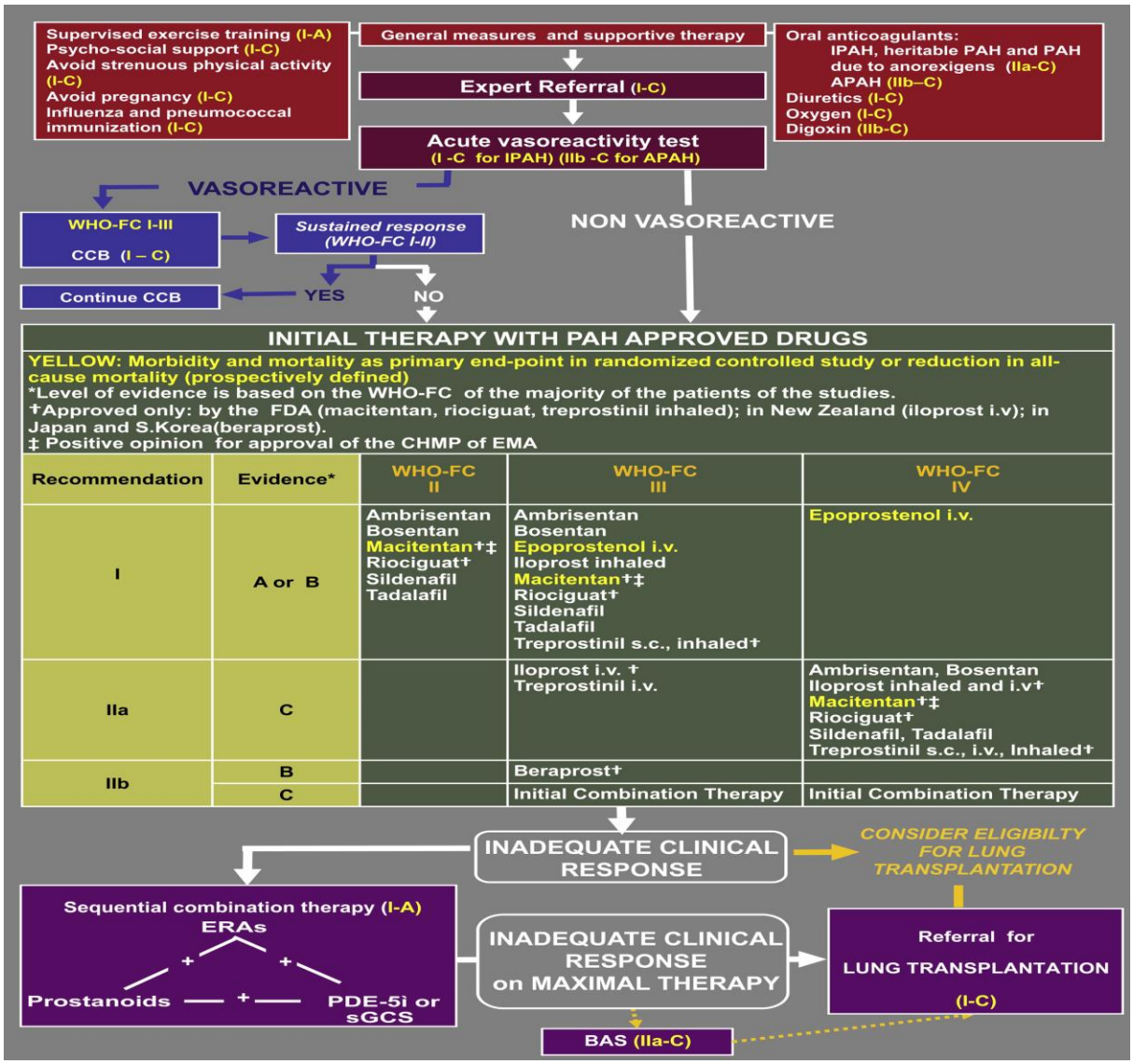
1.2.5 Treatment

Treatment for pulmonary arterial hypertension is determined by the classification system and approached according existing international guidelines. (Figure 2) (Galie et al., 2013) The majority of therapies has only been licenced for Group 1 disease. Chronic thromboembolic pulmonary hypertension (Group 4) should be assessed at a specialist centre performing pulmonary endarterectomy to determine if surgery would be beneficial. Recently, Riociguat (soluble guanylate cyclase inhibitor) has been licenced for patients with inoperable or distal CTEPH.(Ghofrani et al., 2013)

Patients are provided with general lifestyle advice. These include provision of low graded aerobic exercise, avoiding exposure to high altitudes, need for some patients to have inflight oxygen, and avoidance of pregnancy. (Badesch et al., 2004)

Conventional treatment in all patients should include the provision of diuretics to manage fluid status, maintaining oxygen levels above 90% to avoid hypoxic pulmonary vasoconstriction, and anticoagulation in certain groups including those with idiopathic, familial and anorexigen-induced PAH. (Olsson et al., 2014)

Figure 2: Current treatment algorithm from the 5th World Pulmonary Hypertension Symposium.



APAH – associated pulmonary arterial hypertension; BAS – balloon atrial septostomy; CCB – calcium channel blockers; ERA – endothelin receptor antagonist; sGCS – soluble guanylate cyclase stimulators; IPAH – idiopathic pulmonary arterial hypertension; i.v – intravenous; PDE-5i – phosphodiesterase type 5 inhibitor; s.c – subcutaneous; WHO-FC – World Health Organization functional class. Figure reproduced from (Galie et al., 2013)

1.2.5.1 PAH -Targeted Treatment

In the last two decades, a number of treatments have been approved that target specific pathways leading to PAH. All approved medications focus on the three main pathways described earlier and work by increasing nitric oxide production through inhibition of phosphodiesterase enzymes, blocking the action of endothelin at the receptor or as prostacyclin analogues. (Galie et al., 2013). The current treatment algorithm in treating PAH is shown in Figure 2.

The current guidelines suggest the goal of treatment is to achieve a low-risk status in patients. (Galie et al., 2016) This would include achieving with appropriate treatment an exercise capacity (6MWD >440m), WHO functional class II, maintaining RV function and improving quality of life. This may require escalation of treatment from single to combination targeted therapy if these goals are not met. If the patient's functional level is poor at diagnosis than this may require continuous prostacyclin analogues, upfront combination treatment or triple therapy. (Sitbon et al., 2014, Galie et al., 2015)

1.2.5.2 Atrial Septostomy

Atrial septostomy is used for palliation or as a bridge to transplantation. This procedure results in a right to left interatrial shunt, increasing the cardiac output, decreasing the pulmonary arterial pressure and ultimately increasing systemic oxygen transport despite a fall in oxygen saturations. (Sandoval et al., 1998)

1.2.5.3 Lung Transplantation

Lung transplantation is an accepted management strategy in selected patients with progressive PAH where medical options have been exhausted as determined by international consensus. (Galie et al., 2013) Most PAH patients on the waiting list undergo double-lung transplantation apart from patients with complex congenital heart disease who may receive heart-lung transplantation. Among all indications for lung transplantation, PAH patients have the highest 3-month and 1-year mortality. If survival is achieved beyond the first year, the median survival time is 9.3years. (George et al., 2011)

1.3 Pathophysiology of Exercise Limitation

1.3.1 Mechanisms of exercise

Exercise intolerance is a predominant complaint afflicting patients with cardiovascular, pulmonary, or musculoskeletal conditions. Patients with PAH report symptoms of shortness of breath and musculoskeletal fatigue on exertion. (Sun et al., 2001) This leads to an inability to perform daily activities including those required to maintain employment and the basic necessities of daily living leading to poorer quality of life. (Flattery et al., 2005) Addressing the factors causing exercise intolerance has always been the focus of treatment for both symptom control and improving prognosis in diseases causing diminished exercise capacity. Further, pathology affecting one system commonly has sequelae on other body systems. As a result, each individual patient has multifactorial causes for their exercise intolerance and fatigue. Skeletal muscle is commonly recognised to be affected by cardiopulmonary conditions including chronic obstructive pulmonary disease (COPD) and left heart failure. (Gea et al., 2016, Kennel et al., 2015)

1.3.2 Mechanisms affecting exercise capacity in PAH

1.3.2.1 Introduction

There are multiple mechanisms that are now recognised contributing to impaired exercise capacity in PAH affecting the pulmonary, cardiovascular and musculoskeletal systems. The changes in skeletal muscle in PAH are discussed later in the chapter. An overview of the mechanisms of exercise intolerance in PAH will be described below.

1.3.2.2 Right ventricle function in PAH

The resistance to blood flow in the pulmonary vessels is increased due to impaired vasodilation, vasculopathy and remodelling. (McLaughlin and McGoon, 2006) The pulmonary vessels demonstrate reduced distensibility and progressive increase in resistance. (Vonk Noordegraaf and Galie, 2011) The right ventricle pumps the same volume of blood as the left ventricle against low resistance, high capacitance pulmonary vessels. The RV is much more compliant than the left ventricle (LV) and able to adapt to the increased afterload in PAH. Therefore, in early development of PAH, RV contractility increases and cardiac index is maintained. (Vonk Noordegraaf and Galie, 2011)

As the pulmonary vascular resistance increases, RV undergoes hypertrophy, remodelling and eventually dilation. (Benza et al., 2015) In most patients with PAH, cardiac index is reduced at diagnosis. (Benza et al., 2010) The response and changes in the RV to the increased hydraulic afterload is very patient specific. The reduced RV contractility and impaired RV – pulmonary artery coupling seen in PAH patients is important to the overall outcome and prognosis of the patient. (van Wolferen et al., 2007, Grapsa et al., 2015, Raymond et al., 2002)

The increase in RV end-systolic and end-diastolic volumes affects the left ventricle function during the cardiac cycle due to the shift in the interventricular septum to the left and reducing LV preload. (Gan et al., 2006) There is additional evidence cardiomyocyte atrophy within the LV occurring in patients with PAH. (Manders et al., 2014) This leads to decreased LV function, with evidence of reduced LV contractility, and acting as another factor impairing systemic oxygen delivery. (Benza et al., 2015)

During exercise, patients with PAH have an impaired ability to augment stroke volume (Holverda et al., 2006) and chronotropic incompetence.(Sun et al., 2001) The diminished ability to increase stroke volume and heart rate reduces the cardiac output during exercise. The currently available targeted treatments for PAH acts by pulmonary artery vasodilation. The decreased afterload leads to increased cardiac output at rest. (Kuhn et al., 2004, Sasayama et al., 2005) The effect of treatment on maximum exercise performance leads to increased oxygen consumption with some limited evidence of improvement in exertional cardiac output. (Arena, 2011, Provencher et al., 2008)

The right ventricle function and cardiac output in PAH can be assessed using non-invasive and invasive methods. These include transthoracic echocardiography, cardiac magnetic resonance imaging, and right heart catheterisation. All three methods are used at the diagnostic stage to evaluate patients depending on availability at individual centres.

1.3.2.3 Respiratory impairment in PAH

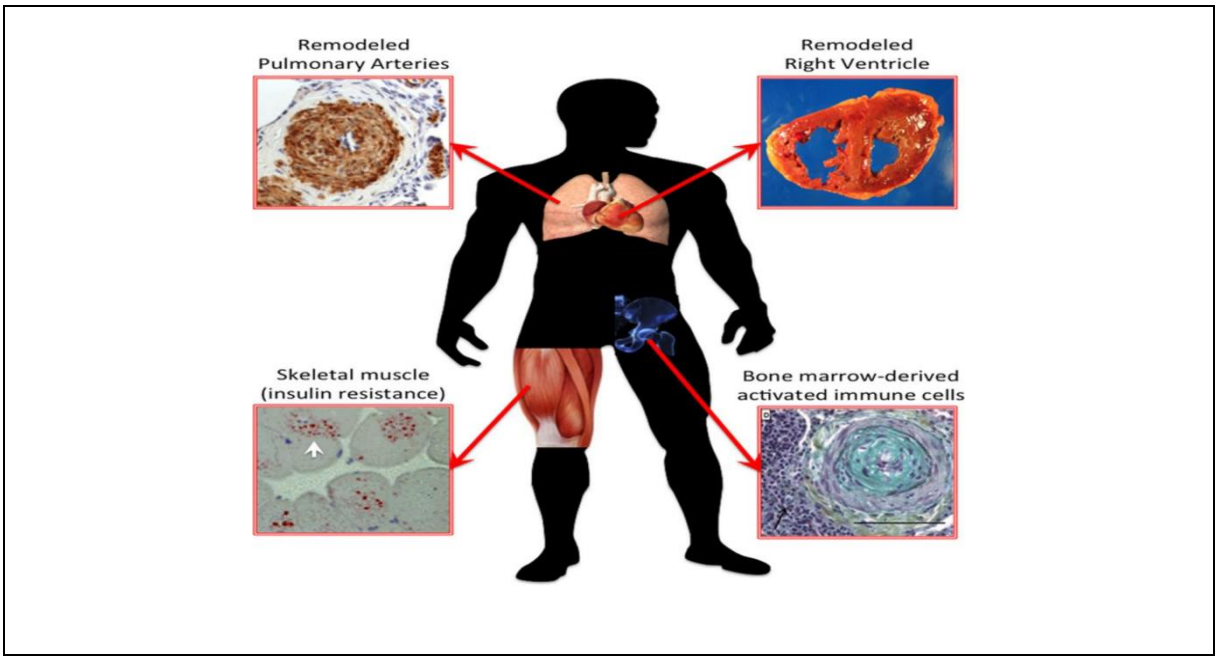
There are pulmonary factors contributing to reduced exercise intolerance. There is evidence of respiratory muscle weakness in PAH including diaphragmatic involvement affecting alveolar ventilation. (Panagiotou et al., 2015) The reduced transfer factor of carbon monoxide

(TLCO) is due to the pulmonary membrane diffusing capacity, ventilation/perfusion mismatch and rapid red cell transit time leading to hypoxaemia. (Fowler et al., 2012) (Chandra et al., 2010)

1.3.2.4 Extrasystematic features in PAH

PAH is recognised as a disease that affects other organs in the body. (Figure 3) There are factors beyond the cardiopulmonary systems contributing to exercise intolerance in PAH. Iron deficiency is common in PAH and may contribute to the pulmonary vasculopathy. (Rhodes et al., 2011) Iron supplementation resulted in improvement in exercise endurance and aerobic capacity in PAH. (Ruiter et al., 2015) Increasing accumulation of evidence suggests skeletal muscle dysfunction in PAH could also be contributing to exercise intolerance and this will be discussed later on in the chapter. (Manders et al., 2015)

Figure 3: Beyond the resistance in pulmonary arteries, other organs are involved in PAH.



There is remodelling of the pulmonary arteries and right ventricle. Immune cells that are activated in the bone marrow are found in the lung parenchyma contributing to the pathology. There are metabolic changes in the skeletal muscle including the presence of lipid droplets suggesting evidence of insulin resistance. Reprinted from (Paulin and Michelakis, 2014)

1.3.3 Peripheral oxygen extraction

Exercise capacity can be assessed using submaximal testing (eg 6MWD) or maximal testing in particular CPEX. The use of CPEX allows for an integrative assessment of cardiac, pulmonary and skeletal function of the patient. (Wasserman et al., 2005) The oxygen consumption (VO_2) is an assessment of cardiac output (CO) and the difference arterial-venous oxygen difference (AVO_2 diff) – Fick principle. (Wasserman et al., 2005)

$$\mathbf{VO_2 = CO \times AVO_2\ diff}$$

From the equation above, not only is exercise capacity is dependent on cardiac output but also on the ability of the peripheral tissues to extract oxygen from the blood. AVO_2 diff is the difference in oxygen content between the arterial supply to the tissues and the mixed venous return to the right heart taking into account haemoglobin, oxygen saturation and the partial pressure of oxygen. Oxygen content at any one point in the blood supply system can be calculated by the following equation. (Finch and Lenfant, 1972)

$$\mathbf{Oxygen\ content\ (mL/dL) = (Haemoglobin \times 1.39 \times O_2\ saturation) + (0.003 \times partial\ pressure\ O_2)}$$

Therefore the relative values in the arterial and venous system can be assessed to calculate the peripheral oxygen extraction. During peak exercise, we can assume the majority of the blood supply is diverted to the working skeletal muscle. Therefore, assumption is made that AVO_2 diff is a reflection on the peripheral oxygen extraction of skeletal muscle. At rest the normal AVO_2 diff is 5mL/dL and at peak exercise about 15mL/dL in a healthy patient based on haemoglobin of 15 g/dL. (Beck et al., 2006) Normally, the average AVO_2 diff is equal to the haemoglobin value of the individual. (Dhakal et al., 2015)

There are four steps that are involved in the transport of oxygen to the tissues: 1) ventilation 2) diffusion into the lungs 3) perfusion (convection) and 4) diffusion into the tissues. (Wagner, 1996) Loss of function in any one of the components causes significant impact on oxygen consumption. The diffusion and utilization of oxygen into the skeletal muscle is dependent on a number of factors. These include local sympathetic function, vasodilatory capacity of the small resistance vessels and microvascular control mechanisms to match

oxygen supply to demand. (Dhakal et al., 2015) In chronic heart failure, there is evidence of reduced muscle blood flow despite adequate arterial blood pressure, leading to increased oxygen extraction but reduced utilization, thus resulting early anaerobic metabolism. (Sullivan et al., 1989) In patients with heart failure with preserved ejection fraction, peripheral oxygen extraction has been shown to be reduced and markedly contributing to impaired oxygen consumption during upright exercise testing. (Dhakal et al., 2015) A potential mechanism discussed is that diffusion rather than convection of oxygen had the greatest impact on the reduced exercise capacity in these patients. These studies show that consideration of peripheral oxygen extraction as part of exercise capacity is an important part of the integrative assessment of the mechanism that underlie exercise limitation.

1.3.4 Measuring components of oxygen consumption

Oxygen consumption is measured using breath by breath gas analyser during physical exertion either on a treadmill or cycle ergometer. Cardiopulmonary exercise testing is an established and validated method of assessing cardiopulmonary function in both healthy and disease patients. (Wasserman et al., 2005) The components of VO_2 can be assessed by invasive and non-invasive methods.

Invasive methods include the placement of radial and pulmonary artery catheter into the patient prior to exercising. This allows for sampling of the arterial and mixed bloods on a minute by minute basis allowing for the cardiac output can be calculated using the Fick principle. (Maron et al., 2013) Furthermore, it allows continuous assessment arterial blood pressure and pulmonary artery pressures changes. However, this method is hampered by the invasive nature leading potential problems in recruitment, complications and resource intensive. (Maron et al., 2013)

Non-invasive methods of assessing cardiac output are now available that can be performed during CPEX testing. (Jones et al., 2015) Therefore, AVO_2 diff can be calculated from the Fick equation. This has the advantageous of being non-invasive without the associated complications and resources needed. There are number of methods of assessing cardiac output including doppler echocardiography, inert gas rebreathing technique, electrical impedance, and pulse contour analysis (Siebenmann et al., 2015) A number of these methods

due to the non-invasive nature have inherent problems with accuracy and reliability. (Warburton et al., 1999)

Bioreactance is a non-invasive method of continuously measuring cardiac output that has been to be accurate, reliable and reproducible on exercise. (Jones et al., 2015) Bioreactance works by measuring the frequency in the phase shift of transthoracic electrical currents as a reflection of blood flow to determine cardiac output. (Keren et al., 2007) Bioreactance has been studied in PAH patients in comparison to thermodilution and Fick methods, and has been shown to be accurate, comparative to Fick's method and responsive to treatment. (Rich et al., 2013) Furthermore, bioreactance has been used in other pathologies to demonstrate impaired peripheral oxygen extraction as contributing to impaired exercise capacity. (Jakovljevic et al., 2012b) Bioreactance is an accepted validated non-invasive technology to accurately measure cardiac output at rest and on exercise. (Jones et al., 2015)

1.3.5 Exercise training in PH

Despite optimised drug based therapies, most patients remain symptomatic with a low quality of life. Prior to 2006, the impact of lifestyle changes particularly exercise was thought to have a negative impact that may contribute to the progression of PH and harmful to patients. Exercise training and rehabilitation have been utilised in other cardiac and pulmonary disease in the last two decades to improve symptom control and physical functioning with beneficial outcomes, that has been proven repeatedly in randomized controlled and meta-analysis studies. (van Tol et al., 2006, de Blasio and Polverino, 2012) Exercise training in PH in both animal and human studies has yielded positive outcomes leading to improved exercise capacity. The current guidelines recommend strongly of the benefits of supervised graded aerobic exercise training. (Galie et al., 2013)

Preclinical animal studies using both chronic hypoxia and monocrotaline-induced PH rats demonstrated that exercise training led to a number of benefits. First of all, they demonstrated a lack of pulmonary vasoreactivity alterations to exercise. (Goret et al., 2005) This was followed by improved exercise endurance, capillary density within the myocardium and promoting positive changes in the right ventricle and pulmonary artery remodelling. (Handoko et al., 2009, Colombo et al., 2013)

A seminal paper by Mereles *et al* 2006, using a supervised aerobic exercise programme first showed the additional benefit of exercise in terms of cardiopulmonary fitness and quality of life. (Mereles et al., 2006) The exercise prescription involved 3 weeks inpatient stay with skeletal and respiratory muscle training. Aerobic training consisted of interval cycle ergometer at moderate heart-rate intensity which was followed by another 12 weeks of home exercise programme predominantly involving the use of bicycle ergometer as set by the researchers for each patient. On follow up assessment compared to the control group they found improvement in 6MWD, quality of life scores, WHO functional class, peak oxygen consumption, anaerobic threshold and achieved workload. The study reported no adverse effects.

Since then several studies have reinforced and supported aerobic exercise training as a beneficial therapy in patients with PH of different causes with improvement in 6MWD, WHO functional class, and peak oxygen consumption. (Handoko et al., 2009, Grunig et al., 2011, Grunig et al., 2012a) More pertinently, the studies have repeatedly shown that moderate intensity exercise is safe in this patient population group contradicting previously accepted views that exercise was unsafe in PH patients. (Grunig et al., 2012a)

A recent polled meta-analysis and systematic review of the available exercise training studies in PH including observational and controlled studies revealed a number of positive outcomes for patients. This included a significant increase in 6MWD of 62m (95% CI: 45 -78) at 12 weeks of exercise that can be considered clinically significant. Furthermore, CPEX testing demonstrated improvement in peak oxygen consumption and workload both at 3 and 12/15 weeks after training. Quality of life measured using SF-36 score improved in the areas of physical, social and emotional functioning. There was no alteration in body pain, general health or mental health scores. (Yuan et al., 2015)

The studies have shown limited correlation between improved cardiopulmonary fitness and quality of life scores with cardiovascular haemodynamic values as assessed by transthoracic echocardiography. (Mereles et al., 2006) The benefits of exercise as an add-on therapy has been proven in PH of different causes including connective tissue disease (CTD), congenital heart disease (CHD) and inoperable chronic thromboembolic pulmonary hypertension (CTEPH) group. (Grunig et al., 2012b, Nagel et al., 2012, Becker-Grunig et al., 2012)

1.3.6 Summary

PAH is a disease that impacts multiple systems. As a result the symptoms the patients experience cannot be addressed by targeting treatments on any one system. The remarkable benefits shown by exercise training beyond optimized drug therapy, suggest further understanding of the pathophysiological changes that occurs within the skeletal muscle is warranted. An overview of skeletal muscle structure and function will now be discussed.

1.4 Skeletal Muscle Structure and Function

1.4.1 Introduction

Skeletal muscle in humans comprises 40% of body weight, with up to 75% of body proteins and encompassing maximum of 50% of body protein turnover. (Frontera and Ochala, 2015) The structure of muscle consists of highly arranged muscle fibres with associated connective tissue. Muscle size is due to the number and size of muscle fibres. They contribute to multiple bodily functions including converting chemical to mechanical energy to generate power that contributes to movement and activity, functional independence and enhancing health. The two main properties of skeletal muscle are to provide strength and endurance. (Frontera and Ochala, 2015)

1.4.2 Extracellular matrix

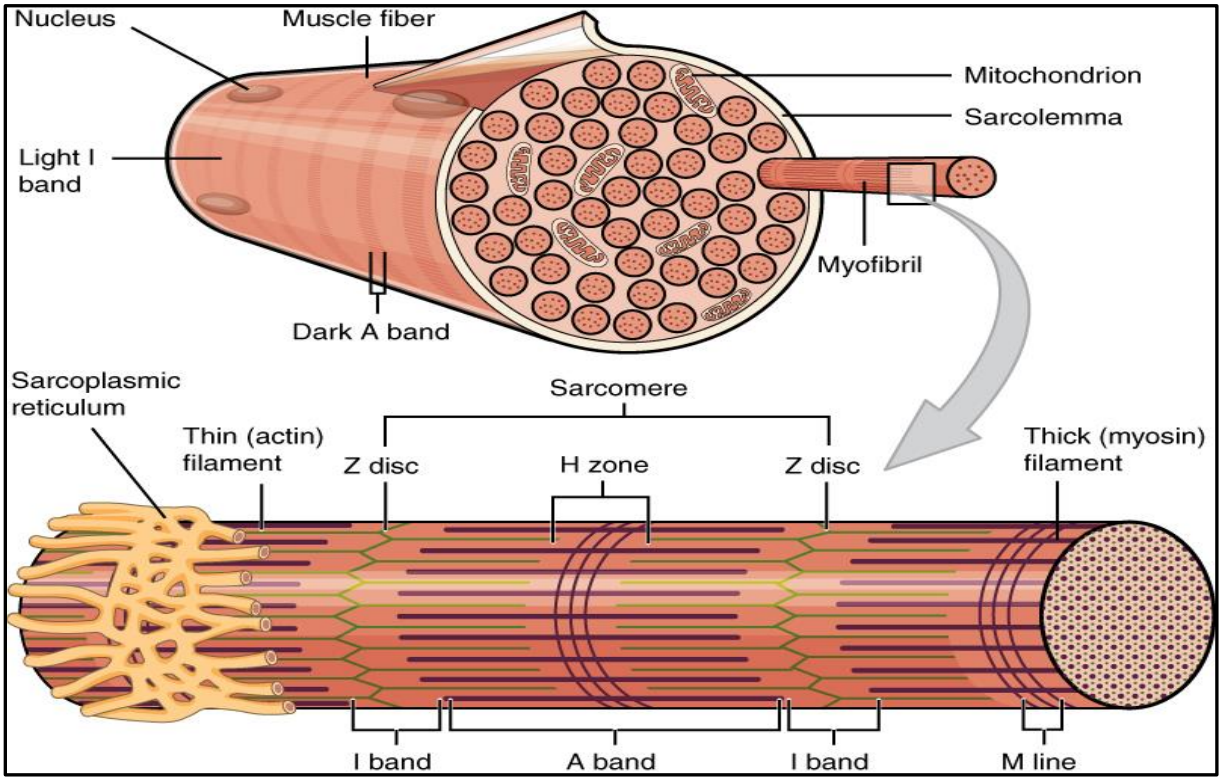
The epimysium is a layer of connective tissue surrounding individual muscle and is continuous with the outer layer of the tendon. Within the muscle, group of fibres are arranged in parallel bundles and surrounded by the perimysium. Each muscle fibre is approximately 1cm in length and 100μm in diameter. (Ganong, 2003, Gillies and Lieber, 2011) Muscle fibres are surrounded by the sarcolemma otherwise known as the cell membrane. The sarcolemma consists of the basement membrane and plasmalemma. The basement membrane consists of a glycoprotein complex involved in the organization of neuromuscular junction, termination of synaptic transmission and connecting the endomysium with the plasmalemma. (Borg and Caulfield, 1980, Passerieux et al., 2006)

1.4.3 Muscle fibre ultrastructure

Each muscle fibre is a multinucleated single cell with a cylindrical structure. The muscle fibre is made of multiple myofibrils, each made of individual filaments. The filaments are made up of contractile proteins. (Figure 4) The muscle proteins make up the contractile mechanism in the skeletal muscle and these include actin, myosin-II tropomyosin and troponin. Troponin is consists of three subunits that are troponin I, troponin T and troponin C. (Ganong, 2003)

The classical cross-striations seen in skeletal muscle can be seen Figure 4. Between two adjacent Z lines is an area call the sarcomere. The thick filaments consists of myosin forming the A band. The thin filaments consisting of actin, tropomyosin, and troponin make up the less dense I bands. The Z line transects the fibrils that connect the thin filaments. (Ganong, 2003)

Figure 4: The gross muscle ultrastructure, the relationship of between the myofibrils, mitochondria and the connective tissue.



(Reproduced from https://courses.candelalearning.com/ap2x1/chapter/skeletal-muscle/)

Myosin-II has two heads and a long tail, forming cross-links to actin. Myosin heads contains two important areas, an actin binding site and the area where adenosine triphosphate (ATP) is hydrolysed. The thin filaments consist of two chains of actin in double helix pattern. Tropomyosins are located in the grove of the double helix, the troponin located at set intervals along the tropomyosin. Troponin T binds the entire troponin structure to tropomyosin. Troponin I role is to inhibit the interaction of myosin with actin whereas troponin C is where calcium binds for the initiation of contraction. (Frontera and Ochala, 2015)

There are further three more important muscle proteins: actinin binds actin to the Z lines, titin links the Z line to the M line and desmin binds the Z lines to the plasma membrane. The sarcotubular system, consists of the T system which are transverse tubules forming a grid around the muscle fibrils and sarcoplasmic reticulum around each of muscle fibrils. The sarcoplasmic reticulum has a fundamental role in the movement of calcium. (Ganong, 2003) The transmission of action potential is via the T system. The mechanism of muscle contraction will now be discussed.

1.4.4 Muscle contraction

The depolarization of the muscle fibre leads to contraction and this process is called the excitation-contraction coupling. The motor end-plate is the starting point of muscle fibre membrane depolarization. The action potential is transmitted to the muscle fibre leading to muscle contraction. A muscle twitch is the result of a single action potential leading to contraction and then relaxation. The length of muscle twitch differs depending on the type of muscle fibre. (Allen et al., 2008)

Prolonged action potential leads to muscle contraction by the movement of the thin filaments over the thick filaments. The Z lines move closer together during contraction. The sliding action is the result of the myosin heads binding to actin, bending at the neck and then detaching. (Calderón et al., 2014) This repeated action leads to the sliding movement. This bending action of the myosin-II molecules depends on the hydrolysis of . Each so called "power stroke" shortens the sarcomere by 10nm. (Huxley and Niedergerke, 1954)

The T-tubules transmits the action potential to all the muscle fibrils, leading to the release of calcium from the terminal cisterns. (Bezanilla et al., 1972) The calcium binds to troponin C, leading to weakened attachment of troponin I to actin, tropomyosin moving away and exposing the binding sites for the myosin heads on actin. (Dulhunty, 2006, Ganong, 2003)

The calcium diffuses back from the sarcoplasmic reticulum into the terminal cisterns for the next muscle contraction. The muscle then relaxes once the calcium concentration outside the sarcoplasmic reticulum goes below a threshold value. (Dulhunty, 2006)

T tubule depolarization leads to sarcoplasmic reticulum activation through the dihydropyridine receptors (voltage-gated calcium channels), that act as a voltage sensor and triggers the release of calcium from the sarcoplasmic reticulum. (Santulli and Marks, 2015) The ryanodine receptor (non-voltage gated calcium channel) is located on the sarcoplasmic reticulum and is responsible for calcium release. The calcium – magnesium-ATPase moves calcium back into the reticulum, leading to muscle relaxation. (Allen et al., 2008, Ganong, 2003)

1.4.5 Muscle fibre types

Skeletal muscle fibres vary in terms of their myosin ATPase activity, speed of contraction, fatigue resistance, glycolytic and oxidative capacity. (Engel, 1998) Generally, in simplistic classification, they are divided into type I and II. The slow, oxidative type I fibres have a high content of mitochondria, increased capillary density and myoglobin content as opposed to type II fibres that are fast-acting, highly glycolytic, low oxidative capacity and easily fatigable. (Brooke and Kaiser, 1970b, Brooke and Kaiser, 1970a) The latter, type II fibres, can be further subclassified into IIa, IIb and IIx fibres. Many would now regard this as an overly simplistic classification based on histochemical analysis of muscle biopsies, and argue that there is a spectrum of muscle fibre types. (Saltin et al., 1977)

1.4.6 Muscle metabolism

Muscle contraction requires a large amount of energy to produce mechanical work. ATP in muscle can be generated from the metabolism of carbohydrate and lipids as well as organic phosphate compound, phosphocreatine (PCr). (Calderón et al., 2014) During muscle rest, PCr

stores are built by ATP donating phosphate molecule to creatine. During active muscle contraction, PCr donates the phosphate molecule to ADP to build up ATP. Skeletal muscle PCr stores provide the majority of the energy in the first few seconds of heavy exercise and are rapidly depleted. (Haseler et al., 1985, Kemps et al., 2010)

Muscle uses free fatty acids (FFA) to generate energy at rest and during light exercise. Carbohydrates are used as exercise intensity increases due to FFA being unable to provide all the required energy. During adequate oxygen supply, breakdown glucose produces pyruvate entering the Kreb's cycle, otherwise termed aerobic glycolysis. (Ganong, 2003) At times of inadequate oxygen supply, the pyruvate is reduced to lactate rather than entering the Kreb's cycle, termed anaerobic glycolysis. The net production of energy via anaerobic metabolism is much smaller than by aerobic metabolism. (Allen et al., 2008, Putti et al., 2015)

During heavy exertion, ATP is generated through breakdown of phosphocreatine and anaerobic glycolysis. Lactate accumulates in the muscle to the extent exceeding the buffer mechanism, and lead to enzyme-inhibition by depressing muscle pH. After exertion, extra oxygen is consumed to remove the lactate, replace oxygen to the myoglobin and generate ATP and PCr stores. This extra oxygen is called the oxygen debt. (Allen et al., 2008, Kemp et al., 2007)

The changes that occur during exercise in the muscle, otherwise known as bioenergetics can be studied non-invasively using spectroscopy and will be discussed in greater detail later in the chapter.

1.4.7 Muscle changes in PAH

Exercise intolerance in PAH is deemed primarily due to haemodynamic impairment but cardiac output is only partially correlated with exercise capacity. (Mainguy et al., 2010) During cardiopulmonary exercise testing, patients report leg fatigue and dyspnoea as being the common symptoms leading to the cessation of exercise, suggesting the existence of dysfunction within the peripheral skeletal muscles. (Sun et al., 2001) There is significant evidence from other cardiopulmonary diseases of skeletal muscle impairment including in chronic obstructive pulmonary disease and left ventricle systolic failure. (Kim et al., 2008, Piepoli and Crisafulli, 2014)

Meyer *et al*, proceeded to demonstrate weakness in both inspiratory and expiratory muscle function in patients with IPAH. They demonstrated in IPAH patients compared to control populations, that the maximum inspiratory (PI, max) and expiratory (PE, max) pressures generated was lower. There was no correlation between the respiratory muscle function and pulmonary haemodynamics. (Meyer et al., 2005) Building on this work, Bauer *et al*, showed forearm grip strength correlates with maximal inspiratory and expiratory mouth pressures but not with systolic pulmonary artery pressure. This lends further support to the notion and enhancing the concept of a generalised muscle weakness in PAH patients independent of cardiopulmonary haemodynamic severity. (Bauer et al., 2007)

Quadricep non-volitional muscle strength correlates with exercise capacity. Skeletal muscle biopsies demonstrate change in muscle fibre type, with lower proportion of type 1 and increased type 2 fibres. There was evidence of higher potential for anaerobic than aerobic metabolism. More revealing was the presence of association between exercise capacity and muscle biopsy findings including two oxidative enzymes, citrate synthase and 2-hydroxyacyl-CoA-dehydrogenase and capillary to type 1 fibre ratio. (Mainguy et al., 2010)

In animal-models with PH induced by the injection of monocrotaline, the twitch and maximal tetanic force generation of the diaphragm muscle was weaker than control animals with a decrease in cross-sectional area (CSA) of muscle fibres. (Manders et al., 2012, de Man et al., 2011) Possible mechanisms suggested were activation of the ubiquitin-proteasome pathway (UPS) with activation of the E3 ligases, atrogin-1 (MAFbx-1) and muscle ringer protein 1 (MURF-1). Their work lends strong support to the role of impaired diaphragm contractility in PH patients contributing to exercise intolerance.

There is evidence from skeletal muscle biopsies of IPAH patients of decreased Akt activation, with increased atrogin-1 expression and MURF-1 activity. (Batt et al., 2014) This suggests the insulin receptor substrate/phosphatidylinositol 3-kinase/Akt pathway function is impaired. This ultimately leads to the activation of the UPS system resulting in muscle breakdown and wasting, with downregulation of pathways that would stimulate muscle hypertrophy. (Batt et al., 2014) The work of this author supports existing evidence of skeletal muscle atrophy and weakening of the peripheral muscles compared to healthy controls.

The animal and human studies on the skeletal muscle so far have suggested PAH affects the individual patient beyond the function of the RV and pulmonary vessels. They have shown potential mechanisms affecting skeletal muscle function and contributing to the exercise intolerance experienced by patients.

1.5 Mitochondria

Mitochondria are cellular organelles involved primarily in energy metabolism. They create ATP from lipids and carbohydrates for cellular processes. Furthermore, they have a vital role in programmed cell death, redox haemostasis, calcium signalling and cellular metabolism. (Picard et al., 2011) Mitochondria within the lungs act as oxygen sensors by producing reactive oxygen species which control ion channels and enzymes. They are dynamic organelle capable of dividing (fission), joining together (fusion) and moving around the cell in networks. (Papa et al., 2012) Thus the accurate function of the mitochondria is crucial to the cell life and bodily functions.

1.5.1 Structure and Function

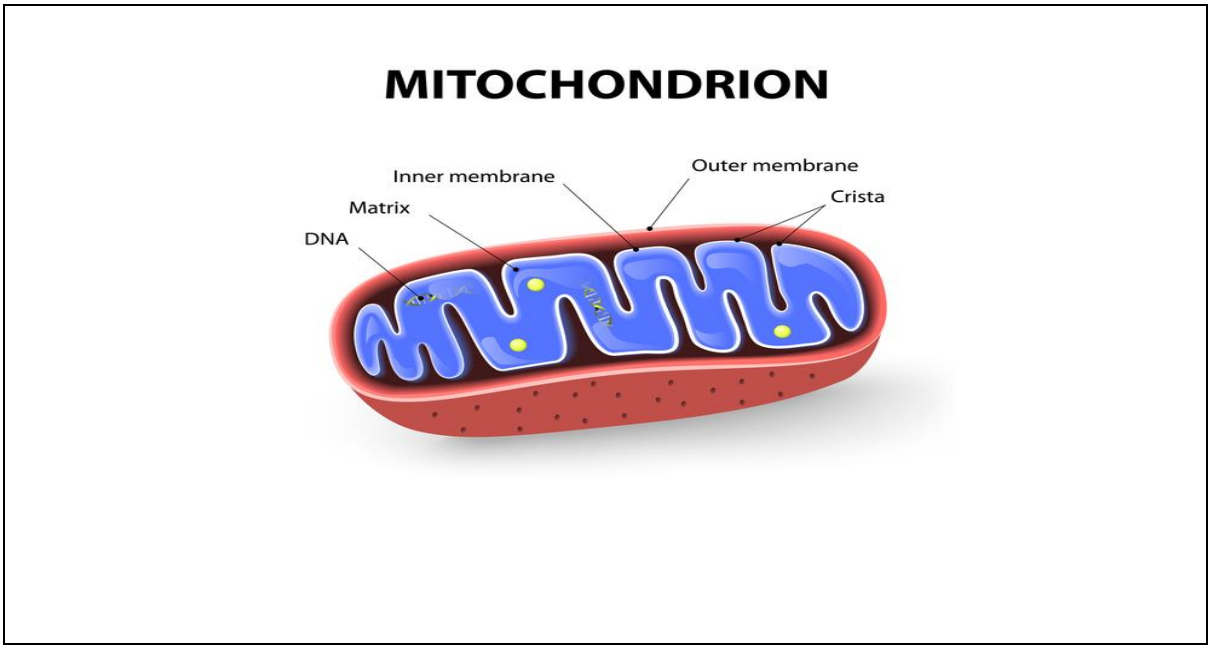
Mitochondria contain two membranes with the space in between termed the intermembrane space. The folded inner membrane form cristae and contains the oxidative phosphorylation system responsible for the generation of ATP. (Kayar et al., 1988) (Figure 5)

The mitochondria contain maternally inherited DNA (mtDNA). The mitochondrial genome contains the coding for a number transfer (22) and ribosomal (2) RNAs as well as 13 subunits of the oxidative phosphorylation system. The nuclear genome encodes for the remaining proteins and subunits of the mitochondria. The nuclear DNA encoded proteins are produced in the cytoplasm and then moved to the mitochondria where the subunits are assembled into functional proteins. (Attardi and Schatz, 1988)

1.5.2 Oxidative phosphorylation system (OXPHOS)

The oxidative phosphorylation system (OXPHOS) is the main mechanism by which mitochondria generates ATP. The electron transport chain (ETC) contains five complexes (I-V) including ATP synthetase on the inner mitochondrial membrane. (Figure 6)

Figure 5: Gross structure of the mitochondria



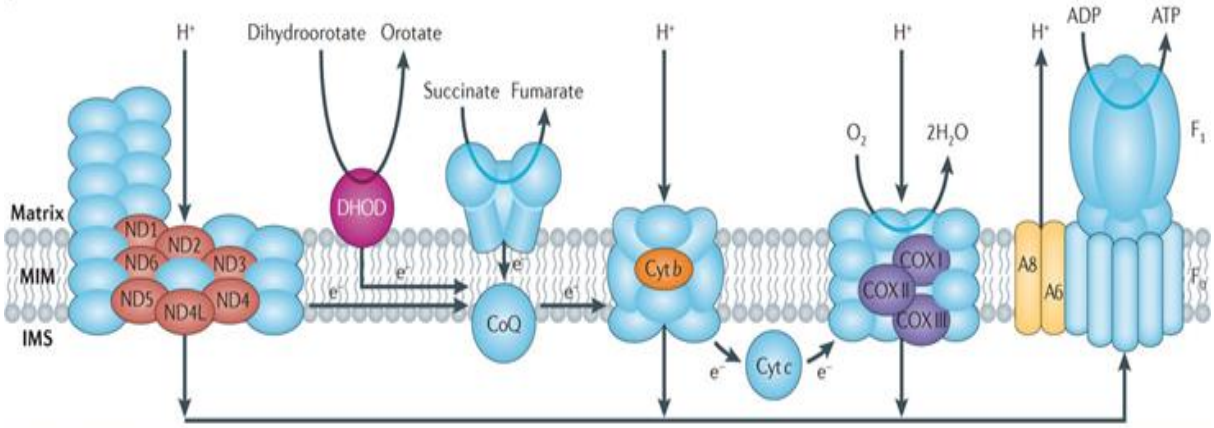
(From the US Genetics Library: https://ghr.nlm.nih.gov/)

Kreb's cycle otherwise known as the tricarboxylic acid cycle, is a series of reactions leading to the transfer of electrons to hydrogen from electron carriers. Therefore, it leads to the formation of nicotinamide adenine dinucleotide (NADH), hydrogen (H+) and flavin adenine dinucleotide ($FADH_2$). Acetyl-coenzyme A, the main intermediary product and a substrate for the Kreb's cycle is generated by two pathways. Glucose is broken down to pyruvate by glycolysis in the cell cytoplasm and transported into the mitochondria by pyruvate dehydrogenase, where it is decarboxylated into acetyl-CoA. Fatty acid is oxidized in the mitochondrial matrix to produce acetyl-CoA. (Papa et al., 2012)

Complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) electrons are donated from NADH and $FADH_2$ at the ETC. Coenzyme Q (ubiquinone) transfers electrons from complex I, II and dihydroorotate dehydrogenase to complex III (cytochrome c reductase). Then, complex III transfers the electrons to complex IV containing an electron acceptor. There is active pumping of electrons into the intermembrane space at complexes I, III and IV, leading to the development of the electrochemical gradient. (Schon et al., 2012) As a result, the gradient allows for the flow of electrons through complex V (ATP synthetase) into the mitochondrial matrix driving the production of ATP. (Papa et al., 2012) Each complex within the OXPHOS consists of multiple subunits with each one either be encoded

by nuclear or mitochondrial DNA. Some of the complexes consist of subunits entirely encoded by nuclear DNA only. (Schon et al., 2012)

Figure 6: The respiratory chain of the OXPHOS system consisting of five complexes



Polypeptides	Complex I	Complex II	Complex III	Complex IV	Complex V
mtDNA-encoded subunits	7	0	1	3	2
nDNA-encoded subunits	~39	4	10	10	~14
Assembly proteins	~11	~2	~9	~30	~3

Complex I – NADH dehydrogenase, II – succinate dehydrogenase, III – cytochrome *c* reductase and IV – cytochrome *c* oxidase) and ATP synthase. CoQ- coenzyme Q, CytC – cytochrome c, e^- - electron. All nuclear encoded subunits of the complexes are blue and mitochondrial encoded subunits are in alternative color. (Figure reprinted from (Schon et al., 2012)

1.5.3 Mitochondria in skeletal muscle

In skeletal muscle, mitochondria are found in the intermyofibrillar (IMF, 80%) and subsarcolemmal (SS, 20%) regions. There are compositional, biochemical and functional differences between these two groups. Mitochondria located in the IMF have a higher rate of protein synthesis, higher complex IV and lower succinate dehydrogenase activity compared to subsarcolemmal mitochondria. (Cogswell et al., 1993)

1.5.4 Evidence of mitochondrial involvement in PAH

Mitochondria are involved in cellular respiration and bioenergetics. They have a role in the signalling between pulmonary artery endothelial cells (PAEC) and pulmonary smooth muscle cells (PSMC). (Freund-Michel et al., 2014) Furthermore, mitochondria play a significant role in cell cycle regulation. In plexiform lesions that are pathognomonic feature of IPAH, apoptosis-impaired proliferation of PAECs/PSMCs has been observed on specimens.(Abe et al., 2010) This suggests defective mitochondrial function within the pulmonary arteries contributes to the pathophysiology of PAH.

Fawn-hooded rats (FHR) are a unique strain of rodents that develop PAH spontaneously. There is decrease in size and fragmented appearances of mitochondria in PSMCs before the development of PAH with pulmonary vasculature remodelling. (Bonnet et al., 2006) In FHR there is evidence of mitochondrial dysfunction with deficient electron transport chain complex I and certain key mitochondrial enzymes. Metabolism as by oxidative phosphorylation is shifted to glycolysis with subsequent normoxic hypoxia-inducible factor (HIF) - 1α activation. This leads to reduced expression of oxygen-sensitive Kv channels with subsequent mitochondrial hyperpolization with associated vasoconstriction and proliferation of PSMCs. Mitochondrial network disruption occurs in the natural history of FHR at the ultrastructural level preceding haemodynamic changes. Mitochondrial dysfunction occurs early in the pathogenesis of PAH either in FHR or human PAH. (Bonnet et al., 2006) HIF-1α over expression has accounted for lower numbers of mitochondria in human endothelial cells from PAH patients. (Fijalkowska et al., 2010)

Impaired skeletal muscle energetics may contribute to physical disability and exercise intolerance in PAH patients. Monocrotaline rat modelling has shown with time, there is impaired mitochondrial respiration and biogenesis in skeletal muscle preceding any changes seen in the RV. (Enache et al., 2013) The exact reason why the change in peripheral muscle mitochondria occur before changes in mitochondrial activity in RV is unknown.

Currently, whether mitochondrial OXPHOS dysfunction in skeletal muscle contributes to exercise intolerance in patients with PAH is uncertain. Early evidence suggests there maybe defects within skeletal muscle mitochondrial fusion but not within the oxidative enzyme pathways. (Batt et al., 2014)

1.5.5 ^{31}Phosphorous –magnetic resonance spectroscopy (^{31}P-MRS)

Skeletal muscle bioenergetics at rest and exercise can be measured non-invasively using ^{31}P-MRS. During exercise, the ATP used is resynthesized by glycolysis and oxidative metabolism by the mitochondria. (Argov et al., 2000) The concentration of ATP is held constant by a number of mechanisms. One of them is the creatine kinase (CK) reaction whereby phosphocreatine (PCr) is broken to down to donate the phosphoryl group to ADP to produced ATP. Therefore during exercise PCr levels fall. (Argov et al., 2000)

$$\mathbf{ADP + PCr + H^{+} \longrightarrow ATP + Cr}$$

During recovery, oxidative phosphorylation by the mitochondria continues at an increased rate whilst glycolysis is stopped, in order regenerate the lost metabolites. Therefore PCr concentration increases due to predominantly mitochondrial work in healthy individuals. (Taylor et al., 1994) Phosphorous spectroscopy allows for the concentration of certain phosphate containing metabolites to be measured in various tissues during rest, exercise and recovery. The recovery kinetics of PCr generated can be measured by fitting best fit line to a single exponential curve to measure the time taken to regenerate half the concentration of PCr ($t_{1/2}$). ^{31}P –MRS has been used in the study of mitochondrial myopathy, and has been shown in severe disease to have increased in PCr recovery times, correlating with muscle weakness. Furthermore, in mitochondrial myopathy ^{31}P-MRS has shown increased recovery of the pH within the muscles due to rapid proton efflux seen in these patients. (Taylor et al., 1994)

1.5.6 Summary

The function of the skeletal muscle is crucial for movement and exercise. PAH seems to have a detrimental effect on muscle structure and function through a number of mechanisms. There have been no studies that have focused on the mitochondria within the skeletal muscle. Mitochondrial dysfunction is an understood part of the pathophysiology of PAH within the pulmonary vessel. Therefore, the question arises of whether there is any evidence of mitochondrial OXPHOS dysfunction in the skeletal muscle contributing impaired oxidative metabolism and leading to impaired exercise capacity. Exercise impairment and daily habitual activity are two ways of assessing the impact of the disease on the patient. Now, an overview of daily physical activity and the ways of measuring are discussed.

1.6 Habitual Physical Activity

1.6.1 Background

The lifestyle led by an individual person can have a profound effect on their health. The level of activity during a day can be broken into four types; sedentary, physical activity, exercise and sleep. The proportion of time spend in each individual section can affect a person's metabolism and energy expenditure; therefore affect the onset and progression of chronic diseases. (Strath et al., 2013)

Physical activity defined by Casperson *et al* is "any bodily movements produced by skeletal muscles that result in energy expenditure". (Wittink et al., 2011) Exercise is a subcategory of physical activity and should not be regarded the same. Exercise involves structured, planned and often repetitive body movements based on exergy expenditure. (Caspersen et al., 1985) (Strath et al., 2013) Physical activity is any bodily movements, therefore includes sitting, socialising, exercise, household chores and transportation. (Caspersen, 1989, Strath et al., 2013) Physical activity can be divided into sedentary, light, moderate and vigorous physical activity. Energy expenditure for activity is the metabolic equivalent of task (MET), with one MET equal to oxygen uptake of 3.5ml/kg/min, and is equivalent to the energy expenditure rate while sitting. (Hill et al., 2015). Light physical activity includes showering or ironing with 1.5-3 METs, with intensity between 20-40% of VO_2max. (Strath et al., 2013)

Maintaining physical activity is important to the overall health in a given population. Physical inactivity is associated with chronic diseases including coronary artery disease, type 2 diabetes mellitus, hypertension, obesity, and depression. (Cloostermans et al., 2015, Bensimhon et al., 2006) A number of societies particularly in the field of coronary heart disease have published guidelines on assessing physical activity in their respective population groups. (Billinger et al., 2014, Strath et al., 2013) They have promoted physically active lifestyle including levels of physical activity that should be achieved in a given week for health benefits. Despite the recommendations and known benefits, the majority of healthy patients fail to maintain regular physical activity levels. They suggest that physical activity should be assessed consistently in clinical and research settings. The benefits of monitoring physical activity levels is to reduce physical inactivity, improve risk factor modifications and for a greater knowledge into the health-related impact. (Tuso, 2015)

Sedentary behaviour can have a profound adverse effect on long-term health. (Wilmot et al., 2012) The energy expenditure of sedentary behaviour is defined by low energy expenditure of less than 1.5 METs. (Sedentary Behaviour Research) Sleep is not considered sedentary behaviour but does include activities such as television watching, reading, driving, and use of computer. (Strath et al., 2013) Excessive sedentary time correlates with being overweight, low or even depressive symptoms, poorer quality of life, and elevated blood parameters of metabolic syndrome. (Tremblay et al., 2010, Bharakhada et al., 2012) The chance of early mortality is increased compared to the least sedentary group. (Wilmot et al., 2012) Even among patients considered physical active, those who spend a considerable amount of time in sedentary behaviour have increased all-cause mortality. (Katzmarzyk et al., 2009) Therefore, reducing the time spent in sedentary behaviour is crucial in improving long-term health in an individual.

1.6.2 Measuring physical activity

There are a number of ways of measuring energy expenditure and daily activity levels. These are discussed below, including subjective and objective methods. .

1.6.2.1 Questionnaires

There are a varied number of physical activity questionnaires that consists of either self-reporting by keeping daily logs, answers to set questions or an interview like format. (Silsbury et al., 2015) Within the answers given in most questionnaires, the investigator can often accurately rank order the most active individuals to the least active. (Strath et al., 2013) However, it is recognised questionnaires are less able to identify activity in the sedentary-low range and can have poor correlation with objective assessments of physical activity. (Strath et al., 2004)

1.6.2.2 Energy expenditure assessment

Indirect Calorimetry - this involves the measurement of energy expenditure within the laboratory setting and is considered the reference standard. Essentially, the participant breathes either room air or mixture of gases with a known concentration and expired gases

are collected. This only measures energy expenditure not actual daily physical activity. (Strath et al., 2013)

Doubly Labelled Water Method – measures total energy expenditure in living individual over 1 to 3 week period. Two stable isotopes, oxygen -18 (^{18}O) and deuterium (^{2}H) are ingested and their respective differential elimination rates is measured representing carbon dioxide production over the period of time. (Strath et al., 2013)

1.6.2.3 Accelerometers

Accelerometers have the capability of capturing body movements in terms of duration, frequency and intensity. The device is attached to the body by a strap to the wrist, hips or ankles. They measure acceleration (body movements) in different planes and modern accelerometers are triaxial (measures movements in three planes). The data is stored and measured in gravity (*g*). This can be transformed into other units including the counts per unit time. The count observed is dependent on the individual accelerometer due to how acceleration data is transformed. (Strath et al., 2013) The measured units using predictive equations can be translated into energy expenditure expressed as kilocalories or METS. This allows certain threshold for particular activities and assessing how long an individual spends in a day or week doing activities of particular intensity.

Accelerometers that generate count-based units have intrinsic advantageous of requiring lower computational memory, however, different accelerometers can generate different counts for the same acceleration signal. (Marschollek, 2013) This makes comparison of studies that employ different accelerometers difficult to interpret. The measurement of raw acceleration data has meant that acceleration data are no longer stored as propriety counts and can be transformed into values that are comparable between studies. (Bakrania et al., 2016, John et al., 2013)

Acceleration signal consists of three components including movement, gravitational and noise. (van Hees et al., 2013) The noise and gravitational components have to be separated out or corrected from the acceleration signal. This is complicated by rotational movements making the separation of gravity and movement more difficult. There are a number of different mathematical ways this can be achieved. One of them is the Euclidean Norm Minus

One (ENMO). The Euclidean Norm is the vector magnitude of the orthogonal acceleration. From this value one gravity unit is subtracted. (Bakrania et al., 2016) ENMO values are calculated for each 5 seconds epoch. From these, the proportion of time spent by an individual undertaking various physical activities can be determined.

The GenieActiv® wrist-worn accelerometer measures raw acceleration and has been validated in a number of studies. (Bakrania et al., 2016, Pavey et al., 2016, Powell et al., 2016, Schaefer et al., 2014, Zhou et al., 2015) Acceleration values for moderate to vigorous physical activity has been determined using ENMO as approximately more than 100 *m*g. (Hildebrand et al., 2014) Acceleration between 0-40 represents inactive behaviour, whilst those between 40-80 and 80-120 represents phases between inactive to active behaviour. (Charman et al., 2016) The GenieActiv® monitor is easy to use in patients studies without having to be removed, and accurately reflects daily habitual physical activity.

1.6.3 Physical activity, metabolic syndrome and PH

Metabolic syndrome can result systemic vascular dysfunction through clinical and biochemical abnormalities. (Grundy et al., 2004) Physical inactivity has deemed as factor associated with increased risk of developing illnesses such as type 2 diabetes and cardiovascular disease.(Hill et al., 2015) There is an increasing recognition that metabolic syndrome is associated with pulmonary hypertension. (Paulin and Michelakis, 2014) Glucose intolerance is increasingly seen at diagnosis in PAH. (Pugh et al., 2011) Furthermore, pulmonary hypertension develops in peroxisome proliferator activated-receptor-γ (PPARγ) and adiponectin – deficient mice, suggests there is potential role of insulin-resistance in the development of PAH. (Wilkins, 2012) As the age of diagnosis of PAH increases and existing patients are living longer with treatment are likely to develop co-morbidities, this is an area that requires further investigation. (Ling et al., 2012)

1.6.4 Physical activity in PH

Physical activity research in PAH has been limited so far. Mainguy *et al*, demonstrated reduced daily physical activity levels compared to controls with reduced number of daily steps, decreased daily energy expenditure and decreased time spent performing physical activities needing greater than three METs. The number of daily steps correlates with 6MWD

and WHO functional class. (Mainguy et al., 2011) Pugh *et al* showed that PAH patients spend an increased proportion of time undertaking sedentary activities (Pugh et al., 2012) Whilst proven targeted treatment leads to improved submaximal and maximal exercise capacity, their respective effects of habitual daily activity is unknown.

1.7 Fatigue

Fatigue is a commonly applied term in both healthy and diseased individuals. Fatigue is a complaint upon physical exertion such as after running or a manifestation of an underlying disease. (Finsterer and Mahjoub, 2014). In healthy people fatigue improves with rest, whilst diseased individuals report fatigue at rest and exertion, limiting their daily activities. (Davis and Walsh, 2010). There are both central and peripheral mechanisms that may underlie patient reported fatigue. (Finsterer and Mahjoub, 2014) These include peripheral muscle function including the role of skeletal bioenergetics and the coupling that leads to muscle contraction. (Davis and Walsh, 2010) Understanding the factors leading to fatigue in individual diseases may help to develop interventions as part of multi-focused treatment plans.

Fatigue in PAH is a common complaint among patients that persists despite targeted therapy. (Matura et al., 2012) The mechanisms underlying fatigue in PAH remains uncharacterised, but exercise intervention seems to improve patient reported fatigue scores. (Weinstein et al., 2013a) Furthermore, as discussed previously physical activity levels is reduced in PAH and the fatigue maybe contributing to this particular aspect of daily living. (Pugh et al., 2012, Weinstein et al., 2013b) There needs to be a greater understanding of the impact, mechanisms and potential viable treatment of fatigue for patients with PAH.

1.8 Summary of literature review

Pulmonary Arterial hypertension has been introduced as a multi-systemic disease that has pathophysiological effects beyond the pulmonary vessels. Skeletal muscle dysfunction in PAH has gained increased prominence as a potential independent factor contributing to functional and exercise limitations in recent publications. Mitochondrial dysfunction in the pulmonary vessel plays a significant role in the pathology of PAH leading to impaired apoptosis and proliferation of cells. There is some early evidence of potential mitochondrial dysfunction in the skeletal muscle. The physiology that leads to exercise limitation in PAH patients was discussed. The majority of research has focused on central resting and exercise haemodynamic particularly on supine exercise. Peripheral factors that affect the oxygen pathway further downstream from the cardiac pump function have gained little attention. Patients report fatigue as a common symptom of exercise and activity limitation. Compared to healthy controls, PAH patients have reduced activity levels and spend a greater proportion of time undertaking sedentary tasks of living. Therefore, there needs to be a further understanding of skeletal muscle function, factors affecting exercise capacity, and how current treatments influence daily activity levels in PAH patients.

1.9 Aims of Thesis

Therefore, with consideration of the information above, the aims of the thesis were to answer following questions:

1. **Is there any evidence that impaired skeletal muscle mitochondrial function in idiopathic pulmonary arterial hypertension contributes to exercise intolerance?**
2. **What are the relative roles of central and peripheral limitations to exercise in pulmonary hypertension?**
3. **What is the effect of PH-targeted treatment on habitual daily activity levels in patients?**
4. **What is the impact of patient reported fatigue on daily activity levels and quality of life?**

Chapter 2 Material and Methods

2.1 Recruitment Strategy and Informed Consent Process

All participants with pulmonary hypertension were actively recruited from the National Pulmonary Hypertension Service (Newcastle). Potential patients were identified prior to outpatient clinics and then approached during their regular clinic visit. They were screened according to the inclusion and exclusion criteria. The study including any potential risks were explained and participant information sheet was provided. They were phoned after a minimum of 48 hours to determine if they wished to participate and any further questions answered. Studies underwent ethical approval by relevant organisations and the patient consent forms are given in the appendices C and D.

2.2 Baseline Clinical Characteristics

All the participants in the various studies had established diagnosis of pulmonary hypertension made at a designated specialist centre (Newcastle). Background clinical information data were retrieved including age, gender, height, weight, WHO-FC, aetiology of pulmonary hypertension, recent cardiopulmonary haemodynamics by right heart catheterisation, current targeted therapies, 6MWD and NT-proBNP. These data are presented in the respective chapters.

2.3 ^{31}Phosphorous –Magnetic Resonance Spectroscopy (^{31}P-MRS)

Chapter 3 involves a pilot study of six participants with idiopathic pulmonary arterial hypertension (IPAH) who underwent ^{31}P-MRS. The use of ^{31}P-MRS allows us to study skeletal muscle bioenergetics by assessing the changes in the concentration of phosphate metabolites during rest, exercise and recovery. The spectroscopy data can be used to determine the relevant concentration of a variety of metabolites and calculate the intracellular pH handling.

2.3.1 Basic principles of ^{31}P-MRS

^{31}P-MRS is method of measuring the resonance frequency of phosphate metabolites in a number of organs during stress using non-invasively. (Kemp et al., 2007) The mitochondria

in any cell has a vital role in the production of phosphate metabolites particularly adenosine triphosphate (ATP) to be used by the cell as a source of energy. ^{31}P-MRS is able to detect the signal of unbound phosphate metabolites with a concentration of at least 1mM. As the method is non-invasive, it can be used to monitor changes in the concentration of phosphate metabolites during rest, exercise and recovery. Therefore, the monitoring of oxidative and glycolytic metabolism is possible. (Kemp et al., 2007) Basic principles of skeletal muscle bioenergetics are shown in Figure 7 (below) (Prompers et al., 2014)

Individual cells have three sources of ATP generation:

a) Oxidative phosphorylation by mitochondria of adenosine diphosphate (ADP) and phosphate metabolite (Pi) to produce ATP.

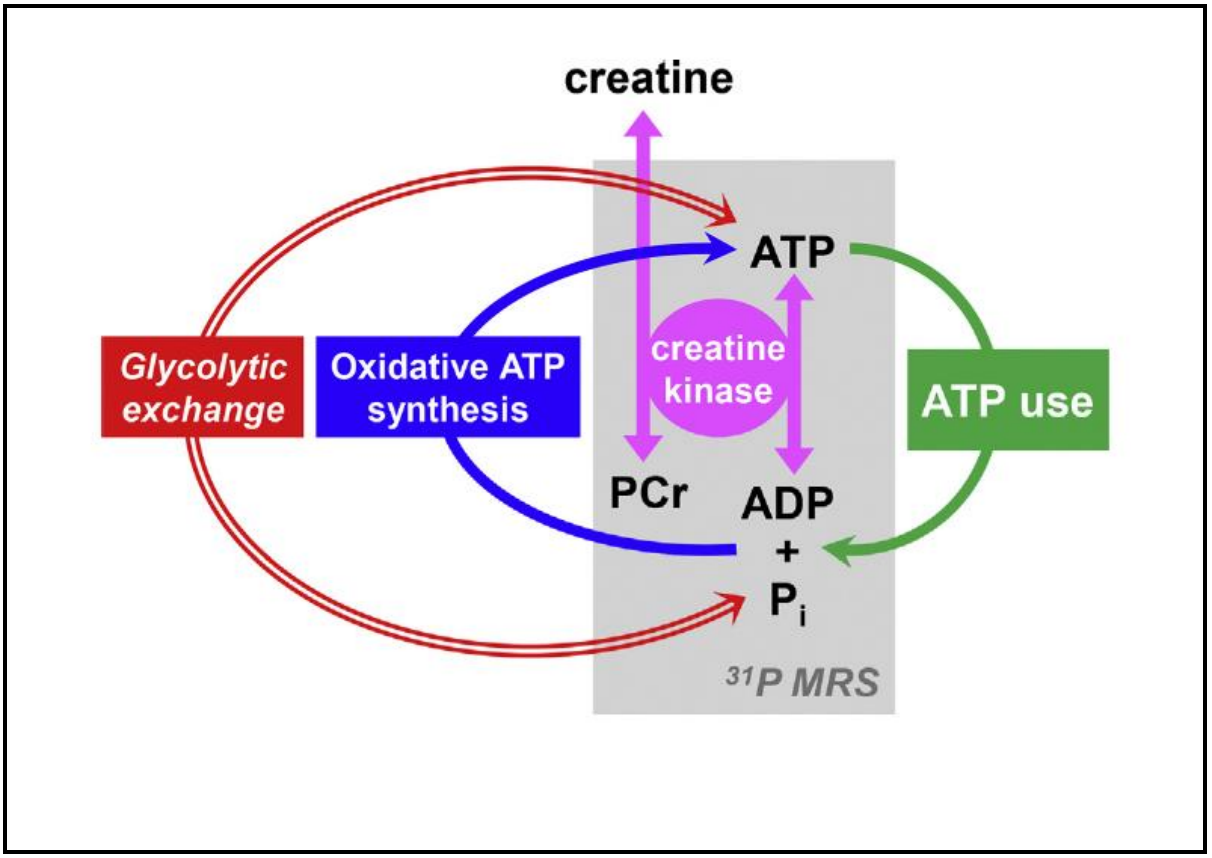
b) Hydrolysis of phosphocreatine (PCr) to produce ATP, requiring hydrogen ion.

c) Glycogenolysis leading to lactate and ATP

To assess mitochondrial function, we can observe a number of parameters including changes in PCr recovery time (PCr $t_{1/2}$), ADP recovery time (ADP $t_{1/2}$), and pH during exercise and relative concentration of the metabolites, to assess any suggestion of mitochondrial dysfunction in skeletal muscle.

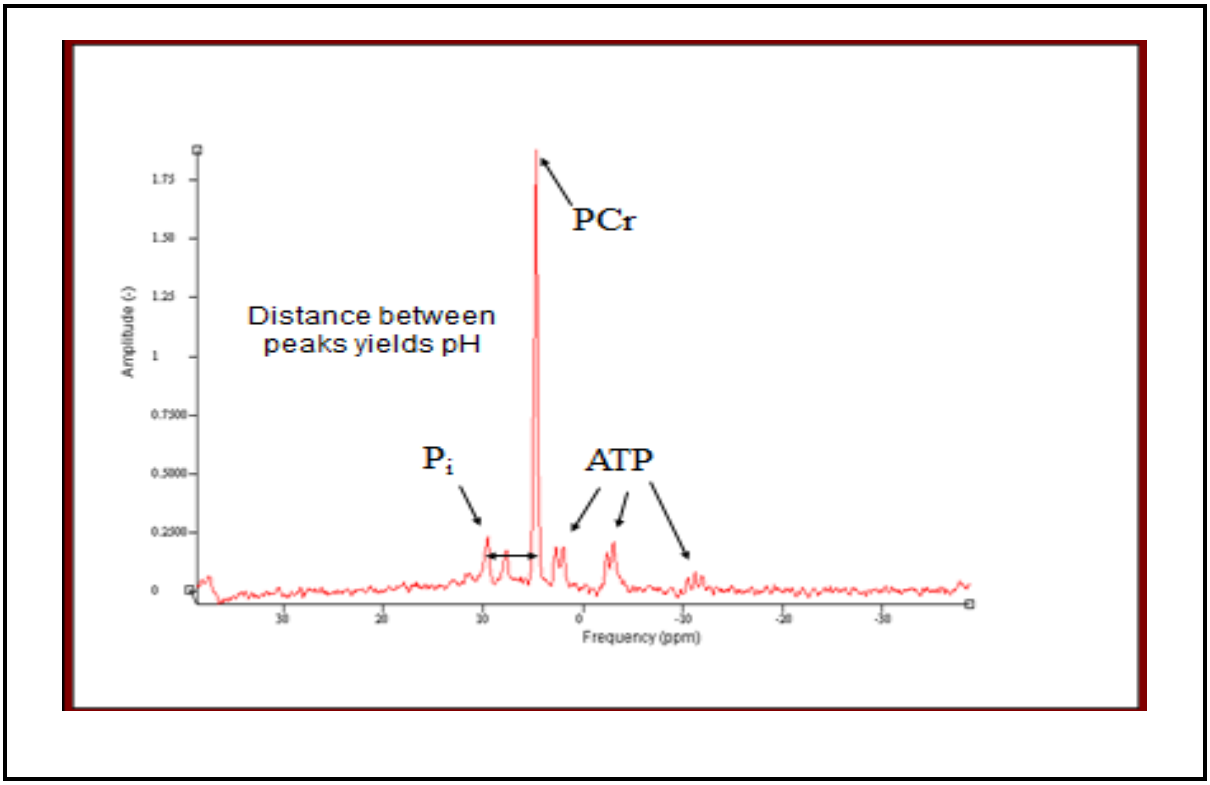
Skeletal muscle oxidative capacity which is an assessment of mitochondrial function can be monitored in terms of recovery of PCr after exercise by the use of ^{31}P-MRS. The re-synthesis of PCr in recovery is a result of mitochondrial ATP production as the CK reaction is stable or rather in equilibrium (Lanza et al., 2011). ADP recovery is calculated indirectly as most ADP is bound therefore cannot be measured directly (Arnold et al., 1984). Muscle pH during exercise is calculated using the gap between the spectra between PCr and Pi as shown in Figure 7. (Prompers et al., 2014)

Figure 7: Basic principles of energy production in skeletal muscle.



ATP hydrolysis leads to ADP and Pi. ATP production is driven by demand (e.g. muscle contraction). During steady state, ATP demand (green) is matched by production (blue). During supply-demand mismatch, the CK reaction (purple) helps to supply the extra ATP. During short duration exercise the mitochondrial oxidative phosphorylation system generates the required ATP to meet demands during and in recovery. The shaded grey block represents what is visible to the ^{31}P-MRS to be detected; PCr, Pi and ATP, with ADP and pH calculated indirectly. (Figure from Pompers *et al*, 2014)

Figure 8: Spectral peaks of ^{31}P-MRS.



MR signal can detect peaks of PCr, Pi and three peaks of ATP (γ, α and β in that order). The gap between PCr and Pi can be used to calculate the pH. (Figure from Dr K Hollingsworth)

2.3.2 ^{31}P-MRS protocol used and analysis of data

Six participants underwent ^{31}P-MRS scan with a 3T Intera Achieva Scanner (Philips, MA, USA). A 14cm diameter phosphorus coil was used for the transmission and reception of signal as well as for the coil used for anatomical imaging. The ^{31}P-MRS protocol was designed by the Newcastle MR centre and processing of data was automated by systems developed by Drs Jehill Parikh and Kieren Hollingsworth. The methodology has been described in previous literature and is described below (Hollingsworth et al., 2008, Payne et al., 2014) .

Each subject performed two periods of exercises at 25% and 35% maximum voluntary contraction (MVC). The MVC was determined before the start of spectroscopy. There was three minutes of rest, three minutes of plantar flexion at 0.5Hz (using a metronome) and 5 minutes of rest to measure recovery in equilibrium. (Hollingsworth et al., 2008) In the first

instance a 25% MVC at a fixed load was used to measure oxidative metabolism in recovery aiming to change the pH levels as little as possible. The second period, after ten minutes of rest, 35% MVC at a fixed load was used generating greater anaerobic metabolism and resulting in measurement of pH handling. Phosphorous spectra was collected at 10s intervals throughout the exercise to identify signals to the gastrocnemious and soleus muscles. (Prompers et al., 2014) (Figure 8) The exercise was performed using MR compatible plantar flexion exercise apparatus, with the use of restraining straps to prevent the aid of other muscle groups (e.g. quadriceps). (Hollingsworth et al., 2008)

The jMRUI processing software was used to measure values for PCr, inorganic phosphate and pH. There was an assumption that a value of 8.2mM ATP at rest and as well as correction of the values of spectroscopy at relative saturation at rest. ADP concentrations were calculated using PCr and pH measurements, with the use of the creatine kinase equation, at each time point. Standard methods as described in published literature were employed to assess oxidative metabolism and pH handling (Kemp and Radda, 1994). Exponential fits to the recovery data were made to estimate the half-times for recovery to equilibrium of ADP and PCr. All spectroscopy analysis for the six participants presented in Chapter 3 was performed by Dr Jehill Parikh.

2.4 Skeletal Muscle Biopsy and Laboratory Analysis

Skeletal muscle biopsies were obtained to study the *in vitro* mitochondrial oxidative phosphorylation function. The process of obtaining, storage and the laboratory techniques used at our centre are described below. The results of the study are reported in Chapter 4. The biopsy was undertaken at the Clinical Research Facility, Royal Victoria Infirmary, Newcastle upon Tyne.

2.4.1 Obtaining and storage of the muscle biopsy

Biopsy of the vastus lateralis muscle was performed under local anaesthesia using a modified ethmoid needle (conchotome forcep), as previously described. (Taivassalo et al., 2006) In brief, using aseptic technique, 2% lignocaine of 10ml was used to anaesthetize the area,

without infiltrating the muscle. A small incision was made up to the fascia, and using the conchotome needle, 2 to 3 pieces of skeletal muscle tissue was obtained. Manual pressure was applied for minimum of 10 minutes, with incision closed with steri-strips and a waterproof dressing. The quadricep muscle was wrapped using a crepe bandage for 1 hour after which it was removed and the dressing inspected.

Extracted muscle tissue was snap frozen in liquid nitrogen and stored at -80^{o}C. For laboratory analysis, sections of 10µM thickness muscle tissue was obtained using a cryostat at -20^{o}C and fixed on positively charged slides (Superfrost®), air dried for 1 hour at room temperature and stored at -80^{o}C.

2.4.2 Histochemical staining

Slides containing 10µm cut sections of transversely orientated muscle sections, were allowed to thaw for 1 hour at room temperature. Individual activities of mitochondrial enzymes consisting part of the oxidative phosphorylation system was assessed. Thus, histochemical staining as previously described for cytochrome C oxidase (COX, complex IV), succinate dehydrogenase (SDH, complex II), and then sequential assay of COX/SDH activity (Old and Johnson, 1989) were undertaken with aid of Mr G Falkous.

Haematoxylin and Eosin Staining: Muscle sections placed in Harris' haematoxylin for 3 minutes, washed in tap water, differentiate in 0.2% acid alcohol, place in 1% eosin for 15-20 seconds, wash in distilled water, dehydrate in ascending alcohol series and mount in synthetic resin (DPX).

COX/SDH staining: Sections were reacted for 45mins at 37^{o}C with COX reaction media (4mM diaminobenzidine tetrahydrochloride, 100µM, cytochrome c and 20µg/ml catalase in 0.2M phosphate buffer, pH 7.0) and 40 min at 37^{o}C with SDH media (1.5mM nitroblue tetrazolium, 1mM sodium azide, 200µM phenazine methosulphate, 130mM sodium succinate, in 0.2 M phosphate buffer, pH 7.0) Sequential COX/SDH staining highlights individual myofibres that are COX-deficient which appear as blue staining myofibres under light microscopy. (Old and Johnson, 1989)

Gomori trichrome: GT staining was undertaken to assess for the presence of ragged-red muscle fibres due to subsarcolemmal accumulations of mitochondria. Muscle sections are placed in Harris' haematoxylin for 5 minutes, rise in distilled water, stain in Gomori trichrome mixture for 10 minutes, rinse in tap water, dehydrate in ascending alcohol series and mount in synthetic resin. (Rifai et al., 1995)

2.4.3 Quadruple immunofluorescence

Immunofluorescent staining was undertaken based on methodology developed by the Wellcome Trust Mitochondrial laboratory, Newcastle and described previously (Rocha et al., 2015). This is a highly sensitive and specific technique to assess defects in the mitochondrial OXPHOS pathway in the skeletal muscle, beyond those achieved by visual assessment of muscle fibres using histochemical techniques. Quadruple immunofluorescence allows for appropriate adjustment for mitochondrial mass and analysis of individual muscle myofibre.

Procedure: Snap frozen muscle sections on Superfrost® slides were dried, fixed in 4% paraformaldehyde (Sigma) for 3 minutes and permeabilised in methanol (Fisher) gradient (10mins 70% methanol, 10mins 95% methanol, 20mins 100% methanol, 10mins 95% methanol and 10mins 70% methanol). 10% normal goat serum was used to block non-protein interactions. Incubation overnight in humidified chamber at 4^{o}C with primary antibodies against complex I (subunit NDUFB8), complex IV (subunit COX-I), mitochondrial mass antibody against porin and myofibre boundries were labelled with anti-laminin antibody. After washes in TBST, sections were incubated with secondary antibodies for 2 hours at 4^{o}C. Sections were then incubated for 2 hours at 4^{o}C with streptavidin conjugated with Alexa 647. After washing, the sections were mounted in Prolong Gold (Sigma). No primary control (NPC) sections were incubated with anti-laminin antibody only. (Rocha et al., 2015)

Image acquisition: Fluorescent images were obtained with Zeiss Axio Imager M1 and Zen 2011 software, using a monochrome digital camera (AxioCam MRm) at 20× magnification. Filter cubes at 405nm, 488nm, 546nm and 647nm wavelengths for laminin, COX-I, porin and NDUFB8 respectively were used. (Rocha et al., 2015) Varying number of muscle fibres were imaged according to the size of the muscle sections.

Data analysis: First, the images obtained were analysed using IMARIS software (Bitplane). Individual muscle myofibres were separated manually. Surfaces were created within the myofibre. Optical density readings were measured (COX-I, NDUFB8 and Porin), within each myofibre. No primary controls were used to obtain mean optical density for non-specific fluorescence.

Further statistical analysis was undertaken using the obtained optical density measurement for each myofibre. This was performed using r studio program (version 3.1.3) by Dr John Grady. Values for OXPHOS complex values were corrected for mitochondrial mass and for background signal for each muscle myofibre. Z-values were obtained from the control population (healthy and disease controls) for each subunit of complex I and IV according to the porin level. Fibres were classified for complex I and IV according to standard deviation limits, with normal being Z > -3SD, intermediate with Z between -3SD to -6SD and deficient if Z-score <-6SD.

2.5 Cardiopulmonary Exercise Testing

Incremental symptom-limited CPEX testing was performed using electronically-braked cycle ergometer (Ergoline®). We used the standard exercise protocol established at our institution for all patients with pulmonary hypertension, involving a step protocol increasing the work rate by 10 Watts per minute. All participants were required to pedal at a frequency around 60 revolutions per minute. Borg dyspnoea score was assessed at rest and at peak exercise. Continuous 12-lead electrocardiograph and pulse oximetry monitoring was employed throughout the exercise and recovery period. Gas exchange variables were measured breath by breath using gas analyser (Medgraphics Cardio 2 Ultima). Anaerobic threshold was determined by the V-slope method and lowest ventilator equivalent of oxygen (Beaver et al., 1985). Systemic blood pressure was recorded at rest and peak exercise. Participants stopped exercising due to intolerable peripheral leg fatigue and/or severe dyspnoea. There were no adverse events observed during any CPEX testing of participants. All tests were performed in the presence of respiratory physiologist and a physician.

The healthy control data was used to compare the central and peripheral haemodynamic response to symptom-limited maximum exercise data of patients with pulmonary hypertension. This was undertaken using a variable ramp protocol of increasing work rate of 5 – 20 Watts/min at 60 revolutions per minute. CPEX testing was performed by staff at the MoveLab, Clinical Research Facility, Royal Victoria Infirmary, Newcastle and grateful for allowing for the use of the data. (Dr Djordje Jakovljevic)

2.6 Non-Invasive Central Haemodynamic Monitoring

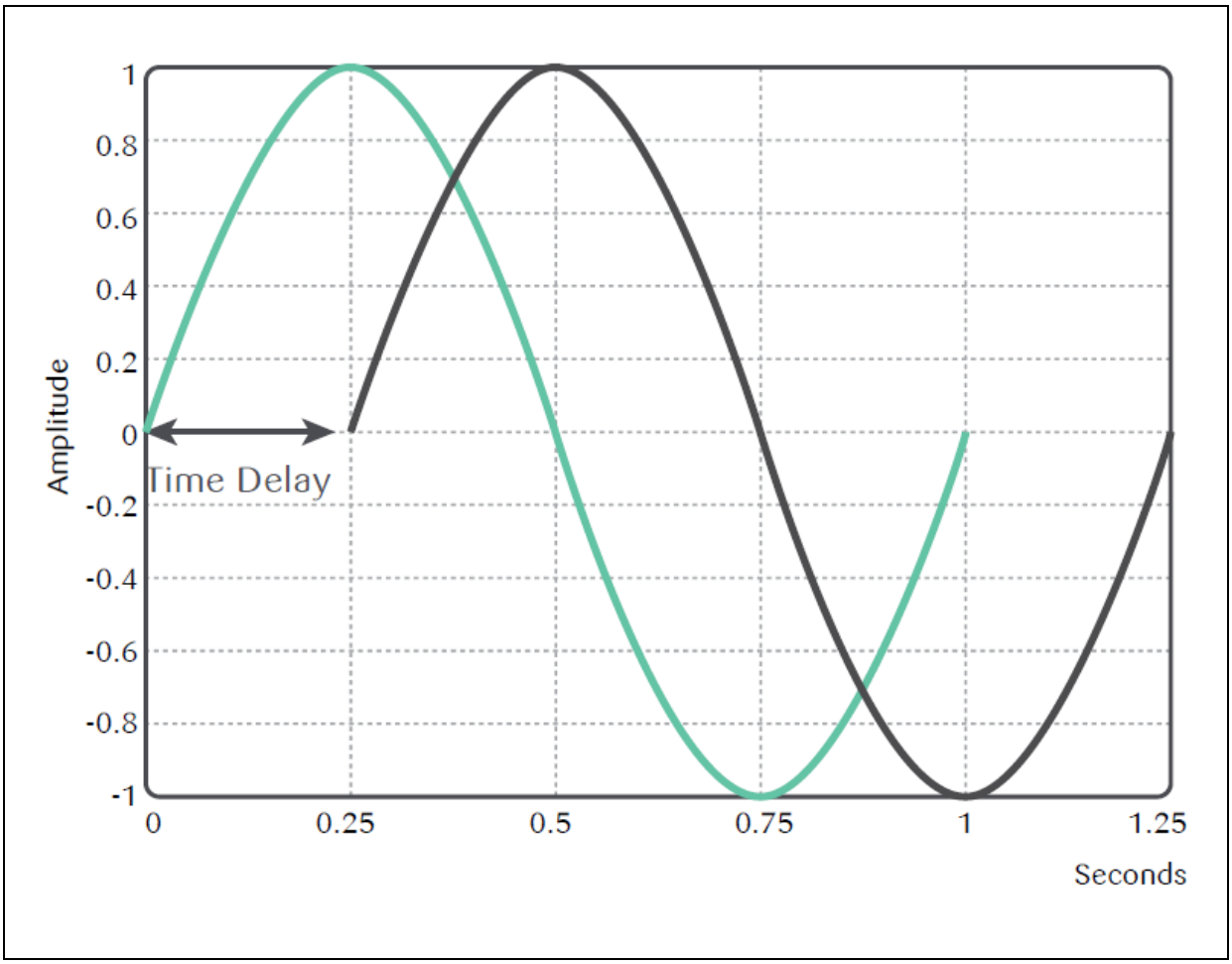
During CPEX testing, central haemodynamics was monitored using non-invasive method, NICOM ® (Cheetah Ltd, Israel). NICOM® allows for continuous monitoring of stroke volume, stroke volume index, heart rate, cardiac output and cardiac index throughout the exercise period. (Jakovljevic et al., 2012a)

This methodology is dependent on bioreactance technology which is when an alternating current (AC) is passed through the thorax leading to time delay (phase shift) to occur. (Jakovljevic et al., 2012a) The patient is attached with four transmitting sensors and additional four receiving sensors. The detection of phase shift in AC current is correlated with stroke volume. The heart rate is detected by the same sensors, and cardiac output can then be calculated. (Figure 9).

The NICOM® has shown good test-retest reliability at rest and throughout graded maximum exercise (Jones et al., 2015). Furthermore, it has been shown in patients with pulmonary hypertension to correlate well with established invasive methods of assessing cardiac output particularly thermodilution during resting right heart catheterisation (Rich et al., 2013).

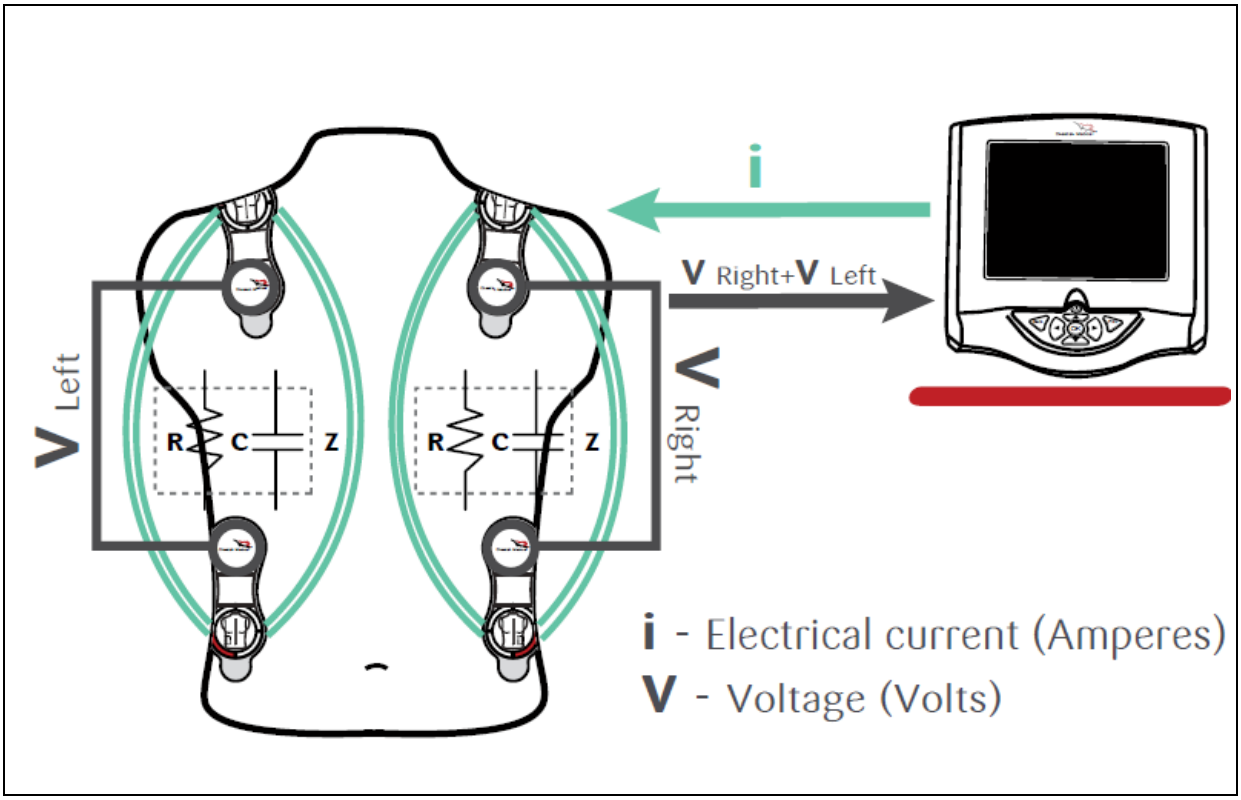
During the CPEX testing, after placing the patient on the upright cycle ergometer, the four double electrodes were placed on the back and connected to the NICOM® device using four wires. (Figure 10) The NICOM® continuously monitored cardiac output every 30 seconds.

Figure 9: Diagram of the basic principles behind NICOM®.



The AC is based on the sine wave. Therefore, the green sine wave starts 0.25seconds before the black sine wave. The black sine wave is phase shifted by 90^{o} or quarter of cycle from the green sine wave. This angle can be used to calculate the stroke volume. (Reproduced from Cheetah Medical®)

Figure 10: Demonstrates the four electrodes (transmitter/sensor) in their respective position.



The monitor induces an electrical current (labelled with i) with a frequency of 75KHz. The returning voltage is detected on both sides (labelled as V). The monitor measures the time delay between i and v, thus detecting the phase shifts. (Reproduced from Cheetah Medical®)

2.7 Habitual Physical Activity Monitoring

In chapter 6, we studied the habitual physical activity (HPA) of patients with pulmonary hypertension in a pilot study. We studied the effect of starting, adding or changing pulmonary hypertension-targeted therapies on physical activity at 3 and 6 months from baseline.

2.7.1 Accelerometer

Accelerometers (GENEActiv, Activinsights Ltd, UK) were used to capture habitual physical activity over a 7-day period under free-living conditions. The accelerometers were wrist-worn and each participant activity was recorded at baseline, 3 and 6 months. Instructions were provided to each participant on how to wear the accelerometer. The activity monitor was pre-

programmed to start recording at a specified time point. The activity monitor is a tri-axial accelerometer with a sample frequency configured at 50Hz. The sensor is parallel to the longitudinal axis of the radius and the z-sensor is perpendicular to the skin surface when attached to the skin surface. The dynamic range of the sensors is ±8*g* and store data at resolution of 12bit. There is temperature and light sensor on the accelerometer. Furthermore, it is waterproof to 10 metres and weighs 16grams without the straps. (Charman et al., 2016)

2.7.2 Data analysis

The process has been described by MoveLab, Newcastle University in previous publications and is described below as such. The data was processed in R (www.cran.r-project.org) by Dr Sarah Charman. The first and last hour of data were disregarded due to monitor disruption of distribution and collection. Only days with a minimum of 16 hours collected were kept for analysis. (Charman et al., 2016)

The average acceleration on the wrist per 5 second epoch was determined using ENMO. The ENMO is in m*g* (1mg = 0.001*g* = 0.001 × 9.8 m/s^2 = 0.001 × gravitational acceleration). (van Hees et al., 2013) Accelerometer non-wear was detected as described before. (van Hees et al., 2013) If the participants took off the monitor deemed non-wear time, the data was replaced from same time points from other days of measurement. The data from the time series was used to calculate the total time in particular acceleration categories per day. (Charman et al., 2016) Category 0 -50 m*g* is inactive behaviour; category 50-100m*g* is a marginal zone between active and inactive behaviour (e.g. such as sitting but moving your arms or slow walking but not moving your arms); categories greater than 100 – 150 m*g* is moderate and vigorous physical activity (MVPA). Moderate-to-vigorous physical activity was determined by using the ≥ 100 m*g* cut-off. (Hildebrand et al., 2014) Furthermore, the least active 5 hour period (L5) and the most active 5 hour period (M5) along with the difference between the two periods (ΔM5L5) were calculated in mean acceleration (m*g*). (Innerd et al., 2015, Charman et al., 2016)

2.8 Quality of Life and Fatigue Severity Questionnaires

We assessed the quality of life and fatigue severity of patients with pulmonary hypertension in the studies involving all the chapters. We monitored response and change in quality of life and fatigue severity with targeted therapy whilst monitoring habitual physical activity in chapter 6.

2.8.1 EmPHasis -10 questionnaire

This questionnaire has been validated for PH patients in assessing health-related quality of life (HRQL). It consists of 10 questions, presented as a six-point differential scale (0-5), giving a total score out of 50, with a higher range reflecting worse quality of life. The questionnaire has shown good correlation with WHO functional class, dyspnoea and psychological distress (Yorke et al., 2014).

2.8.2 Fatigue severity scale (FSS)

The questionnaire measures patient's view of how fatigue affects their physical and social behaviour. There are nine situations where patients' are asked to make a response of how fatigue affects these situations. (Rosa et al., 2014) The scoring is based on Likert scale where 1 for strong agreement to 7 for strong disagreement. The scoring is completed by calculating a mean response and is a validated tool. (Valko et al., 2008) Furthermore, it has been used to assess response to exercise training in PH patients. (Weinstein et al., 2013b)

Chapter 3 *In Vivo* Study of Skeletal Muscle Mitochondrial Function in Idiopathic Pulmonary Arterial Hypertension

3.1 Introduction

Idiopathic pulmonary arterial hypertension (IPAH) results in obstruction to blood flow in the pulmonary arteries leading to right ventricle failure. (McLaughlin and McGoon, 2006) Increasingly, IPAH is being recognised as a multi-systemic disease process with the role of insulin resistance, skeletal muscle dysfunction, bone-marrow derived mononuclear cells and iron-deficiency contributing to exercise intolerance experienced by IPAH patients. (Paulin and Michelakis, 2014) Currently available therapies include phosphodiesterase V inhibitors, endothelin receptor antagonists and prostacyclin analogues which act on targets within the pulmonary artery. (Humbert et al., 2014) Aside from primary pathophysiology, there is increased focus in understanding causes of exercise intolerance and fatigue beyond the pulmonary vasculature. In the last decade, exercise rehabilitation has shown to be safe and beneficial in patients with IPAH by increasing their exercise capacity and quality of life. (Grunig et al., 2012a)

Skeletal muscle dysfunction contributes to exercise intolerance and reduced muscle strength in IPAH patients. (Mainguy et al., 2010, Bonnet et al., 2006, Archer et al., 2008) Mitochondrial dysfunction is recognised to be contributing to the pathophysiology of IPAH in the pulmonary arteries. (Ryan and Archer, 2015) More recently, there is evidence of a potential role of mitochondrial oxidative phosphorylation dysfunction in the skeletal muscle of IPAH patients contributing to exercise intolerance. (Malenfant et al., 2015) However, the proteomic analysis methods used have their limitations including a "shot-gun" approach to analysis, lack specificity to individual expression of specific proteins and failure to adjust for mitochondrial mass. Furthermore, their *ex vivo* analysis does not reveal *in vivo* performance.

^{31}Phosphorous –magnetic resonance spectroscopy (^{31}P-MRS) is a non-invasive method of measuring the phosphate metabolites *in vivo*. ^{31}P-MRS is able to detect the signal of unbound phosphate metabolites with a concentration of at least 1mM. (Kemp et al., 2007) As the method is non-invasive, it can be used to monitor changes in the concentration of phosphate metabolites during rest, exercise and recovery, making it possible to monitor oxidative and

glycolytic metabolism. Further principles and mechanisms relating to ^{31}P-MRS are discussed in chapter 2.

The primary aim of this pilot study was to assess *in vivo* mitochondrial function in the skeletal muscles of patients with IPAH compared to healthy controls without mitochondrial disease. The secondary aims were: 1) To explore skeletal muscle intracellular pH handling during and after exercise in IPAH 2) To determine any association between skeletal muscle bioenergetics, and self-reported quality of life and fatigue severity.

3.2 Methods

3.2.1 Study design

Six IPAH patients underwent ^{31}P-MRS scan. All six participants were clinically stable, in WHO functional class 2 or 3 and established on PAH-targeted therapies. They had no contraindications to undergoing magnetic resonance scans. They did not have any other cardiovascular, pulmonary, neurological and musculoskeletal co-morbidity. All six participants completed quality of life questionnaires (EmPHasis-10) and fatigue severity (FSS).

The study was approved by the Newcastle and North Tyneside 2 ethics committee, 13/NE/0353. All participants gave informed written consent for the study. The scans were performed by trained radiographers at the Newcastle Magnetic Resonance Centre. The spectral analyses were performed by physicists at the MR centre. (Dr Jehill Parikh and Dr Kieren Hollingsworth). The main principles of the ^{31}P-MRS scan and methodology are described in Chapter 2. The primary outcome variable was phosphocreatine recovery half-time (PCR $t_{1/2}$) as a marker *in vivo* mitochondrial function. The secondary outcome variable included adenosine diphosphate recovery half-time (ADP $t_{1/2}$), intracellular pH, patient-reported quality of life and fatigue severity.

3.2.2 Statistical analysis

Data was assessed to be non-parametric due to the small number involved and by Shapiro – Wilk test. Comparison to healthy control data were analysed using Kruskal-Wallis test. *P*-value less than 0.05 were considered significant. All data analysis was performed using SPSS version 21(Chicago, IL). All continuous data are given as mean $\pm$ standard deviation. This was a pilot study, to stimulate a main trial into skeletal muscle bioenergetics in IPAH. Based on a power calculation 90% chance of detecting a difference, with 0.05 alpha and 0.1 beta value, nine patients would be needed in the main trial.

3.3 Results

3.3.1 Baseline characteristics

Individual patient characteristics of all six patients are shown in Table 1. No participants were on intravenous prostacyclin infusion as the pump was ferromagnetic and therefore contraindication to MR scanning.

3.3.2 Skeletal muscle mitochondrial function

^{31}P-MRS data are presented in Table 2. The main outcome measure was the recovery kinetics of phosphocreatine (PCr) as an *in vivo* marker of mitochondrial oxidative phosphorylation function. There was observed delayed recovery in four participants with the remaining two participants having normal PCr recovery kinetics compared to healthy controls (40.0 ± 8.8 vs 27.2 ± 7.1 seconds, p = 0.013). There was greater inter-individual variation in PCr recovery kinetics among IPAH patients than with healthy normal controls (range 30.67-56.03 vs control range 18.42-39.04). Phosphocreatine, inorganic phosphate resting content, and ADP recovery half-time were not different to controls (Table 2, P > 0.05). There was no close relationship between the kinetics of ADP and PCr recovery times (Figure 11, below r^2 =0.027, p = ns) compared to literature healthy controls (r^2=0.78, p < 0.005).

3.3.3 Intracellular pH handling in skeletal muscle

IPAH patients had on average significantly lower minimum pH values during exercise than healthy controls (7.00 ± 0.02 vs 6.85 ± 0.10, p = 0.001). The start and end-pH were not different to controls. (Table 2, P > 0.05). IPAH patients demonstrated evidence of an increase in pH recovery times compared to controls (197 seconds, range 130-240 vs 58 seconds, range 0-160).

Table 1: Baseline Characteristics

Patient	Age/Gender	BMI (kg/m2)	WHO FC	mPAP (mmHg)	CO (L/min)	CI (L/min/m2)	PVR (Wood units)	Treatment	6MWD (m)
1	29F	40	3	42	5.9	2.5	6.4	PDE5i	420
2	52M	32	2	20	6.8	3.0	2.5	PDE5i	402
3	48F	28	2	31	7.5	4.1	2.3	PDE5i and ERA	374
4	69F	26	2	46	4.0	2.5	10.1	ERA	376
5	40M	28	2	37	4.8	2.5	6.8	PDE5i	520
6	43F	24	2	33	4.9	2.8	5.1	PDE5i and ERA	455

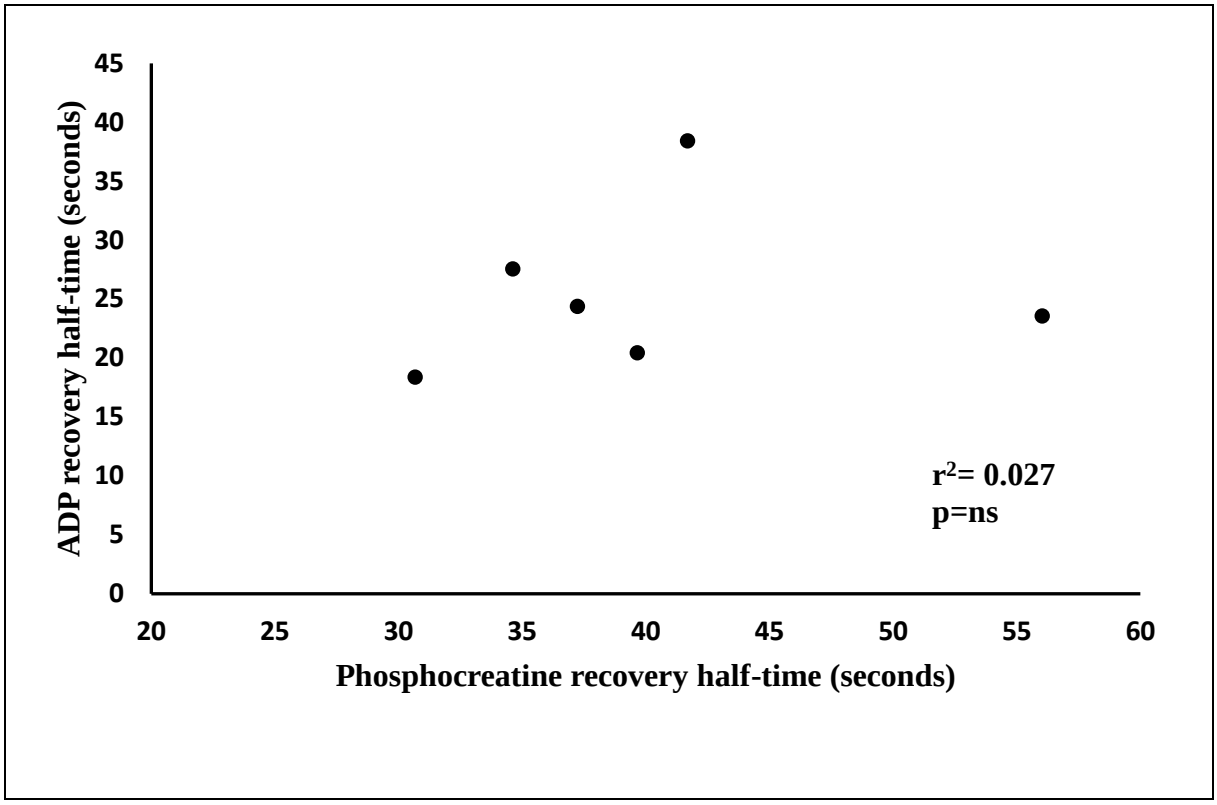
Abbreviations: BMI – Body Mass Index, WHO FC – World Health Organization Functional Class, mPAP – mean pulmonary artery pressure, CO – cardiac output, CI – cardiac index, PVR – pulmonary vascular resistance, 6MWD – six minute walk distance. PDE5i – Phosphodiesterase -5-inhibitor (Sildenafil or Tadalafil), ERA – endothelin receptor antagonist (Bosentan or Ambrisentan)

Table 2: 31P-MRS readings obtained from PAH and healthy control participants

31P-MRS readings	Healthy Controls (n=8)	PAH (n=6)	*P*-value
PCr $t_{1/2}$	27.2 ± 7.1	40.0 ± 8.8	0.013
ADP $t_{1/2}$	20.9 ± 5.1	25.5 ± 7.1	0.228
Rest pH	7.04 ± 0.02	7.03 ± 0.02	0.573
End Exercise pH	7.02 ± 0.02	7.02 ± 0.04	0.573
Minimum pH	7.00 ± 0.02	6.85 ± 0.10	0.001
Rest PCr (mM)	33.6 ± 2.7	32.1 ± 1.8	0.345
Resting Pi (mM)	3.13 ± 0.81	3.05 ± 0.36	0.950

PCr $t_{1/2}$ – phosphocreatine recovery half-time, ADP $t_{1/2}$ – adenosine diphosphate recovery half-time, Pi – inorganic phosphates, mM - millimoles

Figure 11: Scatter plot of ADP and PCr recovery half times of individual IPAH patients. There is no significant correlation between the two values.



3.3.4 Phosphocreatine recovery kinetics, quality of life and fatigue severity

Given the small number of participants, formal statistical analysis was not undertaken to look for correlation between indices derived from 31P-MRS, quality of life and fatigue severity. Table 3 shows individual patients PCr recovery times, scores given for emPHasis-10 and fatigue severity scale. Overall, there is no clear pattern emerging to suggest correlation of what was measured in terms of skeletal muscle bioenergetics and patient-reported symptoms.

Table 3: Individual patient phosphocreatine recovery half time, quality of life and fatigue severity score.

Patient	PCr $t_{1/2}$ (s)	emPHasis-10	FSS
1	56.0	35	3.9
2	30.7	6	2.9
3	34.6	29	4.9
4	41.7	7	1.0
5	39.7	29	6.0
6	37.2	25	5.3

Abbreviations: PCR $t_{1/2}$ – phosphocreatine recovery half time in seconds, FSS – fatigue severity scale. Any value greater than 4 on FSS is deemed the patient suffers from severe fatigue.

3.4 Discussion

This is the first pilot study to have used ^{31}P-MRS to assess skeletal muscle bioenergetics in mitochondrial function in patients with idiopathic pulmonary arterial hypertension. The current pilot study has shown 1) IPAH patients have abnormal PCr recovery half-time and achieve lower minimum pH during exercise 2) The pattern of skeletal muscle bioenergetics observed is not consistent with an underlying mitochondrial dysfunction 3) There is no clear association between recovery kinetics of phosphocreatine and fatigue severity or quality of life scores.

These data demonstrate that IPAH patients have significantly prolonged PCr recovery half-time compared to control population without IPAH. In the recovery period after exercise, the regeneration of PCr is determined mainly by the oxidative capacity of the mitochondrial OXPHOS function in ATP resynthesis. (Thompson et al., 1985, Quistorff et al., 1993) Therefore, PCr $t_{1/2}$ has been used as a surrogate *in vivo* marker of skeletal muscle mitochondrial function. Certainly in established mitochondrial myopathy, PCR $t_{1/2}$ is increased in absolute terms due to delayed oxidative ATP synthesis and in relation to end exercise ADP concentration. (Taylor et al., 1994) Furthermore, PCr recovery half-times have a close correlation with *in vitro* mitochondrial function measured by respirometry. (Lanza et al., 2011) The extended PCr recovery is indicative of mitochondrial impairment, although given the pathophysiology of IPAH it is important to understand the physiological context of this. Using PCr $t_{1/2}$ has its inherent limitation as mitochondrial function can be affected beyond defects in the OXPHOS system. PCr recovery is prolonged in patients with peripheral vascular disease or artificially induced limb ischaemia, where blood flow is reduced on exercise. (Hands et al., 1990) In COPD patients with reduced resting partial pressure of oxygen at rest, PCr $t_{1/2}$ is increased but improves with oxygen supplementation during exercise. (Payen et al., 1993) Mitochondrial oxidative capacity can be affected by both blood flow to the skeletal muscle and oxygen partial pressure.

In pulmonary hypertension, the progressive increase in pulmonary vascular resistance to blood flow, leads to increase in right ventricle afterload and a decrease in cardiac output. (McLaughlin and McGoon, 2006) Patients with IPAH have impaired cardiac output at rest and during exercise due to impaired stroke volume augmentation and chronotropic incompetence. (Holverda et al., 2006, Riley et al., 2000) This leads to a decrease in systemic

oxygen delivery to peripheral tissues including the skeletal muscle. In IPAH there is impaired muscle angiogenesis, capillarisation and microcirculatory alterations that can affect local oxygen delivery. (Potus et al., 2014, Dimopoulos et al., 2013b) The recovery kinetics of PCr is dependent on extra-mitochondrial factors including oxygen delivery, therefore not representative purely of muscle mitochondrial capacity. (Kemp, 2004) Impaired oxygen delivery either due to central or peripheral haemodynamics to the skeletal muscle can affect PCr recovery times. (Kemps et al., 2010, Kemp, 2004) In chronic heart failure, prolonged PCr $t_{1/2}$ is associated with decreased recovery kinetics of deoxygenated haemoglobin in the post-exercise period, suggesting inadequate oxygen delivery rather than oxygen utilization as the cause of exercise intolerance. (Kemps et al., 2010) Furthermore, the literature values of PCr $t_{1/2}$ in mitochondrial myopathy is more prolonged, between 64-92seconds, although these were performed in different muscle groups using different 31P-MRS protocol. (Taylor et al., 1994) Therefore, the delayed PCr $t_{1/2}$ times observed in this study is due to impaired oxygen delivery rather a primary mitochondrial dysfunction.

In the current study, there is evidence of abnormal muscle acid handling in IPAH with significantly lower pH during exercise and prolonged pH recovery times after exercise. At low intensity exercise, intracellular pH control is predominantly by sodium/proton antiporters. (Juel, 1998) PCr recovery times can be affected by pH and end-exercise conditions, whereas ADP recovery times is less affected. (Argov et al., 2000) There was no significant difference in ADP recovery time compared to healthy controls in the current study. Moreover, in mitochondrial myopathy the rate of recovery of pH is faster than healthy patients due to upregulation of proton efflux, further supporting the argument of impaired oxygen delivery rather than mitochondrial dysfunction as the cause of prolonged PCR $t_{1/2\ in}$ IPAH patients. (Taylor et al., 1994) The rapid proton efflux allows for a high concentration of ADP in the presence of lactic acid production without depleting PCr in primary mitochondrial diseases. (Taylor et al., 1994, Chen et al., 2001). In primary biliary cirrhosis there is abnormal peripheral muscle pH handling after exercise with impaired proton efflux, which is associated with perceived fatigue levels. (Hollingsworth et al., 2008) Studies have suggested that autonomic nervous system play a role in acid handling by driving the sodium/proton antiporter. (Kemp et al., 1997, Syme et al., 1991). In chronic fatigue syndrome, there is association between autonomic dysfunction and abnormal pH handling. (Jones et al., 2010) Certainly, in IPAH there is evidence of impaired autonomic dysfunction associated with exercise capacity. (Wensel et al., 2009) Although the muscle pH handling is a complex

process, the question arises as to whether autonomic dysfunction affects muscle pH handling, and perceived fatigue experienced by pulmonary hypertension patients.

3.5 Limitations

The current study has a number of limitations. This was a pilot study, hence the number of participants recruited were small, and therefore should be cautious in our interpretation of the findings. The study cohort was a very specific group within pulmonary hypertension with relatively mild disease (n = 5 in WHO-FC 2). Patients on intravenous therapy by definition have severe disease could not be studied due to the intravenous pump being ferromagnetic. The results cannot necessarily be extrapolated to other patient groups with pulmonary hypertension of differing aetiology and severity. Skeletal bioenergetics was studied in the supine position in response to submaximal exercise and therefore interpreting ^{31}P-MRS results in view of skeletal muscle bioenergetics in whole-body exercise is difficult. (Kemps et al., 2010) Whilst the ^{31}P-MRS is used for research purposes to assess mitochondrial function due to its inherent advantage of being non-invasive, there is limited application of this technique to detect mitochondrial myopathies in the clinical environment due to a lack of sensitivity. (Jeppesen et al., 2007) Although the assumption is PCr recovery is due to oxidative ATP synthesis, a small component of this recovery is due to the glycolytic pathway. (Vinnakota et al., 2006, Forbes et al., 2009) As discussed earlier, mitochondrial oxidative capacity as assessed by PCR $t_{1/2}$ can be affected by blood flow and arterial oxygen content.

3.6 Clinical Perspective

The study lends support to the notion that PH is a multi-systemic disease, with implications beyond the pulmonary vasculature. The impaired skeletal muscle bioenergetics is due to lack of oxygen delivery. Thus, the individual patient needs a more holistic approach to treatment, with a greater explanation of the impact the disease will have of them. In clinical practice, PH patients report observationally significant peripheral muscle fatigue on exertion. This could be due to impaired acid handling in the muscle that was observed in current study and these patients may benefit from focused resistance exercise training.

The study should stimulate further work on understanding the mechanisms of skeletal bioenergetics, exercise intolerance and muscle fatigue in pulmonary hypertension. Assessing

central and peripheral factors affecting local oxygen delivery during exercise should also be explored. This can include right heart catheterisation or cardiac magnetic resonance scans whilst undertaking supine aerobic or resistance exercises. Near-infrared spectroscopy (NIRS) simultaneously with ^{31}P-MRS scan or during measuring central haemodynamics allows for the possibility of determining local oxygen kinetics during and after exercise. (Kemps et al., 2010). Moreover, further work should explore the possibility of improving muscle performance and fatigue in pulmonary hypertension with focused resistance exercise training.

3.7 Conclusions

In summary, there is evidence of abnormal skeletal muscle bioenergetics in IPAH patients. The prolonged PCr recovery is secondary to impaired oxygen delivery rather than primary mitochondrial dysfunction. IPAH patients showed abnormal handling of muscle intracellular acidosis during exercise, a pattern inconsistent with mitochondrial dysfunction and could be contributing to perceived muscle fatigue on exercise. Further studies are needed to carefully examine factors affecting oxygen delivery during and after exercise that can affect exercise intolerance and be contributing to muscle fatigue in IPAH patients.

Chapter 4 *In Vitro* Skeletal Muscle Mitochondrial Function in Idiopathic Pulmonary Arterial Hypertension

4.1 Introduction

The clinical spectrum of IPAH extends beyond the pulmonary vasculature and cardiac function. Patients suffer from iron-deficiency, skeletal myopathy and increased prevalence of co-morbidities of increasing age at diagnosis contributing to exercise intolerance. (Ruiter et al., 2015, Panagiotou et al., 2015, Ling et al., 2012) Improvement in functional status and exercise capacity with targeted treatments including phosphodiesterase 5 inhibitors, endothelin receptor antagonists and prostacyclin analogues corresponds with better long-term prognosis. (Barst et al., 2013) Understanding the cellular and molecular processes affecting exercise capacity are crucial in the development future novel therapies.

Mitochondria are the key organelles for the generation of energy in the form of adenosine triphosphate (ATP) using oxygen. Oxidative phosphorylation (OXPHOS), otherwise known as the respiratory electron transport chain, is the main mechanism by which ATP is generated through series of complexes creating an electrochemical gradient. (Freund-Michel et al., 2014) The mitochondria can initiate apoptosis and provide metabolites for the proliferating cell. (Zamzami and Kroemer, 2001). In the pulmonary arteries, the mitochondria act as oxygen sensors and has a role in mediating hypoxic pulmonary vasoconstriction. (Ward and McMurtry, 2009) There is evidence of metabolic dysfunction in the pulmonary vasculature with \impaired mitochondrial function contributing to the pathobiology of pulmonary hypertension. (Paulin and Michelakis, 2014)

Mitochondrial dysfunction in the pulmonary artery contributes to the proliferation and impaired apoptosis of pulmonary artery smooth muscle (PASMC) and endothelial cells (PAECC). (Archer et al., 2013) . The mitochondrial-reactive oxygen species (ROS) – HIF-1α-Kv1.5 oxygen sensing pathway is altered. There is decreased superoxide dismutase 2 (SOD2) levels leading to reduced ROS production, contributing to increased hypoxia inducible factor-1α (HIF-1α) levels, despite the presence of adequate oxygen. (Archer et al., 2010) This ultimately results in inhibition of pyruvate dehydrogenase (PDH), and with downregulation in the expression of Kv1.5 potassium channel. (Bonnet et al., 2006) As a result, there is calcium

overloading and depolarisation of the PASMCs. The altered mitochondrial metabolism and dynamics leads to apoptotic impairment and proliferation of PASMC.

Skeletal muscle mitochondrial dysfunction appears to contribute to exercise intolerance in patients with proven mitochondrial myopathy. (Taivassalo et al., 2003) Skeletal muscle mitochondria in IPAH have reduced ability to undergo fusion with downregulation of mitofusin 1 and 2 suggesting this may contribute to muscle atrophy and mitochondrial inefficiency. (Batt et al., 2014) Malenfant *et al*, using proteomic analysis highlighted nine proteins of skeletal muscle that were down regulated including subunits of complex I, III and V of the mitochondrial OXPHOS pathway. (Malenfant et al., 2015) The authors concluded that mitochondrial dysfunction found in the pulmonary vasculature was also evident in the skeletal muscle of patients with IPAH, contributing to their exercise intolerance.

The primary aim of this study is to objectively assess skeletal muscle mitochondrial oxidative phosphorylation protein expression and function in IPAH. The secondary aims are to 1) To determine structural morphology of the skeletal muscle in IPAH, and 2) To assess any association between exercise capacity, fatigue severity, quality of life and skeletal mitochondrial OXPHOS function.

4.2 Patients and Methods

4.2.1 Study design

This was a cross-sectional, pilot study. Nine participants with IPAH were recruited from the National Pulmonary Hypertension Service (Newcastle). IPAH diagnosis were made on results of right heart catheterisation of mean pulmonary artery pressure ≥ 25mmHg, mean pulmonary capillary wedge pressure ≤ 15mmHg with an elevated pulmonary vascular resistance > 3 Wood units. All participants completed two questionnaires (emPHasis-10 and Fatigue Severity Scale) and underwent a vastus lateralis skeletal muscle biopsy was obtained. Clinical characteristics including age, gender, World Health Organization functional class (WHO FC), body mass index (BMI), cardiopulmonary haemodynamics, 6MWD and current treatments were collected.

All patients were on anticoagulation (warfarin) that was stopped two weeks prior to the biopsy, with international normalized ratio (INR) checked 1-2 days before the biopsy. The study was approved by the local research ethics committee (Newcastle and North Tyneside 2: 13/NE/0353). All patients gave written informed consent to participate in the study.

The primary outcome is the expression of subunits of complex I and IV after adjustment for mitochondrial mass. The secondary outcomes are skeletal muscle histology, mitochondrial OXPHOS histochemical analysis, exercise capacity, quality of life, and fatigue severity.

4.2.2 Inclusion and exclusion criteria

Inclusion Criteria:

- Patients' aged greater than 18 years and less than 75 years;
- Underlying diagnosis of IPAH confirmed by the Northern Pulmonary Vascular Service;
- Clinically stable and compensated on medical therapy for at least 3 months;
- World Health Organization (WHO) functional Class II to IV.

Exclusion Criteria

- Patients who are unable to provide informed consent;

- Patients on steroid therapy – as these drugs are known to cause myopathy;
- Patients with recent syncope;
- Patients with known skeletal muscle abnormalities;
- Patients with ischaemic heart disease.
- Patients with claustrophobia;
- Patients with active cancer;

4.2.3 Cardiopulmonary exercise testing

Each participant completed symptom-limited CPEX exercise testing using a step-protocol with breath-by-breath gas analysis was performed at rest and during exercise in all studies (Medgraphics, St Paul, MN, USA) to measure oxygen consumption. Six participants underwent non-invasive central haemodynamics measurements continuously throughout the test. (NICOM®)

Peak VO_2 was highest O_2 uptake, averaged over last 30 seconds of exercise. Arterio-venous oxygen content difference (AVO_2 diff) was obtained indirectly using the Fick method as the ratio between VO_2 and CO.

4.2.4 Muscle biopsy

All participants underwent muscle biopsy at the Clinical Research Facility, Royal Victoria Infirmary, Newcastle. Aseptic technique using modified ethmoid needle was employed to obtain 2-3 pea sized muscle biopsies from the vastus lateralis. The biopsies were snap frozen in liquid nitrogen and stored at -80^{o}C.

Cryostat was used at -20^{o}C, to cut transversely orientated sections at 10µM thickness onto positively charged Superfrost® slides and air dried for 1 hour. The slides were then stored at -80^{o}C.

4.2.5 Laboratory analysis

All muscle biopsies underwent initial screening analysis of basic structure and mitochondrial oxidative phosphorylation function using non-quantitative method. This included structural

analysis of muscle samples using haematoxylin/eosin (HE) staining and modified Gomori trichrome. Using histochemistry, analysis for complex II (succinate dehydrogenase, SDH), complex IV (cytochrome C oxidase, COX) and combined SDH/COX to determine mitochondrial OXPHOS dysfunction was determined. Images were analysed using a modified light microscope (Olympus, Japan) with a motorized stage. These techniques are described in further detail in chapter 2 and performed with aid from Mr Gavin Falkous. (Old and Johnson, 1989, Reichmann et al., 1996)

In order to accurately corroborate the findings, quadruple immunofluorescence was performed. Primary antibodies to detect complex I (NDUFB8), complex IV (COX-1), mitochondrial mass (porin) and delineate muscle myofibre boundaries (laminin) was used. Each patient sample had a muscle section for no-primary antibody controls (NPC), incubated with anti-laminin antibody only. Image acquisition was performed and acquired at ×20 magnification using a Zeiss Axio Imager M1 and Zen 2011(blue edition) software. Further details are described in chapter 2. (Rocha et al., 2015)

4.2.6 Statistical analysis

Analyses were performed using R 3.1.3 program to generate z-values for the protein expression of COX-1 and NDUFB8 subunits after adjustment for mitochondrial mass. R-program coded by Dr John Grady. (Rocha et al., 2015) Mitochondrial respiratory chain expression profile plots were generated using GraphPad Prism Version 5.02®. Full details of analysis are described in chapter 2.

4.3 Results

4.3.1 Baseline characteristics

Individual patient clinical and exercise capacity characteristics are shown in Table 4.

4.3.2 Skeletal muscle mitochondrial OXPHOS function

Initial histochemical analysis determined the function of complex II (SDH) and IV (COX). (Old and Johnson, 1989) Observational analysis of the muscle sections showed normal function of complex II and IV. The COX/SDH sequential staining show COX-deficient fibres as blue. All patients had less <1% fibres that were COX-ve, which were within normal range. (Figure 13)

In order to corroborate our results quadruple immunofluorescence was undertaken. The results compared to healthy and disease control samples, showed normal expression of subunits of complex I and IV in the majority of myofibres analysed after adjustment for mitochondrial mass. The expression profiles of all patients including control samples are shown in figures 14-17.

4.3.3 Structural morphology in skeletal muscle

Haematoxylin and eosin sections were visually inspected, with all nine samples demonstrating peripheral position of nuclei, with no necrosis of myofibres, absence of inflammatory cells with no basophilic granularity. There was no accumulation of adipose tissue, fibrosis or longitudinal fibre splitting.

Gomori trichrome failed to demonstrate any myofibres with ragged-red appearance or rimmed vacuoles in all nine patients. Ragged-red fibres are seen in primary mitochondrial myopathies due to sub-sarcolemmal peripheral accumulation of abnormal mitochondria. (Old and Johnson, 1989)

4.3.4 Exercise capacity, fatigue severity and quality of life

Nine participants had a 6MWD between 374-520 metres. They demonstrated peak oxygen consumption (VO_2) between 38-84% of predicted value (10 -19 ml/kg/min). In six patients

central haemodynamics were measured. Cardiac index at peak exercise varied between 4.4-9.2 L/min/m^2 with peripheral oxygen extraction at peak exercise 9.2-12.8 ml O_2/dl. There was a significant association between fatigue severity and quality of life (r^2=0.486, p = 0.037, Figure 12). Three patients reported severe fatigue levels (FSS>4). (Table 5)

Table 4: Baseline characteristics.

Patient	Age/Gender	BMI (kg/m^2)	WHO FC	mPAP (mmHg)	CO (L/min)	CI ($L/min/m^2$)	PVR (Wood units)	Treatment	BMPRII status
1	29F	39	3	42	5.9	2.5	6.4	PDE5i	+
2	52M	32	2	20	6.8	3.0	2.5	PDE5i	-
3	38F	18	3	45	3.8	2.5	10.5	IV prostacyclin	-
4	31F	20	3	65	5.6	3.0	10.0	IV prostacyclin	-
5	67M	33	2	46	4.0	2.1	10.5	PDE5i and IV prostacyclin	-
6	48F	28	2	31	7.5	4.1	2.3	PDE5i and ERA	-
7	69F	26	2	46	4.0	2.5	10.1	ERA	-
8	40M	28	2	37	4.8	2.5	6.8	PDE5i	+
9	43F	24	2	33	4.9	2.8	5.1	PDE5i and ERA	-

Abbreviations: BMI – body mass index, WHO FC – world health organization functional class, mPAP – mean pulmonary artery pressure, CO – cardiac output, CI – cardiac index, PVR – pulmonary vascular resistance, BMPRII – bone morphogenetic protein receptor type II mutation, PDE5i – phosphodiesterase type 5 inhibitor, ERA – endothelin receptor antagonist, IV prostacyclin- intravenous prostacyclin (iloprost)

Table 5: Quality of life, fatigue severity and exercise capacity for each patient

Patient	EmPHasis-10	Fatigue Severity Scale	6MWD (m)	Peak VO_2 (ml/kg/min)	Predicted (%)	Peak CO (L/min)	Peak CI ($L/min/m^2$)	AVO_2 diff (ml O_2/dl)
1	35	3.88	420	10.0	52			
2	6	2.88	402	14.5	57	16.6	7.0	9.9
3	28	3.22	423	12.5	41			
4	26	5.44	520	14.8	38	10.9	5.8	9.2
5	29	4.77	396	10.8	69			
6	29	4.88	374	18.2	84	12.7	7.0	10.8
7	7	1.00	376	12.9	63	7.3	4.4	11.5
8	29	6.00	520	18.9	61	17.8	9.2	9.0
9	25	5.33	455	16.2	65	8.6	5.2	12.8

Abbreviations: 6MWD – six-minute walk distance, peak VO_2 – peak oxygen consumption, CO – cardiac output, CI – cardiac index AVO_2 diff – arterio-venous oxygen difference

Figure 12: Scatter plot with trendline to demonstrate the association with quality of life assessed by standardised questionnaire for pulmonary hypertension (emPHasis-10) and fatigue severity (FSS).

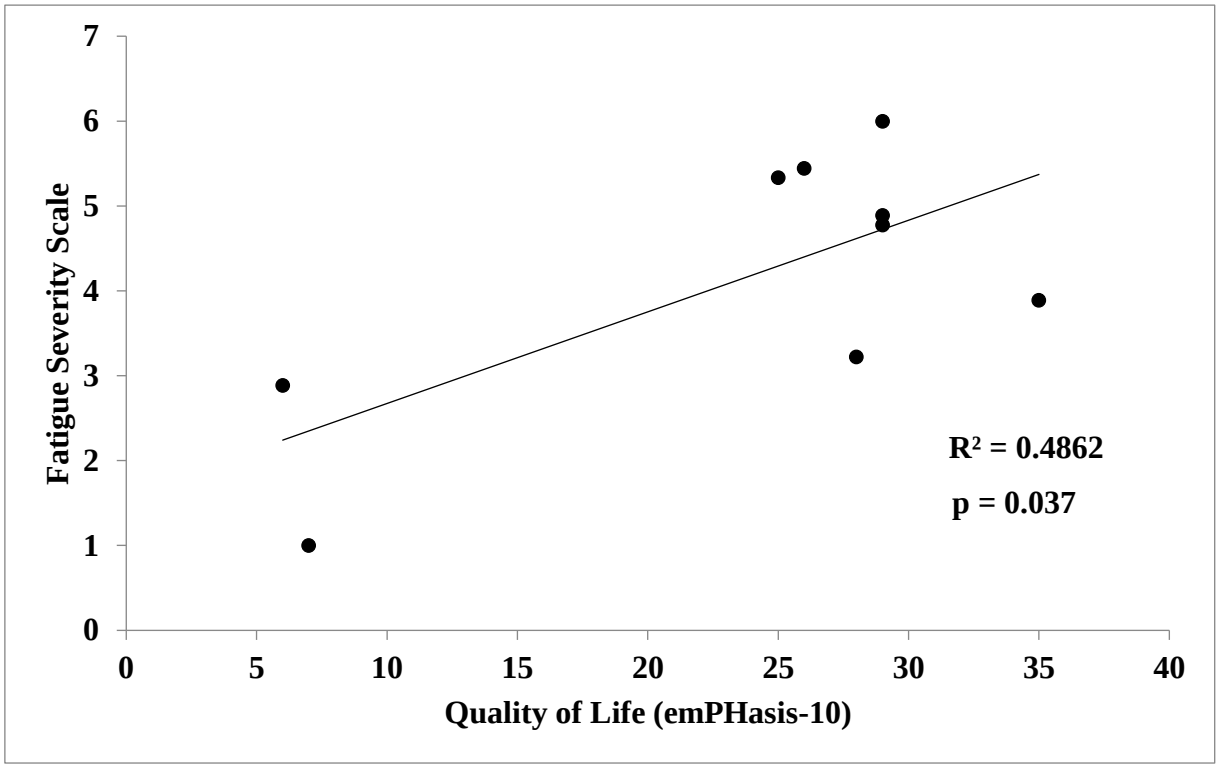
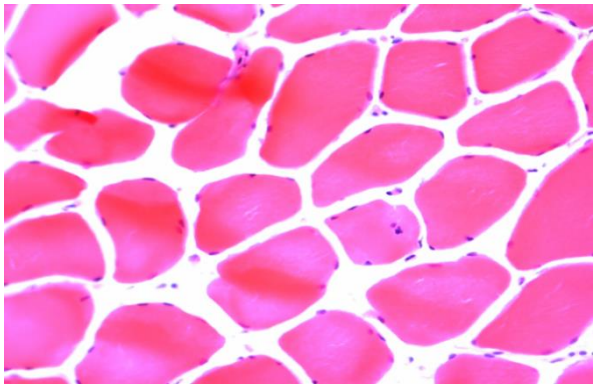


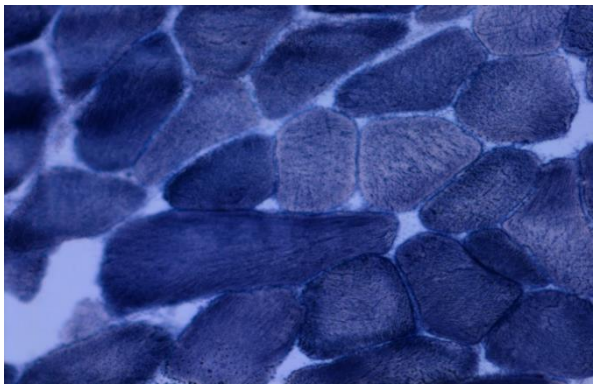
Figure 13: Example of histology and histochemistry slides from an IPAH patient at ×20 magnification.

Structural assessment of skeletal muscle by haematoxylin/eosin staining (H & E) and Gomori trichrome (GT). Histochemistry assessed respiratory chain function of complex II (succinate dehydrogenase, SDH), complex IV (cytochrome C oxidase, COX), and sequential COX/SDH staining. In COX/SDH staining slides, COX deficient fibres will appear blue.

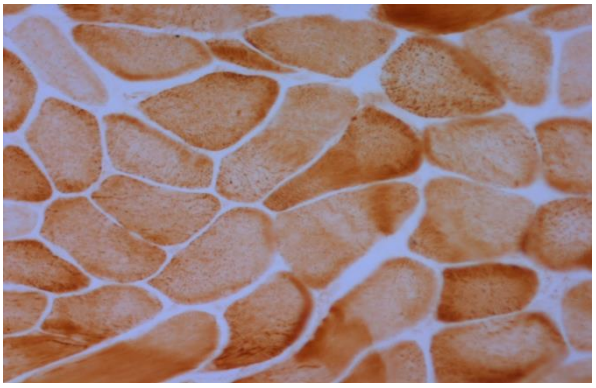
H & E



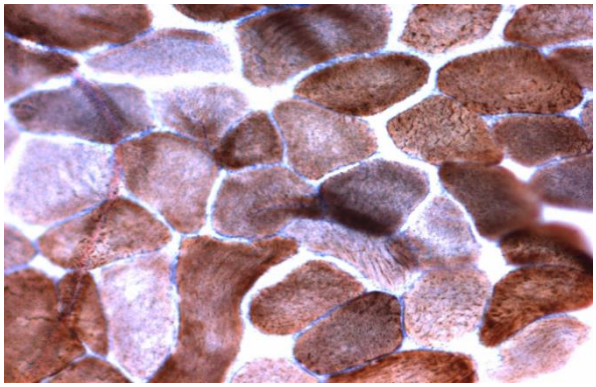
SDH



COX



COX/SDH



GT

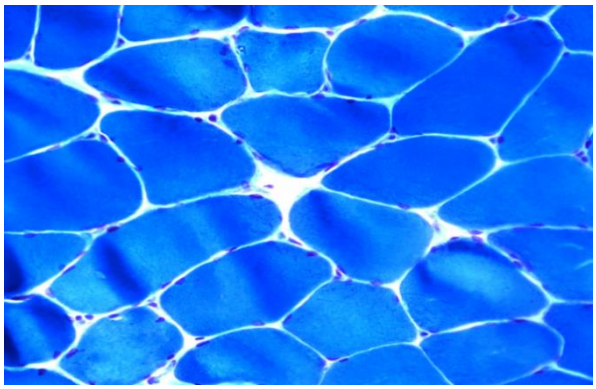


Figure 14: Representative images from quadruple immunofluorescence.

Complex I and IV abundance in skeletal muscle from healthy control, IPAH patient and disease control. Images acquired through fluorescent detection: COX –I – green, NDUFB8 – purple, porin – red and laminin – white.

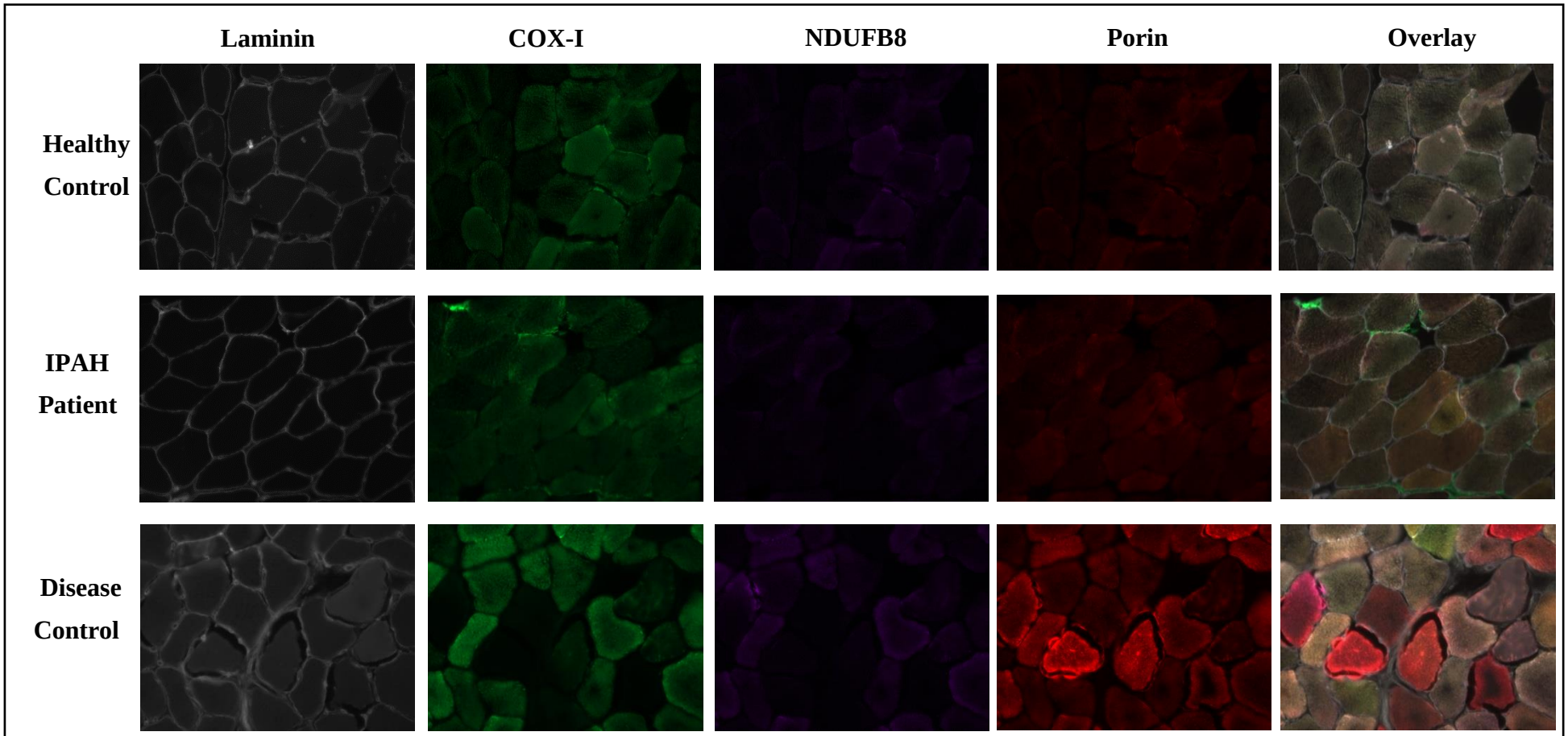
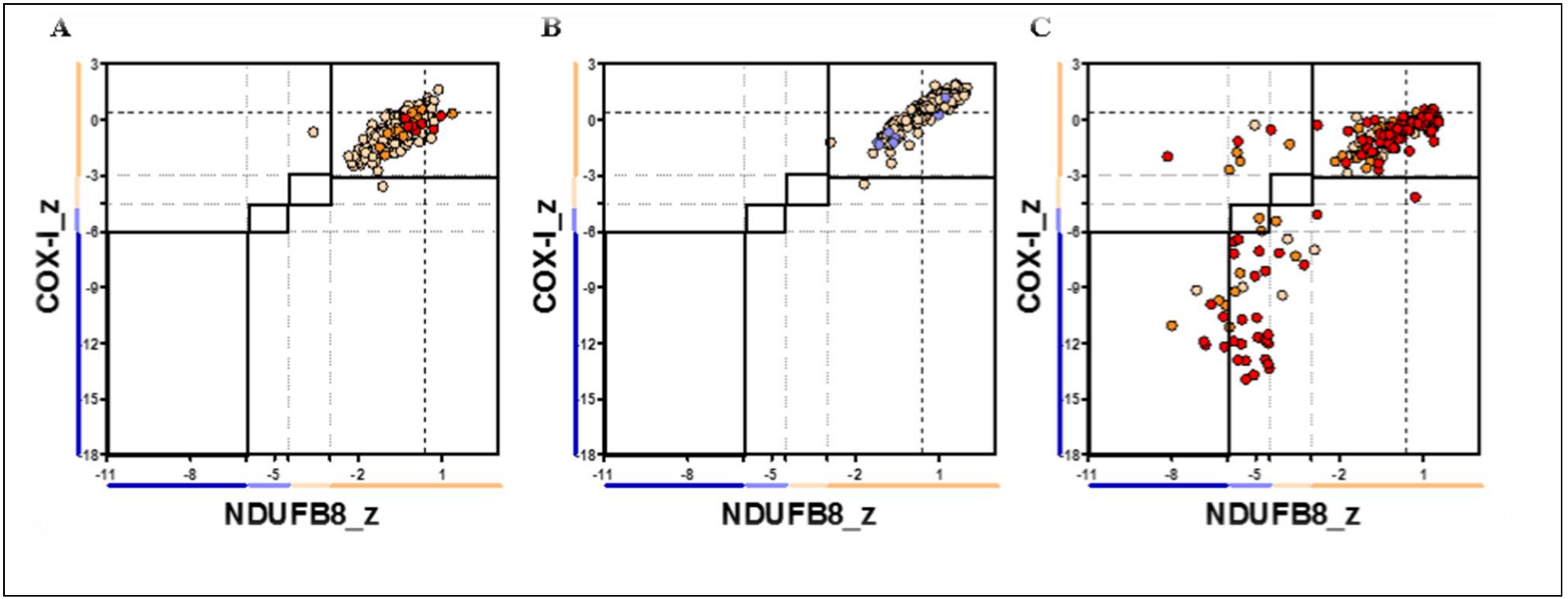


Figure 15. Mitochondrial oxidative phosphorylation respiratory chain expression profile, linking complex I, complex IV and porin levels in patients.



(A) Healthy control, 51F (n = 318) (B) Healthy control, 49M (n = 238) and (C) Disease control, 60M (n= 188). As described before by Rocha *et al*, each dot represents the measurement from individual muscle fibre, colour coded according to its mitochondrial mass (very low: blue, low: light blue, normal: light orange, high: orange and very high: red). The x and y axis indicate the levels of complex I and IV, respectively, in standard deviations. Colours on the axis indicate levels of deficiency: beige: normal, light beige: intermediate (+), light blue: intermediate (-) and blue: deficient. The number (n) in brackets gives the number of myofibres analysed for each patient.

Figure 16: Plots show complex I and IV expression in patients with IPAH.

(D) Patient 1 (n = 166) (E) Patient 2 (n = 514) (F) Patient 3 (n = 497)

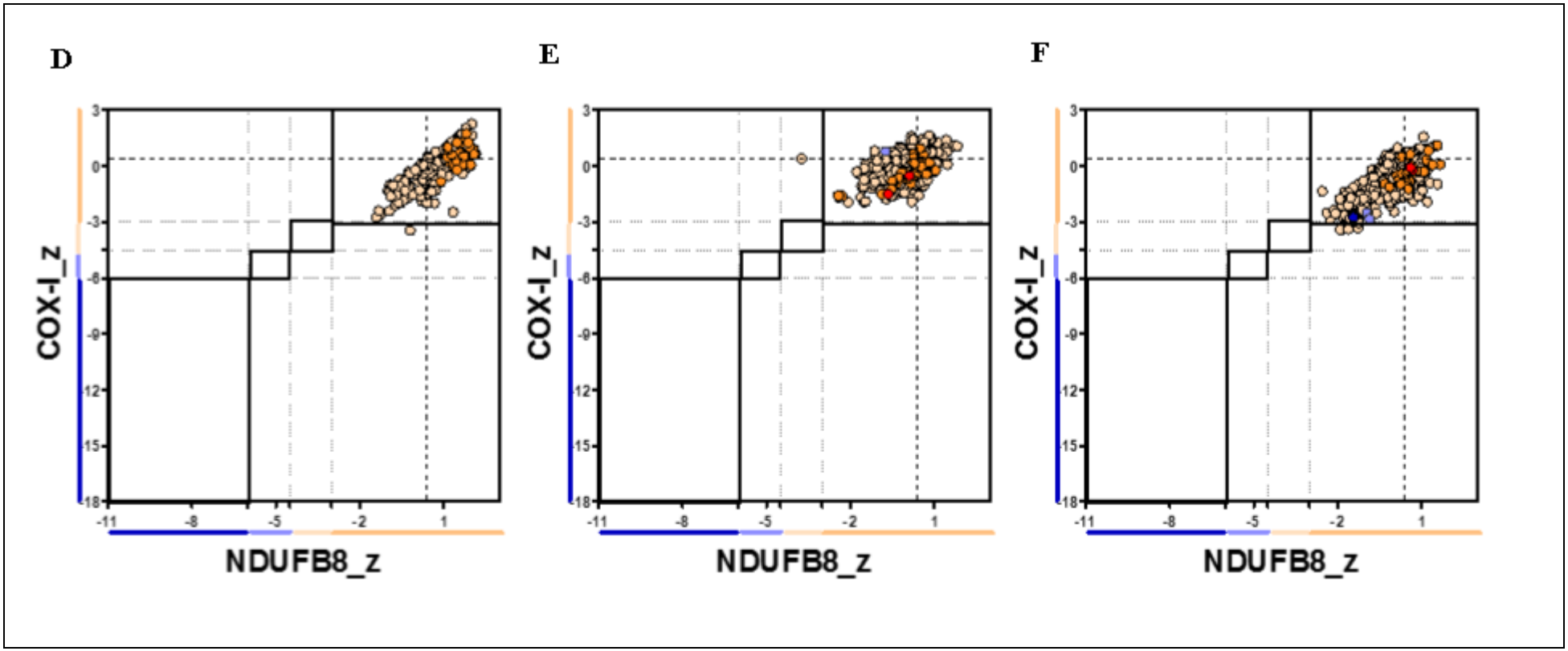


Figure 17: Continuation of Figure 14.

Plots show complex I and IV expression in patients with IPAH. (G) Patient 4 (n = 317) (H) Patient 5 (n = 449) (I) Patient 6 (n = 394)

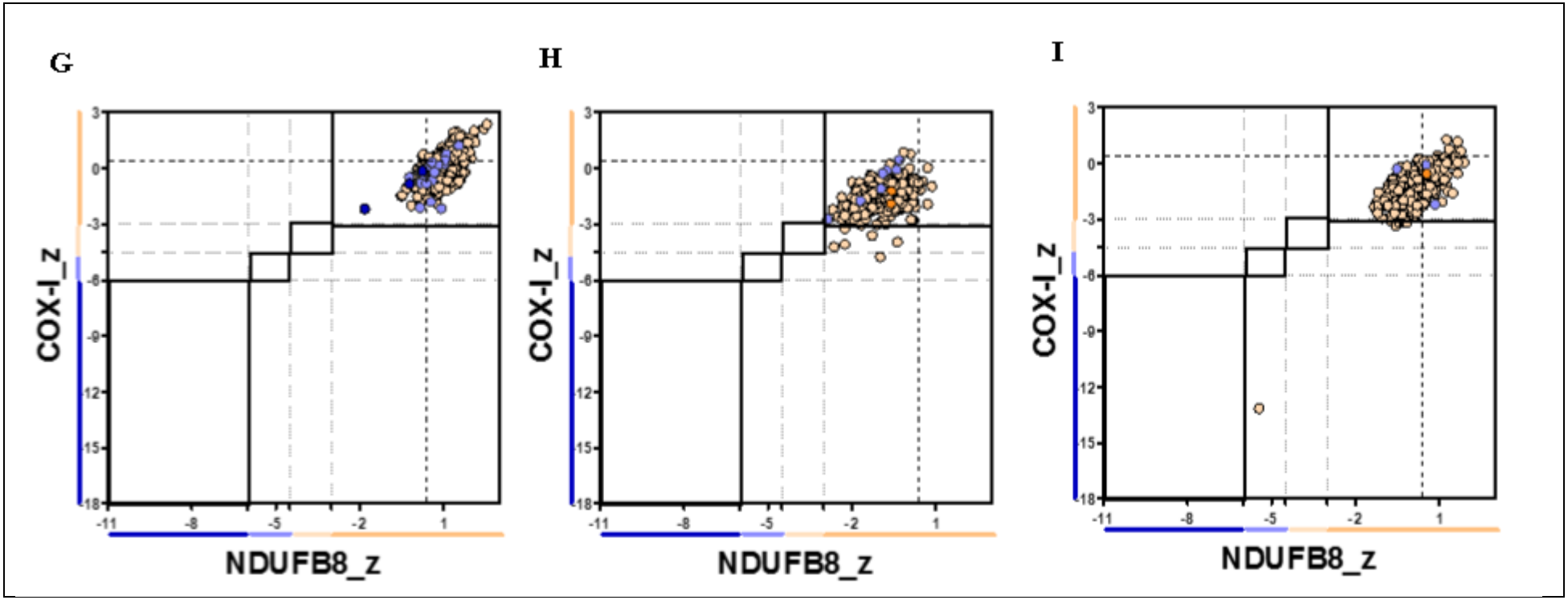
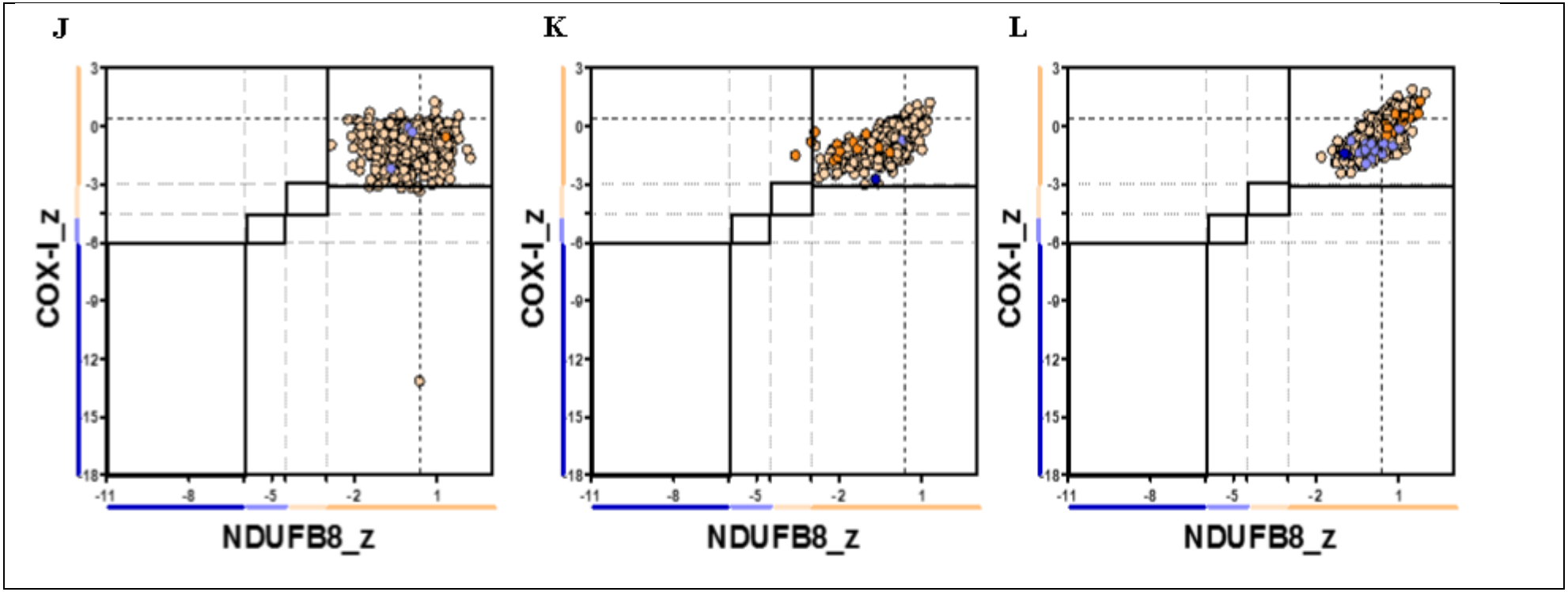


Figure 18: Continuation of Figure 14.

Plots show complex I and IV expression in patients with IPAH. (J) Patient 7 (n = 431) (K) Patient 8 (n = 405) (L) Patient 9 (n = 409)



4.4 Discussion

This is the first study to objectively assess *in vitro* skeletal muscle mitochondrial OXPHOS capacity and function in IPAH. The study shows 1) there is normal skeletal muscle mitochondrial OXPHOS protein expression and function 2) no gross structural abnormality noted on visual assessment of the skeletal muscle 3) IPAH patients demonstrated heterogeneous exercise capacity, peak cardiac index, quality of life and fatigue severity in patients with consistently normal skeletal muscle mitochondrial function.

Using the two laboratory methods, data revealed that IPAH patients have normal skeletal muscle mitochondrial OXPHOS function. Quadruple immunofluorescence is a new state-of-the-art technology that accurately quantifies expression of subunits of complex I and IV with adjustment for mitochondrial mass. In comparison to current histochemical methods, advantages of immunofluorescence includes the avoidance of subjective assessment of mitochondrial dysfunction, the presence of tissue-specific artefacts leading to uneven labelling and individual assessment of all myofibres in a given transverse cross-section of muscle biopsy. (Rocha et al., 2015) In IPAH, skeletal muscle oxidative enzyme expression has been shown to be reduced, with an association between exercise capacity and muscle contractile strength. (Mainguy et al., 2010) In animal models, expression of genes involved in mitochondrial biogenesis within the skeletal muscle was decreased earlier than in cardiomyocytes from the right ventricle during the disease course (Enache et al., 2013) In PH patients, skeletal muscle mitochondria has diminished ability to undergo fusion with reduced levels of mitofusin 1 and 2. (Batt et al., 2014) Skeletal muscle mitochondrial content and biogenesis were assessed to be similar between IPAH and healthy controls. (Batt et al., 2014) Malefont *et al,* suggested mitochondrial dysfunction evident in the pulmonary vasculature was also present in the skeletal muscle of IPAH patients and contributing to their exercise intolerance. First, they showed decreased mitochondrial density using electron microscopy and second, demonstrated downregulation of subunits of complexes I, III and IV by proteomic analysis. The methodology used , proteomic analysis, has a number of limitations including lacking specificity and values obtained were unable to be adjusted for mitochondrial mass. The current study results indicate that one of the reasons for exercise intolerance in IPAH is not due to skeletal muscle mitochondrial OXPHOS ability to generate ATP during exercise. The findings are corroborated by the *ex vivo* results of ^{31}P-MRS in chapter 3, where the obtained data pattern would be inconsistent with the presence of a true skeletal muscle

mitochondrial dysfunction. These results supports the assertion that factors further upstream along oxygen transport pathway, rather than oxygen utilization, are responsible for impaired exercise capacity in IPAH.

The oxygen transport pathway from the air to the skeletal muscle mitochondria is a complex system. There are number of potential mechanisms that can affect oxygen delivery to the muscle mitochondria in IPAH including central and peripheral factors. (Wagner, 2012) Central factors include chronotropic incompetence and impaired stroke volume augmentation during exercise affects peak VO_2. (Holverda et al., 2006, Fowler et al., 2012). At the skeletal muscle level, there is evidence of reduced capillarisation, angiogenesis and abnormal peripheral microcirculation. (Mainguy et al., 2010, Potus et al., 2014, Dimopoulos et al., 2013a) During the onset of heavy exercise, there is an imbalance between oxygen delivery to utilization can lead to ATP generation through oxygen-independent pathway, by-products of which could cause muscle fatigue.(Barbosa et al., 2011) All these factors can affect oxygen delivery to the exercising skeletal muscle and impairing exercise capacity. Furthermore, muscle ability to extract oxygen from the capillaries to the mitochondria through diffusion could be altered, but currently, there is limited understanding to what extent this plays a role in IPAH.

Available therapies focus on predefined targets in the pulmonary artery known to be affected in IPAH including the nitric oxide, endothelin and prostaglandin pathways. (Humbert et al., 2014) They work by reducing the resistance to blood flow by vasodilatation, therefore the afterload is decreased and resting cardiac output is increased. (Humbert et al., 2014) There is some evidence that targeted therapies in pulmonary hypertension improves cardiac output during exercise but the effect on peripheral factors affecting oxygen delivery is unknown. (Provencher et al., 2008) The current study showed in clinically stable IPAH patients with variable peak oxygen consumption, cardiac output and peripheral oxygen extraction at peak exercise, all had normal ability to utilize oxygen in the skeletal muscle. (Table 5)

Peak cardiac index achieved during symptom-limited cardiopulmonary exercise testing has been shown to be powerful prognostic marker of outcome. (Blumberg et al., 2013) Exercise training improves submaximal and maximal exercise performance beyond those achieved by medications in clinically stable patients. (Mereles et al., 2006) Resting central haemodynamics has been shown to improve with aerobic exercise training. (Ehlken et al.,

2016) Muscle oxidative enzyme activity and capillarisation is improved by a combination of aerobic and resistance training. (de Man et al., 2009) Therefore it would seem exercise training is a potential way of targeting central and peripheral mechanisms of oxygen delivery to the skeletal muscle.

4.5 Limitations

The present pilot study is not without limitation. Being a pilot study the number of patients recruited was small, but the patient numbers are on par with published studies that focused on the skeletal muscle in IPAH, where 8-10 patients were recruited. (Mainguy et al., 2010, Batt et al., 2014, Malenfant et al., 2015) Furthermore for the main trial, recruiting patients in great numbers would be difficult due to invasive nature of the study, the use of anticoagulation for reasons beyond IPAH, and presence of co-morbidities will restrict numbers from any one pulmonary hypertension centre. The patients recruited were clinically stable and all were in WHO FC 2 or 3. Nevertheless, three of the patients were on intravenous therapy, including one patient who underwent lung transplantation within a year of the muscle biopsy. Therefore, the study recruited patients with a wide spectrum of disease severity and demonstrated consistent results in terms of primary outcome. Skeletal muscle mitochondrial oxidative phosphorylation function could be affected by the aetiology causing pulmonary arterial hypertension and care should be sought if there was extrapolation of the current data beyond idiopathic cases. There is considerable inter-observer variability in terms of counting COX-ve fibres on the COX/SDH histochemistry sections. (Rocha et al., 2015) No attempt was made to quantify COX-intermediate fibres in COX/SDH sections due to the subjective nature of this assessment. (Murphy et al., 2012) However, the use of quadruple immunofluorescence supersedes the methodological flaws with the use of histochemistry.

4.6 Clinical Perspective

The current study raises a number of questions regarding exercise intolerance and fatigue in IPAH. There is a need to understand: 1) peripheral factors affecting oxygen delivery and extraction in the skeletal muscle during exercise 2) to what extent does this contribute to impaired exercise capacity beyond central haemodynamics and 3) how can fatigue be improved in daily clinical practice. Although this study showed that skeletal muscle mitochondrial OXPHOS system is intact, there are still structural and functional changes

present that would affect physical performance and quality of life. The role of exercise training in maintaining or reversing changes in skeletal muscle requires further investigation. Aerobic training under supervision has been shown to be relatively safe; no study has focused purely on resistance training in maintaining physical conditioning in IPAH. (Grunig et al., 2012a) Resistance training is known to increase protein synthesis, muscle hypertrophy and strength. (Damas et al., 2015) Therefore research is needed into how the skeletal muscle will change at the molecular, structural and functional levels in response to resistance training in IPAH. This would stimulate further work into the safety, dose, and intensity of resistance training programmes that would prove beneficial. Resistance training would not be expected to lead to increased exercise capacity, but it may improve ability to perform activities of daily living, quality of life and decrease perceived muscle fatigue levels.

4.7 Conclusions

In vitro analysis of skeletal muscle in IPAH has showed normal protein expression and function of mitochondrial oxidative phosphorylation system. The study demonstrates that one of the reasons for diminished exercise capacity in IPAH is not due to primary mitochondrial OXPHOS dysfunction. These same patients in the study cohort demonstrated variable subjective and objective effects of the disease on their daily life including quality of life, fatigue and exercise capacity. Three patients where on intravenous treatment with one requiring lung transplantation within a year of their muscle biopsy. Further research is warranted into understanding peripheral factors affecting oxygen delivery and extraction in IPAH. This will hopefully stimulate research into potential treatments that target central and peripheral factors affecting oxygen transport to the muscle mitochondria.

Chapter 5 Central and peripheral limitations to exercise intolerance in pulmonary hypertension and the relationship with fatigue

5.1 Introduction

Pulmonary hypertension (PH) is marked by exercise intolerance that becomes progressively debilitating as the disease progresses. (McLaughlin and McGoon, 2006) Peak oxygen uptake, a marker of maximal exercise capacity, has been shown to be reduced in PH compared to healthy subjects and associated with poor prognosis. (Sun et al., 2001, Deboeck et al., 2012) Improving exercise capacity is a recognized target and clinical biomarker of response to PH targeted therapy. Cardiopulmonary exercise stress testing aides in determining whether escalation of treatment is warranted. (Wensel et al., 2013, Paolillo et al., 2012)

Understanding the physiological limitations of physical performance and oxygen consumption may allow a more targeted approach to therapy. According to the Fick principle, oxygen consumption (VO_2) is determined by three components; (1) the stroke volume (SV), (2) heart rate (HR) and (3) arterio-mixed venous oxygen content difference (AVO_2 diff). (Murias et al., 2013) In people without cardiorespiratory disease, in response to maximal exercise, VO_2 increase is due to significant increases in heart rate and AVO_2 diff, with a smaller percentage increase in stroke volume. (Higginbotham et al., 1986, Proctor et al., 1998) Studies of people with PH demonstrate evidence of chronotropic incompetence and impaired ability to augment stroke volume in response to exercise. (Holverda et al., 2006, Provencher et al., 2006a) PH is also associated with a number of skeletal muscle changes that may result in impaired peripheral oxygen extraction. (Panagiotou et al., 2015) Currently, whether peripheral oxygen extraction increases proportionately to exercise to compensate for impaired cardiac output or acts as another component limiting VO_2 in pulmonary hypertension remains unclear.

The primary aim of this pilot study is to determine central and peripheral factors that affect oxygen uptake and utilisation during upright symptom-limited exercise in patients with pulmonary hypertension compared to age- and gender-matched controls. The secondary aims are to 1) to determine central and peripheral reserve capacity from rest to peak exercise and 2) to determine association between patient-reported fatigue and quality of life with derived exercise parameters.

5.2 Methods

5.2.1 Study population and design

Eighteen patients with pulmonary hypertension (n=18) and eighteen people without cardiorespiratory disease took part in this study. Patients were recruited from the National Pulmonary Hypertension Service (Newcastle) and controls from ongoing studies from within the MoveLab team. All patients were able to undergo symptom-limited cardiopulmonary exercise testing. Patients with significant musculoskeletal disease or cardiopulmonary diseases including patients with coronary artery disease evident on coronary angiogram were excluded. None of the subjects were on supplemental oxygen.

All participants underwent cardiopulmonary exercise testing (CPEX) with continuous non-invasive gas exchange and central haemodynamic measurements. Patients also completed self-reported fatigue severity and quality of life questionnaires. Patients had up to date 6 minute walk distance (6MWD), WHO functional class and N-terminal pro brain naturietic peptide (NT-proBNP). Most recent resting right catheter measurements were retrieved.

Ethical approval was given by the local regional ethics committee (Westminister – 15/LO/0144) and the study complies with the Declaration of Helsinki.

The primary outcome measure was the AVO_2 diff between the two study groups. Secondary outcome measures include difference in peak cardiac index, peak cardiac power output, the reserve capacity of the individual components of VO_2 and correlation of exercise variables with patient-reported fatigue.

5.2.2 Cardiopulmonary exercise testing

Patients and controls undertook a progressive cycle test with termination limited by clinical symptom (patients) or self-terminated (patients/controls). Expired gases were collected to assess gas-exchange (Medgraphics, St Paul, MN, USA) and cardiac haemodynamic measures by bioreactance (NICOM®, Cheetah Medical, USA) at rest and during exercise.

Bioreactance has been shown to be superior to other non-invasive methods of assessing cardiac output at rest and on exercise. (Jakovljevic et al., 2012a) Furthermore, at rest NICOM® has been correlated with thermodilution method in PH patients. (Rich et al., 2013)

The NICOM® determines the phase shift of current across the thorax using surface electrodes. After the skin is prepped, two electrodes placed in the upper torso and two over the lower torso. The sensors on either side generate signals that are integrated for final signal analyses. The NICOM® processing unit determines the phase shift ($\Delta\varphi$) between output signal relative to the input signal, reflecting blood flow changes in the aorta. The cardiac output is equivalent to $C \times VET \times \Delta\varphi dt_{max}$, where C is the constant of proportionality, VET is the ventricular ejection fraction time.(Jones et al., 2015) The stroke volume is calculated from the cardiac output and heart rate. (Jones et al., 2015, Squara et al., 2007)

Peak VO_2 was highest O_2 uptake, averaged over last 30 seconds of exercise. AVO_2 diff was obtained indirectly using the Fick method as oxygen consumption divided by cardiac output. Maximum predicted heart rate was defined as 220 minus age in years. Cardiac power output was obtained by multiplying the mean arterial blood pressure with cardiac output and a constant (0.002222) and gives a value in watts. (Jakovljevic et al., 2012c) Physiological reserves were calculated as the percentage change from resting to peak values divided by resting values of each component of oxygen uptake. (Dhakal et al., 2015)

Further information on cardiopulmonary exercise testing and bioreactance technology is provided in Chapter 2.

5.2.3 Fatigue severity and quality of life

Each participant completed two questionnaires including fatigue severity scale (FSS) and quality of life (emPHasis-10) prior to exercise testing.(Yorke et al., 2014, Valko et al., 2008) Questionnaires were not completed by healthy controls.

5.2.4 Statistical analysis

SPSS version 22 (Chicago, IL) was used for statistical analysis. Normality of distribution of the data was assessed by the Shapiro-Wilk test. Continuous data are presented as mean ± standard deviation. Comparison between groups is by either unpaired 2-sample student *t* test,

Mann Whitney U test or chi-squared test. Correlation was assessed by Pearson or Spearman tests with 2-tail test for significance. Partial R^2 values were obtained after adjustment for age and gender by multiple linear regression as described previously. (Dhakal et al., 2015) P-values less than 0.05 were considered significant. Post-hoc power analysis for the sample n = 18 revealed statistical power of 71.4%.

5.3 Results

5.3.1 Baseline characteristics

Population characteristics of all PH (n = 18) and control subjects (n = 18) are listed in Table 6. The two groups were well matched in terms of age, gender, weight, height and body surface area. All patients exercised beyond their respective anaerobic threshold and achieved respiratory exchange ratio greater than 1. Patients were established on clinical pulmonary hypertension targeted treatments. (Table 6) There were no adverse effects in any subjects during exercise testing. diff in patients.

Table 7 and Table 8 show the resting and peak exercise characteristics of the two groups. Compared to control subjects, PH patients showed no significant difference in resting oxygen uptake or heart rate. (Table 7) Exercise capacity, assessed by peak VO_2 was significantly reduced in PH group compared to control. (Table 8)

5.3.2 Baseline and exercise haemodynamics

At rest, PH patients demonstrated significantly lower mean arterial blood pressure, cardiac output, cardiac index, stroke volume, stroke volume index, cardiac power output and cardiac power output index. (Table 7) At peak exercise, there were significant difference in heart rate, mean arterial blood pressure, stroke volume index, cardiac output, cardiac index, cardiac power output and cardiac power output index, with patients not performing as well as controls. Stroke volume at peak exercise was not significantly different between PH and control subjects. (Table 8)

Table 6: Baseline Characteristics

	PH	Control	*p* - value
Age (years)	**53 ± 15**	**49 ± 16**	**0.378**
Gender (M:F)	**8:10**	**8:10**	**1.000**
Height (cm)	**167 ± 10**	**168 ± 11**	**0.861**
Weight (kg)	**72 ± 16**	**71 ± 11**	**0.795**
BSA (m^2)	**1.83 ± 0.25**	**1.82 ± 0.19**	**0.911**
PH subtype			
- **IPAH**	**11**		
- **PoPH**	**1**		
- **PAH-CHD**	**4**		
- **CTEPH**	**2**		
WHO FC			
- **II**	**10**		
- **III**	**8**		
NT-proBNP, pg/ml	**606 ± 758**		
Haemoglobin (gm/dL)	**14.2 ± 1.0**		
Haemodynamics§			
- **mPAP (mmHg)**	**41 ± 13**		
- **sPAP (mmHg)**	**69 ± 23**		
- **RAP (mmHg)**	**4 ± 3**		
- **PCWP (mmHg)**	**6 ± 4**		
- **PVR (Woods)**	**8.4 ± 4.4**		
- **CO (L/min)**	**4.7 ± 1.4**		
- **CI ($L/min/m^2$)**	**2.6 ± 0.8**		
- **SvO_2, %**	**68 ± 8**		
Treatment*			
- **PDE5i**	**16**		
- **ERA**	**4**		
- **Prostacyclin**	**1**		

*Three patients were on combination treatment of PDE5i and ERA or PDE5i and prostacyclin. § - right heart catheter data in n = 17, mean time from CPEX of 1.43 ± 0.84 yrs

Abbreviations. BSA – body surface area, IPAH – idiopathic pulmonary arterial hypertension, PoPH – portopulmonary hypertension, PAH-CHD – Pulmonary arterial hypertension secondary to congenital heart disease, CTEPH – chronic thromboembolic pulmonary hypertension, WHO FC – World Health Organization Functional class, NT-proBNP – N-terminal pro Brain Naturietic peptide, mPAP – mean pulmonary arterial pressure, sPAP – systolic pulmonary arterial pressure, RAP – right atrial pressure, PVR – pulmonary vascular resistance, CO – cardiac output, CI – cardiac index, SvO_2- mixed venous oxygen saturation, PDE5i – phosphodiesterase type 5 inhibitor, ERA – endothelin receptor antagonist

5.3.3 Peripheral oxygen extraction at rest and peak exercise

At rest, PH patients had higher, but not statistically significant, AVO_2diff compared to control subjects (5.6 ± 1.6 vs 4.8 ± 1.4 mL/dL, p = 0.134). At peak exercise, PH patients had a significantly lower AVO_2 diff (9.3 ± 2.3 vs 12.4 ± 4.8 mL/dL, p = 0.012).

There was a significant correlation between cardiac output and peak oxygen uptake in patients (r = 0.510, p = 0.031) and controls (r = 0.504, p = 0.028). There was no relationship between AVO_2diff and peak oxygen consumption in patients (r = 0.265 p = 0.289) whilst controls demonstrated a strong relationship (r = 0.753, p < 0.001). At peak exercise, there was no correlation between mass-adjusted peak O_2 and cardiac index (r = 0.248, p = 0.321) in PH patients. Ventilatory efficiency slope (V_E/VCO_2) showed trend in correlation with peak cardiac index (r = -0.458, p = 0.056) and cardiac power output (r = -0.483, p = 0.043) but with no relationship with stroke volume, heart rate and AVO_2diff in patients.

Table 7: Resting metabolic and haemodynamic parameters

Characteristics	Control	PH	*p*-value
O_2 (L/min)	0.28 ± 0.84	0.25 ± 0.07	0.252
O_2 (L/min/kg)	4.12 ± 1.66	3.63 ± 0.86	0.273
RER*	**0.90 ± 0.05**	**0.82 ± 0.16**	**0.030**
V_E (L/min)*	**8.0 ± 1.7**	**11.3 ± 3.5**	**0.003**
HR (bpm)	68 ± 8	72± 12	0.297
SBP (mmHg)	**137 ± 15**	**127 ± 15**	**0.043**
DBP (mmHg)	**84 ± 11**	**74 ± 12**	**0.019**
MAP (mmHg)	**101 ± 9**	**92 ± 11**	**0.008**
CO (L/min)	**6.0 ± 1.2**	**4.8 ± 1.0**	**0.002**
CI (L/min)	**3.3 ± 0.5**	**2.6 ± 0.5**	**< 0.001**
SV (mL/beat)	**89 ± 21**	**65 ± 17**	**0.001**
SVI (mL/m2)	**49 ± 10**	**36 ± 8**	**< 0.001**
AVO_2 diff (mL/dL)	4.8 ± 1.4	5.6 ± 1.6	0.134
CPO (Watts)	**1.36 ± 0.32**	**0.98 ± 0.21**	**< 0.001**
CPOI (Watts/m^2)	**0.74 ± 0.12**	**0.53 ± 0.10**	**<0.001**

*Control data based on n = 14.

Abbreviations: O_2 –oxygen uptake, RER – respiratory exchange ratio, V_E – minute ventilation, HR – heart rate, SBP –systolic blood pressure, DBP – diastolic blood pressure, MAP – mean arterial blood pressure, CO – cardiac output, CI – cardiac index, SV – stroke volume, SVI – stroke volume index, AVO_2diff – arterio-venous oxygen difference, CPO – cardiac power output, CPOI – cardiac power output index

Table 8: Peak exercise gas-exchange and haemodynamic variables

Characteristics	Control	PH	*p*-value
O_2 (L/min)	**2.25 ± 1.05**	**1.01 ± 0.31**	**< 0.001**
O_2 (L/min/kg)	**25.3 ± 5.7**	**14.0 ± 3.3**	**< 0.001**
RER*	**1.21 ± 0.08**	**1.10 ± 0.07**	**< 0.001**
V_E (L/min)*	**65.8 ± 22.0**	**51.9 ± 14.2**	**0.037**
V_E/VCO_2 slope*	**28.7 ± 2.4**	**49.6 ± 14.2**	**<0.001**
BR (%)*	34.9 ± 4.6	37.3 ± 15.6	0.583
HR (bpm)	**163 ± 21**	**123 ± 24**	**< 0.001**
HR predicted (%)	**95 ± 9**	**74 ± 14**	**< 0.001**
SBP (mmHg)	**200 ± 14**	**148 ± 27**	**<0.001**
DBP (mmHg)	88 ± 16	93 ± 29	0.903
MAP (mmHg)	**125 ± 12**	**111 ± 26**	**0.046**
SV (mL/beat)	118 ± 41	95 ± 33	0.92
SVI (mL/m2)	**64.0 ± 17.4**	**52.1 ± 15.9**	**0.037**
CO (L/min)	**18.4 ± 5.4**	**11.5 ± 4.0**	**<0.001**
CI (L/min/m^2)	**10.0 ± 2.2**	**6.2 ± 1.9**	**<0.001**
CPO (Watts)	**5.21 ± 1.85**	**2.77 ± 1.06**	**< 0.001**
CPOI (Watts/m^2)	**2.81 ± 0.78**	**1.52 ± 0.55**	**<0.001**
AVO_2 diff (mL/dL)	**12.4 ± 4.8**	**9.3 ± 2.3**	**0.012**
$\Delta AVO_2diff/\Delta VO_2$ (min/dL)	4.0 ± 1.8	5.3 ± 3.6	0.193
$\Delta CO/\ \Delta VO_2$ (L blood/L O_2)	7.1 ± 3.6	9.8 ± 6.0	0.121

* Control data based on n = 14.

Abbreviations: O_2 –oxygen uptake, RER – respiratory exchange ratio, V_E – minute ventilation, BR – breathing reserve, HR – heart rate, SBP –systolic blood pressure, DBP – diastolic blood pressure, MAP – mean arterial blood pressure, CO – cardiac output, CI – cardiac index, SV – stroke volume, SVI – stroke volume index, AVO_2diff – arterio-venous oxygen difference, CPO – cardiac power output, CPOI – cardiac power output index

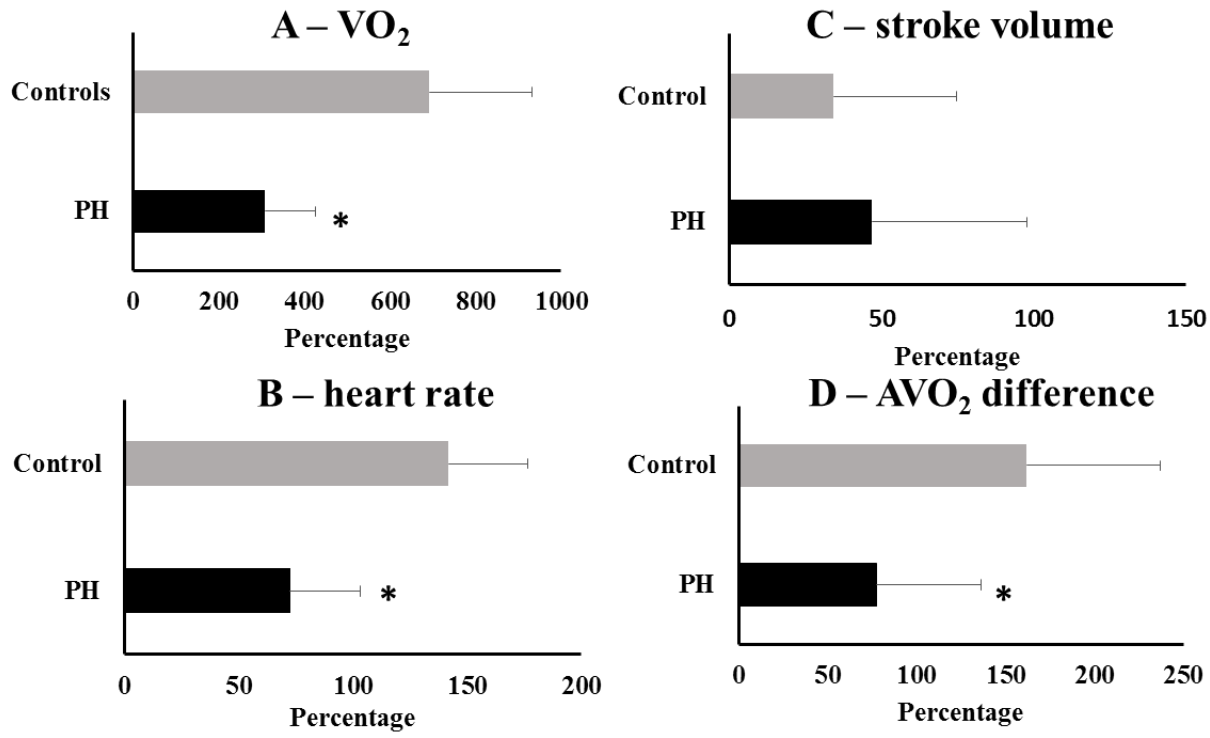
5.3.4 Integrated response of exercise haemodynamics

The reserve capacity of each component of VO_2 was assessed compared to resting values in percentages. In healthy controls, peak VO_2 increased by 679 ± 247 %. This increase was due to changes in heart rate of 141 ± 36%, stroke volume 34 ± 42% and AVO_2 diff 162 ± 79%. In PH subjects, peak VO_2 increased by 308 ± 120% due to increase in heart rate of 72 ± 32%, stroke volume 48 ± 52% and AVO_2 diff of 78 ± 62%. In both healthy controls and PH patients, the increase in AVO_2 difference made significant contribution to the increase in VO_2, with the percentage increase being the largest of the three components of VO_2. (Figure 19) There was correlation in absolute change in cardiac output as a function of oxygen uptake and was on par with controls, whereas there was no correlation between absolute increases in ΔAVO_2diff as a function of oxygen uptake in PH patients in comparison to controls. (Figure 20)

Partial R^2 values were obtained for each component of peak VO_2, including heart rate, stroke volume and AVO_2 diff after adjusting for age and gender. In healthy controls, peak VO_2 was related to AVO_2 diff ($R^2 = 0.397$, $p = 0.028$) but heart rate ($R^2 = 0.151$, $p = 0.211$) and stroke volume ($R^2 = 0.05$, $p = 0.467$) were not significant at maximum exercise. In PH patients, peak VO_2 was related to peak HR ($R^2 = 0.284$, $p = 0.033$), with a non-significant trend towards association with AVO_2 diff (R^2=0.212, $p = 0.073$) and no association with SV ($R^2 < 0.001$, $p = 0.922$).

Figure 19: The increase from baseline to peak exercise in percentage of VO_2 and each of its components in PH and control subjects.

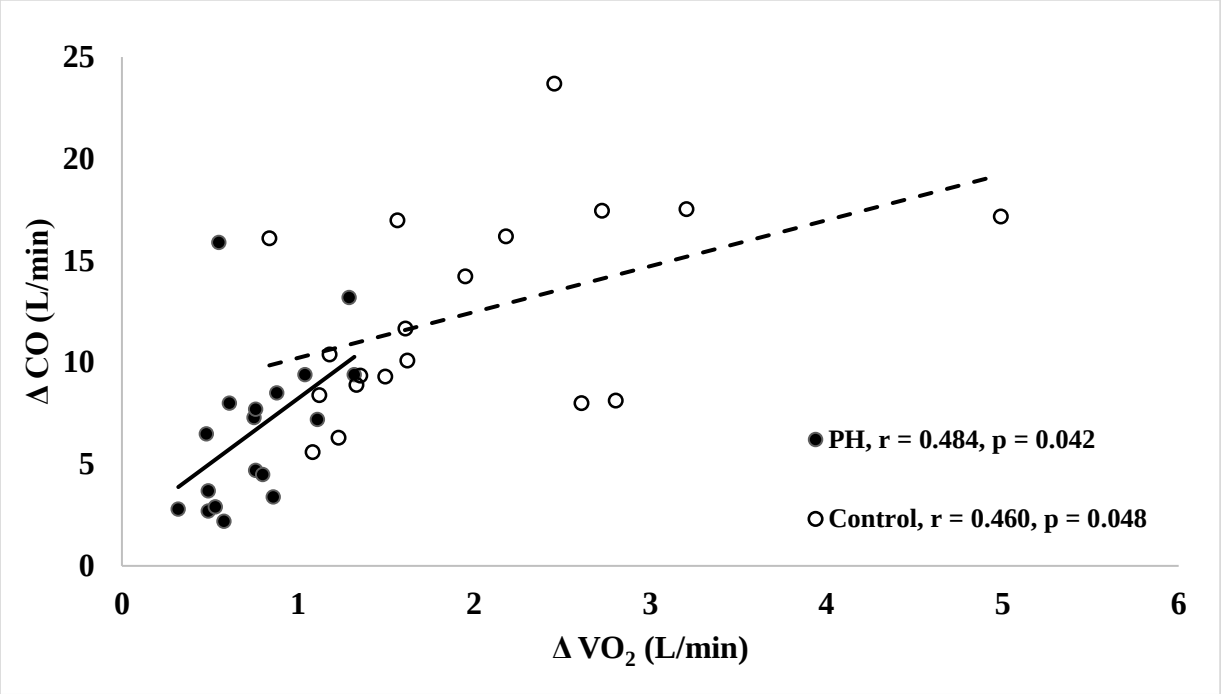
A) VO_2 B) heart rate C) stroke volume and D) AVO_2 difference. * P-value less than 0.05.



5.3.5 Fatigue, quality of life and exercise haemodynamic in PH

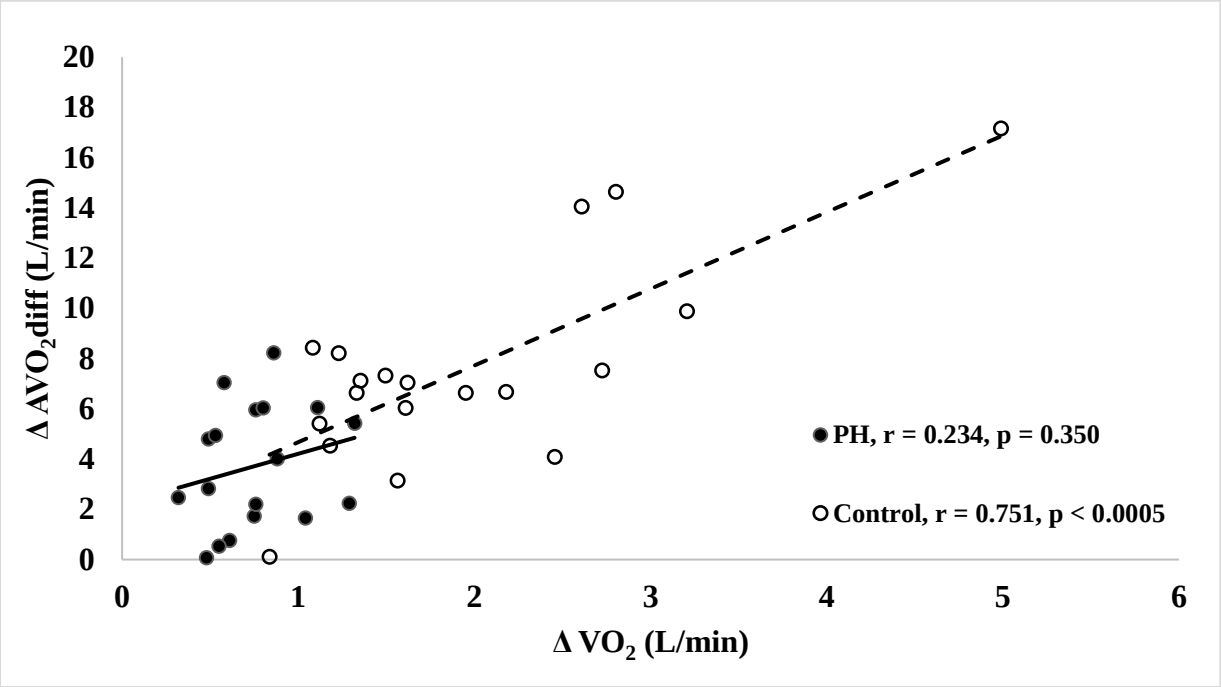
Patient quality of life and fatigue were significantly correlated ($r = 0.806$, $p < 0.001$). Quality of life scores did not correlate significantly with any of the peak exercise parameters. In the 18 PH patients, severe fatigue as deemed by FSS score >4 was evident in 11 with a mean of 4.3 ± 1.5. Fatigue levels showed significant negative correlation with percentage predicted heart rate ($r = -0.474$, $p = 0.047$) and peak AVO_2 diff ($r = -0.542$, $p = 0.020$). After adjusting for age and gender, only AVO_2 diff remained negatively correlated with fatigue ($r = -0.645$, $p = 0.007$). There was no significant correlation between fatigue scores and 6MWD, peak oxygen uptake, cardiac output, cardiac index, and NT-proBNP.

Figure 20: Change in cardiac output (ΔCO) as a function of oxygen uptake (ΔVO_2).



This showed steeper increase in PH patients (solid line, p = 0.042) than controls (dashed lines, p = 0.042).

Figure 21: Change in arterio-venous oxygen difference (AVO_2 diff) as a function of oxygen uptake (ΔVO_2)



This demonstrates that in PH patients (solid line, p = 0.350) have a less relative increase compared to controls (dashed line, p <0.001)

5.4 Discussion

In this study, continuous haemodynamic response to upright exercise was assessed using non-invasive methodology in pulmonary hypertension. The study showed that PH patients in comparison with matched healthy controls demonstrate 1) impaired central cardiac output response to exercise predominantly due to chronotropic incompetence, 2) impaired peripheral oxygen extraction despite reduced cardiac output at peak exercise, 3) lack of association between change in peak oxygen uptake and change in peripheral oxygen extraction, 4) that fatigue severity was associated with peak peripheral oxygen extraction. These findings suggest the potential important role of targeting peripheral limitations in PH to augment aerobic exercise capacity beyond central cardiac limitations.

Compared to healthy controls, PH patients had significant impairment in maximum exercise capacity as shown by reduced peak oxygen uptake. (Table 8) Pulmonary hypertension is marked by progressive exercise intolerance affecting quality of life and daily activity levels. (Fowler et al., 2012, Pugh et al., 2012) The body mass-adjusted peak VO_2 achieved in the current study is similar to reported literature values for PH patients, with a mean respiratory exchange ratio of 1.10 suggesting our patients exercised to exhaustion. (Deboeck et al., 2012, Sun et al., 2001, Ferreira et al., 2014). Peak VO_2 achieved on maximum exercise is determined by number of factors including cardiac output, peripheral oxygen extraction, patient effort or musculoskeletal problems. The strength in the current study is that stress testing was performed in the upright position with continuous measurement of cardiac output allowing for assessment of aerobic exercise capacity. Therefore, the relative contribution of the different components of oxygen uptake from rest to peak exercise was analysed to determine the factors affecting exercise intolerance in PH patients.

The study demonstrates impaired peak cardiac output in both absolute values and adjusted for body surface area. Using the NICOM®, the relative contribution of heart rate and stroke volume were able to be assessed from rest to peak exercise. There was evidence of chronotropic incompetence at peak exercise compared to controls, with impaired heart rate reserve. None of the patients were on beta blockers. Stroke volume index at rest and peak exercise were significantly lower in PH patients but there was no significant difference in relative percentage increase in stroke volume between the two groups from rest to peak

exercise. The study suggests impaired ability to augment heart rate in PH patients with exercise has the most significant contribution to cardiac output.

In previous studies, both chronotropic incompetence and lack of stroke volume augmentation in response to exercise limits cardiac output in PH. (Holverda et al., 2006) PH is associated with sympathetic over activity with impaired cardiac autonomic control that correlates with disease severity. (Wensel et al., 2009) Moreover, impaired recovery of heart rate after exercise has been associated with a worse exercise capacity and predictive of clinical deterioration. (Minai et al., 2012, Ramos et al., 2012) The significant difference in percentage increase in heart rate from rest to peak exercise between the groups as opposed to percentage increase in stroke volume suggest chronotropic incompetence is the predominant factor limiting cardiac output response to exercise in PH. (Figure 19). In support, withdrawal of beta-blockers in patients with portopulmonary hypertension leads to improved exercise capacity with increase in resting cardiac output driven by the rise in heart rate. (Provencher et al., 2006b) Studies have shown in PH, there is impaired ability of the right ventricle (RV) to increase contractility in response to submaximal exercise. (Spruijt et al., 2015) This has raised the possibility of whether inotropic medications will be ineffective and suggests that investigations into preventing maladaptive changes in the right ventricular are important in order restore contractility. (Spruijt et al., 2015) The impaired right ventricular contractile reserve and chronotropic incompetence could both be due to downregulation beta-adrenoreceptors in the myocardium in PH patients. (Bristow et al., 1992) The evidence suggests developing treatments that prevent right ventricular maladaptation and restoring chronotropic response to exercise would result in improved aerobic exercise capacity in PH.

Peripheral oxygen extraction (AVO_2 diff) was significantly reduced at peak exercise in PH patients compared to controls. Furthermore, the percentage increase in AVO_2 diff was significantly lower in PH subjects from rest to peak exercise. AVO_2 diff is a function of oxygen content and systematic oxygen extraction. Oxygen content in the blood is dependent on the haemoglobin level, oxygen saturation and partial pressure of oxygen. The current study findings are supported by previous evidence of impaired systematic oxygen extraction in PH patients using invasive methods in comparison to patients with left ventricle systolic dysfunction. (Tolle et al., 2008)

There are a number of potential explanations for these findings in this current study. Peripheral oxygen extraction is dependent on first, convective oxygen delivery to skeletal muscle, then passive diffusion of oxygen from the capillary to skeletal muscle mitochondria along a pressure gradient. (Wagner, 2012) Abnormalities present in skeletal muscle morphology and enzyme activities could in part, potentially explain the reduced AVO_2 diff including muscle fibre type switch, reduced enzyme oxidative capacity, decreased capillary to fibre ratios, alteration in pathways favouring protein breakdown leading to muscle atrophy and impairment in electrical-contraction coupling affecting muscle contractility. (Mainguy et al., 2010, Batt et al., 2014) Factors affecting oxygen diffusion from the capillaries to the muscle mitochondria may also determine in part reduced peripheral oxygen extraction as observed in heart failure with preserved ejection fraction. (Dhakal et al., 2015). The increased sympathetic activity observed in many PH patients, could lead to increased local muscle vasoconstrictor activity and impaired vasodilation required during exercise to accommodate the increased blood flow to skeletal muscle during peak exercise. In support of this, there is evidence of impaired skeletal muscle microcirculation present in PH. (Dimopoulos et al., 2013a) Another possible explanation is that potentially PH subjects could have terminated exercise prematurely before maximum peripheral oxygen extraction is achieved. All our patients demonstrated RER > 1.0 and the body-weight adjusted peak VO_2, which was similar to previous studies in PH. (Sun et al., 2001, Ehlken et al., 2016) Furthermore, the increase in AVO_2 diff with exercise is hyperbolic in both genders with increasing VO_2 values therefore the relative contribution to the absolute values obtained is likely to be minimal if there was poor effort.(Murias et al., 2013, Tolle et al., 2008) The mean haemoglobin in PH subjects was 14.2 ± 1.0gm/dL suggesting there was no evidence to suggest anaemia to confound the results. Due to the non-invasive nature of the study, direct measurement of oxygen saturation and partial pressure was not possible during exercise and must be factored into the interpretation of the results. However, within the population of patients with LV systolic dysfunction, have a higher than normal AVO_2 diff that becomes wider as exercise increases to compensate for the impaired cardiac output. (Shelton et al., 2010, Agostoni et al., 2000). Therefore at peak exercise even compensating for a greater exercise induced hypoxia would not explained the reduced AVO_2 diff observed in PH patients.

Further studies are warranted in understanding the mechanisms affecting peripheral oxygen extraction during exercise in pulmonary hypertension.

The mean score in the FSS scale suggests PH patients suffer from a high degree of fatigue and higher than literature reported values for healthy subjects. (Valko et al., 2008) Fatigue scores correlated strongly with PH specific patient-reported quality of life scores. Peak peripheral oxygen extraction values negatively correlates with fatigue severity after adjustments for age- and gender- in PH patients and was the only measured exercise parameter to do so. Fatigue is a concept with multidimensional components that is recognized by the World Health Organization to have profound effect on patients secondary to the disease. (de Vries et al., 2010) Studies have shown fatigue to be disabling symptom in chronic disease resulting in lower activity and engagement of patients. (de Vries et al., 2010) Fatigue is a common compliant among patients with PH. The observed association between fatigue and peripheral oxygen extraction is clearly in a small number of patients and further research is needed to confirm these preliminary findings. Nevertheless in chronic fatigue syndrome sufferers, peripheral oxygen extraction has been shown to be reduced compared to healthy participants with normal central cardiac response at peak exercise. (Vermeulen and Vermeulen van Eck, 2014) Importantly, peripheral muscle fatigue can be due impaired convective oxygen delivery leading to accumulation of metabolites generated through oxygen-independent energy pathways affecting electrical-contraction coupling performance in the skeletal muscle.(Amann and Calbet, 2008) Although objectively measured skeletal muscle fatigue is not the same as patient-reported fatigue levels affecting exercise performance, however, it is noticeable that 10-weeks of aerobic exercise training in PH is associated with reduced self-reported fatigue levels in patients.(Weinstein et al., 2013a) . Using 31P-MRS, in a small number of PH patients there is preliminary evidence of abnormal peripheral acid handling during exercise and this may contribute to skeletal muscle fatigue during exercise. (Chapter 3) Further mechanistic studies are needed to carefully dissect out the pathophysiology of fatigue affecting PH patients in order to achieve improved quality of life beyond what can be achieved through pharmacology

5.5 Limitations

There are a number of limitations to the study. The numbers of subjects was relatively small, with certain CPEX exercise parameters being available in only 14 control subjects. There were no available haemoglobin values for the control subjects although they were healthy

patients with no symptoms to suggest anaemia. The PH population were heterogeneous in terms of their underlying aetiologies, with potential variations in cardiac and pulmonary physiology affecting exercise performance. This however reflects real-world experience. The effect of exercise induced arterial hypoxaemia on AVO_2 diff could not be measured due to the non-invasive nature of the study. AVO_2diff can be measured directly during maximum upright exercise with placement of a right heart catheter and a radial arterial line. Therefore, serial measurements of arterial and venous samples during exercise measuring haemoglobin content, oxygen saturations and partial pressures would allow for more definitive understanding of the limitations to exercise in patients with pulmonary hypertension. However, invasive procedures have their inherent limitations including the risks of bleeding, infection and trauma to underlying structures. A number of the PH subjects (n = 12) were on anticoagulation as part of their treatment. Further, the presumption was made that during peak exercise, the majority of blood flow is diverted to the skeletal muscle and therefore, AVO_2 diff is influenced predominantly due to skeletal muscle oxygen extraction.

The study includes four patients with pulmonary arterial hypertension secondary to congenital heart disease. The use of non-invasive technology to assess cardiac output and intracardiac shunting affecting peak VO_2 could potentially be a limiting factor in the interpretation of the results. In paediatrics, the potential for NICOM as a more accurate way of measuring cardiac output than echocardiography in congenital heart disease is being explored as the technology is dependent on pulsatile blood flow causing phase shift in the current.(Sun et al., 2015) The use of NICOM has been shown to have good correlation with cardiac output assessed at rest during paediatric cardiac surgery in patients with congenital heart disease in comparison to Fick method. (Tirotta et al., 2017) In subgroup analysis of the 14 PH patients without congenital heart disease compared to controls, at peak exercise, there was significantly reduced peak cardiac output (11.5 ± 4.3 vs 18.3 ± 5.5 L/min, p = 0.001) and peripheral oxygen extraction (9.6 ± 2.4 vs 12.9 ± 4.6 mL/dL, p = 0.021). The percentage change from rest to peak in peripheral oxygen extraction remained significantly reduced in PH compared to control subjects (170 ± 71 vs 87± 65 %, p = 0.002). Fatigue severity adjusted for age and gender remained negatively correlated with AVO_2 diff (r = -0.679, p = 0.015). These findings suggest the inclusion of patients with congenital heart disease do not have bearing on the statistical findings of the study and would not affect the conclusions of the study.

5.6 Clinical Perspective

The study has a number of important implications for clinical practice. There needs to be a paradigm shift away from assessing resting cardiopulmonary haemodynamics to those that can be measured with exercise. Further, non-invasive cardiac output measurements have a number of important advantageous. Non-invasive technologies allow for the assessment of cardiac output with CPEX testing on an outpatient basis. Therefore it can be performed in selected patients within the clinic setting, prior to consideration of escalation of treatment. Right heart catheterisations (RHC) in most PH centres in the UK are only able to assess resting cardiopulmonary haemodynamic. Moreover, RHC carries inherent procedural risks with the procedure, as well as needing inpatient stay and monitoring after completion. CPEX on other hand, on maximum exercise in PH patients is relatively safe if the patient is adequately monitored. (Eloara V.M. Ferreira et al., 2016) The measurement of cardiac output in addition to conventional CPEX, would allow clinicians greater information in clinical decisions prior prescribing/escalating treatment that have significant cost-burden to the currently strained NHS budget.

The study also shows that peripheral oxygen extraction is impaired in PH patients and associated with perceived fatigue levels. Further research is needed to confirm these findings but offers another avenue to focus in order to improve exercise tolerance beyond cardiac output. This supports the notion that PH is a multi-systemic disease and focusing on purely on improving cardiopulmonary haemodynamics would result in the under-treatment of the patient. In addition, it would seem there is a lack of appreciation that chronotropic incompetence during stress has an important and significant impact in limiting cardiac output on exercise. Therefore further research in understanding and importantly correcting this impairment would seem fundamental in improving exercise capacity.

5.7 Conclusions

The current study shows that aerobic exercise capacity is affected by central and peripheral limitations during maximal exercise in patients with pulmonary hypertension. From rest to peak exercise, cardiac output increases in proportion to change in oxygen uptake, but there is

no clear change in peripheral oxygen extraction with oxygen uptake in PH subjects as seen in healthy subjects. Finally, peak AVO_2diff was the only exercise parameter to show association with patient-reported fatigue levels. Further carefully designed laboratory and clinical studies are needed to elucidate the mechanisms affecting peripheral oxygen extraction and fatigue in PH.

Chapter 6 Physical activity, Fatigue and Exercise capacity in Pulmonary Hypertension

6.1 Introduction

Pulmonary hypertension (PH) is a progressive debilitating condition leading to exercise intolerance, poor quality of life and premature death. (McLaughlin and McGoon, 2006) The last two decades has been marked by significant progress in the development of effective pharmacological targeted therapies that has revolutionised the management resulting in improved exercise capacity and survival. (Galie et al., 2013, Benza et al., 2012) Patients remain afflicted with symptoms that interferes with their daily activities and quality of life. (Mathai et al., 2016) Patients are assessed by monitoring their exercise capacity by 6 minute walk distance (6MWD), commonly used in many of the clinical drug trials in PH. (McLaughlin et al., 2009) Clinical assessments performed during hospital visits to monitor response to treatment and disease activity may not reflect real-world effect of the disease on the individual patient.

Physical activity during daily living is increasingly recognised as an important marker of long-term cardiovascular health. (Crichton and Alkerwi, 2014) PH patients have a lower level of activity compared to controls and spend a greater proportion of their time in sedentary behaviour. (Pugh et al., 2012, Mainguy et al., 2011) Exercise training has shown self-reported activity scores increase in PH patients. (Weinstein et al., 2013a) Physical activity as a biomarker of clinical response to pharmacological intervention has not been assessed in PH patients. Furthermore, the trend in activity level over a period of time has not been studied.

Accelerometers are the most commonly used method for the objective assessment of daily physical activity. The majority of these validated accelerometers measure daily energy expenditure or intensity of physical activity as measured by proprietary count. (Strath et al., 2013) More recently, accelerometers can record raw acceleration expressed in gravity (g) units from three orthogonal axes. This allows for greater control over data processing and for comparison between different accelerometers to be made. (van Hees et al., 2013) Accelerometers are non-invasive, easy to wear devices that can accurately assess activity levels during daily life. (Pavey et al., 2016)

The primary aim of this pilot study is to determine the change in physical activity with the initiation or addition of PH-targeted treatment in comparison to current standard of objective exercise response by using the 6MWD at baseline, three and six-months. The secondary aims are 1) to determine the change in physical activity over period of six months, 2) assess the relationship between physical activity and exercise variables and 3) assess the correlation between fatigue, quality of life and activity levels.

6.2 Methods

6.2.1 Study population

Fifteen patients with PH were recruited from the National Pulmonary Hypertension Service (Newcastle) and who held a clinical diagnosis of pulmonary hypertension. Patients with a new diagnosis or required optimisation of targeted therapies who were able to exercise without any adverse response were approached. Patients with other significant cardiovascular, pulmonary or peripheral comorbidities were excluded. The study was approved by the local regional ethics committee (Westminister - 15/LO/0144) and all participants provided written informed consent to participate in the study

All patients at baseline and three months following treatment change underwent baseline exercise capacity assessment by use of 6 minutes' walk distance (6MWD), cardiopulmonary exercise testing (CPEX) ± with non-invasive haemodynamic measurement, wearing a physical activity monitor, and completion of two questionnaires. All patients had their World Health Organization functional class (WHO FC) assigned and N-terminal pro-brain naturietic peptide (NT-proBNP) measured.

6.2.2 Physical activity assessment

Physical activity monitoring was undertaken by all participants at baseline, three and six months using a triaxial, raw accelerometer (GeneActiv, Unilever, UK) on their wrist continuously for 5-7 days for at least 3 valid weekdays and 2 valid weekend days. Valid days were counted as wear time ≥ 16hours per day. (Charman et al., 2016) From the raw data, physical activity were deemed as the mean acceleration (milli*g*) during the most active (ΔM5) and least active (ΔL5) five-hour period of each day and the difference between these periods (ΔM5L5). (Innerd et al., 2015) Daily sedentary - low and moderate-vigorous (MVPA) activity time from the acceleration was calculated based on metabolic equivalents (METS) from values derived from previous studies. (Hildebrand et al., 2014)

6.2.3 Exercise capacity assessment

Each participant with pulmonary hypertension completed symptom-limited CPEX testing using a step-protocol with breath-by-breath gas analysis performed at rest and during exercise (Medgraphics, St Paul, MN, USA) to measure oxygen consumption. Simultaneously with gas-exchange, central haemodynamic variables were measured at rest and during CPEX using the bioreactance method (NICOM®, Cheetah Medical, USA).

Peak VO_2 was highest O_2 uptake, averaged over last 30 seconds of exercise. Arterio-venous oxygen content difference, AVO_2 diff was obtained indirectly using the Fick method as the ratio between oxygen consumption and cardiac output. Maximum predicted heart rate was defined as 220 minus age in years.

6.2.4 Fatigue severity and quality of life

Each participant completed two questionnaires being fatigue severity scale (FSS) and quality of life (emPHasis-10) prior to exercise testing.

6.2.5 Statistical analysis

Analysis was performed using SPSS software package version 22 (Chicago, IL). All continuous data is presented as mean ± standard deviation. Normality of data was determined by Shapiro-Wilk test. Parameteric data analysed by paired sample T test and non-parametric data analysed by Wilcoxon signed rank test. Categorical data analysed using Fisher's exact test. Correlation was assessed either Pearson or Spearman testing. A p-value less than 0.05 was considered significant.

6.3 Results

6.3.1 Population characteristics

The baseline clinical characteristics of the 15 participants are provided in Table 9.

Table 9: Baseline Characteristics

	Patients
Age (years)	**58 ± 15**
Gender (M:F)	**5 :10**
Height (cm)	**165 ± 10**
Weight (kg)	**68 ± 14**
BSA (m^2)	**1.76 ± 0.22**
PH subtype	
- **IPAH**	**8**
- **PoPH**	**1**
- **PAH-CHD**	**5**
- **CTEPH**	**1**
WHO FC	
- **II**	**3**
- **III**	**12**
NT-proBNP, pg/ml	**892 ± 1015**
Haemoglobin (gm/dL)	**14.2 ± 1.0**
Haemodynamics[§]	
- **mPAP (mmHg)**	**42 ± 11**
- **sPAP (mmHg)**	**73 ± 24**
- **RAP (mmHg)**	**4 ± 3**
- **PCWP (mmHg)**	**6 ± 4**
- **PVR (Woods)**	**9.7 ± 4.3**
- **CO (L/min)**	**4.2 ± 1.2**
- **CI (L/min/m^2)**	**2.4 ± 0.8**
- **SvO_2, %**	**65 ± 8**
Baseline Treatment	
- **PDE5i**	**8**
- **PDE5i and ERA**	**3**
- **No Therapy**	**4**
Change in Treatment	
- **Initiated PDE5i**	**4**
- **Added ERA to existing PDE5i therapy[†]**	**8**
- **Switched ERA[§]**	**3**

Abbreviations: BSA – body surface area, IPAH – idiopathic pulmonary arterial hypertension, PoPH – portopulmonary hypertension, PAH-CHD – Pulmonary arterial hypertension secondary to congenital heart disease, CTEPH – chronic thromboembolic pulmonary hypertension, WHO FC – World Health Organization Functional class, NT-proBNP – N-terminal pro Brain Naturietic peptide, mPAP – mean pulmonary arterial pressure, sPAP – systolic pulmonary arterial pressure, RAP – right atrial pressure, PVR – pulmonary vascular resistance, CO – cardiac output, CI – cardiac index, SvO_2- mixed venous oxygen saturation, PDE5i – phosphodiesterase type 5 inhibitor, ERA – endothelin receptor antagonist

§ - switched from bosentan to macitentan † - macitentan was added to sildenafil

6.3.2 Physical activity and adherence

Fifteen patients wore the wrist-based activity monitor continuously for at least 5 days with only one patient failing to wear the monitor for the more than 16 hours a day. Therefore, the patient's results were removed from the final analysis. No patient reported adverse effects or discomfort with only one patient reporting the activity monitor was a nuisance. The average valid days per patient at baseline 7.2 ± 1.2 days, 3 months 8.5 ± 2.4 days and 6 months 13.3 ± 1.9 days. At baseline, time spent in MVPA on the weekday was 77 ± 73 and weekend 72 ± 64 minutes. At three months, time spend in MVPA on the weekday was 84 ± 76 and weekend 80 ± 76 minutes. There was strong correlation between baseline and three months in time spend in MVPA on weekday ($r = 0.927$, $p < 0.001$) and weekend ($r = 0.959$, $p < 0.001$).

6.3.3 Change in exercise capacity and physical activity with treatment

From baseline to three months, 6MWD increased significantly by 41 ± 37 m ($p = 0.001$), with significant improvement in WHO FC (2.8 vs 2.2, $p < 0.001$) and non-significant improvement in NT-proBNP (1015 ± 262 vs 584 ± 151 pg/ml, $p = 0.336$). In terms of physical activity, we noted no significant change in the most active 5 hours, least active 5 hours or the difference between them (ΔM5L5), with no significant change in time spend in sedentary activities. (Table 10) There was no correlation between Δ6MWD and ΔM5L5 ($r = 0.393$, $p = 0.147$) or ΔMVPA ($r = -0.236$, $p = 0.398$) from baseline to 3 months. The ΔM5L5 and time spend in

MVPA remained consistent with strong correlation between baseline, 3 and 6 month suggesting activity levels over this period of time in individual PH patients did not change. Figure 22 shows time spend in at different activity levels at the three time points categorised by acceleration in m*g*.

Table 10: Activity level classification at the three time points

	Baseline	**3 months**	**6 months**
Rest/active analysis (milli*g*)			
Mean ENMO	**23 ± 10**	**24 ± 11**	**22 ± 19**
M5	**42 ± 19**	**46 ± 19**	**41 ± 22**
L5	**5 ± 1**	**4 ± 1**	**5 ± 1**
ΔM5L5	**38 ± 18**	**41 ± 68**	**36 ± 22**
Activity intensity classification (min/day)			
$PA_{sedentary/low}$	**1364 ± 70**	**1357 ± 76**	**1369 ± 73**
$PA_{mod/high}$ (MVPA)	**74 ± 68**	**82 ± 58**	**70 ± 70**

Abbreviations: ENMO – Enclidean Norm Minus One, M5- most active five hours ENMO value, L5 – least active 5 hours ENMO value, PA – physical activity

6.3.4 Exercise capacity and physical activity

The study determined whether there was any correlation between current measures of exercise capacity and physical activity. There was no correlation between mean ENMO, ΔM5L5 or time spend in MVPA and peak oxygen uptake (VO_2), cardiac output, cardiac index or AVO_2 diff. There was a negative correlation between age and physical activity: mean ENMO ($r = -0.539$, $p = 0.038$) ΔM5L5 ($r = -0.600$, $p = 0.018$) and time in MVPA ($r = -0.618$, $p = 0.014$). There was no significant difference between the genders (M vs F) in terms of ΔM5L5 (27 ±10 vs 43 ± 19, $p = 0.108$) and MVPA (1407 ± 23 vs 1343 ± 76, $p = 0.092$), with no significant difference in age between the genders.

6.3.5 Quality of life, fatigue and physical activity

Patient reported quality of life scores did not correlate with any parameter of physical activity including ΔM5L5 ($r = -0.279$, $p = 0.371$) and time in MVPA ($r = -0.162$, $p = 0.565$). Fatigue severity scores also did not correlate with any parameter of physical activity including ΔM5L5 ($r = -0.170$, $p = 0.544$) and time in MVPA ($r = -0.162$, $p = 0.565$). Fatigue severity demonstrated non-significant decrease from baseline to three months (4.1 ± 1.4 vs 3.8 ± 1.3, $p = 0.149$). There was no correlation between Δ6MWD and ΔFSS score ($r = -0.127$, $p = 0.651$). (FiFigure 24) There was no correlation between individual itemized effect of fatigue on motivation, physical function and activities of daily living and any marker of physical activity measured objectively.

Figure 22: Acceleration categories according to three time points.

Data are Mean ± SD.

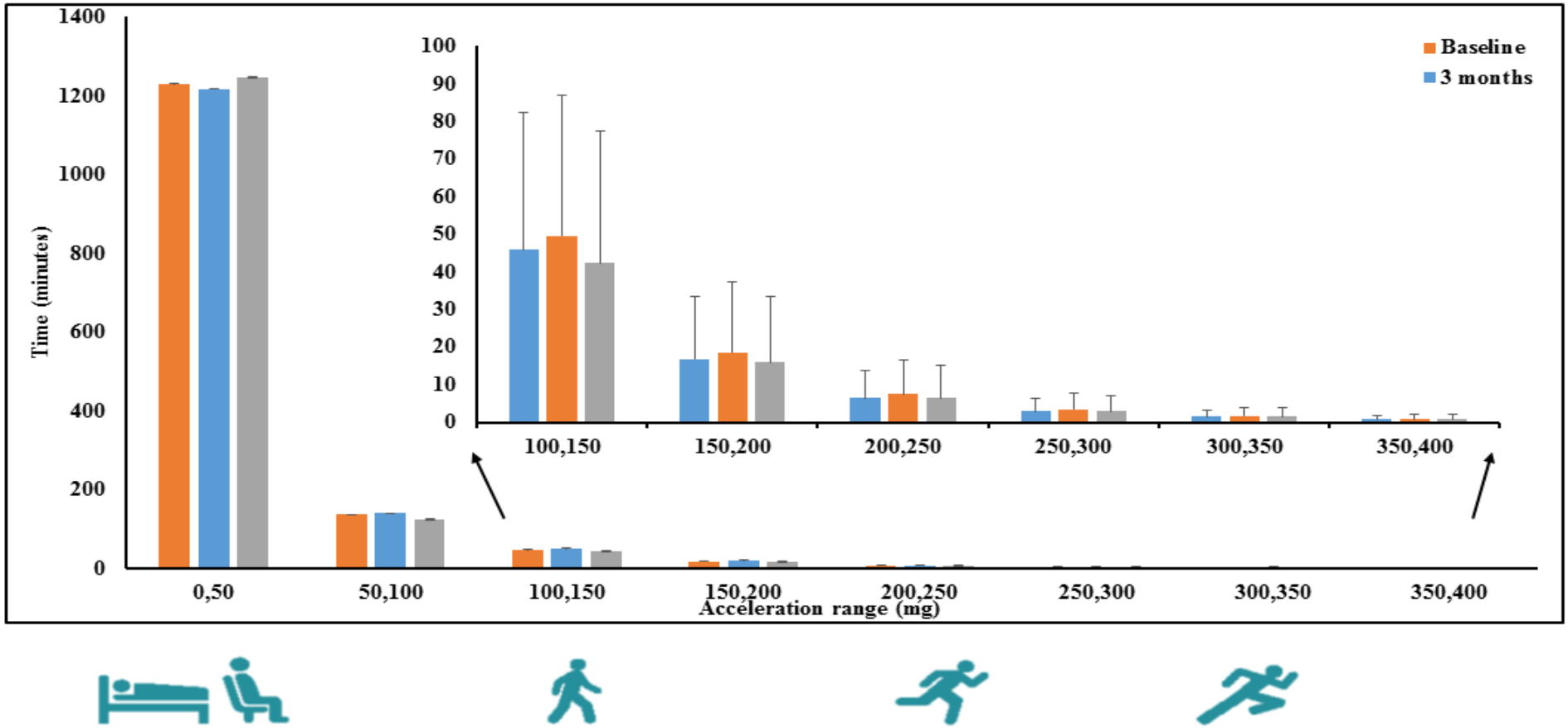


Figure 23: Graphical representations of the change in 6MWD and change in time spend in moderate to vigorous physical activity in minutes from baseline to three months.
($R^2 = 0.154$)

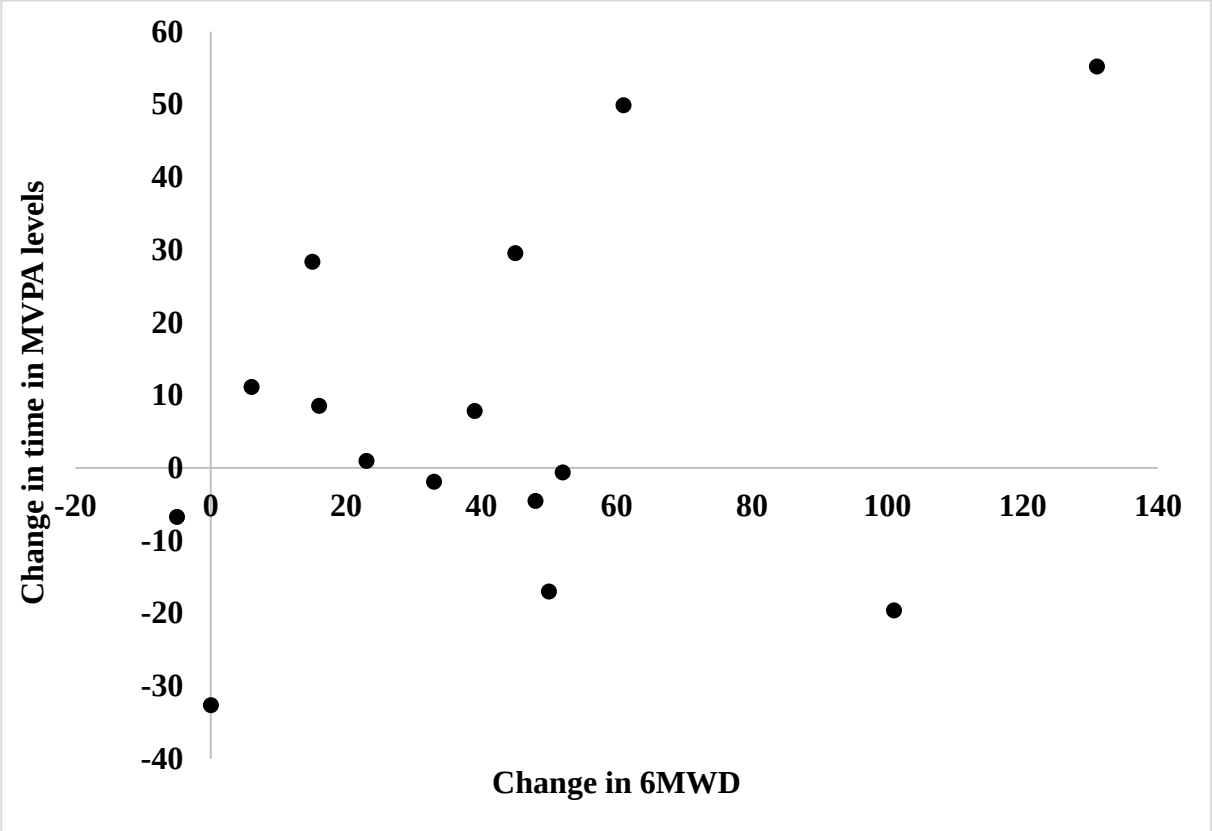
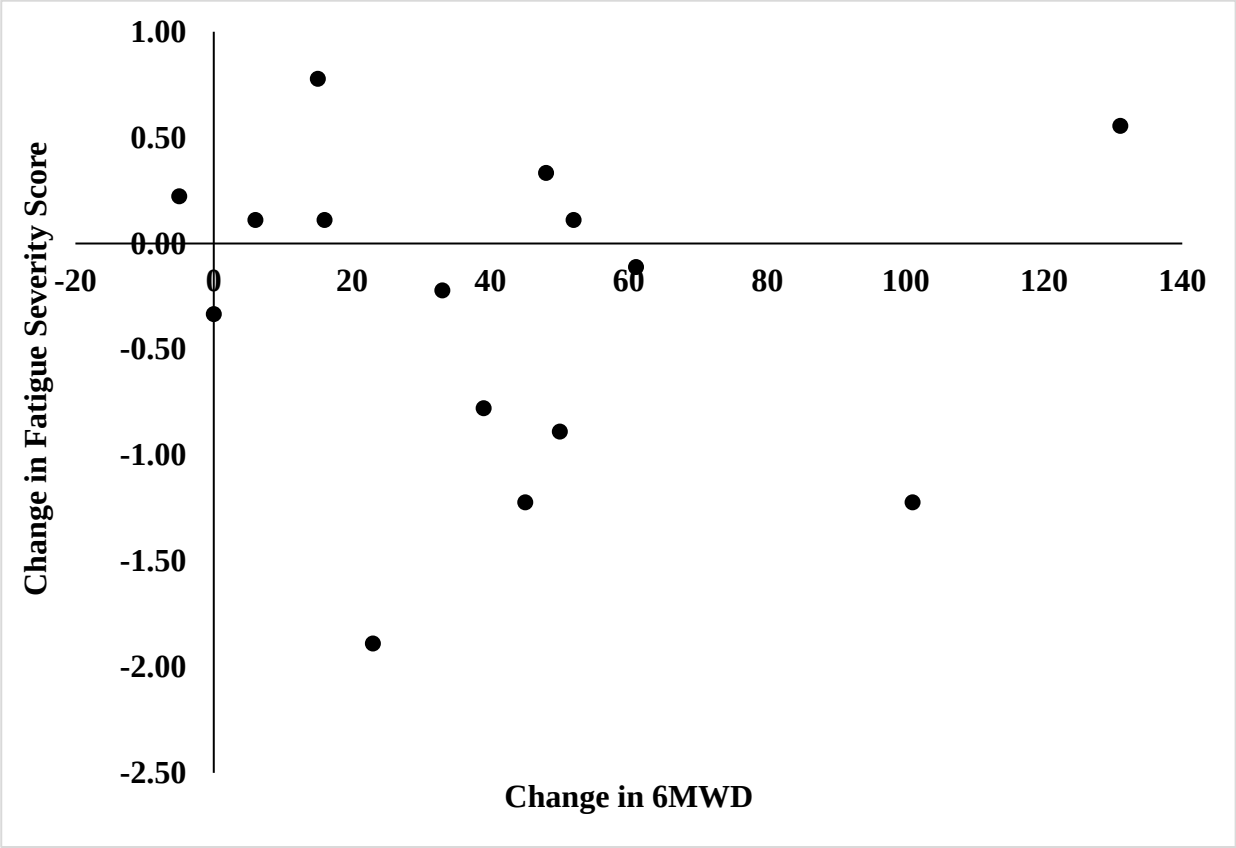


Figure 24: Graphical representation of change in 6MWD and change in fatigue severity score with treatment at three months.

($R^2 = 0.03$)



6.4 Discussion

In this pilot study, objective raw accelerometry data was collected over a period of six months in patients with pulmonary hypertension who underwent initiation or change in their targeted treatment. The study showed 1) there was no change in daily physical activity despite significant improvement in 6MWD from baseline to three months, 2) daily physical activity did not correlate with exercise and haemodynamic variables, 3) patient-reported quality of life and fatigue severity scores had no bearing on daily physical activity level and 4) physical activity level remains consistent without significant fluctuations over a period of six months. The study has shown preliminary evidence that pharmacological treatment for PH leading to improvement in exercise capacity does not result in change in daily physical activity. Furthermore, daily physical activity does not correlate with measured self-reported fatigue scores, exercise and haemodynamic variables.

The study showed no change in activity levels in acceleration measured in milli*g* from baseline to three months after initiation or change of treatment in targeted therapies. In this current study, raw accelerometery data was collected using wrist-worn devices allowing for greater control over the processing of the information by the user. The activity change was compared with currently accepted clinical biomarkers of disease severity where at three months there were significant improvements noted in WHO FC and 6MWD. Activity levels remained consistent and unchanged throughout the six month period. Furthermore, at baseline and three months, activity levels did not correlate with 6MWD. These preliminary results suggest habitual daily activity were not altered by pharmacological intervention in pulmonary hypertension.

Physical activity is a result of complex interaction of health, social and behavioural factors affecting daily function. There has been extensive research into physical activity in ischaemic heart disease, left ventricular failure and COPD. (Schonmann et al., 2015, Spruit et al., 2015, Backshall et al., 2015, Echouffo-Tcheugui et al., 2015) Research has focused on exercise intervention and increasing the proportion of daily time undertaking moderate-vigorous physical activity. In pulmonary hypertension, exercise training has been shown to be beneficial in terms of improving exercise capacity, quality of life and fatigue. (Mereles et al., 2006, Weinstein et al., 2013b) Whilst supervised exercise training in selected population with

PH is clearly beneficial, trying to improve daily physical activity level in the long term in patients with essentially a chronic disease affecting their daily life has received limited attention.

Using accelerometer that generates proprietary counts (steps per day), have shown that subjects with PH compared to healthy controls have less activity level in terms steps taken per day and spend greater proportion of their day undertaking sedentary activity. (Pugh et al., 2012) These studies also demonstrated daily physical activity correlated with WHO FC and 6MWD. (Mainguy et al., 2011, Pugh et al., 2012) There has been no study regarding the prognostic impact of low activity levels in PH or whether exercise intervention does lead to increase in long-term daily activity levels.

In the study, 6MWD distance improved with treatment, whilst daily physical activity levels demonstrated no such response. The 6MWD is the current accepted biomarker of exercise capacity along with clinical assessment to assess the effect of treatment on individual patients. Until recently, almost all drug trials involving PH have used the 6MWD as the primary outcome measure of effectiveness. The reason for the common use of the 6MWD is the low-cost, reproducibility and the ability to be performed by all centres involved in the care of PH patients by personnel without the requirement of significant experience. Maintaining an appropriate 6MWD has been associated with long-term survival.(Sitbon et al., 2002, Barst et al., 1996) Objectively measuring physical activity relays the real-world effect of the disease on the individual patient that is not reflected in submaximal exercise testing or clinical assessment. The 6MWD test has its inherent limitations including failing to adjust for the height of the patient, and the ceiling effect. (Gaine and Simonneau, 2013) Recently published multicentre clinical drug trials in PH, have moved away from the 6MWD and used the occurrence of clinical events as the primary outcome measure. (Pulido et al., 2013, Galie et al., 2015) The question arises as to whether measured daily activity levels reflects a better biomarker encompassing multi-dimensional factors that may reflect future clinical events and long-term prognosis in pulmonary hypertension is unclear. In COPD, low physical activity has been shown as independent factor associated with mortality and maintaining a high level of physical activity is associated with survival. (Waschki et al., 2011, Vaes et al., 2014) Physical activity is influenced by other factors beyond exercise tolerance including the effect of co-morbidities and increasing age, both of which are increasingly recognised and dominate

the cohort of patients seen with PH in the modern era. (Ling et al., 2012) In the current study, age is significant factor negatively associated with activity levels, therefore addressing the needs of the aging PH population is an important factor to consider and how best to keep them as active as possible is clearly an important subject.

The study analysed the effect of PH-targeted treatments on quality of life (emPHasis-10) and fatigue severity. Pharmacological treatment of PH failed to show any significant effect on both quality of life and fatigue severity in the present study. This may simply reflect the lack of adequate study power to detect to such a significant difference to both patient-reported questionnaires. At baseline, fatigue effect on daily and physical functioning did not correlate with any marker of activity levels. Mental and physical fatigue levels correlated with day-to-day variability in physical activity levels in a recent study of 15 patients with IPAH. (Matura et al., 2016) The authors also showed relationship between activity bouts and energy levels, although the current study failed to demonstrate such an association. (Matura et al., 2016) There is considerable difference between the study designs, including different questionnaires used to assess fatigue and QoL, with different accelerometer with differing final markers of activity levels. Furthermore, both studies enrolled relatively small number of patients (n = 15). The interrelationship between subjective symptoms and objectively measured activity levels in PH patients' needs further study, as our understanding of this area remains poor. Whilst the focus of recent clinical trials in PH has been on long-term morbidity and mortality outcomes, real-world clinical practice is focused in improving the symptoms and QoL on an individual basis. Many of these symptoms are beyond the reach of existing targeted therapies and therefore, how best to address these issues pertaining to patients with PH needs to be considered on an individual basis.

6.5 Limitations

The present pilot study is not without limitation. This is a pilot single-centre study, with a small number of participants, with a wide range of diagnosis, age and gender. The lack of improvement in activity levels with treatment and the absence of correlation of activity with peak exercise variables maybe due to the size of the study. Age as demonstrated in our study

has an impact on activity levels. There may be differing factors involved in improving habitual physical activity levels of patients of different age groups and gender beyond treatment of their pulmonary hypertension. Whilst our inclusion and exclusion criteria was far less restrictive than in multicentre randomised, placebo-controlled pharmaceutical drug trials in PH, the study population does reflect modern clinical practice in pulmonary hypertension in the United Kingdom. There was no control group to compare activity levels with PH subjects, however, that was not the aim of the study and has been previously undertaken by other authors. There was limited consideration of other factors that may affect activity levels including social circumstances, depression, anxiety and motivation levels. No one patient was taking medications for depression or anxiety. Currently the delineation between sedentary and low activity levels has not been clearly defined using the GenieActiv® monitor; therefore we cannot see a change from sedentary to low activity levels with treatment. In our population, reducing the time spend in sedentary behaviour and increasing the time in low rather than in moderate-vigorous physical activity is a more plausible response to intervention. Furthermore, minimum change in activity levels that reflects meaningful clinical benefit in patients with chronic disease has not been investigated using raw accelerometer data.

6.6 Clinical Perspective

The study failed to show any consistent improvement in habitual physical activity with treatment despite improvement in other accepted clinical and biochemical biomarkers. Therefore alternative ways to improve activity levels in PH patients is needed beyond pharmacological therapy. Exercise rehabilitation with an educational component to increase activity levels and improve symptoms is a possibility that could address some of the needs of PH patients. The recent European Society of Cardiology guidelines recommend aerobic exercise training under supervision in selected PH patients. (Galie et al., 2016) Exercise training improves exercise capacity, cardiopulmonary haemodynamics and quality of life. (Grunig et al., 2012a, Ehlken et al., 2016, Yuan et al., 2015) Potentially, exercise could help to reduce perceived fatigue levels and increase activity levels. (Weinstein et al., 2013b) Further research is needed, in order develop exercise programmes that could be tailored to

individual needs that is both safe and efficacious to be undertaken locally or at home, without the requirement for inpatient stays that was a requisite for many of the studies on exercise training in PH. From the chronic obstructive pulmonary disease pulmonary rehabilitation studies, it should be noted that patients fail to maintain their activity levels in their home environment after the sessions finishes. Current financial and resource limitations within the National Health Service would mean inpatient stays for exercise training wouldn't be feasible beyond the remit of research. Therefore, the focus should be on research that can be initiated and monitored within the outpatient setting.

There has been a significant shift in the last 20 years to improve physical activity in the general population. Strategies to improve daily physical activity in PH patients may be more successful than in the general population due to their frequent interaction with health care professionals. This maybe include the monitoring physical activity at home with commonly available accelerometers, continuous education providing reinforcement and further research to understand prognosis as a result of differing activity levels but well defined patients.(Tuso, 2015)

6.7 Conclusions

This pilot study shows that objectively measured daily physical activity do not change to reflect changes in exercise capacity or WHO FC with treatment. Activity levels remained consistent throughout the study period of six months, with no correlation with fatigue or QoL scores. Activity levels did not correlate with variables of maximum exercise testing. Further larger studies are needed into first validating accepted measurements of activity levels and patient-reported fatigue severity questionnaires in PH. This can be used in order to investigate different interventions to address daily physical activity and perceived fatigue by patients. However, data from this pilot study suggest that clinical care teams and research investigators should be aware of the decoupling of physical capacity (6MWT) and physical function (everyday physical activity) in therapeutic trials in PH.

Chapter 7 General Discussion

The work presented in this thesis attempts to further our understanding of the role of skeletal muscle function, peripheral oxygen extraction and habitual physical activity in PH. This has included the application of ^{31}P-MRS to assess *ex*-vivo skeletal muscle bioenergetics, skeletal muscle biopsy to determine *in vitro* mitochondrial function, peripheral oxygen extraction limitations to maximal exercise capacity and the effect of targeted therapy on habitual daily physical activity levels. PAH is a rare, heterogeneous disease with varying aetiologies, where individual patients present to clinical service at differing stages of severity. Developing treatments acting beyond the pulmonary vasculature is important as we begin to understand the concept of PAH being a multi-systemic disease. Recent studies have demonstrated convincing evidence of the benefits of exercise training for improving aerobic exercise capacity and quality of life in stable patients established on targeted therapies. In order to understand further the limitations to exercise, basic science and clinical studies were undertaken.

Assessing skeletal muscle bioenergetics and *ex*-vivo mitochondrial function to stress (exercise) is important in determining altered energy generation and acid handling. Chapter 3 uses ^{31}P-MRS in a pilot study of six patients with idiopathic pulmonary arterial hypertension. There have not been any previous studies employing ^{31}P-MRS to assess skeletal bioenergetics in PAH. From spectroscopy analysis and compared to healthy control values, there was evidence of an increase in the time taken for phosphocreatine recovery. This effect is most likely due to limitations of oxygen delivery as a result of the underlying PAH physiology rather than skeletal muscle mitochondrial dysfunction. There was no decrease in the time of pH recovery to suggest rapid proton efflux, further supporting the evidence against mitochondrial dysfunction. The results of this study indicate normal skeletal muscle mitochondrial function in PH.

There was a preliminary signal of altered muscle acid handling during exercise with significantly lower pH during exercise and increase in pH recovery times. Altered peripheral muscle acid handling is associated with perceived fatigue levels in other chronic conditions such primary biliary cirrhosis, with links to autonomic function controlling the sodium/proton antiporters. There is some published evidence of impaired autonomic function in PAH and

the question arises as to whether this could affect muscle acid handling leading to fatigue so commonly reported by patients on exertion. This would require carefully considered basic clinical studies to determine if autonomic dysfunction has a significant effect on acid handling in PH in the skeletal muscle. Furthermore, what is the role of altered acid handling has on the development of muscle fatigue and exercise limitation in PAH remains unexplained.

In order to corroborate the results from ^{31}P-MRS, in chapter 4 *in vitro* analysis of skeletal muscle mitochondrial oxidative phosphorylation protein abundance and function using established and state-of-the-art techniques were undertaken. Quadruple immunofluorescence (QF) allows for assessment OXPHOS subunit expression and abundance within each individual myofibre with adjustment for mitochondrial-mass. Histochemistry showed normal complex II and complex IV function using a more subjective approach, whilst QF showed consistently normal complex I and IV subunit protein abundance after adjustment for mitochondrial mass. These results support the findings from chapter 3 that intrinsic mitochondrial function in the skeletal muscle of PAH patients' is normal, and therefore do not limit exercise capacity.

There are certainly changes within the skeletal muscle in PAH patients that may contribute to fatigue and difficulties of performing everyday tasks. This includes decrease in type 1 fibre expression, capillary to muscle fibre ratio, imbalance between pathways related to muscle protein atrophy and synthesis as well as altered intrinsic muscle contractility. These changes have an impact on functional assessments as evident by a decrease in muscle strength and correlation with exercise capacity. There are many studies within the literature to support the role of exercise training in improving exercise tolerance. Out of these, only one randomised study so far supports the notion that exercise can help to stem and reverse the skeletal muscle changes by increasing the expression of oxidative enzymes and muscle capillarity in PAH. All studies involving analysis of muscle biopsies are limited by small number of participants. This is inevitable due to the strict inclusion criteria to avoid patients with confounding co-morbid conditions as well as the invasive nature of the study prohibiting the recruitment of patients.

The impairments to exercise in PAH are evidently upstream from skeletal muscle mitochondria. Therefore factors affecting oxygen delivery to the exercising skeletal muscle needs further clarification, including the role of impaired cardiac output and peripheral oxygen extraction. Significant focus of research in PAH has been on the right ventricle adaptation to chronic pressure overload. There has been some research to assess cardiac output using right heart catheterisation and supine exercise testing. This has its inherent limitations of failure to recruit and exercise to maximal performance using large muscle mass that is representative of everyday living. Non-invasive haemodynamic monitoring allows for cardiac output to be measured continuously and in conjunction with oxygen uptake during cardiopulmonary exercise testing. Therefore, allowing for an indirect assessment of peripheral oxygen extraction using Fick principle at maximum exercise.

Chapter 5 demonstrates evidence of impaired chronotropic response and peripheral oxygen extraction due to maximal upright exercise. Whilst cardiac output in response to exercise does improve in response to treatment and exercise training in PAH, limited work has been undertaken as to whether peripheral oxygen extraction has a significant role in limiting exercise. The reasoning for the impaired peripheral oxygen extraction would require a greater understanding of the pathophysiology including the role of autonomic function, oxygen diffusion capacity and perfusive oxygen delivery in the peripheries of PAH patients. The non-invasive nature of the current study does limit our interpretation due to the inability to accurately assess partial pressure of oxygen and exercise induced desaturation during testing.

To study the role of blood flow, blood pressure, and temperature to the peripheral muscle mass during exercise can be achieved through the use of femoral arterial and venous lines but recruitment into such studies maybe difficult even on the assumption that ethical approval is obtained. However, this would allow for a greater understanding of the factors affecting peripheral limitations to exercise in PAH. Whilst such invasive tests cannot be undertaken in routine clinical practice, developing the role of non-invasive methods to assess central haemodynamics in response to treatment is important. The NICOM® technology is easy to use and validated to assess cardiac output during cardiopulmonary exercise testing, therefore could be transferred into routine clinical practice pending clear evidence of benefit in the management of patients.

Finally, Chapter 6 assesses the effect of targeted PAH treatment on daily activity levels using raw accelerometer data. This pilot study demonstrates a lack of significant effect of targeted treatment on activity levels and shifting patients from sedentary to low and moderate-vigorous activity levels. Leading an active lifestyle has come to be recognised as an important factor in the overall health of a person. Whilst there are many factors affecting activity, PAH patients have reduced activity levels and spend proportionally greater amount of time performing sedentary tasks compared to controls. Changing this behaviour would seem unlikely with pharmaceutical agents. The study also showed a lack of treatment effect on patient-reported fatigue levels. The study has shown that in PAH, fatigue and quality of life are closely correlated, therefore treatments to improve fatigue in PAH patients would seem appropriate. To achieve such a desired effect education and regular exercise training are potential ways to address both physical inactivity and fatigue. Clearly further research is needed to prove that exercise can address fatigue and activity levels in a heterogeneous population with PAH that reflects everyday clinical practice. In addition any exercise prescription must be safe to be carried out without supervision in order to be realistically achievable in the long-term.

In summary, this thesis increases our understanding to the limitations of exercise capacity in PAH beyond the pulmonary vasculature. Impaired oxygen delivery by means of diminished cardiac output no doubts plays a key role, but limited peripheral oxygen extraction could be a contributory mechanism. Current PAH therapies would seem ineffective to address factors such as fatigue and activity levels. Therefore, there is a fundamental need to develop alternative additional treatments to address symptoms of PAH to improve exercise capacity, fatigue and daily activity levels in order to achieve a more successful holistic management of patients with an incurable and life-limiting disease.

Chapter 8 Appendices

8.1 Appendix A – Fatigue Questionnaire

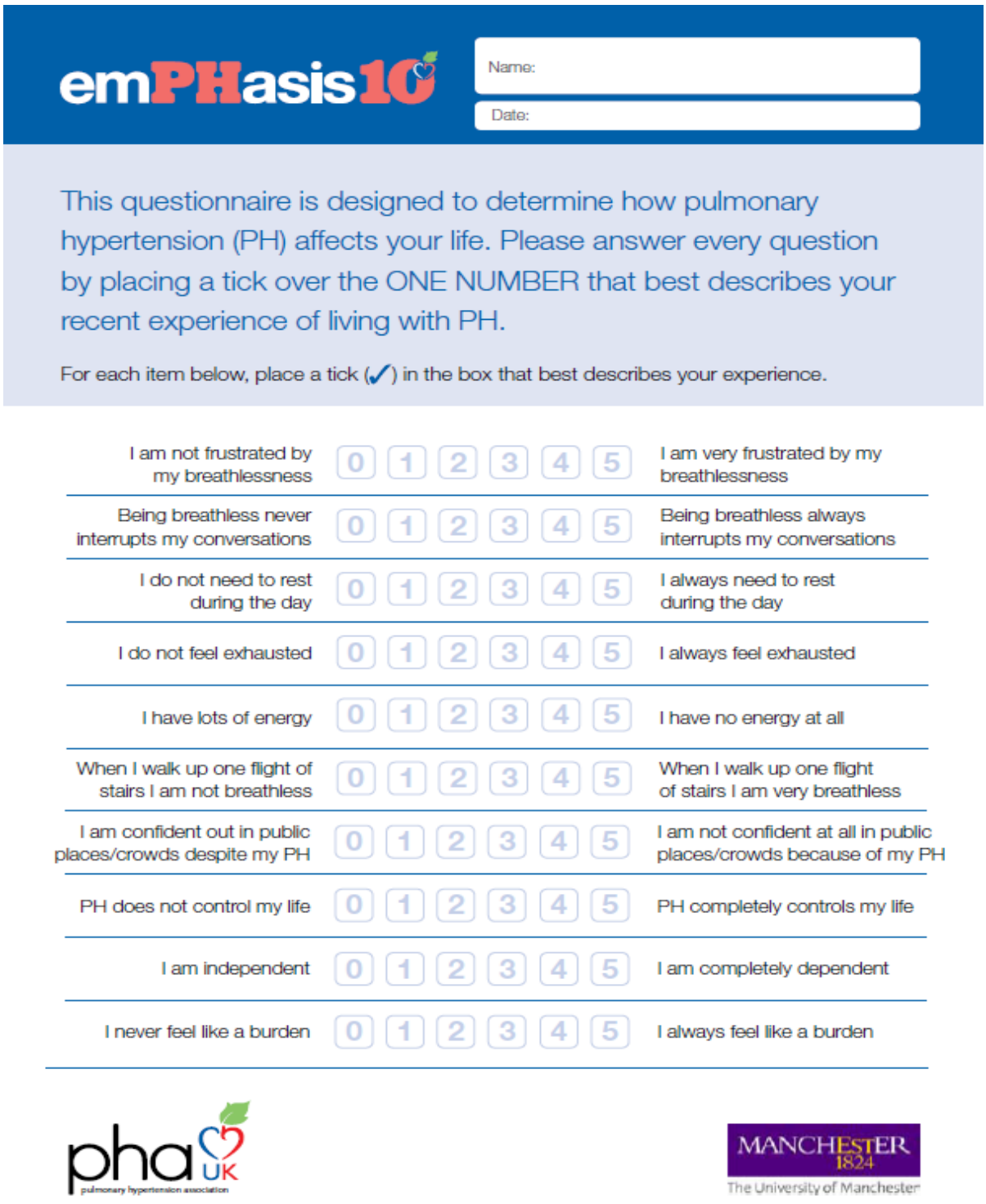
Date: **Name:**

Please circle the number between 1 and 7 which you feel best fits the following statements. This refers to your usual way of life within the last week. 1 indicates "strongly disagree" and 7 indicates "strongly agree."

Read and circle a number	Strongly Disagree → Strongly Agree						
1. Exercise brings on my fatigue.	1	2	3	4	5	6	7
2. I am easily fatigued.	1	2	3	4	5	6	7
3. Fatigue interferes with my physical functioning.	1	2	3	4	5	6	7
4. Fatigue causes frequent problems for me.	1	2	3	4	5	6	7
5. My fatigue prevents sustained physical functioning.	1	2	3	4	5	6	7
6. Fatigue interferes with carrying out certain duties and responsibilities.	1	2	3	4	5	6	7
7. Fatigue is among my most disabling symptoms.	1	2	3	4	5	6	7
8. Fatigue interferes with my work, family or social life.	1	2	3	4	5	6	7
9. My motivation is lower when I am fatigued	1	2	3	4	5	6	7

Total Score:

8.2 Appendix B – emPHasis-10 Questionnaire



emPHasis10

Name:

Date:

This questionnaire is designed to determine how pulmonary hypertension (PH) affects your life. Please answer every question by placing a tick over the ONE NUMBER that best describes your recent experience of living with PH.

For each item below, place a tick (✓) in the box that best describes your experience.

I am not frustrated by my breathlessness	0 1 2 3 4 5	I am very frustrated by my breathlessness
Being breathless never interrupts my conversations	0 1 2 3 4 5	Being breathless always interrupts my conversations
I do not need to rest during the day	0 1 2 3 4 5	I always need to rest during the day
I do not feel exhausted	0 1 2 3 4 5	I always feel exhausted
I have lots of energy	0 1 2 3 4 5	I have no energy at all
When I walk up one flight of stairs I am not breathless	0 1 2 3 4 5	When I walk up one flight of stairs I am very breathless
I am confident out in public places/crowds despite my PH	0 1 2 3 4 5	I am not confident at all in public places/crowds because of my PH
PH does not control my life	0 1 2 3 4 5	PH completely controls my life
I am independent	0 1 2 3 4 5	I am completely dependent
I never feel like a burden	0 1 2 3 4 5	I always feel like a burden

pha UK
pulmonary hypertension association

MANCHESTER 1824
The University of Manchester

8.3 Appendix C - Patient Consent Form Study

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Newcastle upon Tyne
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CONSENT FORM for participants

Title of Study: Skeletal Muscle Mitochondrial Function in Pulmonary Hypertension

Researchers: Dr S Sithamparanathan, Professor R Taylor, Dr G Gorman, Professor P Corris

Please initial box

1. I confirm that I have read and understand the information sheet date 14th January 2014 (Version 1.1) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I have the right to withdraw my data from the study any time before and up to 24 hours following assessment, without giving reasons and without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS Trust, where it is relevant to my part in this research. I give permission for these individuals to have access to my records. I agree to this research being recorded in the Newcastle Upon Tyne Hospitals NHS Trust clinical notes by authorised staff. ☐
4. I understand and agree that genetic analysis will be undertaken on the muscle samples. ☐
5. I agree to my General Practitioner being informed of my participation in the study. ☐
6. I agree for any surplus, anonymous samples of muscle that are collected as part of this study to be transferred to a REC approved tissue storage bank and used for future research. (If you prefer not to consent for this it will not preclude your participation in the main study). ☐
7. I agree to take part in the above study. ☐

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

Consent Form Version 1.2, 5th February 2014

8.4 Appendix D – Patient Consent Form Study 2

Institute of Cellular Medicine
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The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

CONSENT FORM for participants

Title of Study: Habitual Physical Activity and Exercise Capacity in Pulmonary Hypertension

Researchers: Dr S Sithamparanathan, Dr D Jakovljevic, Dr G Gorman, Professor M Trenell, Professor P Corris

Please initial box

1. I confirm that I have read and understand the information sheet date ..... (Version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I have the right to withdraw my data from the study any time before and up to 24 hours following assessment, without giving reasons and without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS Trust, where it is relevant to my part in this research. I give permission for these individuals to have access to my records. I agree to this research being recorded in the Newcastle Upon Tyne Hospitals NHS Trust clinical notes by authorised staff. ☐
4. I agree to my General Practitioner being informed of my participation in the study. ☐
5. I agree to take part in the above study. ☐

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

Consent Form Version 1.0, 12th December 2014

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