Mechanisms of exercise intolerance in chronic heart failure and type 2 diabetes mellitus

Jack Oliver Garnham

Submitted in accordance with the requirements for the degree of

Doctor of Philosophy

The University of Leeds

Faculty of Medicine and Health

School of Medicine

Leeds Institute of Cardiovascular and Metabolic Medicine

September 2018

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Manuscripts arising from this thesis:

Gierula J, Paton MF, Lowry JE, Jamil HA, Byrom R, Drozd M, **Garnham JO**, Cubbon RM, Cairns DA, Kearney MT & Witte KK (2018). Rate-response programming tailored to the force-frequency relationship improves exercise tolerance in chronic heart failure. *JACC Heart Fail*, **6**(2), 105-113.

Ferguson C, Birch KM, **Garnham JO** & Witte KK (2017). Exercise training: The under developed elixir vitae of chronic disease? *Diab Vasc Dis Res*, **14**(2), 113-115.

Abstracts arising from this thesis:

Garnham JO, Boyle JP, Roberts LD, Peers C, Kearney MT, Bowen TS & Witte KK (2018). Diabetic heart failure patients demonstrate a mitochondrial complex I dependent impairment in skeletal muscle. *FASEB J*, **32**(S1): 903.10.

Allen L, Jamil HA, Gierula J, Paton MF, **Garnham JO**, Kearney MT, Bowen TS & Witte KK (2015). Factors affecting exercise capacity in diabetic heart failure patients. *Circulation*, **132**(S3): A19397.

Acknowledgements

First and foremost I wish to thank my primary supervisor Dr Klaus Witte for his unrelenting passion, enthusiasm, and commitment to both myself and this project. His inimitable ebullience has both inspired and motivated me throughout this PhD and I shall always be grateful for his persistent belief in my potential, which has flourished under his careful support and guidance. Furthermore his aphoristic musings on life have left an indelible mark on me and I believe that adopting a similar philosophy will allow me to successfully progress my career and develop further as an individual. I will always retain many fond memories of our times together in the "cath lab" and hope to maintain a close personal and professional relationship with him in the future.

I wish to extend this gratitude to my three co-supervisors, Dr John Boyle, Professor Stuart Egginton, and Dr Scott Bowen, all of whom provided invaluable scientific insight throughout this project. Their input has successfully developed my research acumen, critical thinking skills, and scientific writing. I am particularly grateful for their patience, allowing me the time and freedom to develop my laboratory techniques at my own pace.

Additionally, I wish to thank Professor David Beech and the Multidisciplinary Cardiovascular Research Centre (MCRC) within the Leeds Institute of Cardiovascular and Metabolic Medicine (LICAMM) for giving me the opportunity to undertake this PhD and funding me for four years. I wish to also thank both Dr Carrie Ferguson and Dr Lee Roberts, who have provided timely and appropriate feedback throughout this PhD. Furthermore I also acknowledge the support of

Abstract

Chronic heart failure (CHF) and type 2 diabetes mellitus (DM) remain primary causes of mortality. Furthermore ~25-30% of CHF patients have DM, and these diabetic heart failure (D-HF) patients have an adverse prognosis. However, as yet, very few studies have examined the D-HF population in comparison to matching CHF, DM, and control patients. Therefore the current thesis described four experimental studies examining exercise intolerance and skeletal muscle abnormalities in these patient populations.

The first study of this thesis employed a novel exercise test (RISE-95) to better evaluate exercise intolerance in CHF and D-HF patients. This study confirmed previous clinical findings indicating that D-HF patients have a lower peak pulmonary oxygen uptake ($\dot{V}O_{2peak}$). Furthermore this study also demonstrated that nearly half of the cohort acutely increased $\dot{V}O_{2peak}$, the mechanism of which may elucidate potential therapeutic targets.

To examine potential mechanisms contributing to exercise intolerance, the second study investigated skeletal muscle mitochondrial function in chest muscle biopsies obtained from control, DM, CHF and D-HF patients. This study identified that D-HF patients exhibit both quantitative and qualitative mitochondrial impairments, with the latter residing at complex I. The next study corroborated these findings in leg biopsies from the same patients.

The final study uncovered potential mechanisms possibly contributing to skeletal muscle mitochondrial impairments, including an increased mitochondrial reactive oxygen species (ROS) production and downregulated expression of key mitochondrial genes. Furthermore this study revealed that D-HF patients exhibit a type II fibre-specific atrophy and capillary rarefaction.

Collectively, these studies expand our current knowledge regarding exercise intolerance in D-HF patients and how skeletal muscle impairments may contribute to the worse symptoms and outcomes seen in this growing population. The findings from this thesis are expected to guide future research endeavours, which may identify potential therapeutic targets by which exercise intolerance may be ameliorated in these patients.

Table of Contents

List of Tables

List of Figures

Abbreviations

^{31}P MRS	^{31}phosphorous magnetic resonance spectroscopy
ACEi	angiotensin converting enzyme inhibitor
ADP	adenosine diphosphate
AF	atrial fibrillation
Ang II	angiotensin II
ANOVA	analysis of variance
ARB	angiotensin receptor blocker
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BCA	bicinchoninic acid
β-HAD	β-hydroxyacyl CoA dehydrogenase
B_f	breathing frequency
BIOPS	relaxing and biopsy preservation solution
BMI	body mass index
BNP	brain natriuretic peptide
BSA	bovine serum albumin
Ca^{2+}	calcium
CABG	coronary artery bypass graft
CD	capillary density
C:F	capillary to fibre ratio
CHF	chronic heart failure
CI_{95}	95% confidence interval
CO_2	carbon dioxide
CoA	coenzyme A
CPX	cardiopulmonary exercise
CRT	cardiac resynchronisation therapy
DCM	dilated cardiomyopathy
DM	type 2 diabetes mellitus
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
E_{I+II}	complex I+II substrates in the maximal ETS state
E_{II}	complex II substrates in the maximal ETS state
eGFR	estimated glomerular filtration rate
ETS	electron transfer system
$FADH_2$	reduced form of flavin adenine dinucleotide

FCCP	carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone
FCR	flux control ratio
FCSA	fibre cross-sectional area
FOXO	forkhead box O
H_2O	water
H_2O_2	hydrogen peroxide
HbA1c	glycated haemoglobin
HFrEF	heart failure with reduced ejection fraction
HFpEF	heart failure with preserved ejection fraction
HR	heart rate
HRP	horseradish peroxidase
ICD	implantable cardioverter defibrillator
L_I	complex I substrates in the Leak state
LCD	local capillary density
LCFR	local capillary-to-fibre ratio
LT	lactate threshold
LVEF	left ventricular ejection fraction
LVIDd	left ventricular internal diameter at diastole
LVSD	left ventricular systolic dysfunction
MaFbx	muscle atrophy F-box
MHC	myosin heavy chain
MiR05	mitochondrial respiration medium
MuRF1	muscle RING finger 1
NADH	reduced from of nicotinamide adenine dinucleotide
NT-proBNP	N-terminal prohormone of BNP
NYHA	New York Heart Association
O_2	oxygen
$O_2^{\cdot-}$	superoxide anion
OXPHOS	oxidative phosphorylation
P	statistical significance value
P_I	complex I substrates in the OXPHOS state
P_{I+II}	complex I+II substrates in the OXPHOS state
PBS	phosphate buffered saline
PCr	phosphocreatine
PGC-1α	PPARγ coactivator-1α
pH	acid/base scale

P_i	inorganic phosphate
PPM	permanent pacemaker
$\dot{Q}$	cardiac output
R^2	coefficient of determination
RAAS	renin-angiotensin-aldosterone system
RCR	respiratory control ratio
RER	respiratory exchange ratio
RI	ramp-incremental
RISE	ramp-incremental step-exercise
ROS	reactive oxygen species
ROUT	robust regression and outlier removal
ROX	residual oxygen consumption
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
SE	step-exercise
SEM	standard error of the mean
SERCA	sarco(endo)plasmic reticulum Ca^{2+} ATPase
SNS	sympathetic nervous system
SOD	superoxide dismutase
SpO_2	arterial oxygen saturation
SR	sarcoplasmic reticulum
SUIT	substrate, uncoupler, and inhibitor titration
TMPD	*N,N,N',N'*-tetramethyl-*p*-phenylenediamine
$\dot{V}CO_{2peak}$	peak pulmonary carbon dioxide output
$\dot{V}_{Epeak}$	peak ventilation
$\dot{V}_E/\dot{V}CO_2$	ratio of ventilation to carbon dioxide output
$\dot{V}O_2$	pulmonary oxygen uptake
$\dot{V}O_{2peak}$	peak pulmonary oxygen uptake
$\dot{V}O_{2max}$	maximal pulmonary oxygen uptake
vs.	versus
V_{Tpeak}	peak tidal volume
Ww	wet weight
χ^2	chi-squared

Chapter 1 Introduction

1.1 Prologue

Chronic heart failure (CHF) and type 2 diabetes mellitus (DM) remain primary causes of morbidity and mortality despite recent advances in management and treatment. Aside from the debilitating human consequences, these conditions also impose a substantial burden on healthcare resources (estimated to be ~$108 billion USD and ~$673 billion USD for CHF and DM, respectively) (Ziaeian & Fonarow, 2016; Zheng *et al.*, 2018). A concerning trend to recently emerge is the increasing prevalence of DM within CHF patients (Lehrke & Marx, 2017), that shall henceforth be termed diabetic heart failure (D-HF). Several recent epidemiological studies have shown that ~25-30% of CHF patients secondary to left ventricular systolic dysfunction (LVSD) present with coexisting DM (MacDonald *et al.*, 2008a; MacDonald *et al.*, 2008b; Cubbon *et al.*, 2013; van Deursen *et al.*, 2014; Dei Cas *et al.*, 2015; Seferovic *et al.*, 2018). Importantly, D-HF an adverse prognosis characterised by an increased risk of CHF hospitalization, as well as all-cause and cardiovascular mortality (Shindler *et al.*, 1996; de Groote *et al.*, 2004; Gustafsson *et al.*, 2004; Cubbon *et al.*, 2013; Dauriz *et al.*, 2017; Seferovic *et al.*, 2018).

Given that exercise intolerance is the most powerful predictor of mortality (Myers *et al.*, 2002), there is much interest as to what mechanisms contribute towards exercise intolerance in CHF, DM and D-HF. In the context of CHF, there is substantial evidence to indicate that peripheral perturbations within the skeletal muscle system influence the archetypal symptoms of exercise intolerance

observed in these patients (Mettauer *et al.*, 2006; Middlekauff, 2010; Okita *et al.*, 2013). DM is also characterised by compromised skeletal muscle metabolism, one outcome of which is insulin resistance (Abdul-Ghani & DeFronzo, 2010). Thus, it may be postulated that the lower exercise capacity previously described in D-HF patients (Guazzi *et al.*, 2002; Tibb *et al.*, 2005; Ingle *et al.*, 2006) may be attributed to aberrant skeletal muscle abnormalities resulting from the presence of both conditions. However, as yet, very few studies have assessed skeletal muscle alterations in D-HF patients. Therefore, it is still unclear as to how the additional comorbidity of DM may influence skeletal muscle and potentially worsen symptoms of exercise intolerance in the context of CHF.

The current thesis aimed to identify and establish the cellular mechanisms within the skeletal muscle system underpinning exercise intolerance in the context of both CHF and DM, and how these factors may contribute to the worse prognosis observed in D-HF patients. This study has also focused on measuring skeletal muscle alterations in both locomotor and postural muscle groups to discriminate the systemic effects of these two conditions from the potentially confounding influence of detraining/disuse that frequently occurs in these clinical conditions (Olsen *et al.*, 2005; Reynolds *et al.*, 2007; Rehn *et al.*, 2012).

1.2 Chronic heart failure

1.2.1 Aetiology

Chronic heart failure (CHF) secondary to left ventricular systolic dysfunction (LVSD) is a complex and progressive clinical condition (Metra & Teerlink, 2017). Heart failure with reduced ejection fraction (HFrEF) accounts for ~50% of all CHF

cases (Bloom *et al.*, 2017), and is characterised by a reduced capacity to provide sufficient blood flow, and hence oxygen (O_2) delivery, to bodily tissues commensurate with metabolic demands (Mudd & Kass, 2008; Kemp & Conte, 2012). Heart failure with preserved ejection fraction (HFpEF) accounts for the remaining 50% of CHF cases; however, as the aetiology, progression, and treatment for these patients remains poorly understood, the current thesis is focused purely on patients with HFrEF, henceforth termed as CHF for simplicity.

CHF arises from congenital or acquired alterations that deleteriously affect the structure and/or function of the heart such that there is a reduction in ventricular filling or ejection (Shah & Mann, 2011; Bloom *et al.*, 2017). While multiple divergent aetiologies may contribute to this final common syndrome - including hypertension, coronary artery disease, cardiomyopathies, myocardial infarction, myocarditis, and valvular disease - they are each underscored by an adverse left ventricular remodelling (McMurray *et al.*, 2012; Bloom *et al.*, 2017). Specifically, this left ventricular remodelling occurs due to a loss of myocyte cells and increased myocardial strain, which lead to left ventricular hypertrophy and dilatation as well as fibrosis and functional mitral regurgitation (Metra & Teerlink, 2017). Consequently, these increase myocardial O_2 consumption and decrease myocardial contractile efficiency, which cause LVSD and a reduction in left ventricular ejection fraction (LVEF) (Moss, 2010; Metra & Teerlink, 2017). Thus, LVSD in CHF occurs as a maladaptive compensatory response to myocardial injury. However, the symptomatic progression to overt CHF occurs due to systemic overactivation of the neurohormonal systems, the renin-angiotensin-aldosterone system (RAAS) and the sympathetic nervous system (SNS) (Hartupee & Mann, 2017). These systems result in numerous cardiovascular,

renal and neurohormonal adaptive responses that initially aim to preserve cardiovascular homeostasis but ultimately progress the condition to a whole-body syndrome by chronically exerting deleterious effects (Kemp & Conte, 2012; Hartupee & Mann, 2017). Therefore, CHF is considered a multifaceted syndrome, which originates as a result of a central haemodynamic disorder, but propagates a multitude of maladaptive consequences and symptoms due to altered haemodynamic, neural, hormonal and renal responses.

CHF patients typically present with a constellation of signs and symptoms which impair quality of life primarily by inducing symptoms of exercise intolerance, including breathlessness (dyspnoea) and skeletal muscle fatigue (Ziaeian & Fonarow, 2016). However, as many of these symptoms are common in other conditions, such chronic obstructive pulmonary disease, CHF can only be diagnosed from an echocardiogram combined with measurements of the serum concentrations of brain natriuretic peptide (BNP) and/or the N-terminal prohormone of BNP (NT-proBNP) (Cowie, 2017; Metra & Teerlink, 2017). A BNP level > 100 pg·mL^{-1} and a NT-proBNP level > 400 pg·mL^{-1} is considered indicative of CHF (Cowie, 2017; Metra & Teerlink, 2017). Symptomatic status is often assessed subjectively in CHF patients using the New York Heart Association (NYHA) functional classification system (Ponikowski *et al.*, 2016). This groups patients into one of four groups (classes I-IV) based on the severity of their symptoms during daily activities. Those in class I have no symptoms whereas classes II, III, and IV correspond to mild, moderate, and severe symptoms, respectively (McMurray *et al.*, 2012). Despite being a subjective measure, this classification system has been shown to be a good predictor of mortality (Scrutinio *et al.*, 1994).

1.2.2 Epidemiology

CHF currently afflicts about 2% of the world's adult population (Metra & Teerlink, 2017) with over half a million of these individuals currently living in the UK (Townsend *et al.*, 2014). CHF prevalence increases with age and is more common in males (Ho *et al.*, 1993). Although the incidence of CHF has been stable or even decreased in recent years (Conrad *et al.*, 2018), the prevalence has increased and is forecast to rise due to an expanding and ageing demographic as well as improvements in survival and treatment of individuals suffering from acute coronary events (Heidenreich *et al.*, 2013; Kearney, 2015; Bloom *et al.*, 2017; Conrad *et al.*, 2018). Importantly, CHF confers a poor prognosis with frequent hospitalizations and readmissions as well as a high mortality (Krum & Abraham, 2009; Bui *et al.*, 2011; Metra & Teerlink, 2017). Indeed, 6-7% of patients with stable CHF will die within 1-year (Metra & Teerlink, 2017). Additionally, treatment costs contribute to ~2% of healthcare expenditure (Cowie, 2017; Metra & Teerlink, 2017). Although several developments in recent years, such as evidence-based pharmacotherapy and device implantations, have improved outcomes (Mudd & Kass, 2008; Ziaeian & Fonarow, 2016), CHF continues to remain a major global concern.

1.2.3 Treatment

Neurohormonal antagonists, diuretics and implantable devices are the mainstay of CHF treatment (Metra & Teerlink, 2017). Neurohormonal antagonists block the RAAS and SNS systems to prevent adverse remodelling from overactivation (Krum & Abraham, 2009). Several CHF drugs that specifically target each of these systems have proven effective in improving morbidity and mortality rates

(Krum & Teerlink, 2011). These drugs include angiotensin converting enzyme inhibitors (ACEis), angiotensin receptor blockers or antagonists (ARBs), aldosterone antagonists, mineralocorticoid receptor antagonists, and beta-blockers (Bristow, 2012; Metra & Teerlink, 2017). CHF patients are also often afflicted with oedema, or fluid retention, in the lower extremities (peripheral oedema), or the lungs (pulmonary oedema), which often exacerbate symptoms of exercise intolerance, particularly breathlessness (Clark & Cleland, 2013). Therefore diuretic treatment aims to achieve and maintain euvolaemia by ameliorating congestion associated with CHF (Krum & Abraham, 2009).

Although left ventricular assist devices have recently emerged as a novel therapy (Fang, 2009; Abraham & Smith, 2013), implantable devices, including both implantable cardioverter defibrillators (ICDs) and cardiac resynchronisation therapy (CRT) devices, remain the cornerstone of surgical treatment options for CHF patients (Holzmeister & Leclercq, 2011). ICDs prevent sudden cardiac death by terminating arrhythmias, such as ventricular tachycardia and ventricular fibrillation (DiMarco, 2003; Betts & de Bono, 2010). ICD therapy effectively reduces mortality risk in CHF patients compared to conventional medical therapy alone (Moss, 2010). CRT devices improve symptoms and prognosis by overcoming intraventricular dyssynchrony, which most commonly manifests as left bundle branch block (Jarcho, 2005; Betts & de Bono, 2010; Abraham & Smith, 2013). CRT devices simultaneously pace both the right and left ventricles (biventricular pacing), and have been shown to reduce the interventricular delay, improving functional haemodynamic indices, including an increase in LVEF (Abraham *et al*., 2002; Cleland *et al*., 2005). Furthermore, CRT device therapy has been shown to improve symptomatic status (NYHA classification), exercise

capacity, and quality of life, as well as reduce hospitalizations and decrease mortality, above conventional medical therapy alone (Abraham *et al.*, 2002; Cleland *et al.*, 2005; Abraham & Smith, 2013). Additionally, the combination of a CRT-ICD device has been shown to improve quality of live, functional status, and exercise capacity in moderate to severe CHF patients (Young *et al.*, 2003) as well as reduce the risk of CHF events in patients with a low LVEF (Moss *et al.*, 2009).

1.3 Type 2 diabetes mellitus

1.3.1 Aetiology

Type 2 diabetes mellitus (DM) is characterised by elevated plasma glucose, or hyperglycaemia, in both the fasting and postprandial states. This condition develops due to dysregulation in the action and production of insulin, one of two hormonal homeostatic peptides that regulate plasma glucose concentrations. In healthy individuals, insulin and glucagon both tightly control plasma glucose concentrations by encouraging glucose disposal in peripheral tissues in the postprandial state and releasing glucose in relation to energetic demand, such as during exercise or fasting (Muoio, 2014). However, in DM, overt hyperglycaemia occurs as a result of a reduction in insulin sensitivity in peripheral target tissues (insulin resistance), which precedes the decrease in insulin secretion from pancreatic β cells (Kahn *et al.*, 2014). Clinically, DM is diagnosed as a fasting plasma glucose concentration ≥ 7 mmol·L^{-1} or a 2-hour postprandial plasma glucose concentration ≥ 11.1 mmol·L^{-1}. Glycated haemoglobin (HbA1c) is also used as a marker of longitudinal glycaemic control with an HbA1c concentration ≥ 6.5% (≥ 48 mmol·L^{-1}) being indicative of DM.

Insulin resistance in peripheral tissues is evident several years before the development of overt hyperglycaemia and is also present in both obese and lean offspring of DM patients and in obese non-DM individuals (Abdul-Ghani & DeFronzo, 2010). Insulin resistance is a relative impairment in the ability of insulin to exert its effects on glucose and lipid metabolism in target tissues (Turner & Heilbronn, 2008), as measured by a euglycaemic-hyperinsulinaemic clamp test to assess insulin sensitivity and glucose uptake (Kim, 2009). These target tissues primarily include the liver, adipose tissue (adipocytes) and skeletal muscle. In skeletal muscle, insulin resistance is evident from a reduction in insulin-stimulated glucose uptake and glycogen synthesis during postprandial hyperinsulinemia (Szendroedi *et al.*, 2011). Indeed, as skeletal muscle accounts for ~80% of postprandial insulin-stimulated glucose uptake (Shulman *et al.*, 1990), it is considered to be the primary site of insulin resistance in DM (Abdul-Ghani & DeFronzo, 2010). Insulin resistance also reduces fatty acid metabolism in the fasting state and promotes 'metabolic inflexibility', which refers to the inability to adjust between the oxidation of different nutrient fuel substrates within the prevailing extracellular milieu (Kelley & Mandarino, 2000). The persistent hyperglycaemic environment associated with DM promotes the development of several macro- and microvascular complications that increase morbidity and mortality (Stratton *et al.*, 2000). The macrovascular complications include an increased risk of intermittent claudication, stroke, heart failure, and coronary heart disease (Kannel & McGee, 1979). The microvascular complications of DM include retinopathy (eye damage), neuropathy (nerve damage) and nephropathy (renal failure). Additionally, DM patients often present with high blood pressure (hypertension) and hyperlipidaemia. Given these complications, it is perhaps

unsurprising that DM patients suffer greatly from cardiovascular morbidity and mortality, with cardiovascular disease being the primary cause of death in DM patients (Abdul-Ghani *et al.*, 2017). Furthermore, the treatment and management of DM and its associated complications is a substantial burden on national healthcare costs and resources.

1.3.2 Epidemiology

The global prevalence of DM has quadrupled in the last 30 years and will soon effect half a billion people (Chatterjee *et al.*, 2017; Zheng *et al.*, 2018). Although a genetic predisposition is associated with an increased risk of DM (Fuchsberger *et al.*, 2016), the predominant factors promoting this dramatic increase in prevalence include an ageing population and obesity, the latter of which has been fuelled by metabolic derangements owing to modern lifestyle changes in physical activity and diet (Zheng *et al.*, 2018). These lifestyle alterations have partially been fostered by changes in the environments of industrialised and economically-developed societies, such as urbanisation, mechanised transportation, and sedentary occupations, all of which have fostered an increasingly inactive population. Simultaneously, there has been an increase in excessive energy intake, particularly as a result of the increased consumption of energy-dense calorific foods high in sugar and fat content (Zheng *et al.*, 2018). Although obesity is the best predictor of DM development, there is evidence that DM incidence is greater in normal weight individuals presenting with insulin resistance than overweight individuals without insulin resistance (Chen *et al.*, 2012). Therefore metabolic perturbations may be of more clinical significance for predicting DM risk than body weight *per se*. Aside from these modifiable lifestyle

factors, unmodifiable risk factors, such as ethnicity and familial history, have combined to contribute to the recent upsurge in these disorders. Although originally believed to only develop in adults, evidence now indicates that DM is becoming increasingly common in young adults, adolescents and, in some instances, children (Chen *et al.*, 2012). Owing to the chronic nature of the disease and its associated comorbidities, this reduction in the age of onset is likely to result in considerable burden both for the individual and healthcare resources.

1.3.3 Treatment

The primary aim of DM treatments is to optimally maintain plasma glucose concentrations within the normal range whilst still accounting for diurnal and postprandial fluctuations (Kahn *et al.*, 2014). Metformin, which reduces hepatic glucose output and increases peripheral insulin sensitivity, remains the primary treatment for DM patients as it is effective at reducing HbA1c concentrations as well as cardiovascular disease risk without increasing the likelihood of a potentially adverse episode of hypoglycaemia (Chatterjee *et al.*, 2017). Treatment with short- and long-acting insulin analogues are most effective at improving glycaemic control but increase the risk of hypoglycaemia (Chatterjee *et al.*, 2017). Often these treatments are combined with sulfonylureas and meglitinides, both of which act to increase insulin secretion from pancreatic β-cells (Chatterjee *et al.*, 2017).

1.4 Diabetic heart failure

Several recent epidemiological studies have shown that ~25-30% of CHF patients present with coexisting DM (MacDonald *et al.*, 2008a; MacDonald *et al.*, 2008b; Cubbon *et al.*, 2013; van Deursen *et al.*, 2014; Dei Cas *et al.*, 2015; Seferovic *et al.*, 2018). This prevalence is higher when prediabetic individuals are included (Suskin *et al.*, 2000) and may even be underestimated given that DM frequently goes undiagnosed (Beagley *et al.*, 2014). This prevalence of DM in CHF is substantially higher than the non-CHF population and is expected to rise with an ageing population and improved survival rates after myocardial infarction (MacDonald *et al.*, 2008a; Dei Cas *et al.*, 2015; Kearney, 2015). This high prevalence is driven by a bidirectional relationship between DM and CHF whereby either condition increases the risk of developing the other (MacDonald *et al.*, 2008a). Indeed, a recent prospective cohort study with over 1.9 m people indicated that the presentation of heart failure was one of the most common initial manifestations of cardiovascular disease in DM patients (Shah *et al.*, 2015). Similarly, follow-up studies on CHF patients have also shown that the prevalence of DM increases over time (Vermes *et al.*, 2003), particularly in older individuals (From *et al.*, 2006).

Insulin resistance and poor glycaemic regulation in both DM (Iribarren *et al.*, 2001) and non-DM (Ingelsson *et al.*, 2005) individuals is associated with a greater propensity of CHF incidence. Indeed, every 1% increase in HbA1c concentrations is associated with an 8% increased risk of developing CHF (Iribarren *et al.*, 2001). Similarly, higher fasting plasma glucose (Khan *et al.*, 2014) and HbA1c concentrations (Matsushita *et al.*, 2010) have both been associated with the development of CHF even in non-DM individuals. Indeed, a

recent meta-analyses discovered that higher HbA1c and fasting plasma glucose concentrations as well as insulin use were all independently associated with the incidence of CHF (Wang *et al.*, 2015). Significantly, higher HbA1c concentrations are associated with worse outcomes in D-HF patients (Iribarren *et al.*, 2001; Shah *et al.*, 2012; Dauriz *et al.*, 2017) and, even in non-DM CHF patients, insulin resistance is independently associated with a worse prognosis (Doehner *et al.*, 2005). Furthermore, insulin resistance is associated with a reduced exercise capacity in CHF patients (Swan *et al.*, 1997) and patients with a higher NYHA functional classification present with higher glucose and insulin concentrations (Suskin *et al.*, 2000). Collectively, these findings suggest that insulin resistance and glycaemic control may play a pathophysiological role in the development and severity of CHF.

Compared to CHF patients, D-HF patients have an adverse prognosis characterised by an increase in CHF hospitalization, as well as all-cause and cardiovascular mortality (Shindler *et al.*, 1996; de Groote *et al.*, 2004; Gustafsson *et al.*, 2004; Cubbon *et al.*, 2013; Dauriz *et al.*, 2017; Seferovic *et al.*, 2018). Indeed, one-third of D-HF patients will die within 1-year and 50% within 3 years (Gustafsson *et al.*, 2004). Furthermore, D-HF patients have worse NYHA symptomatic status and exercise capacity despite comparable cardiac function (Suskin *et al.*, 2000; Cubbon *et al.*, 2013). Similarly, adverse left ventricular remodelling following myocardial infarction does not explain the increased incidence of CHF in DM patients (Solomon *et al.*, 2002). Therefore, given these findings, it is plausible to suggest that peripheral, non-cardiac factors, such as skeletal muscle abnormalities, may promote the increased incidence and poor prognosis of D-HF patients.

As yet, little is known regarding the underlying, and likely complex, pathophysiological pathways mediating the intrinsic relationship between CHF and DM. Given that exercise capacity is lower in D-HF (Guazzi *et al.*, 2002; Tibb *et al.*, 2005; Ingle *et al.*, 2006), and this is closely associated with mortality (Myers *et al.*, 2002), it is likely that the mechanisms mediating exercise intolerance in D-HF contribute to the worse prognosis in these patients. Therefore a greater understanding of the mechanisms contributing to exercise intolerance in D-HF patients may uncover potential therapeutic targets to ameliorate symptoms and improve outcomes in this population.

1.5 Exercise intolerance in CHF and D-HF

Compared to CHF patients, exercise intolerance is exacerbated in D-HF patients (Guazzi *et al.*, 2002; Tibb *et al.*, 2005; Ingle *et al.*, 2006), despite comparable left ventricular ejection fraction (Cubbon *et al.*, 2013). Importantly, exercise intolerance is associated with an increased risk of premature mortality in both CHF (Swank *et al.*, 2012; Keteyian *et al.*, 2016) and DM patients (Wei *et al.*, 1999; McAuley *et al.*, 2007; Nylen *et al.*, 2010). Furthermore the physical symptoms of exercise intolerance are of clinical significance as they reduce quality of life (Bekelman *et al.*, 2007) and are associated with a decline in functional capacity resulting in an inability to fulfil daily activities (McMurray *et al.*, 2012). Therefore this produces a vicious cycle in which reduced exercise capacity decreases physical activity levels which further exacerbate exercise intolerance.

1.5.1 Assessment of exercise intolerance

To provide an objective assessment of exercise intolerance, it is most common for patients to undertake a non-invasive symptom-limited incremental cardiopulmonary exercise (CPX) test, performed with continuous breath-by-breath measurements. The latter provide data relating to individual symptoms of breathlessness and fatigue and are considered more valuable in the clinical evaluation of exercise intolerance in CHF patients (Piepoli *et al.*, 2006; Corra *et al.*, 2014; Malhotra *et al.*, 2016). Indeed, the prognostic significance of the measurements obtained from incremental CPX tests with metabolic gas exchange lies in the ability to identify and discriminate the pathophysiological origins potentially contributing to the symptoms of exercise intolerance (Corra *et al.*, 2014; Malhotra *et al.*, 2016). CPX tests are often conducted on either a treadmill or cycle ergometer and consist of patients exercising from a relatively low-intensity before the workload is incrementally increased until the patient achieves volitional exhaustion at the limit of tolerance.

The peak pulmonary oxygen uptake ($\dot{V}O_{2peak}$) measured at the limit of tolerance is one of the most clinically significant variables obtained from CPX tests. This measure reflects the integrative whole-body capacity of the pulmonary, cardiovascular, and neuromuscular systems to uptake, transport, extract, and utilise atmospheric O_2 for the cellular respiration that provides the necessary energy for contractile activity. $\dot{V}O_{2peak}$ is dictated by the products of the Fick equation, cardiac output ($\dot{Q}$) and skeletal muscle O_2 extraction. Compared to age-matched controls, $\dot{V}O_{2peak}$ is reduced by ~40% in CHF patients (Clark *et al.*, 2000; Witte *et al.*, 2003) and ~20% in DM patients (Regensteiner *et al.*, 1995). Importantly, $\dot{V}O_{2peak}$ is prognostically predictive of all-cause (Keteyian *et al.*,

2016) and cardiac-related mortality and hospitalisations in CHF patients (Arena *et al.*, 2004). Furthermore, $\dot{V}O_{2peak}$ is also used to guide clinical interventions in CHF patients, such as cardiac transplantation in those patients with a $\dot{V}O_{2peak} \leq$ 14 mL·kg^{-1}·min^{-1} (Mancini *et al.*, 1991; Mehra *et al.*, 2016). In DM patients, a lower $\dot{V}O_{2peak}$ is associated with an increased risk of cardiovascular events (Seyoum *et al.*, 2006), as well as cardiovascular (Church *et al.*, 2005) and all-cause mortality (Wei *et al.*, 1999; McAuley *et al.*, 2007; Nylen *et al.*, 2010). Furthermore, prospective studies show that a low $\dot{V}O_{2peak}$ is an independent risk factor for the development of DM (Wei *et al.*, 1999). The reduction in $\dot{V}O_{2peak}$ in DM patients is also independently associated with the microvascular complications associated with DM, including retinopathy and neuropathy (Estacio *et al.*, 1998). Additionally, exercise capacity is inversely correlated with DM duration, HbA1c concentrations and body mass index (BMI) (Fang *et al.*, 2005) as well as insulin sensitivity (Seibæk *et al.*, 2003).

Despite the clinical and prognostic utility of a CPX test to volitional exhaustion, CHF patients invariably terminate tests prematurely due to symptoms of breathlessness and fatigue (Piepoli *et al.*, 2006). Therefore the term symptom-limited $\dot{V}O_{2peak}$ is most commonly used, rather than the term maximal pulmonary oxygen uptake ($\dot{V}O_{2max}$), which is evidenced by a plateau in the $\dot{V}O_2$ response at maximal exercise, thus providing evidence that the upper limit of aerobic function has been attained (Hill & Lupton, 1923). As such, verification phases at the end of a peak exercise test have been advocated to overcome any confusion between $\dot{V}O_{2peak}$ and $\dot{V}O_{2max}$, rather than using poorly discriminate secondary criteria such as predictive maximal heart rate, respiratory exchange ratio (RER), blood lactate levels and ratings of perceived exertion (Bowen *et al.*, 2012a; Poole

& Jones, 2017). One such alternative protocol is the RISE-95 protocol, which involves a ramp-incremental (RI) phase followed by a constant-load step-exercise (SE) phase at 95% of the peak work rate achieved during RI (Rossiter *et al.*, 2006). A non-significant difference in $\dot{V}O_{2peak}$ between RI and SE can confirm $\dot{V}O_{2max}$ attainment by satisfying the traditional criterion plateau (Rossiter *et al.*, 2006). This protocol recently demonstrated, albeit in a small cohort of CHF patients, that $\dot{V}O_{2max}$ is often underestimated, with a significant proportion able to acutely increase $\dot{V}O_{2peak}$ between the RI and SE phases by ~20% (Bowen *et al.*, 2012a). However, it is not known whether these findings translate to a large clinical CHF cohort, what mechanisms may facilitate this increase in $\dot{V}O_2$, and why certain patients are able to access this so called "aerobic reserve". A better understanding of the mechanisms allowing the increase in $\dot{V}O_2$ with prior exercise warrants further investigation as this may uncover potential therapeutic targets by which $\dot{V}O_{2peak}$ can be pharmacologically increased and thus improve symptoms, prognosis and outcomes in exercise intolerant patients.

1.5.2 Assessing mechanisms of exercise intolerance

Historically, given that CHF is characterised by LVSD, it was intuitively presumed that the severity of cardiac dysfunction would relate to the degree of exercise intolerance in patients. Therefore, many early studies hypothesised that central cardiovascular factors mediated exercise intolerance in CHF. Indeed, two studies concluded that reduced skeletal muscle blood flow to the legs resulted in muscular fatigue and exercise termination during incremental maximal cycle exercise (Wilson *et al.*, 1984a; Wilson *et al.*, 1984b). Thus, it was presumed that a reduction in $\dot{Q}$ resulted in an underperfusion of the exercising skeletal

musculature and an earlier onset of intramuscular acidosis, which subsequently induced volitional exhaustion (Okita *et al.*, 2013).

Subsequent experimental study designs sought to increase $\dot{Q}$ and improve skeletal muscle blood flow. However, several studies showed that inotropic agents were unable to improve exercise capacity despite improving $\dot{Q}$ and peripheral O_2 delivery (Maskin *et al.*, 1983; Wilson *et al.*, 1984a; Mancini *et al.*, 1990). This led to the conclusion that impaired O_2 extraction or utilisation within the muscle was the primary cause of exercise intolerance in CHF patients. In addition to these interventional studies, correlational studies have failed to demonstrate a significant relationship between resting cardiac function and exercise capacity in CHF patients (Franciosa *et al.*, 1981). Furthermore, symptom severity does not correlate with the degree of LVSD in CHF patients (McMurray *et al.*, 2012). This lack of correlation suggests that therapeutic strategies targeted towards improving cardiac function may not necessarily ameliorate exercise intolerance symptoms (Middlekauff, 2010). Collectively, these findings suggest that cardiac dysfunction may not be the primary cause of exercise intolerance in CHF patients. Consequently, it has been proposed that peripheral factors, particularly skeletal muscle abnormalities, may represent a common underlying pathology contributing to the aetiology of exercise intolerance. Therefore a better understanding of the underlying skeletal muscle physiology that mediates exercise intolerance has the potential to elucidate therapeutic targets in the context of CHF and also D-HF.

Given that both CHF and DM contribute to exercise intolerance, it is perhaps unsurprising that several studies have sought to determine exercise intolerance in D-HF patients. Indeed, it has been shown that the additional comorbidity of DM exacerbates exercise intolerance in CHF with several studies indicating that D-HF patients have a lower exercise capacity than those presenting with CHF alone (Guazzi *et al.*, 2002; Tibb *et al.*, 2005; Ingle *et al.*, 2006). One of the earliest studies to investigate the additional comorbidity of DM in CHF showed that D-HF patients exhibited a lower $\dot{V}O_{2peak}$ and a steeper increase in ventilation for a given carbon dioxide output ($\dot{V}_E/\dot{V}CO_2$) during ramp incremental exercise tests compared to CHF alone (Guazzi *et al.*, 2002). Follow-up studies expanded on these findings by demonstrating, in a larger equally matched cohort of 78 D-HF and 78 CHF patients, that $\dot{V}O_{2peak}$ prospectively measured during a symptom-limited treadmill exercise test was significantly lower in the D-HF cohort (Tibb *et al.*, 2005). This finding persisted even after accounting for several confounding baseline characteristics, such as LVEF, physical activity, hypertension and HbA1c. Furthermore, there were no significant between group differences in peak heart rate or ventilatory threshold in this cohort. Based on these findings, the authors concluded that the additional comorbidity of DM further exacerbated skeletal muscle metabolic perturbations that ultimately reduced $\dot{V}O_{2peak}$ (Tibb *et al.*, 2005).

Comparable results have also been demonstrated in patients undertaking the 6-minute walk test (6-MWT), with D-HF patients covering less distance compared to CHF (Ingle *et al.*, 2006). Interestingly, there is also evidence that D-HF patients have reduced peripheral skeletal muscle strength as measured by a handgrip dynamometer and isokinetic dynamometry of the knee extensors and flexors

(Izawa *et al.*, 2013). Furthermore, even in non-DM CHF patients, insulin resistance is highly prevalent (79 out of 129 patients) and this correlated with a higher symptomatic status, as determined by NYHA functional classification, as well as a reduced exercise capacity and $\dot{V}O_{2peak}$ (AlZadjali *et al.*, 2009). This study is supported by the finding that acute insulin infusion can increase $\dot{V}O_{2peak}$ and time to anaerobic threshold as well as lower the $\dot{V}_E/\dot{V}CO_2$ slope (Guazzi *et al.*, 2003). Therefore these studies suggest that DM lower exercises capacity and this is associated with poor glycaemic control. Thus, it is of increasing clinical interest to determine the pathophysiology of exercise intolerance in the context of D-HF.

1.6 Skeletal muscle structure, function and metabolism

1.6.1 Structure

Skeletal muscle makes up ~40-50% of body mass in normal weight individuals and is composed of long multinucleated cells bound tightly together within fascicles that attach to bones via ligaments and tendons. During skeletal muscle contractions, these attachments transfer force generation from the muscles to the bones and thereby facilitate locomotion. Individual muscle fibres consist of cylindrical strands of proteins known as myofibrils, which are segmented into sarcomeres, and consist of thick myosin filaments and thin actin filaments. Myosin filaments consist of a heavy chain, which is the most abundant protein within the muscle, while actin contains myosin binding sites that are covered by tropomyosin at rest.

Human skeletal muscle contains three different myosin heavy chain (MHC) isoforms, which are used to differentiate individual muscle fibres into type I, type IIa and type IIx in humans (Schiaffino & Reggiani, 2011). The consequence of the different isoform phenotypes expressed in skeletal muscle is a preferential shift in the metabolic substrate pathway and underlying differences in contractile properties. At one end of the spectrum, the slow-twitch type I fibres have fatigue-resistant characteristics underpinned by a greater reliance upon aerobic metabolism and low force but sustainable contractions. At the other end of the spectrum, the fast-twitch type IIx fibres rely heavily on anaerobic pathways to rapidly generate high force contractions but are easily fatigued. Oxidative type I fibres have a smaller diameter, appear red due to the higher concentration of iron-containing myoglobin, and contain more mitochondria and oxidative enzymes. Type II fibres are characterised by an increased glycogen content and greater myosin adenosine triphosphatase (ATPase) activity. The type IIa "fast oxidative" glycolytic fibres may be considered a hybrid isoform containing elements of both type I and IIx isoforms. Human skeletal muscle may be composed of various proportions of these three isoforms depending on several factors, such as contractile activity, gender and age. Capillaries are interspersed throughout the skeletal musculature to facilitate O_2 delivery and removal of metabolites and other waste products, which helps sustain repeated contractions.

1.6.2 Function

Skeletal muscle primarily functions by coordinating metabolic processes and neuronal inputs to facilitate force generation and contractions. Contractions

occur when the individual myofilaments, actin and myosin, interact to produce cross-bridges within skeletal muscle sarcomeres. Downstream of the neuromuscular junction, this process is reliant upon two processes: 1) the release (and reuptake) of calcium (Ca^{2+}) from the sarcoplasmic reticulum (SR) via ryanodine receptors in response to action potential propagation from efferent neurons; and 2) the resynthesis of adenosine triphosphate (ATP), which is used as an "energy currency", facilitating the conversion of stored chemical energy in the form of metabolic substrates to the mechanical energy required for myofilament cross-bridge formation. The released Ca^{2+} from the SR binds to troponin C, which shifts tropomyosin from the actin molecule, thus allowing actin-myosin binding and cross-bridge cycling. Therefore the binding of actin to myosin results in the contractile process of the individual sarcomere, which collectively results in the coordinated contraction of the muscle. During relaxation of the muscle, SR reuptake of Ca^{2+} is facilitated by sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA).

1.6.3 Metabolism

The hydrolysis of ATP to inorganic phosphate (P_i) and adenosine diphosphate (ADP) by the enzyme myosin ATPase releases the free energy (Δ) required for the actin-myosin interaction. Given that the concentration of muscle ATP is finite (~8 mM) (Kemp *et al.*, 2007), repeated muscular contractions for exercise continuation are reliant upon ATP resynthesis, predominantly via the phosphorylation of ADP with Pi. This resynthesis can occur via several different metabolic pathways and substrates throughout the myocyte depending on the rate and capacity of ATP required. Specifically, the metabolic pathways for ATP

resynthesis rely upon the oxidation of both stored and circulating carbohydrates and fats. The intrinsic differences in the molecular structure of these different substrates dictate the rate and capacity at which ATP can be resynthesized. ATP resynthesis generally occurs via three metabolic pathways in skeletal muscle: 1) the phosphocreatine (PCr) system; 2) cytosolic glycolysis; and 3) mitochondrial oxidative phosphorylation (OXPHOS). The PCr pathway is capable of providing an immediate and instantaneous source of ATP from the catalytic breakdown by creatine kinase of PCr to creatine and P_i, which is used to phosphorylate ADP. However, given that intramuscular PCr stores are limited (~33 mM) (Kemp *et al.*, 2007), this pathway is only able to sustain ATP resynthesis for a few seconds. Glycolysis occurs in the cytosol and this pathway facilitates the conversion of glucose to pyruvate. This process releases free energy to resynthesise ATP and the pyruvate formed is then transported into the mitochondrial matrix via pyruvate dehydrogenase to produce acetyl-coenzyme A (CoA), which initiates the Krebs cycle. However, the predominant source of ATP resynthesis during daily activities performed at a relatively low intensity is from mitochondrial OXPHOS. Thus, the oxidative phosphorylation substrate pathway is of most clinical significance as it provides the majority of ATP required for daily living.

1.6.3.1 Mitochondrial oxidative phosphorylation

The ability to resynthesise ATP by mitochondrial OXPHOS is underpinned by: 1) the supply of metabolic substrates; 2) the delivery of atmospheric O_2 to myocyte mitochondria; and 3) adequate mitochondrial content and function. The delivery of O_2 to skeletal muscle mitochondria is reliant on several integrative pathways that may provide a limitation. Firstly, in normoxic conditions, O_2 uptake is

dependent upon the capacity of the lungs and pulmonary capillaries to facilitate gaseous exchange; secondly, the capacity of the cardiovascular system to effectively deliver oxygen-rich blood to the skeletal muscles; and finally, the successful diffusion of O_2 from capillary to skeletal muscle mitochondria.

Mitochondria are organelles found in the majority of eukaryotic cells that primarily function to resynthesise ATP via oxidative phosphorylation. Within skeletal muscles, mitochondria are organized in a reticular network throughout the sarcoplasm and are structurally distinct within different muscle fibre types to facilitate energy distribution (Ogata & Yamasaki, 1997; Glancy *et al.*, 2015). Mitochondria comprise ~6% of deep oxidative muscles and only 2-3% of superficial glycolytic muscles (Garnier *et al.*, 2003). Mitochondria contain both an inner and outer mitochondrial membrane, owing to their evolutionary origin from an endosymbiotic relationship formed between a host archaebacterium engulfing an α-proteobacterium (Gray *et al.*, 1999). The inner mitochondrial membrane encloses the mitochondrial matrix and is separated from the outer membrane by the intermembrane space. The mitochondrial matrix is the primary site of substrate metabolism (via β-oxidation and the Krebs cycle) and the inner mitochondrial membrane is embedded with five multi-subunit respiratory enzyme complexes along a series of invaginations, or folds in the membrane, termed cristae. These five enzyme complexes (denoted complexes I-V) – NADH:ubiquinone oxidoreductase (Complex I), succinate dehydrogenase (Complex II), cytochrome bc_1 complex (Complex III), cytochrome *c* oxidase (Complex IV), and ATP synthase (Complex V) - resynthesise ATP from the chemical energy of reduced substrates and the acceptance of electrons by O_2.

Mitochondrial OXPHOS involves the coupling of substrates derived from the Krebs cycle for entry into the electron transfer system (ETS) that allow subsequent ATP resynthesis at complex V. Initially, glycolysis and β-oxidation produce substrates derived from carbohydrates and fats that enter the Krebs cycle as acetyl-CoA. The enzymatic reactions of the Krebs cycle produce two high-energy reducing equivalents, reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$), which donate electrons to complexes I and II, respectively. These electrons are then sequentially transferred along to complexes III and IV via a series of redox reactions that are facilitated by the electron carriers, ubiquinone/co-enzyme Q_{10} and cytochrome *c*, respectively. At complex IV, O_2 acts as the final electron acceptor and is reduced to water, as a metabolic by-product. Coupled with this electron transfer is the translocation of H^+ protons from the mitochondrial matrix to the intermembrane space at complexes I, III and IV, respectively. Specifically, complexes I and III translocate 4 protons for each pair of electrons whilst complex IV transfers 2 protons per pair.

This proton translocation creates an electrochemical gradient across the inner mitochondrial membrane. The gradient provides the necessary proton-motive force to facilitate ATP resynthesis via a process known as chemiosmotic coupling (Mitchell, 1961). More specifically, the free energy released for every 4 protons that dissipate back down their chemical gradient through ATP synthase (complex V) is coupled with the phosphorylation of 1 ADP molecule to ATP. This proton-motive force, or electrochemical potential (ΔPmt), has both an electrical

component, determined by the inner mitochondrial membrane potential ($\Delta\Psi$mt), and a chemical component, governed by the pH difference (ΔpH) across the membrane. Matrix-formed ATP is then exchanged for cytosolic ADP by adenine nucleotide translocators that are also situated within the inner mitochondrial membrane. Similarly, the mitochondrial isoform of creatine kinase is coupled to oxidative phosphorylation by controlling the transfer of matrix-formed ATP to the myosin cross-bridge heads. Given the close coupling of these processes, skeletal muscle O_2 consumption closely reflects ATP resynthesis via mitochondrial OXPHOS. As such, skeletal muscle O_2 consumption is dependent on both mitochondrial density as well as the functional capacity of mitochondria to successfully couple the transport of electrons with the phosphorylation of ADP to ATP.

1.7 Skeletal muscle abnormalities in CHF and DM

Although CHF is initiated by cardiac dysfunction, it is now recognised as a multifactorial condition affecting several physiological systems, including the renal and vascular systems. Numerous studies now indicate that the skeletal muscle system is also perturbed in CHF patients (Table 1.1), which include: muscle fibre atrophy; a phenotypic shift from type I oxidative fibres to type II glycolytic fibres in limb muscle; reduced capillarisation; impaired Ca^{2+} dynamics; and mitochondrial dysfunction. Many of these factors are also evident in skeletal muscle samples from DM patients (Table 1.1). In this context, these abnormalities contribute to the reduced insulin-stimulated glucose uptake that characterise insulin-resistance. However, as yet, there is limited research on how D-HF *per se* impacts the skeletal muscle system (Table 1.1).

Table 1.1. Summary of studies assessing skeletal muscle function in DM, CHF and D-HF patients. Appropriate references are included in the text.

	DM	CHF	D-HF
Fibre cross-sectional area	↓	↓	?
Proportion of type II to type I fibres (phenotype)	↑	↑	?
Capillarisation	↑, ↓ and ↔	↑, ↓ and ↔	?
Ca^{2+} dynamics	↓	↓	?
In vivo skeletal muscle metabolism	↓	↓	?
Mitochondrial enzyme activity levels	↓	↓ and ↔	?
Mitochondrial content	↓	↓	↓
Mitochondrial function	↓ and ↔	↓ and ↔	?
Mitochondrial ROS production and oxidative stress	↑	↑	↑

↑, increase; ↓, decrease; ↔ unchanged; ?, unknown

1.7.1 Skeletal muscle structure

1.7.1.1 Atrophy

Both CHF (Anker *et al.*, 1997; Harrington *et al.*, 1997; Anker *et al.*, 2003; Fulster *et al.*, 2013; von Haehling *et al.*, 2017) and DM (Park *et al.*, 2007; Park *et al.*, 2009; Kim *et al.*, 2010; Leenders *et al.*, 2013; Kim *et al.*, 2014) patients exhibit a loss of muscle mass (atrophy) that is greater than what is observed naturally with the ageing process. In CHF, this loss of muscle mass is termed cardiac cachexia and is estimated to effect ~10% of all patients, equating to ~1.2 million people within Europe alone (von Haehling & Anker, 2014). In DM, ~16% of patients present with atrophy (Kim *et al.*, 2010) and the risk of low muscle mass is ~2-4-fold higher in elderly patients with DM compared to age-matched controls (Kim *et al.*, 2014). Importantly, muscle wasting is independently associated with reduced $\dot{V}O_{2peak}$ (Fulster *et al.*, 2013) and is also an independent risk factor for hospitalisations and mortality (Anker *et al.*, 1997; Anker *et al.*, 2003; Rossignol *et al.*, 2015). Furthermore this loss of muscle mass reduces muscle strength (dynapenia) (Cetinus *et al.*, 2005; Park *et al.*, 2006; Park *et al.*, 2007; Fulster *et*

al., 2013; Almurdhi *et al.*, 2016; Almurdhi *et al.*, 2017), endurance (Minotti *et al.*, 1993) and impairs the ability to perform routine activities (Leenders *et al.*, 2013), thus contributing to a vicious cycle of inactivity and exercise intolerance in patients. Studies have also shown that muscle size is a correlate and predictor of $\dot{V}O_{2peak}$ (Mancini *et al.*, 1992; Harrington *et al.*, 1997; Fulster *et al.*, 2013) and altered skeletal muscle metabolism (Mancini *et al.*, 1992). Furthermore, reduced muscle mass and strength has been associated with insulin resistance, poor glycaemic control, and increased intramuscular fat accumulation in CHF (Doehner *et al.*, 2015), DM (Park *et al.*, 2006; Almurdhi *et al.*, 2016) and impaired glucose tolerant patients (Almurdhi *et al.*, 2016). Collectively, these studies indicate that the reduced muscle mass and strength seen in CHF and DM contribute to insulin resistance, exercise intolerance and mortality.

Many studies in both CHF (Lipkin *et al.*, 1988; Mancini *et al.*, 1989; Sullivan *et al.*, 1990; Massie *et al.*, 1996; Delp *et al.*, 1997; Xu *et al.*, 1998; Larsen *et al.*, 2002; Li *et al.*, 2007; Yu *et al.*, 2008) and DM (Almond & Enser, 1984; Kemp *et al.*, 2009) have corroborated that this loss of muscle mass is the result of individual fibre atrophy by demonstrating a reduction in fibre cross-sectional area (FCSA) in histological samples of skeletal muscle biopsies. Interestingly, the majority of these studies have shown that atrophy selectively occurs in type II fibres (Almond & Enser, 1984; Mancini *et al.*, 1989; Sullivan *et al.*, 1990; Massie *et al.*, 1996; Xu *et al.*, 1998; Larsen *et al.*, 2002; Li *et al.*, 2007; Yu *et al.*, 2008; Kemp *et al.*, 2009). However, the exact mechanisms behind this selective atrophy remain to be elucidated. Furthermore the reduction in FCSA has been strongly correlated with reduced $\dot{V}O_{2peak}$ and muscle strength (Massie *et al.*, 1996). Muscle fibre atrophy occurs when catabolic protein degradation

(proteolysis) pathways exceed anabolic protein synthesis pathways (Perry *et al.*, 2016). In both CHF and DM, it has been postulated that chronically elevated concentrations of circulating pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), may induce oxidative stress and upregulate proteolysis via the ubiquitin-proteasome and autophagy-lysosome pathways as well as apoptosis (Fulster *et al.*, 2013; Bowen *et al.*, 2015b; Perry *et al.*, 2016; von Haehling *et al.*, 2017). However, as yet, it is unknown whether D-HF patients exhibit greater muscle fibre atrophy.

1.7.1.2 Phenotype

In locomotor skeletal muscle, several previous studies have documented a fibre type shift from type I oxidative to type II glycolytic fibres in both CHF (Lipkin *et al.*, 1988; Mancini *et al.*, 1989; Sullivan *et al.*, 1990; Drexler *et al.*, 1992; Schaufelberger *et al.*, 1995; Massie *et al.*, 1996; Simonini *et al.*, 1996; Schaufelberger *et al.*, 1997; Sullivan *et al.*, 1997; Middlekauff *et al.*, 2013) and DM patients (Marin *et al.*, 1994; Nyholm *et al.*, 1997; Gaster *et al.*, 2001; Oberbach *et al.*, 2006; Mogensen *et al.*, 2007). Furthermore, these findings have been corroborated in well controlled animal models of CHF (De Sousa *et al.*, 2000) and DM (Eshima *et al.*, 2017). Although the magnitude of this phenotypic shift as well as the predominant type II isoform (IIa or IIx) are not consistent amongst these studies, given that type II fibres contain fewer mitochondria (Schiaffino & Reggiani, 2011), these findings have been interpreted as evidence of a reduction in oxidative capacity in both CHF and DM. Indeed, some of these studies have demonstrated an inverse correlation between the percentage of II fibres and $\dot{V}O_{2peak}$ and a positive correlation between $\dot{V}O_{2peak}$ and the percentage

of type I fibres (Mancini *et al.*, 1989; Schaufelberger *et al.*, 1995), thereby suggesting that a shift in muscle phenotype may contribute to exercise intolerance in these patients. Furthermore some studies have shown that the proportion of type IIx fibres is strongly correlated with circulating free fatty acids and insulin resistance in obese DM and non-DM subjects (Mogensen *et al.*, 2007). Therefore, collectively, these studies indicate that a shift in muscle fibre type may contribute to exercise intolerance and insulin resistance in CHF and DM patients. However, the effect of D-HF on muscle phenotype has yet to be elucidated.

1.7.1.3 Capillarisation

Measures of skeletal muscle capillarisation in CHF and DM studies have identified a reduction in microvascular O_2 perfusion may contribute to exercise intolerance. One of the most frequently reported indices of capillarisation is capillary density (CD), which is the number of capillaries within a region of interest obtained from a histological stain (Egginton, 1990b). CD is reduced in animal models of CHF (Nusz *et al.*, 2003) as well as some (Drexler *et al.*, 1992) but not all (Mancini *et al.*, 1989) patient studies. Similarly, CD is reduced in DM patients (Marin *et al.*, 1994; Groen *et al.*, 2014) and strongly correlates with insulin resistance (Lillioja *et al.*, 1987; Marin *et al.*, 1994).

One of the issues with CD is that it is highly sensitive to changes in FCSA, as seen with atrophy, which often accompany pathophysiological conditions such as CHF and DM (Ahmed *et al.*, 1997). Therefore many studies also cite the numerical capillary-to-fibre ratio (C:F), which is relatively independent of changes

in FCSA (Egginton, 1990b). Interestingly, some of these studies have then shown that CD is not changed whereas C:F is reduced in both CHF patients (Schaufelberger *et al.*, 1995; Schaufelberger *et al.*, 1997; Williams *et al.*, 2004) and animals (De Sousa *et al.*, 2000). Additionally, others have reported equivocal findings in terms of how C:F is changed with respect to the individual fibre isoforms in CHF, with some suggesting that both type I and II are affected (Sullivan *et al.*, 1990; Williams *et al.*, 2004) and others demonstrating that only type II fibres exhibit a reduced capillarisation (Xu *et al.*, 1998). Furthermore, CHF patient studies have shown that the reduced C:F is inversely correlated with $\dot{V}O_{2peak}$ (Duscha *et al.*, 1999). C:F has also been reported to be reduced in DM patients (Andreassen *et al.*, 2014; Groen *et al.*, 2014) and animals (Machado *et al.*, 2016), with the latter study showing that this difference could be ameliorated through exercise training. Collectively, these studies suggest that CHF and DM appear to have a reduced skeletal muscle capillarisation, and this may impact upon both insulin sensitivity and exercise intolerance due to a reduced area of tissue being supplied by the microvasculature.

1.7.2 Skeletal muscle function

Studies in both CHF (Buller *et al.*, 1991; Minotti *et al.*, 1991; Minotti *et al.*, 1992; Harrington *et al.*, 1997; Toth *et al.*, 2010) and DM (Sayer *et al.*, 2005; Park *et al.*, 2006; Park *et al.*, 2007; Volpato *et al.*, 2012; Leenders *et al.*, 2013; Almurdhi *et al.*, 2016; Bianchi & Volpato, 2016) patients have demonstrated impairments to muscle strength and endurance, which impair physical functional capacity. Specifically, these studies have shown that patients experience skeletal muscle fatigue due to a reduced force generating capacity, as shown by lower torques

and power outputs during isometric, eccentric and isokinetic contractions of single muscle groups (Buller *et al.*, 1991; Minotti *et al.*, 1991; Minotti *et al.*, 1992; Harrington *et al.*, 1997; Sayer *et al.*, 2005; Park *et al.*, 2006; Park *et al.*, 2007; Toth *et al.*, 2010; Volpato *et al.*, 2012; Leenders *et al.*, 2013; Almurdhi *et al.*, 2016). The use of nerve-stimulation to augment voluntary contractions has demonstrated that this fatigue is the result of an intrinsic impairment within the muscle rather than a reduction in central motor drive or neuromuscular junction transmission (Minotti *et al.*, 1992). Furthermore, even when these measures are adjusted to account for differences in muscle size and mass, the reduction in contractile performance persists (Harrington *et al.*, 1997; Park *et al.*, 2006; Park *et al.*, 2007; Toth *et al.*, 2010). This therefore suggests that, in addition to atrophy, intrinsic qualitative abnormalities, a process termed contractile dysfunction, contribute to loss of muscle strength and endurance in CHF and DM patients.

To corroborate these findings and elucidate the mechanisms behind these impairments, several studies have measured contractile activity and Ca^{2+} handling in single muscle fibres isolated from CHF patients (Szentesi *et al.*, 2005; Miller *et al.*, 2010; Middlekauff *et al.*, 2012; Rullman *et al.*, 2013; DiFranco *et al.*, 2014) and animal models of DM (Bayley *et al.*, 2016; Rodriguez-Reyes *et al.*, 2016; Eshima *et al.*, 2017). Collectively, these studies have shown: reduced tetanic force production (Eshima *et al.*, 2017); reduced maximal isometric force production (Bayley *et al.*, 2016; Rodriguez-Reyes *et al.*, 2016); impaired Ca^{2+}-activated acto-myosin ATPase activity (Szentesi *et al.*, 2005); decreased cross-bridge kinetics and Ca^{2+} sensitivity (Miller *et al.*, 2010); decreased SERCA protein content (Middlekauff *et al.*, 2012; Bayley *et al.*, 2016); increased oxidative damage to ryanodine receptors (Rullman *et al.*, 2013); and impaired action

potential-evoked Ca^{2+} release (DiFranco *et al.*, 2014). Many of these findings have also been documented in animal models of CHF (Simonini *et al.*, 1999; Lunde *et al.*, 2001; Lunde *et al.*, 2006; Gillis *et al.*, 2016). Collectively these studies indicate that Ca^{2+} may play a role in exercise intolerance in CHF and DM.

1.7.3 Skeletal muscle metabolism

1.7.3.1 *In vivo* skeletal muscle mitochondrial function

Evidence of aberrant skeletal muscle metabolism in CHF and DM patients was first derived from ^{31}phosphorous magnetic resonance spectroscopy (^{31}P MRS) studies. This technique permits continuous and non-invasive *in vivo* measurements of intracellular concentrations of P_i, PCr, ATP and pH during exercise as a means to evaluate intracellular acidosis (decreased pH) and skeletal muscle metabolism (Kemp *et al.*, 2015). Importantly, measures of oxidative capacity determined from the ^{31}P MRS method have been validated against invasive muscle biopsy methods that measure *in situ* skeletal muscle mitochondrial function using respirometry (Lanza *et al.*, 2011; Ryan *et al.*, 2014). Using ^{31}P MRS, several studies have shown that CHF patients exhibit intramuscular metabolic abnormalities during exercise (Wilson *et al.*, 1985; Wiener *et al.*, 1986; Massie *et al.*, 1987a; Massie *et al.*, 1987b; Rajagopalan *et al.*, 1988; Mancini *et al.*, 1989; Mancini *et al.*, 1990; Kemp *et al.*, 1996; van der Ent *et al.*, 1998; Okita *et al.*, 2001; Nakae *et al.*, 2005) with these effects persisting even after cardiac transplantation (Stratton *et al.*, 1994). Collectively, these studies showed that CHF patients exhibit greater skeletal muscle fatigue at lower exercise intensities, which was accompanied by lower PCr concentrations, an elevated P_i:PCr ratio and an earlier onset of intracellular

acidosis, many of which correlated with the reduced exercise capacity (Wilson *et al.*, 1985; Wiener *et al.*, 1986; Massie *et al.*, 1987a; Massie *et al.*, 1987b; Rajagopalan *et al.*, 1988; Mancini *et al.*, 1989; Mancini *et al.*, 1990; Stratton *et al.*, 1994; Kemp *et al.*, 1996; van der Ent *et al.*, 1998; Okita *et al.*, 2001; Nakae *et al.*, 2005). Interestingly, some of these studies failed to show that these metabolic perturbations were the result of compromised muscle blood flow, thereby indicating impairments with skeletal muscle utilization rather than perfusion (Wiener *et al.*, 1986; Massie *et al.*, 1987a; Massie *et al.*, 1987b; Mancini *et al.*, 1990). More recently, studies in DM patients have also shown similar *in vivo* impairments using ^{31}P MRS, as reflected by a slower rate of PCr recovery following exercise, which is an alternative method for determining mitochondrial oxidative phosphorylation (Petersen *et al.*, 2004; Schrauwen-Hinderling *et al.*, 2007; Phielix *et al.*, 2008; Bajpeyi *et al.*, 2011; Tecilazich *et al.*, 2013). Furthermore, these studies also showed that this impaired *in vivo* mitochondrial function strongly correlated with measures of glycaemic control, including HbA1c and fasting plasma glucose concentrations (Schrauwen-Hinderling *et al.*, 2007) as well as glucose disposal (Bajpeyi *et al.*, 2011). Taken together, these studies show that *in vivo* impairments of mitochondrial oxidative phosphorylation contribute to exercise intolerance and likely impact insulin resistance in CHF and DM patients.

1.7.3.2 Mitochondrial content

These initial ^{31}P MRS data prompted investigators to assess skeletal muscle mitochondrial content in biopsies from CHF and DM patients using transmission electron microscopy (Drexler *et al.*, 1992; Hambrecht *et al.*, 1995; Kelley *et al.*,

2002; Morino *et al.*, 2005; Ritov *et al.*, 2005; Chomentowski *et al.*, 2011; Guzman Mentesana *et al.*, 2014). Early studies showed that mitochondrial volume density and surface density of cristae were reduced by ~20% in the *vastus lateralis* muscle of severe CHF patients (NYHA II-IV) and these measures correlated with $\dot{V}O_{2peak}$ and the lactate threshold (Drexler *et al.*, 1992). Similarly, training studies in CHF patients indicated that 6 months of regular physical activity increased the mitochondrial volume density in the *vastus lateralis* muscle, and this matched improvements in exercise tolerance and leg O_2 consumption (Hambrecht *et al.*, 1995). Transmission electron microscopy has also identified a reduced mitochondrial density in DM patients and insulin-resistant offspring of DM parents compared to control subjects (Kelley *et al.*, 2002; Morino *et al.*, 2005; Ritov *et al.*, 2005; Chomentowski *et al.*, 2011). Interestingly, two of these studies also showed a significant correlation between mitochondrial density and insulin sensitivity, as measured by the euglycaemic clamp method (Kelley *et al.*, 2002; Chomentowski *et al.*, 2011). Similarly, the decrease in mitochondrial content in insulin-resistant offspring has been associated with impairments in insulin signalling (Morino *et al.*, 2005). Collectively, these studies indicate that the reduced mitochondrial content in CHF and DM may contribute to exercise intolerance and insulin resistance.

1.7.3.3 *Ex vivo* skeletal muscle mitochondrial function

Aside from changes in mitochondrial morphometry, studies have also shown that CHF and DM are associated with reductions in mitochondrial oxidative enzyme activity levels which likely impair mitochondrial O_2 consumption. Specifically, these studies have shown reduced activity levels of: β-hydroxyacyl CoA

dehydrogenase (β-HAD), a key mitochondrial enzyme involved in β-oxidation of fatty acids (Mancini *et al.*, 1989; Schaufelberger *et al.*, 1996; Larsen *et al.*, 2009); succinate dehydrogenase, a key enzyme involved in both the Krebs cycle and the electron transport system (Massie *et al.*, 1996; He *et al.*, 2001); citrate synthase, a Krebs cycle enzyme which is often used as a marker of mitochondrial content (Vondra *et al.*, 1977; De Sousa *et al.*, 2000; Mettauer *et al.*, 2001; Kelley *et al.*, 2002; Larsen *et al.*, 2011); and mitochondrial complex IV, where O_2 accepts electrons (Drexler *et al.*, 1992). Importantly, succinate dehydrogenase activity (Massie *et al.*, 1996) as well as β-HAD and citrate synthase activity (Brassard *et al.*, 2006) have been strongly correlated with measures of exercise intolerance in these studies. Taken together, these consistent findings of reduced oxidative enzyme activity levels indicate that reduced skeletal muscle mitochondrial content and function may play a contributory role to exercise intolerance in CHF patients.

Although isolated measurements of muscle enzyme activity provide insight into mitochondrial abnormalities, it remains unclear how such findings translate to the *in vivo* setting, where several rate-limiting enzymes are simultaneously operating within the mitochondria to resynthesise ATP. More recently, the advent of high-resolution respirometers capable of measuring *in situ* O_2 consumption in small permeabilized muscle biopsies has advanced the field of muscle biology (Kuznetsov *et al.*, 2008). Key to this method is the capacity to use multiple substrates, uncouplers, and inhibitors to measure mitochondrial O_2 consumption in several different *ex vivo* respiratory states, thus allowing for functional characterization (i.e. quality) of the individual complexes involved in mitochondrial OXPHOS (Kuznetsov *et al.*, 2008). Furthermore, this method has

the advantage of measuring mitochondrial function in relation to the extracellular milieu, such as the cytoskeleton, nucleus and endoplasmic reticulum, which is not possible when mitochondria are isolated or homogenized (Picard *et al.*, 2011b; Picard *et al.*, 2011c; Larsen *et al.*, 2014). This technique is only viable after incubating dissected skeletal muscle samples in saponin, which has a high affinity for cholesterol-containing membranes, and therefore selectively permeabilizes the sarcolemma whilst maintaining the structural integrity of both the inner and outer mitochondrial membranes for *ex vivo* analysis (Kuznetsov *et al.*, 2008).

Using high-resolution respirometry methods, several studies have demonstrated evidence of mitochondrial dysfunction in animal models of CHF (De Sousa *et al.*, 2000; Garnier *et al.*, 2003; Zoll *et al.*, 2006). These studies have shown that *in situ* mitochondrial respiration is reduced by ~30-40% in limb skeletal muscles, with this effect being most apparent using substrates for complex I (Zoll *et al.*, 2006). Importantly, these effects were only observed over a longer duration of CHF, suggesting that skeletal muscle mitochondrial dysfunction only manifests after prolonged exposure to the condition (Zoll *et al.*, 2006). However, despite these findings in animal models, studies in CHF patients have failed to detect differences in skeletal muscle mitochondrial ATP production rate, oxidative enzyme activity, and mitochondrial function, including both complex I and III activity, when compared to age- and sedentary-matched controls (Mettauer *et al.*, 2001; Williams *et al.*, 2004; Garnier *et al.*, 2005; Toth *et al.*, 2012; Middlekauff *et al.*, 2013; Guzman Mentesana *et al.*, 2014). Nevertheless, one of these studies did demonstrate a correlation between mitochondrial function and both $\dot{V}O_{2peak}$ and $\dot{V}O_2$ at the lactate threshold (LT) in both the control and CHF populations

(Mettauer *et al.,* 2001), thereby suggesting a strong association between muscle mitochondrial function and exercise tolerance. The discrepancies between the human and animal studies may be explained by the large variability inherent with human studies coupled with the relatively small sample sizes studied. Furthermore, these human studies included patients on optimized drug therapy, which may also explain the differences between these modern studies and those initially conducted in the 1980's and 1990's.

Using similar methods, several previous studies have assessed skeletal muscle mitochondrial respiration in biopsies from DM patients. Collectively, these studies have demonstrated that DM patients exhibit a lower O_2 flux with substrates supporting complex I, complex I+II, and uncoupled respiration (Kelley *et al.*, 2002; Ritov *et al.*, 2005; Boushel *et al.*, 2007; Mogensen *et al.*, 2007; Phielix *et al.*, 2008; Larsen *et al.*, 2009; Rabøl *et al.*, 2009; Rabøl *et al.*, 2010; Ritov *et al.*, 2010; Larsen *et al.*, 2011). Interestingly, however, these studies have produced equivocal results, with some (Boushel *et al.*, 2007; Larsen *et al.*, 2009; Rabøl *et al.*, 2009; Rabøl *et al.*, 2010; Larsen *et al.*, 2011) but not all (Kelley *et al.*, 2002; Ritov *et al.*, 2005; Mogensen *et al.*, 2007; Phielix *et al.*, 2008; Ritov *et al.*, 2010), reporting that these differences in mitochondrial respiration are eradicated after adjusting the data to a marker of mitochondrial content, such as citrate synthase activity or mitochondrial DNA copy number. Therefore, although reduced mitochondrial content may be a primary mechanism contributing to mitochondrial deficits in DM patients, associated qualitative functional impairments also likely play a key role.

With regards to D-HF patients, there have only been a limited number of muscle biopsy studies conducted in this population so far (Taub *et al.*, 2012; Ramirez-Sanchez *et al.*, 2013). Despite relatively small sample sizes and the absence of DM, CHF and control groups, these studies have generally demonstrated that D-HF patients could increase mitochondrial protein expression, cristae abundance and volume density following a dietary intervention (Taub *et al.*, 2012; Ramirez-Sanchez *et al.*, 2013). However, they did not measure mitochondrial function *in situ* using high-resolution respirometry so were unable to specifically identify what the functional consequences of these aberrations were in terms of oxidative phosphorylation.

1.7.4 Putative mechanisms of skeletal muscle abnormalities in CHF and DM

In CHF and DM, chronically elevated concentrations of pro-inflammatory cytokines and angiotensin II, as well as mitochondrial substrate overload, are all considered to contribute to oxidative stress, which subsequently induces fibre atrophy and mitochondrial dysfunction. Oxidative stress is often caused by an increase in reactive oxygen species (ROS) and decreased antioxidant defences (Tsutsui *et al.*, 2011). Furthermore, oxidative stress has been implicated in numerous pathologies, including cardiovascular and metabolic diseases (Johannsen & Ravussin, 2009), as well as ageing (Harper *et al.*, 2004). Indeed, animal models of CHF (Tsutsui *et al.*, 2001; Rush *et al.*, 2005; Supinski & Callahan, 2005; Coirault *et al.*, 2007; Okutsu *et al.*, 2014; Martinez *et al.*, 2015; Gomes *et al.*, 2016; Laitano *et al.*, 2016; Mangner *et al.*, 2016) and CHF patients (Linke *et al.*, 2005; Cunha *et al.*, 2012; Guzman Mentesana *et al.*, 2014) exhibit oxidative stress and increased ROS production, which have been associated

with decreased mitochondrial function and exercise intolerance. Similarly, diet-induced hyperlipidaemia and hyperglycaemia animal models of DM demonstrate increased skeletal muscle ROS production and oxidative stress, which result in mitochondrial dysfunction, insulin resistance and exercise intolerance (Bonnard *et al.*, 2008; Anderson *et al.*, 2009; Yokota *et al.*, 2009). Recent studies in D-HF patients have demonstrated that low mitochondrial antioxidant enzyme activity levels are accompanied with increased markers of oxidative stress, including protein carbonylation and nitrotyrosine residue formation (Ramirez-Sanchez *et al.*, 2013). However, further research is required to determine whether oxidative stress and increased ROS production are evident in D-HF patients compared to CHF and DM patients alone.

1.8 Overall aims and objectives of thesis

The overall aim of this thesis was to determine how the specific combination of both CHF and DM, as seen in D-HF patients, impacts upon whole-body exercise tolerance and skeletal muscle structure and metabolism, with a particular emphasis on mitochondrial dysfunction. The rationale for this was based on evidence showing that D-HF patients have exacerbated exercise intolerance compared to CHF patients alone despite comparable cardiac function. Therefore we hypothesised that peripheral skeletal muscle abnormalities may present a key mechanism that contribute, in part, to exercise intolerance in D-HF patients. To answer this question, the present studies in this thesis first aimed to provide a more in-depth evaluation of measuring exercise intolerance in D-HF patients. Thereafter muscle biopsies from the upper (i.e. chest*)* and lower body (i.e. leg) were sampled to directly assess *in situ* mitochondrial function in D-HF patients.

Finally, this thesis ended by examining further muscle-specific mechanistic changes that likely contribute to the worse exercise intolerance D-HF patients.

Therefore the present thesis performed the following studies to achieve these aims:

1. A novel CPX test in a large clinical setting to better evaluate exercise tolerance in CHF patients, that would also provide important mechanistic insight into why D-HF patients suffer worse limiting symptoms on exertion;

2. Assessment of skeletal muscle mitochondrial function using high-resolution respirometry in *pectoralis major* biopsies from control, DM, CHF, and D-HF patients;

3. Assessment of skeletal muscle mitochondrial function using high-resolution respirometry in both *vastus lateralis* and *pectoralis major* biopsies from control, DM, CHF, and D-HF patients. This is to provide insight into the systemic effects of the clinical conditions (which we hypothesise to be more discernible in the *pectoralis major* muscle) and the potential confounding effects of physical activity and detraining, which likely have more of an impact on the *vastus lateralis*, a muscle which is frequently recruited during locomotor activities.

4. Identification of general underlying muscle-specific mechanisms contributing to the muscle pathology in D-HF including gene expression, fibre atrophy, capillarisation, and mitochondrial ROS production.

Chapter 2 General methods

2.1 Research participants

A total of 329 patients were recruited for the current thesis. Figure 2.1 details how these patients were included in each of the Results chapters included. These patients were grouped into four cohorts based on the presence of CHF and/or DM, including: 1) control (CON) patients selected for pacemaker intervention with a LVEF > 50% and no previous diagnosis of LVSD or DM; 2) DM patients with a previous diagnosis > 3 months as defined by a documented history of DM, fasting plasma glucose ≥ 7.0 mmol·L^{-1}, plasma glucose ≥ 11.1 mmol·L^{-1} two hours after the oral glucose tolerance test, and/or an HbA1c ≥ 6.5% (≥ 48 mmol·L^{-1}); 3) CHF patients, with symptomatic (NYHA class I-III) and stable CHF with no changes in medical therapy > 3 months and a LVEF < 50 % secondary to LVSD, as confirmed by echocardiography, with indications for a pacemaker implant procedure of either an ICD, permanent pacemaker (PPM) or CRT device; and 4) diabetic heart failure (D-HF) patients, that met criteria for both CHF and DM outlined above. The physical, demographic, clinical and treatment characteristics of these patients are included in the four individual results chapters.
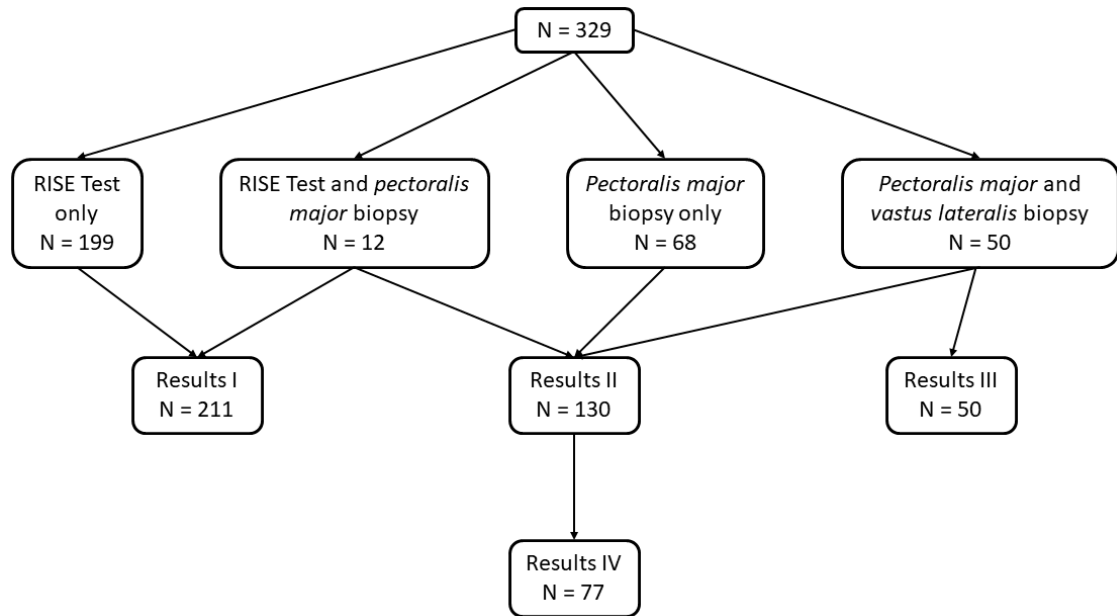
Figure 2.1. Summary of all the patients recruited to each of the Results chapters included in this thesis.

CON and DM patients were prospectively recruited after being referred for device implantation by a consultant cardiologist. CHF and D-HF patients were initially approached about the study after being listed for implant device therapy by a consultant cardiologist at the Heart Failure Clinic at Leeds General Infirmary. Most of the CHF and D-HF patients had previously attended a clinically-indicated symptom-limited peak CPX test. This test was used as evidence of exercise intolerance as demonstrated by a $\dot{V}O_{2peak} < 20$ mL·kg^{-1}·min^{-1}. This threshold was applied as patients with a $\dot{V}O_{2peak} > 20$ mL·kg^{-1}·min^{-1} are asymptomatic during exercise and have a better prognosis (Mancini *et al.*, 1991; Myers *et al.*, 2002). Patients were screened and excluded if they were unable to provide informed consent (i.e. those with cognitive dysfunction e.g. dementia). Additional exclusion criteria included those who had previously been diagnosed with other potentially confounding comorbidities, such as chronic obstructive pulmonary disease, cachexia or cancer. If patients satisfied these criteria, they were progressed to the informed consent stage of the study.

2.1.1 Sample size

The sample size for the skeletal muscle biopsy studies was determined from a power calculation on data previously collected from a pilot study. This calculation suggested that a sample size of at least 22 patients in each of the four cohorts would be required to detect a significant difference in a two-sided test with an alpha of 0.05 and 80% statistical power. Therefore we aimed to enrol 50 patients with CHF and 50 control subjects (25 patients with DM in each group). To account for participant drop-outs and experimental failures, we reasoned that 25 in each group would be a suitable sample target.

2.2 Ethics and informed consent

The studies performed were all approved by the Leeds West Research Ethics Committee (11/YH/0291; Appendix A) and the Leeds Teaching Hospitals R+D committee (CD11/10015) and all procedures were conducted in accordance with the Declaration of Helsinki (Appendix B). All prospective patients were provided with a participant information sheet (Appendix C) which detailed the outline of the study, including any potential risks. Prospective patients were then allowed sufficient time to read the information sheet and ask any questions they may have before deciding whether they wished to participate in the study. Participation was voluntary and patients were not required to provide an explanation for not wishing to participate. Non-participation in the study had no effect on any future treatment(s). Eligible patients elected for pacemaker surgery whom volunteered to participate then provided written informed consent (Appendix D). I took informed consent from the majority of patients after acquiring

certification in Good Clinical Practice (Appendix E) and attending an NIHR informed consent workshop (Appendix F).

All patients were assigned a numerical study identification code, which was used for recording all experimental data. A record linking the study ID to the participant's name was stored on a password-protected spreadsheet within a secure restricted access folder on the Leeds Teaching Hospitals Trust (LTHT) servers. Therefore all patient-identifiable information (e.g. name, NHS number, and date of birth) could not be determined from the experimental data spreadsheets. The data collected in this thesis will remain securely stored for the foreseeable future until no longer required.

2.3 Demographic, clinical and pharmacotherapy treatment factors

Age, sex, and body mass were recorded on the day that patients attended their appointment for an implantable device. Exercise capacity ($\dot{V}O_{2peak}$) was determined from a CPX test performed prior to the implant procedure (details provided in 2.4). NYHA functional classification was determined by a cardiologist based on the symptomatic status of the patient. Documented medical history records were accessed to determine the aetiology of LVSD (i.e. ischaemic or dilated cardiomyopathy (DCM)). These records were also accessed to record any additional comorbidities and/or treatments, including atrial fibrillation (AF), previous coronary artery bypass graft (CABG), and hypertension. Prior to the implant procedure, an accredited sonographer determined LVEF and the left ventricular internal diameter at end diastole (LVIDd) from 2-dimensional

transthoracic echocardiography according to European Society of Cardiology criteria using the Simpson's biplane method (Lang *et al.*, 2015). Blood samples were taken from patients on the day of the implant procedure to measure concentrations of haemoglobin, sodium, potassium, and creatinine. Renal function was determined using the estimated glomerular filtration rate (eGFR) method. Fasting plasma glucose and HbA1c concentrations were also recorded to determine DM status and confirm that non-DM patients (i.e. CON and CHF cohorts) did not show evidence of undiagnosed DM.

All relevant pharmacological treatments were recorded based on previous documented medical history. These included the use of angiotensin converting enzyme inhibitors (ACEi), beta-blockers, loop diuretics, angiotensin receptor blockers (ARB), aldosterone antagonists, statins, aspirin, metformin and insulin. Equivalent daily dosages for each treatment were also recorded. An equivalent dosage of furosemide was recorded for patients receiving bumetanide treatment by assuming 1 mg of bumetanide equates to 40 mg furosemide (Cubbon *et al.*, 2013).

2.4 Exercise testing

All ambulatory CHF and D-HF patients undertook a peak symptom-limited CPX test to volitional exhaustion prior to the implant procedure. These tests were performed under the supervision of trained cardiac physiologists at Leeds General Infirmary. CPX tests were performed on a computer-controlled stationary electromagnetically-braked cycle ergometer that continuously recorded cadence and power output (Excalibur Sport, Lode BV, Groningen, NL). A 12-lead

electrocardiogram (ECG) was used to monitor patients throughout the test and heart rate (HR) was determined beat-by-beat from the R-R interval. Pulse oximetry (Biox 3745, Ohmeda, Louisville, KY) was used to measure arterial oxygen saturation (S_pO_2) from the finger. Patients wore a nose clip and a mouthpiece, which was connected to a pneumotach and a sampling tube that enabled continuous breath-by-breath measurements of pulmonary gas exchange ($\dot{V}O_2$, $\dot{V}CO_2$, RER) and ventilatory variables (ventilation, $\dot{V}_E$; tidal volume, V_T; breathing frequency, B_f) to be recorded throughout the protocol (MedGraphics Ultima™ CardiO$_2$®, Medical Graphics UK Limited, Gloucester, UK). This measurement system was recalibrated prior to each experiment using manufacturer-recommended volume and gas calibration methods.

All tests were started with a 5 minute baseline warm-up period at a work rate of 5 W. Thereafter patients completed a RI test to exhaustion. The RI protocol consisted of a continuous increase in power output over the duration of the test, typically between 5-20 $W \cdot min^{-1}$ depending on the patients' symptomatic status. This ramp rate was selected to induce intolerance within ~8-12 minutes (Buchfuhrer *et al.*, 1983). Exercise was terminated voluntarily by the patient or when a pedal cadence > 50 rpm could no longer be maintained despite strong verbal encouragement, thereby indicating the limit of tolerance. The test was then preceded by a cool-down period at 10 W. Peak pulmonary variables were determined by averaging the final 12 breaths of exercise (~20 s) prior to exercise termination, as previously described (Bowen *et al.*, 2012a).

A selection of patients (Results I) completed a modified protocol, which included RI followed by a SE phase at 95% of the max work rate attained during RI (RISE-95) (Rossiter *et al.*, 2006; Bowen *et al.*, 2012a) to determine if $\dot{V}O_{2max}$ had been attained (Figure 2.2). During this test, patients completed the RI protocol outlined above and then exercised at 10 W for 5 minutes before completing the SE component of the test. The SE phase consisted of an immediate increase in power output, which patients were encouraged to maintain until the limit of tolerance, as defined above. Patients selected for the RISE-95 test were encouraged to attend the test in a postprandial (2-3 hr) state, having refrained from strenuous activity (24 hr), caffeine (3 hr) and alcohol consumption (48 hr) prior to testing.

Ramp-Incremental (RI) **Step-Exercise (SE)**
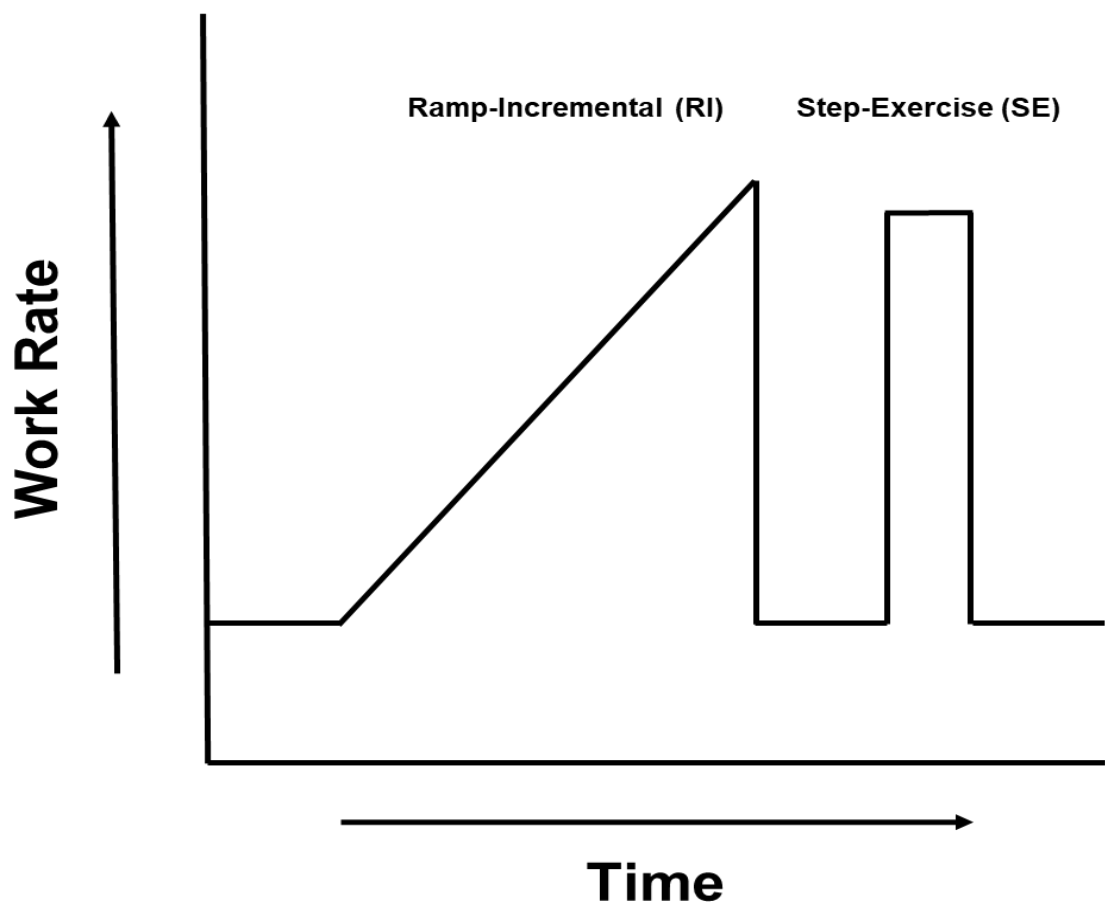
Figure 2.2. Schematic of RISE-95 protocol employed in the current study. Following a warm-up period, patients exercised to volitional exhaustion (RI). They were then allowed to recover for 5 minutes before completing the SE component of the test, which was performed at 95% of the peak work rate achieved during RI.

2.5 Pacemaker implantation and muscle biopsy sampling

2.5.1 *Pectoralis major* sampling

Fresh human skeletal muscle biopsies of the *pectoralis major* muscle were obtained from eligible patients undergoing pacemaker procedures at Leeds General Infirmary (Leeds Teaching Hospitals Trust) (Figure 2.3A). This muscle was chosen as a suitable candidate for analysis for two reasons. Firstly, as we hypothesised that the clinical impact of both CHF and DM have systemic effects on skeletal muscle, the *pectoralis major* muscle was considered a suitable candidate to discriminate these effects from any potentially confounding training effects, which are more prominent in the locomotor muscles of the lower body. Secondly, this muscle can be easily and painlessly accessed during routine pacemaker procedures due to the position of the pacemaker generator in a pre-pectoral pocket. Therefore samples may be procured from this site without burdening patients with additional invasive biopsies from a different muscle. This was considered to be a more ethically sound procedure for obtaining muscle biopsies for research purposes.

In each case, the pacemaker procedure was carried out routinely. Lidocaine was initially injected to anaesthetize the area and a small incision was made under the left clavicle. A pre-pectoral pocket for the pacemaker generator above *pectoralis major* provided a suitable position for the extraction of ~4 small biopsies weighing ~50 mg each (Figure 2.3B). Previous studies have shown that local anaesthetics have no effect on subsequent measures of skeletal muscle mitochondrial respiration (Cardinale *et al.*, 2018).
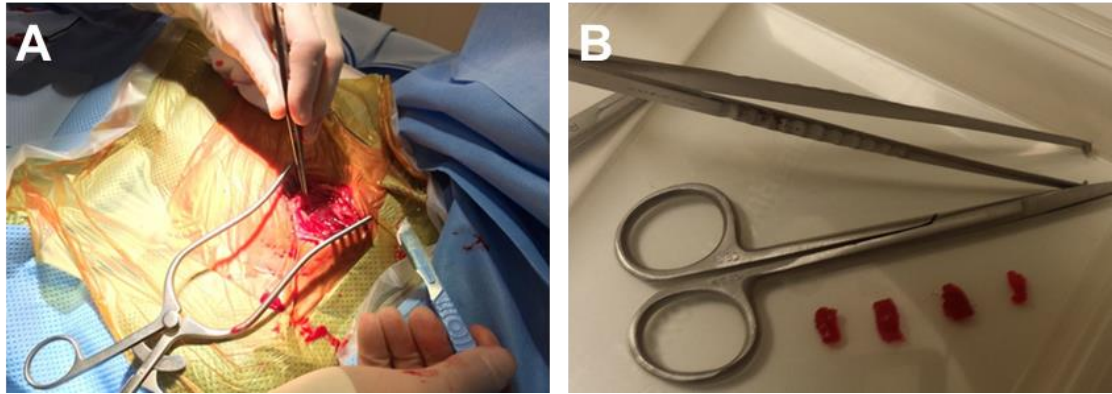
Figure 2.3. Sampling *pectoralis major* muscle from the pre-pectoral pocket during routine device implantations (A). Using this sampling method, we extracted ~4 small biopsies weighing ~50 mg each (B).

2.5.2 *Vastus lateralis* sampling

Percutaneous skeletal muscle biopsies from the *vastus lateralis* muscle of the right thigh were obtained from patients using the Bergström needle method (Bergström, 1962), modified to allow for suction (Evans *et al.*, 1982; Hennessey *et al.*, 1997; Shanely *et al.*, 2014). This suction-modified Bergström needle biopsy method is known to be safe and has a low complication rate (Tarnopolsky *et al.*, 2011). The biopsy was taken from the lateral part of the thigh muscle belly approximately one-third of the distance between the mid-patella of the knee and the femoral greater trochanter (Cardinale *et al.*, 2018). In cases where excessive subcutaneous adipose tissue and/or muscle atrophy made it difficult to identify the *vastus lateralis* muscle, patients were asked to tense their thigh muscles to allow for accurate determination of the sample site. After scrubbing the sample region with iodine, local anaesthetic (1% lidocaine) was injected intradermally in to the incision area and a sterile scalpel was used to make a ≤ 10 mm piercing in the skin and puncture through the subcutaneous adipose tissue and fascia of the muscle. After clearing of blood with sterile swabs, a 6 mm Bergström biopsy needle (Dixons 11420-06, UK) was introduced progressively through the incised

fascia to a depth of 2-3 cm and rotated to match the pennation angle of the muscle (Figure 2.4C). This was included to extract transverse samples for later histological analyses. The end of the needle was attached to a suction catheter, which was appended to a 50 mL syringe that was used to apply the necessary suction to extract samples (Figure 2.4D). Where necessary, several passes were made with the needle to extract sufficient muscle tissue (~4 samples totalling ~100 mg) for all future analyses. Following the procedure, the biopsy wound was closed with surgical tape strips and a soft adhesive sterile dressing (Figure 2.4E).
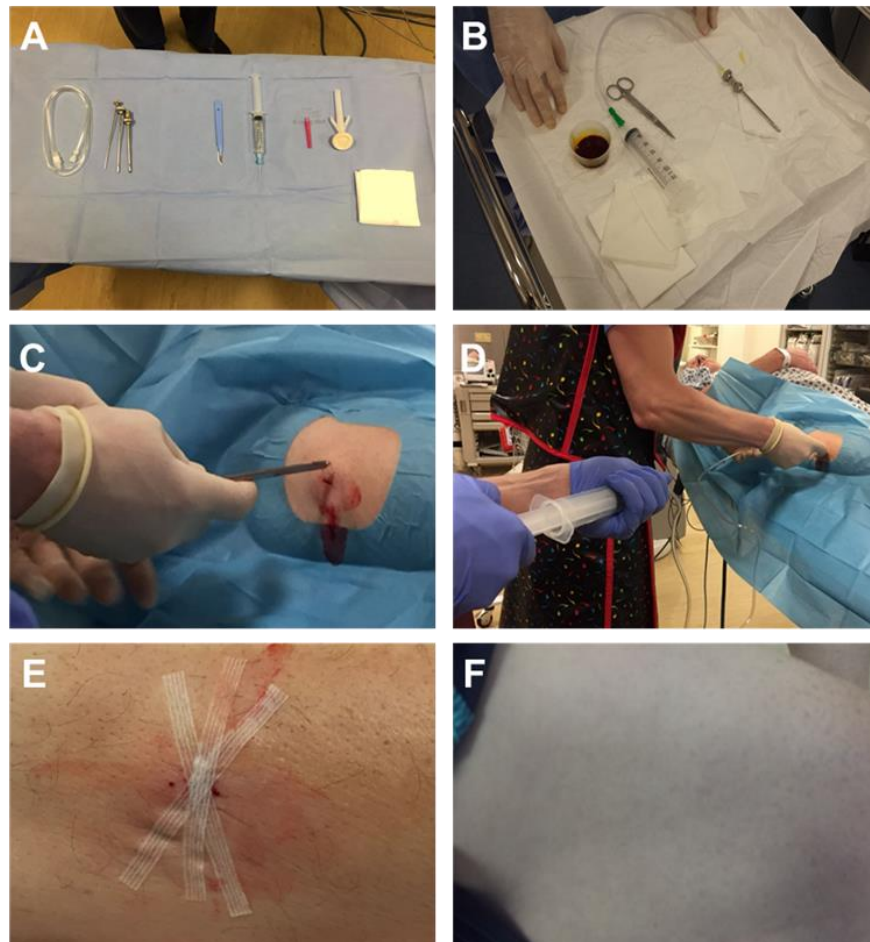
Figure 2.4. Sampling *vastus lateralis* muscle. Sterilised table set up prior to sampling *vastus lateralis* biopsies (A). Bergström biopsy needle attached to a suction catheter and syringe (B). Biopsy sampling method of inserting needle through a small incision of the thigh (C) and applying suction to extract samples (D). The biopsy wound was closed with surgical strips immediately following the procedure (E). Six months after the procedure, there was no visible scarring from the site where the biopsy was extracted (F).

2.5.3 Preparing muscle samples

Of the 4 samples extracted from the *pectoralis major* and *vastus lateralis*, 1 was immediately immersed in a 1.5 mL Eppendorf tube containing 1 mL of ice-cold relaxing and biopsy preservation solution (BIOPS) (Table 2.1). This sample was transported back to the laboratory on ice for high-resolution respirometry and, in some cases, for simultaneous fluorometric analysis. Skeletal muscle samples may be kept in BIOPS solution on ice for up to 5 hours without any impairment on respirometry measures (Cardinale *et al.*, 2018). A second sample was slightly stretched above its slack length and mounted on a pinned cork disc. This sample was orientated with the fibres parallel to the pin and perpendicular to the cork before being embedded in optimum cutting temperature medium (Thermo Fisher Scientific, Waltham, MA) (Figure 2.5A). This sample was snap-frozen in isopentane (2-Methylbutane; Sigma-Aldrich, St Louis, MO) cooled in liquid nitrogen and stored at -80 °C until later analysis. However, in some cases, samples were too big to be pinned up and so were embedded in the optimum cutting temperature medium in a cut-off pipette tip (Figures 2.5B & 2.5C) before being snap-frozen as described above. This sample was used for quantifying MHC isoform composition, fibre cross-sectional area and indices of capillarity. The remaining two samples were immediately snap-frozen in liquid nitrogen and stored at -80 °C for additional molecular biology analyses, including enzyme activity measurements and gene expression analysis.
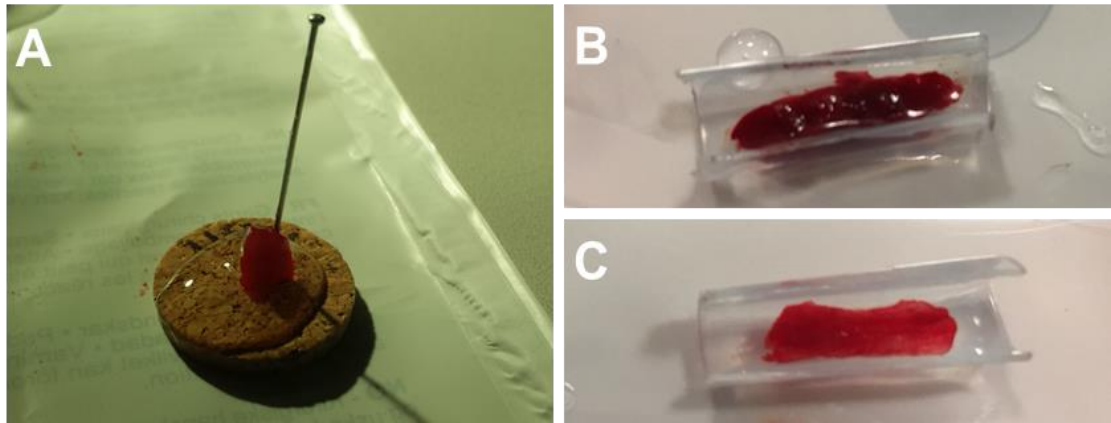
Figure 2.5. Preparing muscle samples for histological staining. Muscle biopsy sample pinned on cork prior to being frozen for later histological staining (A). Bigger samples from both *vastus lateralis* (B) and *pectoralis major* (C) were frozen in cut-off pipette tips.

2.6 Mitochondrial function in saponin-permeabilized fibres

Mitochondrial function was assessed in saponin-permeabilized skeletal muscle fibres using high-resolution respirometry (Oxygraph-2K; Oroboros Instruments, Innsbruck, Austria). High-resolution respirometry is considered the gold standard for assessing mitochondrial function by measuring *in situ* O_2 consumption in different mitochondrial respiratory states (Kuznetsov *et al.*, 2008). Furthermore, the permeabilized fibre method has the advantage of measuring mitochondrial function in relation to the extracellular milieu, such as the cytoskeleton, nucleus and endoplasmic reticulum, which is not possible when mitochondria are isolated (Picard *et al.*, 2011b; Picard *et al.*, 2011c; Larsen *et al.*, 2014).

2.6.1 Stock solutions for respirometry protocol

The relaxing and biopsy preservation solution (BIOPS) for transporting and preparation of permeabilized muscle fibres was made according to previously outlined protocols (Veksler *et al.*, 1987; Letellier *et al.*, 1992; Fontana-Ayoub *et al.*, 2014), and is outlined in Table 2.1 below. The pH was adjusted to 7.1 using

5 M KOH and the final solution was aliquoted into 20 ml universal tubes and stored at -20 °C until required. BIOPS has an ionic strength of 160 mM and contains the following ion concentrations: 0.1 µM free Ca^{2+}, 1 mM free magnesium (Mg^{2+}), and 5 mM MgATP.

Table 2.1. Solutions and compounds used to make BIOPS.

Solution/Compound	Final Con.	Molecular weight	Stock solution	Addition to 1 Litre	Source and product code
CaK_2EGTA*	2.77 mM		100 mM	27.7 ml	
K_2EGTA*	7.23 mM		100 mM	72.3 ml	
Na_2ATP	5.77 mM	551.1		3.141 g	Sigma A2383
$MgCl_2 \cdot 6 H_2O$	6.56 mM	203.3		1.334 g	Sigma M9272
Taurine	20 mM	125.1		2.502 g	Sigma T0625
$Na_2Phosphocreatine$	15 mM	255.1		4.097 g	Sigma P7936
Imidazole	20 mM	68.1		1.362 g	Sigma S6750
Dithiothreitol	0.5 mM	154.2		0.077 g	Sigma D0632
MES hydrate	50 mM	195.2		9.76 g	Sigma M0895

*See below

The CaK_2EGTA and K_2EGTA stock solutions were made up according to the below concentrations and stored at -20 °C until required:

1. CaK_2EGTA was made by dissolving 2.002 g CaCO3 (C4830, Sigma-Aldrich, St Louis, MO) in a 100 mM solution of EGTA (E4378, Sigma-Aldrich, St Louis, MO; 7.608 g/200 mL) while stirring continuously and adding 2.3 g KOH (P 1767, Sigma-Aldrich, St Louis, MO) to adjust the pH to 7.0.

2. K_2EGTA was made by dissolving 100 mM EGTA (7.608 g E4378, Sigma-Aldrich, St Louis, MO) and 200 mM KOH (2.3 g; P 1767, Sigma-Aldrich, St Louis, MO) in 200 ml water (H_2O), and adjusting the pH to 7.0 with KOH.

The mitochondrial respiration medium (MiR05) for respirometry experiments was made according to previously outlined protocols (Fasching *et al.*, 2014a), and is outlined in Table 2.2 below. The pH was adjusted to 7.1 using 5 M KOH (P 1767, Sigma-Aldrich, St Louis, MO) and the final solution was aliquoted into 20 ml universal tubes and stored at -20 °C until required. MiR05 has an ionic strength of 95 mM and contains the following ion concentrations: 2.1 mM free magnesium (Mg^{2+}), 90 mM K^+, and 0.46 mM free EGTA.

Table 2.2. Solutions and compounds used to make MiR05.

Solution/ Compound	Final con.	Molecular weight	Stock solution	Addition to 1 Litre	Source and product code
K-lactobionate*	60 mM		0.5 M	120 mL	
EGTA	0.5 mM	380.4		0.190 g	Sigma E4378
$MgCl_2$·6 H_2O	3 mM	203.3		0.610 g	Sigma, M9272
Taurine	20 mM	125.1		2.502 g	Sigma T0625
KH_2PO_4	10 mM	136.1		1.361 g	BDH Laboratory Supplies
HEPES	20 mM	238.3		4.77 g	Sigma H3375
D-Sucrose	110 mM	342.3		37.65 g	Acros Organics
Bovine serum albumin (BSA)	1 g·L^{-1}	66.5		1 g	Sigma A6003

*See below

The K-lactobionate stock solution was made by dissolving 35.83 g lactobionic acid (L2398, Sigma-Aldrich, St Louis, MO) in 200 ml H_2O and adjusting the pH to 7.0 with KOH.

2.6.2 Preparation of muscle fibres

Permeabilized skeletal muscle fibres for high-resolution respirometry were prepared as previously described (Pesta & Gnaiger, 2011; Pesta & Gnaiger, 2012; Lemieux *et al.*, 2014). Initially samples were mechanically permeabilized under a microscope (Meiji EMT, Tokyo, Japan), in a petri dish on ice containing BIOPS solution (Figure 2.6A). Connective tissue, blood and fat were removed from samples with sharp forceps and individual muscle fibre bundles (containing ~6-8 muscle fibres each) were gently dissected along their longitudinal axis (Figure 2.6B). This mechanical permeabilization allows for increased mitochondrial exposure to exogenous titrations during the respirometry experiment. These fibre bundles were then immediately placed into a new 1.5 mL Eppendorf containing 1 mL BIOPS solution and 50 µg·mL^{-1} freshly prepared saponin. Saponin selectively permeabilizes the cholesterol-containing sarcolemma whilst retaining the structural integrity of the inner and outer mitochondrial membranes as well as other extracellular components (Veksler *et al.*, 1987; Letellier *et al.*, 1992; Saks *et al.*, 1998; Kuznetsov *et al.*, 2008; Picard *et al.*, 2011c). This method consequently provides uninhibited access to the mitochondria for subsequent titrations of substrates, uncouplers and inhibitors added to the respiration medium (Kuznetsov *et al.*, 2008). To allow sufficient time for saponin to exert its effects, samples were left on ice for a 30 minute chemical permeabilization period. These saponin-permeabilized fibre bundles were

subsequently rinsed twice for 10 minutes each on ice in MiR05 (Table 2.2) to remove excess saponin.
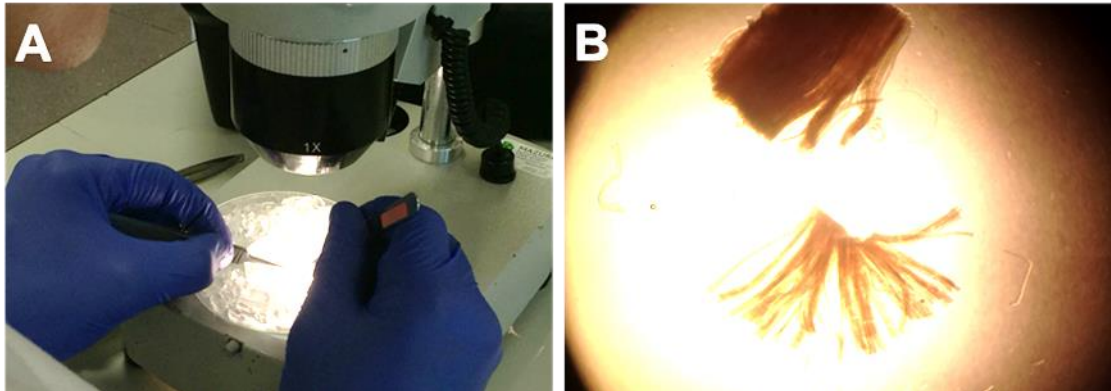
Figure 2.6. Mechanical permeabilization of muscle samples. Samples were initially mechanically permeabilized on ice under a microscope using sharp forceps (A) to gently dissect out individual muscle fibre bundles (containing ~6-8 muscle fibres each) along their longitudinal axis (B).

Samples were thereafter dry blotted on filter paper and carefully weighed (wet weight, W_W) on calibrated digital scales (Mettler Toledo, Greifensee, Switzerland), which measure samples in mg to 2 decimal places (Figure 2.7A). In each case, the sample was split in to two pieces (each weighing the recommended ~2-6 mg (Kuznetsov *et al.*, 2008). Small differences in sample weight within this range do not effect respirometry measures in permeabilized fibres (Cardinale *et al.*, 2018). Fibres were then then immediately transferred to the two internally lit sealed chambers of the high-resolution respirometer, where each chamber contained 2 mL MiR05 (Figure 2.7B).
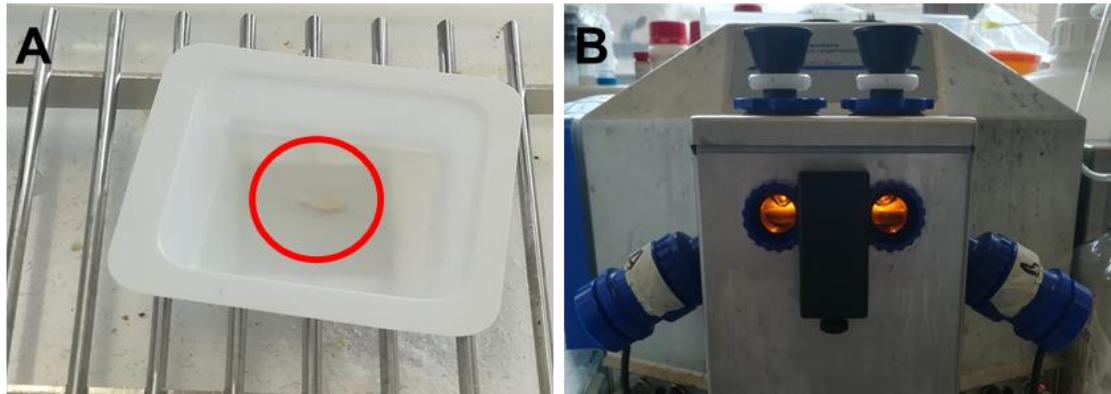
Figure 2.7. Preparing samples for high-resolution respirometry. After samples had been mechanically and chemically permeabilized, they were washed, dried and weighed on digital scales (A – sample circled in red). The Oxygraph-2k contains two internally lit chambers connected to polarographic O_2 sensors (B). Stoppers inserted at the top were used to seal the chambers.

2.6.3 Oxygraph-2k and DatLab software

The two chambers of the Oxygraph-2k are contained within a copper block that maintains a constant temperature of 37 °C and samples are suspended in MiR05 by electromagnetic stirrers rotating at 750 rpm. The dual chamber design of the O2K respirometer allowed each sample to be measured in duplicate on the same machine. Polarographic O_2 sensors are attached to each chamber and continuously measure the O_2 concentration within the sealed chambers over the time course of the experiment. The decline in the O_2 concentration within the chambers was measured over 2-second intervals by the sensors and recorded within specialised software (DatLab5 and DatLab6; Oroboros Instruments, Innsbruck, Austria) connected to the respirometer. These sensors measure partial O_2 pressure, which is then converted to O_2 concentration by accounting for the barometric pressure and an O_2 solubility factor of the respiration medium relative to pure water of 0.92 at 37 °C (Rasmussen & Rasmussen, 2003; Fasching & Gnaiger, 2014). The derivative of the decline in O_2 concentration within the chambers over time was calculated as the O_2 flux of the permeabilized muscle fibres throughout

the duration of the protocol, corrected for Ww of the sample. Therefore all O_2 flux measurements are expressed as O_2 flux per mass, in pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$ Ww. On the day of each experiment, a standard air calibration of the high-resolution respirometer was performed prior to adding samples (Fasching & Gnaiger, 2014).

2.6.4 SUIT protocol using high-resolution respirometry

Mitochondrial respiration was measured using a standard previously described substrate, uncoupler, and inhibitor titration (SUIT) protocol (Holmstrom *et al.*, 2012; Wüst *et al.*, 2012; Distefano *et al.*, 2017; Grassi *et al.*, 2017) to assess mitochondrial function in several different respiratory states. This SUIT protocol reconstitutes physiological conditions which allows functional *ex vivo* evaluation of the mitochondrial complexes in isolation. Each exogenous titration of the SUIT protocol was suitably separated to allow for stabilization of the O_2 flux signal recorded by the DatLab software. These titrations were performed using 10 µL, 25 µL, and 50 µL Hamilton syringes (Hamilton Co., Reno, NV), which are designed to fit the small opening in the stopper used to seal the chambers. Hamilton syringes were cleaned with 70% EtOH and distilled water between titrations (Fasching & Gnaiger, 2014). All the solutions used in the respirometry protocol, including stock and final concentrations, titration volume and dilution, as well as manufacturer's names and product codes are detailed in Table 2.3 below. Aside from saponin and pyruvate, which were always made fresh daily, all solutions were prepared in advanced according to recommended protocols and stored at -20 °C, except ADP (stored at –80 °C) (Saks *et al.*, 1998; Fontana-

Ayoub *et al.*, 2014; Gnaiger, 2014). These solutions were kept on ice for the duration of the experiment.

Table 2.3. Compounds used in the SUIT protocol in order of use.

Compound	Molecular weight	Stock concentration	Titration volume (µL)	Dilution	Final concentration	Source and product code
Saponin	929.1	20 mg·mL^{-1}	N/A	1:400	50 µg·mL^{-1}	S4521, Sigma-Aldrich
Blebbistatin		25 mM	2	1:1000	25 µM	13186, Cayman Chemical
Glutamate	169.1	2 M	10	1:200	10 mM	G1626, Sigma-Aldrich
Malate	134.1	0.4 M	2.5	1:800	0.5 mM	M1000, Sigma-Aldrich
Pyruvate	110.0	2 M	5	1:400	5 mM	P2256, Sigma-Aldrich
ADP	501.3	0.5 M	10	1:200	2.5 mM	A5285, Sigma-Aldrich
Cytochrome *c*	12.4	4 mM	5	1:400	10 µM	C7752, Sigma-Aldrich
Succinate	270.1	1 M	20	1:100	10 mM	S2378, Sigma-Aldrich
FCCP	254.2	10 mM	1	1:2000	5 µM	C2920, Sigma-Aldrich
Rotenone	394.4	0.1 mM	5	1:400	0.25 µM	R8875, Sigma-Aldrich
Antimycin A	540.0	5 mM	5	1:400	12.5 µM	A8674, Sigma-Aldrich
Ascorbate	198.1	0.8 M	5	1:400	2 mM	A4034, Sigma-Aldrich
TMPD	237.2	0.2 M	5	1:400	0.5 mM	T3134, Sigma-Aldrich
Sodium Azide	65.0	4 M	10	1:200	20 mM	BDH Laboratory Supplies

Each chamber contained 5 µM of the myosin II ATPase inhibitor, blebbistatin, which encourages fibre relaxation in order to limit the inhibition of respiration, which can occur in contracting fibres (Perry *et al.*, 2011). After the samples were added to the chambers but prior to any exogenous titrations, pure O_2 (BOC Healthcare, Manchester, UK) was injected in to the chambers with a 50 mL syringe to increase O_2 concentration > 400 nmol·mL^{-1} (µM) and overcome the known O_2 diffusion limitation within the mitochondria of permeabilized skeletal muscle fibres (Gnaiger *et al.*, 1998). Additional O_2 injections were performed during the experiment where necessary to prevent the concentration from decreasing to < 270 nmol·mL^{-1} (µM) (Wüst *et al.*, 2012).

The first respiratory state to be measured in the SUIT protocol was complex I Leak respiration (L_I). This state was measured by titrating in the Krebs cycle intermediates, glutamate (10 mM), malate (0.5 mM) and pyruvate (5 mM), which generate the complex I substrate NADH. This provides a measure of non-phosphorylated O_2 consumption that may be attributed to proton leak across the inner mitochondrial membrane. A bolus saturating concentration of 2.5 mM ADP was subsequently titrated to provide a measure of maximal complex I-supported OXPHOS (P_I). The permeability of the outer mitochondrial membrane was assessed by the addition of 10 µM cytochrome *c*; samples were excluded from the final analysis if this increased respiration > 15% as this is deemed indicative of outer mitochondrial membrane damage (Kuznetsov *et al.*, 2004). In such cases the increase in respiration demonstrates that cytochrome *c* can easily penetrate across the outer mitochondrial membrane. Such damage is considered to not accurately represent *in vivo* function, in which the outer mitochondrial membrane contains numerous ion channels and membrane-

bound co-transporters required for cellular respiration. The addition of 10 mM succinate provided a measure of maximal coupled electron flow with substrates providing convergent electron flow to the Q-junction supported by complex I+II-supported OXPHOS (P_{I+II}). The stepwise titration of 5 µM carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP) yielded an uncoupled state of maximal ETS capacity with complex I+II substrates (E_{I+II}). The addition of the complex I inhibitor rotenone (0.25 µM) provided a measure of complex II-supported ETS capacity in isolation (E_{II}). Finally, 2.5 µM antimycin A, a complex III inhibitor, provided a measure of residual oxygen consumption (ROX) as an estimate of non-mitochondrial (background) respiration, which was subtracted as a correcting factor from all respiratory measurements.

2.6.5 Complex IV activity assay

After the addition of antimycin A, a 2 mM titration of ascorbate in combination with 0.5 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) was used as a measure of complex IV activity. TMPD is an artificial electron donor to complex IV and ascorbate maintains TMPD in a reduced state (Kuznetsov & Gnaiger, 2010). Following this, 20 mM sodium azide was added to completely inhibit mitochondrial respiration (Bowler *et al.*, 2006) and therefore correct for the chemical background O_2 consumption that occurs due to autoxidation of TMPD in the presence of cytochrome *c* (Kuznetsov & Gnaiger, 2010). The difference in O_2 flux between the TMPD+ascorbate titration and that obtained following sodium azide was used as a measure of complex IV activity (Figure 2.9), which has been previously described as a surrogate measure of mitochondrial content (Larsen *et al.*, 2012a). To confirm the validity of this method as a marker of

mitochondrial content, we measured citrate synthase activity in 27 samples and observed that this was significantly correlated with the corresponding measures obtained using this complex IV assay (Figure 2.8). For respirometry analysis, this method has the advantage of being easily incorporated at the end of the protocol using the same sample of tissue. This therefore circumvents the need to do alternative methods on a separate tissue sample, which may have a slightly different mitochondrial content to that used in the respirometry experiment.
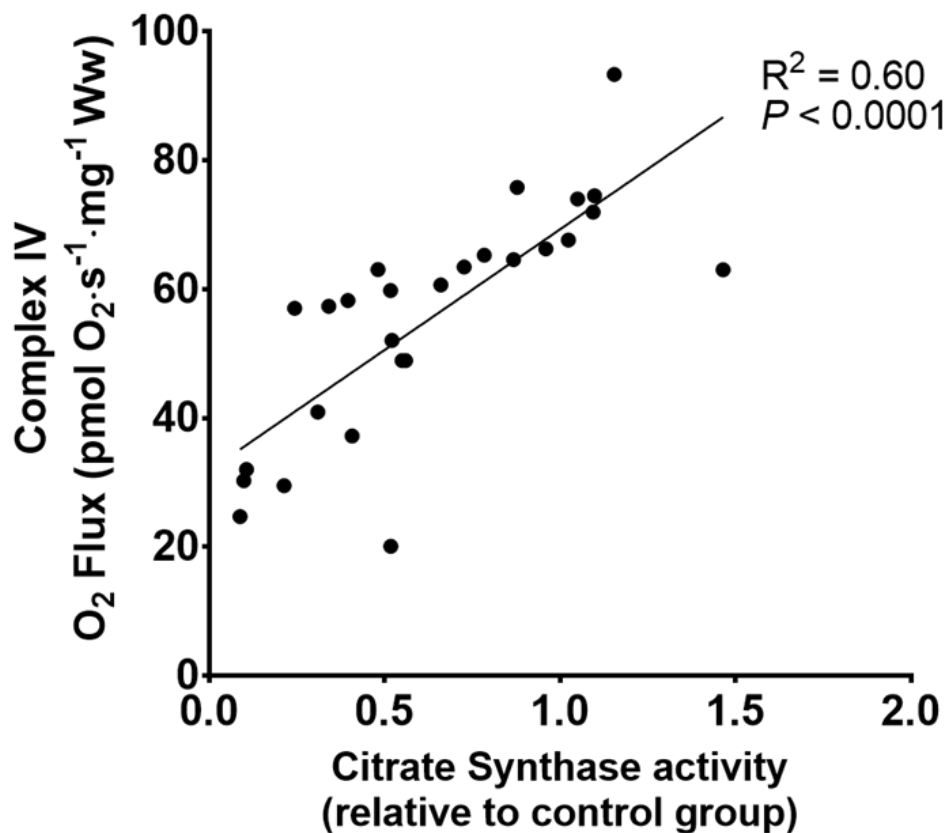
Figure 2.8. Validating complex IV assay against citrate synthase activity. The assay for complex IV activity was validated by a significant correlation with citrate synthase activity, which is a more common and well established method for determining mitochondrial content.

Figure 2.9 shows a typical trace obtained from the Datlab software for the SUIT and complex IV activity protocols outlined above. This trace shows both the O_2 concentration signal and the derived O_2 flux signal as well as the portions of data from which O_2 flux measures were obtained for each of the respective respiratory

states. After correcting for ROX, the recorded measures from each duplicate simultaneous experiment were averaged prior to statistical analysis. Previous studies have shown that measurements from each chamber are in close agreement with each other and there is no difference in measures on the same biopsy sample when conducted by two different investigators (Cardinale *et al.*, 2018). When consecutive experiments were carried out on the same day, the chambers were cleaned for 30 minutes in 100% EtOH and rinsed out with distilled water, otherwise they were left overnight in 70% EtOH (Fasching & Gnaiger, 2014).
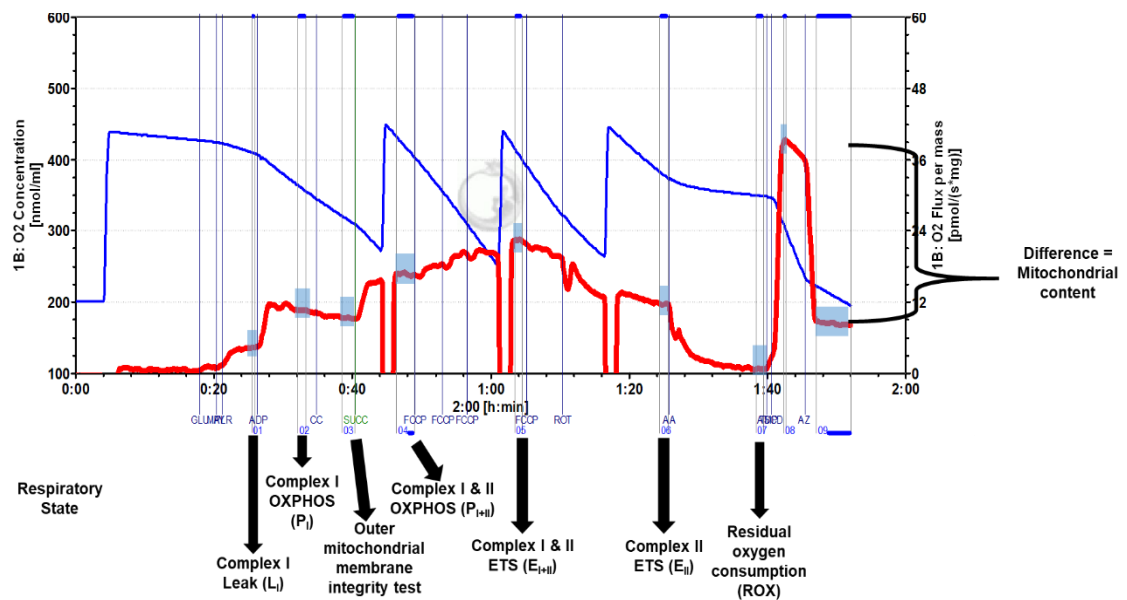
Figure 2.9. Screenshot showing traces obtained from a standard SUIT protocol on the Oxygraph-2k. The blue trace corresponds to the O_2 concentration within the chambers (left *y*-axis) and the red trace represents the O_2 flux of the muscle fibres, corrected for wet weight of the tissue (right *y*-axis), plotted against time (*x*-axis). The light blue shaded boxes show the areas of the red trace which were used to extract O_2 flux data for the corresponding respiratory states identified beneath the graph.

2.6.6 Respiratory and flux control ratios

Respiratory and flux control ratios were calculated to explore limitations within intrinsic mitochondrial respiratory capacity. The respiratory control ratio (RCR), which provides the degree of coupling between the O_2 consumed and the ATP

resynthesized by complex I, was calculated as the ratio of P_I to L_I respiration. Thus, a low RCR would suggest that a greater proportion of electrons leak across the inner mitochondrial membrane without contributing to ATP resynthesis via OXPHOS. Flux control ratios (FCR), which express the O_2 flux of each respiratory state (L_I, C_I, C_{I+II}, E_{II}) as a proportion of the maximal O_2 flux in the uncoupled state (E_{I+II}), were also calculated. These provide an estimate of how limiting complexes I and II are to maximal ETS capacity and therefore may be used as evidence of qualitative abnormalities within the individual complexes. Therefore a low FCR would indicate that there is an intrinsic abnormality within a complex which is limiting electron flow from being the maximal that may be sustained for ATP resynthesis. To relate changes back to a recommended measure of mitochondrial content (Szendroedi *et al.*, 2011), the raw O_2 flux results for each respiratory state were corrected to complex IV activity.

2.7 Citrate synthase enzyme activity levels

2.7.1 Muscle homogenization

Snap-frozen muscle tissue was thawed on ice and roughly minced with scissors in T-PER™ Tissue Protein Extraction Reagent (78510, Thermo Fisher Scientific, Waltham, MA) buffer containing a protease inhibitor tablet (1 tablet per 10 mL) (05892970001, cOmplete™ ULTRA Tablets, Mini, *EASYpack* Protease Inhibitor Cocktail, Roche, Mannheim, Germany). Each ~50 mg sample was homogenized in ~1 mL of homogenization buffer. Roughly chopped tissue was transferred to a Falcon tube stored on crushed ice and homogenized using a handheld homogenizer (IKA® T10 basic Ultra-Turrax®, Oxford, UK), set to maximum speed. Samples were sequentially homogenized for 20 seconds each to avoid

overheating and potentially denaturing enzymes. Each 20 second homogenization was repeated until no visible chunks remained and the handheld homogenizer was washed in distilled water between uses. Thereafter homogenate was transferred to a 1.5 mL Eppendorff and sonicated briefly using a handheld sonicator (Misonix XL-2000, Misonix Inc., Farmingdale, NY). Samples were then centrifuged at 12000 rpm (~12000 × g) for 10 minutes at 4 °C. The supernatant was then aliquot in to a new 1.5 mL Eppendorff, snap-frozen in liquid nitrogen and stored at -80 °C until later analysis.

2.7.2 Bicinchoninic acid protein assay

A bicinchoninic acid (BCA) assay was performed on homogenate samples to quantify total protein content and therefore calculate specific activity measurements for the citrate synthase assay. The BCA assay works on the premise that proteins reduce Cu^{2+} to Cu^+ under alkaline conditions (biuret reaction), producing a colour change from green to purple from the chelation of Cu^+ by bicinchoninic acid that is linearly proportional to the protein content of the sample across a broad range of concentrations (Smith *et al.*, 1985). Homogenate samples were thawed, vortexed and centrifuged for 20 seconds at 6000 rpm before being diluted 1:5 in the homogenization buffer and loaded onto a 96-well plate (3599, Costar, Corning Inc., Corning, NY) in 10 µL triplicates. Eight BSA standards, with a protein concentration range between 0-2000 µg·mL^{-1}, were loaded on to the plate in 10 µL duplicates. The homogenization buffer and distilled water (10 µL each) were loaded in duplicates as reference controls. A 200 µL working reagent buffer from a commercially available kit (23225, Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA) was added to

each well. The plate was then incubated at 37 °C for 30 minutes and then absorbance was read at 562 nm using colorimetry (Promega GloMax®-Multi+ Detection System, Madison, WI). The BSA standards were used to produce a standard curve from which the protein content of the homogenate samples could be determined based on their absorbance. Protein concentrations were typically within the range of 1-4 mg·mL^{-1}.

2.7.3 Stock solutions for citrate synthase assay

All stock solutions used in the citrate synthase assay are detailed below in Table 2.4. Acetyl-CoA was made up in advance by dissolving a 10 mg vial of acetyl-CoA in 1.235 mL distilled water and aliquoting 300 µL into 0.5 mL tubes that were stored at -20 °C or kept on ice during the experiment. 5,5'-Dithiobis-(2-Nitrobenzoic acid) (DTNB) was made fresh each day in a bijou tube by dissolving 2 mg DTNB with 5 mL of a 1M Tris-HCl buffer at pH 8.1. Oxaloacetic acid was made fresh each day in a bijou tube by dissolving 6.6 mg oxaloacetic acid in 4.5 mL distilled water and 0.5 mL 1M Tris-HCl buffer at pH 8.1. A citrate synthase standard was always made fresh each day by diluting 2 µL citrate synthase (C3260, citrate synthase from porcine heart, Sigma-Aldrich, St Louis, MO) in 998 µL (1:500) 0.1 M Tris-HCL buffer at pH 7.0.

Table 2.4. Stock solutions used in citrate synthase assay.

Name	Molecular weight	Stock Solution	Volume per well	Final concentration	Source and product code
Acetyl-CoA	809.57	10 mM	5 µL	0.25 mM	Sigma A2181
DTNB	396.35	1 mM	20 µL	0.1 mM	Sigma D8130
Tris-HCL	157.6	1 M	N/A	N/A	Sigma T3253
Oxaloacetic acid	132.07	10 mM	10 µL	0.5 mM	Sigma O4126

2.7.4 Spectrophotometry assay

Citrate synthase activity was measured spectrophotometrically in a plate reader (Thermo Fisher Scientific Varioskan® Flash, Waltham, MA) at 412 nm using a previously described method (Srere, 1969; Eigentler *et al*., 2015). All samples were measured in triplicate with each well of the clear 96-well plate (3599, Costar, Corning Inc., Corning, NY) containing 1 µL diluted (1:10) homogenate and a 200 µL working buffer containing 0.25 mM acetyl-CoA and 0.1 mM DTNB. The linear change in absorbance following the addition of 0.5 mM oxaloacetic acid was measured every 30 seconds over 10 minutes at 37 °C to give the rate of absorption change (rA = dA/dt). Each plate contained a commercially available citrate synthase standard as a reference control, one blank (no sample) control, and one internal control sample, which was used to correct for inter-assay variability. Specific enzyme activity was quantified by normalising to protein content to give a measure as international units (IU) per mg protein (IU/mg). All data were then expressed as a fold change relative to the control patient cohort.

2.8 Simultaneous high-resolution respirometry with H_2O_2 production

High-resolution respirometry was performed simultaneously with fluorometric measures of hydrogen peroxide (H_2O_2) formation. H_2O_2 is the stable ROS product formed by the dismutation of superoxide anion ($O_2^{\cdot-}$) by manganese superoxide dismutase (MnSOD/SOD2) in the mitochondrial matrix. Measuring H_2O_2 emission is the preferred method for determining mitochondrial ROS production for three reasons: 1) it is a lot more stable than $O_2^{\cdot-}$; 2) the majority of mitochondrial superoxide production is immediately dismutated to H_2O_2 by MnSOD; and 3) H_2O_2 can permeate across the two mitochondrial membranes and react with exogenous fluorogenic probes used to accurately quantify H_2O_2 production (Starkov, 2010). H_2O_2 production was determined using the established Amplex Red assay method (Zhou *et al.*, 1997), which has recently been incorporated to detect H_2O_2 production simultaneously with measurements of O_2 flux using high-resolution respirometry (Eigentler *et al.*, 2013; Fasching *et al.*, 2013; Krumschnabel *et al.*, 2015b; Makrecka-Kuka *et al.*, 2015).

2.8.1 O2K-Fluorescence LED2-Module

Mitochondrial H_2O_2 production and O_2 flux were simultaneously measured in different respiratory states using the Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria) high-resolution respirometer fitted with the O2K-Fluorescence LED2-Module (Oroboros Instruments, Innsbruck, Austria). This module contains two fluorometric sensors, each of which consist of a light-emitting diode, a photodiode, a filter-cap, and a filter set-up specific for detecting the fluorophore resorufin (Fasching *et al.*, 2014b). The filter set-up consists of a green short pass excitation filter (Fluorescence Sensor Green; excitation

wavelength = 520 nm) covering the light-emitting diode and a red long pass

emission filter (emission wavelength = 590 nm) covering the photodiode. The

fluorometric sensors were fitted in to the two front windows of the O2K chambers

and connected to the amperometric O2K-Multisensor channels, which allows for

the signals and corresponding H_2O_2 fluxes to be measured simultaneously with

O_2 flux measurements and recorded within specialised software (DatLab5 and

DatLab6; Oroboros Instruments, Innsbruck, Austria).

2.8.2 Amplex Red assay

The Amplex Red assay measures the production of the red fluorescent

compound resorufin, which is the reaction product formed in a 1:1 stoichiometric

ratio by the H_2O_2-dependent oxidation of Amplex® UltraRed (excitation maxima

= 568 nm; emission maxima = 581 nm) catalysed by the enzyme horseradish

peroxidase (HRP). Resorufin is extremely stable once formed and is detected at

emission/excitation wavelengths of 563 nm/587 nm, respectively (Anderson &

Neufer, 2006). Although the manufacturers of the commercially available

Amplex® UltraRed (A36006, Invitrogen™ Molecular Probes®, Eugene, OR) do

not stipulate how it differs from 10-acetyl-3, 7-dihydroxyphenoxazine, commonly

referred to as Amplex® Red, the reaction is assumed to work the same way. As

a result of this enzymatic reaction, there is an increased concentration of

resorufin formed within the chambers with a concurrent decrease in Amplex®

UltraRed (Krumschnabel *et al.*, 2015b). The concentration of H_2O_2 within the

chambers remains at (or close to) zero as all mitochondrial-derived H_2O_2

immediately reacts with the Amplex® UltraRed in the presence of HRP

(Krumschnabel *et al.*, 2015b).

2.8.3 H$_2$O$_2$ calibrations

Up until 3 µM, there is a linear relationship between the concentration of the accumulated resorufin and the fluorescence signal emitted (Krumschnabel *et al.*, 2015b). Thus, the slope in the fluorescence signal over time (ΔF/s) generated by the accumulation of resorufin is directly proportional to the production and release of mitochondrial H$_2$O$_2$. However, when the accumulated resorufin concentration is expected to exceed 3 µM, such as in prolonged SUIT protocols described in this study, the non-linearity of the relationship has to be corrected by repeated calibrations of external H$_2$O$_2$ to account for the change in sensitivity (V/µM) between the raw voltage recorded by the fluorometric sensors and the actual concentration of resorufin formed within the chambers (Krumschnabel *et al.*, 2015b). Using the previously described SUIT protocol, these calibrations were performed at the start of the experiment without the sample added, and then once in the Leak, OXPHOS, and uncoupled maximal ETS states. In each instance, 3 titrations of 0.1 µM freshly prepared H$_2$O$_2$ (Table 2.5) were added, which resulted in a step-wise increase in the amp raw signal with respect to time that was used to determine the signal change (V) in relation to concentration (µM). The regression equation of these titrations provided a measure of sensitivity (V/µM) that was used as a correction factor when calculating H$_2$O$_2$ production. Low concentrations of H$_2$O$_2$ were used for two reasons: firstly, to prevent excessive consumption of Amplex® UltraRed, which can be depleted during prolonged SUIT protocols; and secondly, to limit unnecessary resorufin formation, which may exceed the capacity of detection by the photodiode, which is within the range of 1-9 V (Fasching *et al.*, 2014b).

2.8.4 Stock solutions for fluorometric measurements

Table 2.5 details the stock and final concentrations, titration volumes and dilutions, plus manufacturer's names and product codes of the additional compounds included in this protocol for measuring H_2O_2 production. Superoxide dismutase (SOD) was optionally included to ensure that all mitochondrial-derived superoxide was dismutated to H_2O_2 (Krumschnabel *et al.*, 2015b). In each case, Amplex® UltraRed, HRP, and SOD were titrated in prior to the sample being added so that the machine could be calibrated in the absence of biological material, as recommended (Krumschnabel *et al.*, 2014). A 40 µM H_2O_2 stock solution was prepared fresh prior to each experiment in order to perform the necessary calibrations during the experiment. Once prepared, the stock solution was immediately aliquoted into 4 (one for each calibration step) 0.5 mL Eppendorf tubes and kept on ice for the duration of the experiment. Aliquots of Amplex® UltraRed, HRP, and SOD were prepared in advance of experiments and stored in the dark at -20 °C. The low concentration of Amplex® UltraRed was used as it is known to exert a degree of toxicity (Makrecka-Kuka *et al.*, 2015).

Table 2.5. Compounds used for fluorometric measurements of mitochondrial H_2O_2 production.

Compound	Stock concentration	Titration volume (µL)	Dilution	Final concentration	Source and product code
Amplex® UltraRed	10 mM	2	1:1000	10 µM	A36006, Invitrogen
Horseradish peroxidase	500 U/mL	4	1:500	1 U/mL	P8375, Sigma-Aldrich
Superoxide dismutase	5 KU/mL	2	1:1000	5 U/mL	S9697, Sigma-Aldrich
Hydrogen peroxide	40 µM	5	1:400	100 nM	H1009, Sigma-Aldrich

2.8.5 Protocol optimisation

Prior to starting experiments on human samples, preliminary experiments were performed to optimise the protocol and settings of the fluorometric sensors. These experiments involved optimising the timing and number of H_2O_2 calibrations as well as determining how the fluorometric sensors responded to reoxygenations and individual exogenous titrations included in the SUIT protocol in the absence of biological material. Additionally, the gain and polarization voltage of the fluorometric sensors were optimised to ensure that the recorded raw voltage signals were within the range of 1-9 V, which is the capacity of detection by the photodiode (Fasching *et al.*, 2014b). This optimisation resulted in a gain of 100 and a polarization voltage of 100 mV being used for each experiment.

2.8.6 SUIT protocol with simultaneous H_2O_2 production

Saponin-permeabilized skeletal muscle fibres for high-resolution respirometry with simultaneous H_2O_2 production were prepared as outlined above. Following the addition of Amplex® UltraRed, HRP, and SOD, the machine was calibrated in the absence of biological material (Krumschnabel *et al.*, 2014). After adding samples to the chambers of the respirometer, a modified version of the SUIT protocol previously outlined was followed. There were four major modifications to this SUIT protocol. Firstly, all experiments were carried out in the dark by switching off the internal chamber lights and using black cover-slips placed on top of black stoppers to prevent any external light from penetrating into the chamber through the injection ports and potentially interfering with the optical fluorometric measurements. The fluorometric sensors attached to the chamber windows also prevented any external light from entering. Secondly, all H_2O_2

production experiments were performed under normoxic conditions (O_2 concentrations of ~200 nmol·mL^{-1}) by maintaining the O_2 concentration within the chambers between 150-250 nmol·mL^{-1} as H_2O_2 production is significantly increased under hyperoxic conditions (Boveris & Chance, 1973). Thirdly, cytochrome *c* was not included in any of the experiments as this strongly redox compound is known to interfere with the measurements and is therefore incompatible with the method (Krumschnabel *et al.*, 2015b). Therefore intactness of the outer mitochondrial membrane had to be assumed during these experiments. Finally, three additional H_2O_2 calibrations were included as outlined above. After the recorded fluorescent signal had been corrected to account for changes in sensitivity, the H_2O_2 production rate was normalized to pmol·s^{-1}·mg^{-1} Ww by correcting to sample wet weight. There is a known "background" H_2O_2 production rate measured after the addition of the sample but prior to titrating in substrates, which was subtracted from all subsequent measurements as a correcting factor (Krumschnabel *et al.*, 2015a). pmol·s^{-1}·mg^{-1}

2.9 Muscle isoform composition, cross-sectional area and capillarity indices

2.9.1 Immunohistochemistry

Isopentane-frozen samples were removed from the -80 °C freezer and allowed to warm up to -20 °C for 30 minutes before cutting sections. Serial cross-sections (10 µm thick) of muscle biopsy samples were cut using a sharp blade (Leica 819 Low Profile microtome blades, Leica Biosystems, Nussloch, Germany) on a cryostat maintained at -20 °C (Leica CM1860 UV cryostat, Leica Biosystems, Nussloch, Germany). Samples were attached to the specimen disc of the

cryostat, which is perpendicular to the blade and therefore provided transverse cross-sections. Cross-sections were mounted on to Polysine®-coated adhesion slides (Thermo Fisher Scientific, Waltham, MA) and stored at -20 °C until staining.

All immunohistochemistry methods outlined below were modified based on a recently published optimised protocol on rat *extensor digitorum longus* muscle (Kissane *et al.*, 2018). Briefly, slides were warmed to room temperature and allowed to air dry for 1 hour before histochemical analysis. Thereafter all subsequent procedures were performed at room temperature on the same day. Each section to be stained was encircled using a wax pen (Pink PAP Pen, Sigma-Aldrich, St Louis, MO) to create a well. Sections were initially fixed for 2 minutes in a 2% paraformaldehyde solution, washed in phosphate-buffered saline (PBS; P4417, Sigma-Aldrich, St Louis, MO), and then blocked for 10 minutes in a 1% BSA (A6003, Sigma-Aldrich, St Louis, MO) solution to prevent any non-specific binding (~150 µL/well). Two monoclonal antibodies specific to different MHC isoforms were used to differentiate muscle fibre types: a mouse BA-D5 (IgG2B, 1:1000) antibody specific to type I (MHC-I) slow oxidative fibres and a mouse SC-71 (IgG1, 1:500) antibody specific to fast oxidative, glycolytic type IIA (MHC-IIa) fibres (Developmental Studies Hybridoma Bank, University of Iowa, IA). The remaining unstained fibres were assumed to be type IIx (fast glycolytic) fibres (Elliott *et al.*, 2016). Laminin, an extracellular matrix glycoprotein within the basement membrane, was stained with a rabbit anti-laminin antibody (1:200; L9393, Sigma-Aldrich, St Louis, MO) to identify muscle fibre boundaries. This dilution was determined after establishing the required concentration needed for the software to detect the fibre boundaries (Figure 2.10). All primary

antibodies were dissolved in PBS and incubated for 1 hour at room temperature. Samples were then washed in PBS and incubated for another hour at room temperature with appropriate polyclonal secondary antibodies also dissolved in PBS: Alexa Fluor® 555-conjugated goat anti-mouse IgG (1:1000; A-21422, Thermo Fisher Scientific, Waltham, MA); Alexa Fluor® 488-conjugated rabbit anti-mouse IgG (1:1000; A11059, Thermo Fisher Scientific, Waltham, MA); and CF™ 405M-conjugated goat anti-rabbit IgG (1:500; SAB4600461, Sigma-Aldrich, ST Louis, MO). Capillaries were simultaneously stained with the carbohydrate binding protein (lectin) biotinylated *ulex europaeus* agglutinin I (1:200; B1065, Vector Labs, Peterborough, UK), which is a specific and established marker for human endothelial cells (Kirkeby *et al.*, 1993; Parsons *et al.*, 1993) that preferentially binds to glycoproteins and glycolipids containing α-linked fucose residues. Cover-slips (VWR International, Radnor, PA) were applied to samples using mounting medium (Fluoromount-G®, SouthernBiotech, Birmingham, AL). Slides were then imaged using the Nikon Eclipse E600 (Nikon, Tokyo, Japan) optical microscope attached to a digital camera (QIMAGING, MicroPublisher™ 5.0 RTV, Surrey, BC, Canada) and analysed using digital image software (AcQuis, Syncroscopy, Cambridge, UK). All slides were imaged at ×10 magnification, which produced images that were 866.33 × 649.75 µm (2560 x 1920 pixels) in size.
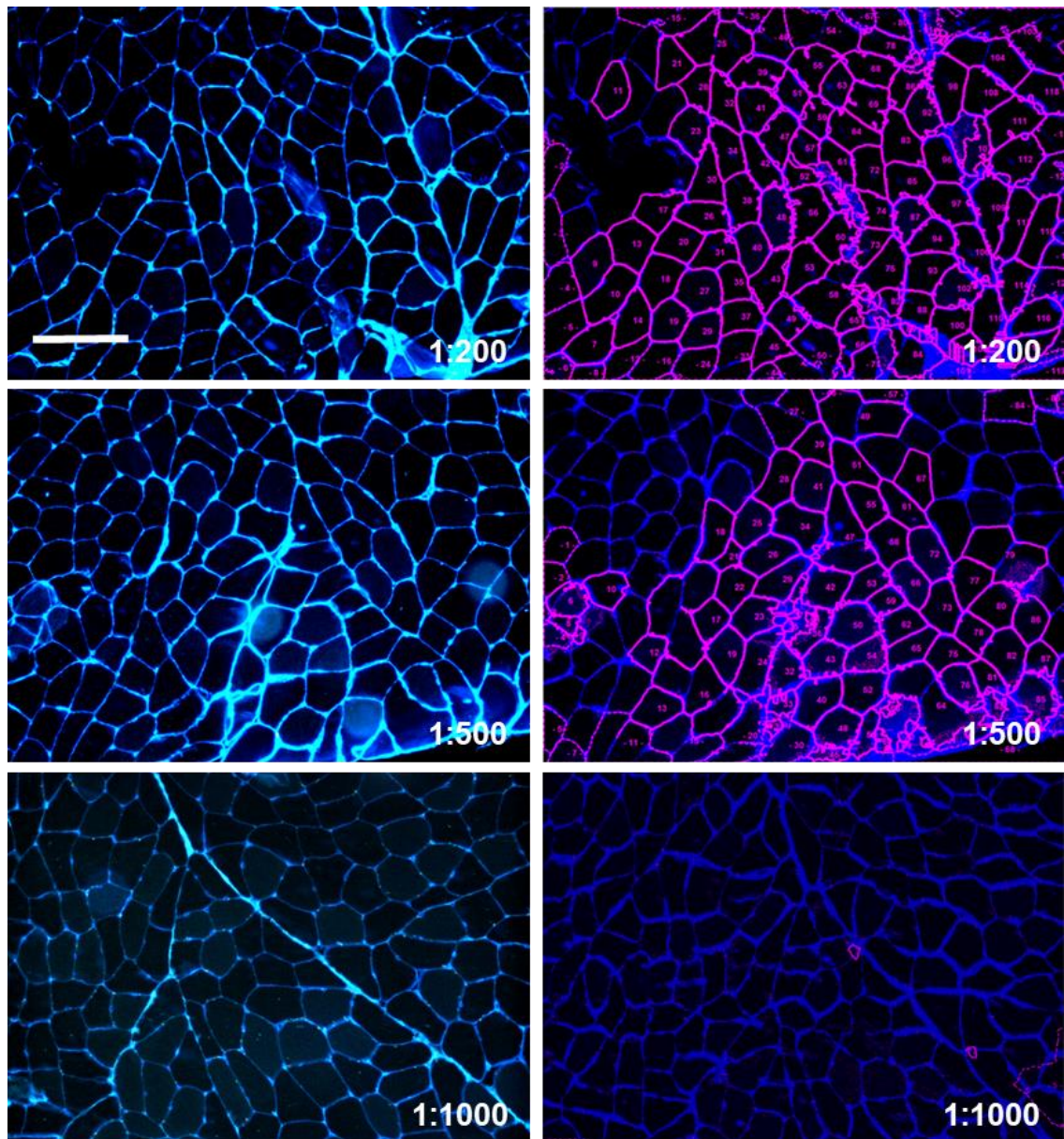
Figure 2.10. Laminin staining optimisation. Serial dilutions of laminin were tested to determine the appropriate concentration to use for the Dtect software to identify the fibre boundary. 1:1000 dilution was undetected by the software. 1:200 gave an appreciable improvement compared to 1:500 and was therefore used throughout all subsequent staining. Scale bar represents 200 μm.

2.9.2 Dtect Analysis

An unbiased sampling method was used to create a quadrat counting frame containing a region of interest (Figure 2.11B) from which quantitative measures of tissue morphometric indices were obtained, as recommended (Egginton, 1990a). Fibre segmentation and isoform identification was performed using a novel semi-automated software, Dtect, which is coded in MATLAB. This software

firstly identifies the blue stained lamina to delineate fibre boundaries with a magenta outline (Figure 2.12A). Secondly, the software uses the different MHC isoform stains to classify all fibres into the three primary human phenotypes (types I, IIa, and IIx) (Figure 2.12B). At each of these stages, the user can make manual adjustments to remove artefacts, improve the demarcation of the fibre boundary and tailor the fibre typing allocation. The third step to this method involves manually identifying individual capillaries on the fibre boundaries (Figure 2.12C), which are then used to generate fibre type-specific morphometric indices of capillarisation.
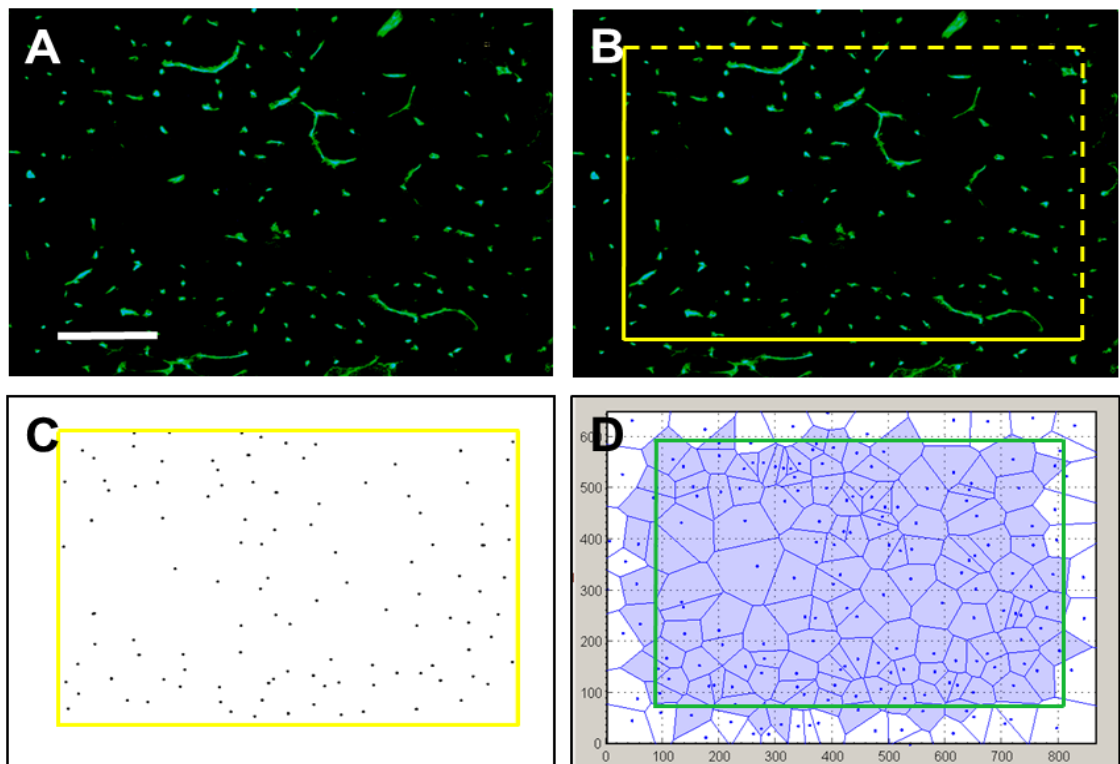
Figure 2.11. Quantifying capillary domain areas using unbiased sampling. Representative image of histological staining with lectin (*ulex europaeus agglutinin I*) (A). A quadrat counting frame was used to overlay histological images to create a region of interest using unbiased sampling (B). Fibres and capillaries overlaying the dotted line were included whilst those overlaying the solid lines were excluded. These histological images may be digitised to compute the number of capillaries within the region of interest (C). MATLAB software was used to create capillary domain areas as tessellating voronoi polygons by bisecting the intercapillary distances between each capillary and adjacent capillaries to produce boundaries (blue borders) that are equidistant from neighbouring capillaries. Scale bar represents 200 μm.
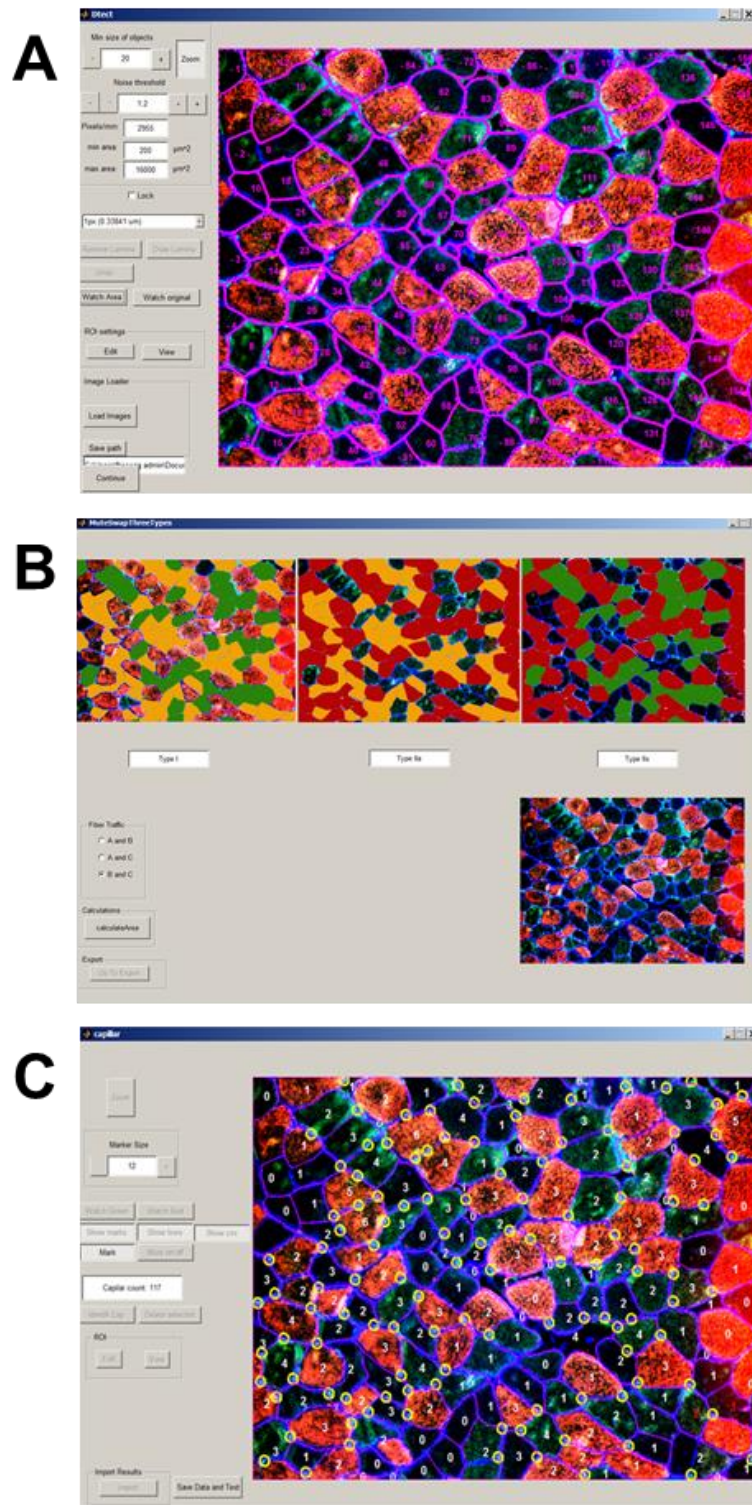
Figure 2.12. Screenshots showing how Dtect software generates fibre-type specific indices of cross-sectional area and capillarity. Firstly, the software identifies the blue laminin staining to create fibre boundaries in magenta (A). Individual fibres are numbered and included in the output file detailing fibre-specific characteristics. Secondly, the software detects the individual fibre types based on the MHC staining (B). Within each box, the only fibres are visible are those corresponding to that fibre type (I, IIa, or IIx). Remaining fibres are filled in colours to show what fibre type they correspond to (type I in red; type IIa in green; type IIx in yellow). Finally, capillaries (in small yellow circles) are identified manually by the user (C). The numbers within each fibre correspond to the number of contiguous capillaries with that specific fibre.

2.9.3 Morphometric analyses

Global average numerical indices of the capillary-to-fibre (C:F, N_N(c,f)) ratio and capillary density (CD, in mm^{-2}; N_A(c,f)) were quantified using standard equations:

$$N_N(c,f) = N(c)/N(f)$$

$$N_A(c,f) = N(c)/n \cdot \bar{a}(f)$$

Both C:F and CD are scale-dependent and are therefore heavily influenced by changes in fibre cross-sectional area (FCSA) (Egginton, 1990b), which can often accompany pathophysiological conditions, such as CHF and DM. Therefore it is more appropriate to provide localised scale-independent measures of capillarity, which are less sensitive to changes in FCSA and can detect very subtle changes in capillary distribution. Moreover, when capillary and MHC isoform markers are used simultaneously, it is possible to quantify fibre type-specific interactions with individual capillaries, which can be used to identify changes in capillary distribution, and thus O_2 supply, within a particular fibre type. To calculate scale-independent measures of capillary distribution, the capillary domain area (in μm^2), which is defined as the 2-dimensional area of a muscle cross-section closest to an individual capillary, was determined using as previously described method (Al-Shammari *et al.*, 2014). By bisecting the intercapillary distances between each capillary and adjacent capillaries, this method produces tessellating voronoi polygons with boundaries that are equidistant from neighbouring capillaries (Al-Shammari *et al.*, 2014). Capillary domain areas were calculated as follows:

$$V_i = \left\{ x \middle| x \in \Omega; \left\| x - x_i \right\| \leq \left\| x - x_k \right\|, K \neq i \right\},$$

Where Ω represents the global domain and V_i and x_i denote the voronoi polygon (capillary domain area) and position of the centroid of the *i*th capillary,

respectively (Al-Shammari *et al.*, 2014). These capillary domains provide an estimate of the area of tissue supported by a particular capillary in terms of O_2 delivery. By overlapping these capillary domain areas with individual muscle fibres, it is possible to derive the fractional areas of each fibre supplied by a particular capillary. This provides a measure of the local capillary-to-fibre ratio (LCFR; Figure 2.13) for each fibre, which was derived from the automated Dtect computational analysis by calculating the sum of these fractional domain areas overlapping each individual fibre using the following equation:

$$LCFR_j^{VP} = \sum_i \frac{A(\Omega_j \cap V_i)}{A(V_i)}$$

Where Ω_j and A represent the region and area of the *j*th fibre, and $LCFR_j^{VP}$ denotes the sum of the fractions of each voronoi polygon (VP) overlapping the *j*th fibre (Al-Shammari *et al.*, 2014). This LCFR method is more sensitive for detecting the heterogeneity of capillary supply and can therefore be more informative with regards to dysfunctional changes that may occur in certain conditions (Egginton, 1990b). The local capillary density (LCD, in μm^{-2}), a normalised measure of CD to account for allometric scaling, was quantified for each fibre in Dtect by dividing the LCFR by the FCSA, accordingly:

$$LCD_j^{VP} = \frac{1}{A(\Omega_j)} \sum_i \frac{A(\Omega_j \cap V_i)}{A(V_i)}$$

These individual measures of LCFR and LCD were then used to provide mean averages for each fibre type according to the MHC staining.
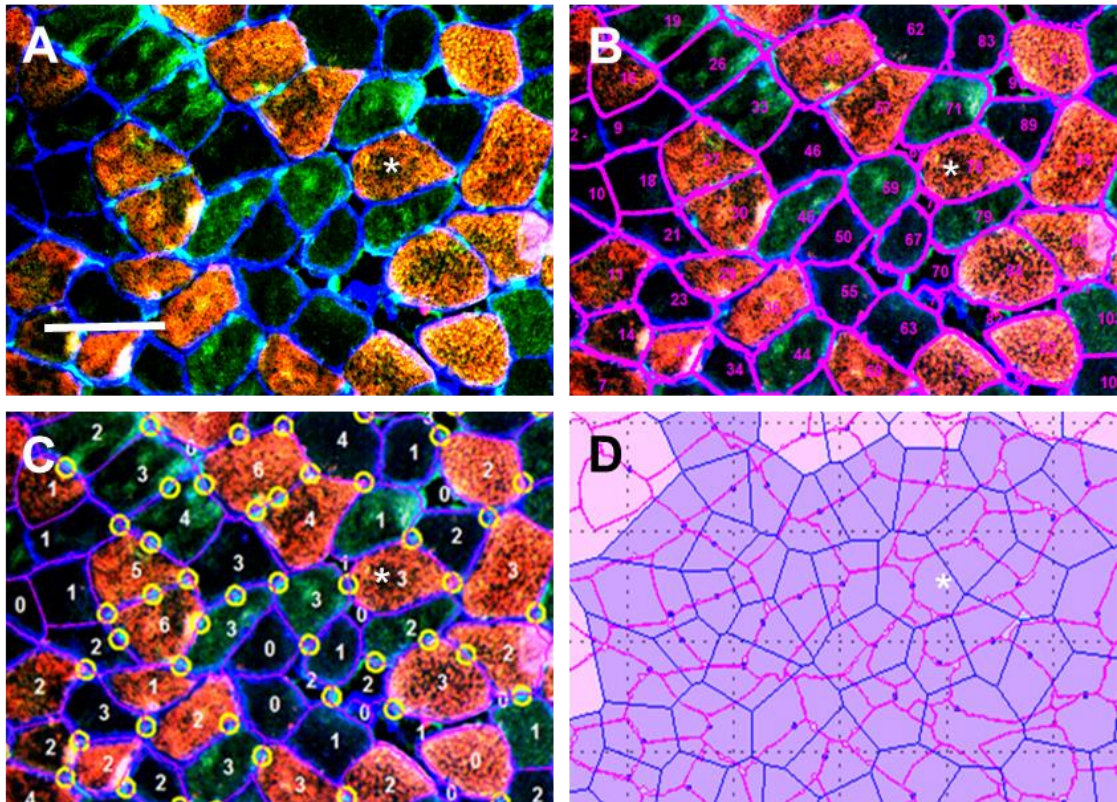
Figure 2.13. Quantifying local capillary-to-fibre ratio. Transverse cross-sectional areas are first simultaneously stained to identify fibre boundaries, capillaries and MHC isoform (A). Fibre boundaries (B) and capillary locations (C) are then identified within specialised software. The tessellating capillary domains (voronoi polygons) overlay the magenta outline of the individual muscle fibres (D). Scale bar represents 50 μm. *Identifies the same corresponding fibre in each image.

The Dtect software also quantified the FCSA (in $μm^2$) for each fibre which was then used to calculate the mean FCSA for each fibre type. The number of fibres as well as the average FCSA for each respective fibre type were then used to compute the corresponding mean fibre numerical and areal densities (both in %) according to previously published equations (Egginton, 1990a). These equations compute total muscle cross-sectional area (A) and number of fibres (N) as follows:

$$A = (n_i \cdot \bar{a}_i) + (n_j \cdot \bar{a}_j) + (n_k \cdot \bar{a}_k)$$

$$N = (n_i + n_j + n_k)$$

Where n and $\bar{a}$ represent the number and mean cross-sectional area of fibre types *i*, *j*, and *k*, respectively. From these values, the total and fibre-type specific mean cross-sectional areas were calculated as follows:

$$\bar{a} = \frac{(n_i \cdot \overline{a}_i) + (n_j \cdot \overline{a}_j) + (n_k \cdot \overline{a}_k)}{N}$$

Accordingly, the fibre numerical density of fibre type *i* could be quantified as:

$$\frac{n_i}{(n_i + n_j + n_k)}$$

Similarly, the fibre areal density of fibre type *i* could be quantified as:

$$\frac{\overline{a}_i}{(\overline{a}_i + \overline{a}_j + \overline{a}_k)}$$

2.10 Gene expression analysis

2.10.1 RNA extraction

RNA molecules longer than 200 nucleotides were extracted and purified from ~30-40mg muscle tissue using the RNeasy® Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. All procedures, including centrifugation steps, were carried out at room temperature and performed as quickly as possible. Firstly, samples were homogenized and lysed in 2 mL microcentrifuge tubes containing 300 μL buffer RLT (with 10 μL·mL^{-1} β-mercaptoethanol) and 5 mm stainless steel beads (QIAGEN, Hilden, Germany) using the TissueLyser II (QIAGEN, Hilden, Germany) operated for 2 minutes at 20 Hz. Lysates were then transferred to new 1.5 mL microcentrifuge tubes and mixed with 590 μL RNase-free water and 10 μL Proteinase K, which digests proteins. After incubating for 10 minutes at 55 °C in a heating block

(AccuBlock™, Labnet International Inc., Edison, NJ), samples were centrifuged for 3 minutes at 10,000 × g to pellet tissue debris. The supernatant was then mixed with 0.5 volumes (~450 µL) 100% ethanol and transferred to RNeasy mini spin columns, placed in 2 mL collection tubes, and centrifuged for 15 seconds at 10,000 × g. Column membranes were washed by centrifuging a further two times for 15 seconds at 10,000 × g, firstly with 350 µL buffer RW1 and then 500 µL buffer RPE (diluted 1:5 with 100% ethanol). Columns were centrifuged again for 2 minutes at 10,000 × g. Flow-through was discarded after each centrifugation step. Columns were centrifuged at maximum speed to dry the membrane. To elute the purified RNA bound to the silica membrane, 30 µL RNase-free water was added to the column and centrifuged for 1 minute at 10,000 × g. RNA eluent concentrations (ng·µL^{-1}) and purities were quantified at 260 nm using a spectrophotometer (NanoDrop™ 2000, Thermo Fisher Scientific, Waltham, MA).

2.10.2 cDNA synthesis

RNA extractions were reverse transcribed to cDNA using the RT^2 First Strand Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Briefly, normalized RNA extractions (to account for differences in RNA concentration) were mixed with 2 µL Buffer GE and RNase-free water in PCR tubes to give a final volume of 10 µL. Tubes were incubated for 5 minutes at 42 °C in a thermal cycler (^{3}Prime, Techne, Cambridge, UK) to eliminate genomic DNA. After adding a 10 µL reverse transcription mix (containing 4 µL buffer BC3, 1 µL control P2, 2 µL RE3 reverse transcriptase mix, and 3 µL RNase-free water), tubes were incubated again in the thermal cycler for 15 minutes at 42 °C followed

by 5 minutes at 95 °C to generate cDNA. All cDNA samples were stored at -20 °C until further analysis.

2.10.3 Real-Time quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis

Triplicate samples of cDNA (20 ng) and a master mix (containing 10 µL RT^2 SYBR® Green ROX™ qPCR Mastermix (QIAGEN, Hilden, Germany), 0.6 µL primer, and RNase-free water) were loaded on to a 96-well plate (MicroAmp™ Optical 96-Well Reaction Plates, Applied Biosystems, Foster City, CA) to give a final volume of 20 µL/well. The qPCR mastermix contains real-time PCR buffer, a DNA *Taq* polymerase, nucleotides, SYBR Green dye, and ROX dye. Plates were sealed with adhesive film (MicroAmp™ Clear, Applied Biosystems, Foster City, CA), centrifuged and loaded on to a standard real-time qRT-PCR system (Applied Biosystems Prism 7900HT, Foster City, CA). The qRT-PCR protocol consisted of 40 thermal cycles of denaturation (95 °C), annealing (60 °C) and amplification (95 °C). All human primers for qRT-PCR were purchased from QIAGEN and are summarized in Table 2.6 below. The difference in threshold cycles (ΔC_T) relative to a control was quantified for each sample and then normalized to an endogenous control, beta-actin (ACTB), to quantify gene expression using the Δ-Δ-C_T method (Schmittgen & Livak, 2008). mRNA expression was then quantified relative to the control cohort.

Table 2.6. Summary of human primers used in real-time qRT-PCR, including their QIAGEN catalogue code and function.

Primer	Catalogue code	Function
ACTB (Beta-actin)	PPH00073G	Part of actin protein family. Housekeeping gene
PPARGC1A (PGC-1α)	PPH00461F	Transcriptional coactivator regulating muscle metabolism and mitochondrial biogenesis
SOD2	PPH01716B	Encodes for the SOD2 antioxidant enzyme in the mitochondrial matrix
NDUFS1	PPH19871A	Largest subunit of complex I forming the iron-sulphur component of the enzyme
NDUFS3	PPH05975A	Core subunit of complex I forming the iron-sulphur component of the enzyme
FIS1	PPH19947A	Regulates mitochondrial morphology by promoting mitochondrial fission
OPA1	PPH12084A	Regulates mitochondrial morphology by promoting mitochondrial fusion

2.11 Statistical analyses

All data were analysed using the IBM Statistical Package for the Social Sciences for Windows (SPSS for Windows Version 25.0; IBM Corporation, Armonk, NY). Outliers were first identified and removed using the robust regression and outlier removal (ROUT) method (Motulsky & Brown, 2006) before proceeding with further analyses. The assumption of homogeneity of variance was confirmed using Levene's test (Levene, 1961). Shapiro-Wilk and Kolmogorov–Smirnov normality tests were employed to confirm the parametric assumption of normal (Gaussian) distribution of continuous data variables from each cohort. In cases where the parametric assumptions of homogeneity and normality were violated, appropriate nonparametric tests were performed instead. For normally distributed data, continuous variables were compared between cohorts using separate one-way (one-factor) 1 × 4 analysis of variance (ANOVA). Where significant differences were detected by the ANOVA, post hoc analyses were performed using Tukey's multiple comparisons test to identify significant

differences between the multiple pairwise comparisons. In instances where only two cohorts were compared against one another, unpaired Student's *t*-tests were employed. Pearson correlations were included where appropriate to examine the relationships between different variables. Categorical variables, such as treatment and clinical factors, were compared using the two-sided Pearson's χ^2 test. Where appropriate, the *P*-value from Fisher's exact test was used i.e. if ≥ 20% of cells had expected counts < 5. Categorical variables are presented as a percentage (%) of the cohort and the number (n). Continuous variables are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. Statistical significance was accepted as $P < 0.05$. Each results chapter details specific statistical tests where appropriate.

Chapter 3 Results I

Chronic heart failure patients have underestimated exercise capacity using standard cardiopulmonary exercise testing

2.1 Introduction

Chronic heart failure (CHF) patients are characterised by symptoms of exercise intolerance, which are exacerbated in the presence of concomitant type 2 diabetes (diabetic heart failure; D-HF) (Guazzi *et al.*, 2002; Tibb *et al.*, 2005; Ingle *et al.*, 2006). This exercise intolerance can be evaluated noninvasively using a symptom-limited treadmill or cycle-ergometer exercise test with continuous measurement of cardiopulmonary responses from rest to peak pulmonary oxygen uptake ($\dot{V}O_{2peak}$) at the limit of tolerance (Piepoli *et al.*, 2006). $\dot{V}O_{2peak}$ is prognostically predictive of all-cause (Keteyian *et al.*, 2016) and cardiac-related mortality and hospitalisations in CHF patients (Arena *et al.*, 2004). Furthermore, $\dot{V}O_{2peak}$ is used to guide clinical interventions, such as cardiac transplantation in those patients with a $\dot{V}O_{2peak} \leq 14$ mL·kg^{-1}·min^{-1} (Mancini *et al.*, 1991; Mehra *et al.*, 2016). Therefore, accurate and reliable measurements of this physiological parameter are essential in CHF and D-HF patients.

Cardiopulmonary exercise tests to volitional exhaustion are invariably terminated prematurely in CHF patients due to symptoms of breathlessness and fatigue (Piepoli *et al.*, 2006). This precludes the confirmation of maximal pulmonary oxygen uptake ($\dot{V}O_{2max}$), defined as a plateau in the $\dot{V}O_2$ response to increasing

work rate (Hill & Lupton, 1923). With insufficient objective data to discriminate between a submaximal $\dot{V}O_{2peak}$ and $\dot{V}O_{2max}$, corroborating secondary criteria, such as blood lactate, peak heart rate (HR), and the peak respiratory exchange ratio (RER_{peak}), are often employed to confirm $\dot{V}O_{2max}$ (Howley *et al.*, 1995). However, the validity of these secondary criteria have been questioned, particularly in elderly and exercise testing naïve patient populations (Poole & Jones, 2017).

Verification phases at the end of a peak exercise test have been advocated to overcome this limitation and circumvent the need to employ secondary criteria (Day *et al.*, 2003). One such alternative protocol is the RISE-95 protocol, which involves a ramp-incremental (RI) phase followed by a short recovery period and a constant-load step-exercise (SE) phase at 95% of the peak work rate achieved during RI (Rossiter *et al.*, 2006). A non-significant difference in $\dot{V}O_{2peak}$ between RI and SE can confirm $\dot{V}O_{2max}$ attainment by satisfying the traditional criterion plateau (Rossiter *et al.*, 2006). This protocol recently demonstrated that $\dot{V}O_{2max}$ is often underestimated in ~20% of CHF patients by ~1.3 $mL \cdot kg^{-1} \cdot min^{-1}$ (Bowen *et al.*, 2012a). However, it remains to be elucidated how D-HF patients, whom represent a significant CHF subpopulation characterised by a worse prognosis (Cubbon *et al.*, 2013), respond to this novel protocol.

Therefore, the purpose of this study was to determine if the RISE-95 protocol is 1) well tolerated in a large, non-selective sample of CHF and D-HF patients; and 2) sufficiently sensitive for identifying clinically significant differences within both

CHF and D-HF patients in terms of prognosis, treatment, symptomatic status and outcomes.

3.1 Methods

3.1.1 Patients

211 unselected and consecutive patients with symptomatic stable CHF, secondary to left ventricular systolic dysfunction, provided written informed consent to participate in the current study. Of these, 195 were able to successfully complete the RISE-95 protocol. Patients were only considered and approached about the study if they were capable and willing to perform the protocol but also had clear evidence of exercise intolerance, as demonstrated by a previous $\dot{V}O_{2peak} < 20$ mL·kg^{-1}·min^{-1}. This threshold was used as patients with a $\dot{V}O_{2peak} < 20$ mL·kg^{-1}·min^{-1} present with symptoms of exercise intolerance and have a poor prognosis (Myers *et al.*, 2002). All patients were referred to undertake the protocol under the supervision of clinical exercise physiologists at Leeds General Infirmary. The investigation followed the guidelines set out in the Declaration of Helsinki (Appendix B) and was approved by the Leeds West Local Research Ethics Committee.

3.1.2 Equipment and measurements

Exercise tests were performed on a computer-controlled electromagnetically-braked cycle ergometer with continuous recording of cadence and power output (Excalibur Sport, Lode BV, Groningen, NL). Patients were monitored throughout each test using a 12-lead electrocardiogram and HR was determined beat-by-beat from the R-R interval. Arterial O_2 saturation (SpO_2) was measured from the

finger using pulse oximetry (Biox 3745, Ohmeda, Louisville, KY). Breath-by-breath measurements of pulmonary gas exchange ($\dot{V}O_2$, $\dot{V}CO_2$, RER) and ventilatory variables ($\dot{V}_E$, V_T, and B_f) were recorded throughout the protocol (MedGraphics Ultima™ CardiO₂®, Medical Graphics UK Limited, Gloucester, UK). This measurement system was recalibrated prior to each experiment using manufacturer-recommended volume and gas calibration methods.

3.1.3 Exercise protocols

All patients completed a symptom-limited RISE-95 cycle ergometry test to the limit of tolerance (Rossiter *et al.*, 2006; Bowen *et al.*, 2012a) during a single visit. A schematic in the General Methods chapter outlines the different components of the RISE-95 protocol (Figure 2.2). Following a 5 minute baseline warm-up period at a work rate of 5 W, patients completed the RI phase of the test at a mean ramp rate of 13.4 ± 0.2 W·min^{-1} and a range of 10-20 W·min^{-1}. After RI, patients exercised at 10 W for 5 minutes before completing the SE component of the test at 95% of the peak work rate achieved during RI. Both the RI and SE phases of the test were terminated when pedal cadence could not be maintained > 50 rpm despite strong verbal encouragement. The test was then preceded by a cool-down period at 10 W. Patients were encouraged to attend the test in a postprandial (2-3 hr) state, having refrained from strenuous activity (24 hr), caffeine (3 hr) and alcohol consumption (48 hr) prior to testing.

3.1.4 Data analyses

The breath-by-breath data were edited in the $\dot{V}O_2$ domain, to exclude occasional breaths that lay outside four standard deviations of the local mean, which may

have resulted from sighs, coughs, swallows etc. (Lamarra *et al.*, 1987). The final

12 consecutive breaths prior to exercise termination were averaged to allow for

a sufficient comparison between the peak pulmonary and ventilatory variables

obtained during RI and SE with a measurement sensitivity of 50 mL·min^{-1} in the

$\dot{V}O_2$ domain. It has been previously demonstrated that a sample size of 12

breaths is sufficient to identify a 50 mL·min^{-1} difference in $\dot{V}O_2$ even in individuals

with the widest breath-by-breath fluctuations (Bowen *et al.*, 2012a). This number

of breaths may also be collected over a sufficiently short duration to allow an

appropriate comparison to be made. Therefore $\dot{V}O_{2peak}$, $\dot{V}CO_{2peak}$, RER_{peak},

$\dot{V}_{Epeak}$, B_f, and V_{Tpeak} were determined from the average of the same 12 breaths

using automated software. Peak HR, SpO_2, and oxygen pulse were averaged

over approximately the last 20 s of exercise. The lactate threshold (LT) was

estimated non-invasively using the V-slope method (Beaver *et al.*, 1986) and

subsequently corroborated by an independent researcher. The $\dot{V}_E/\dot{V}CO_2$ nadir

was determined as the lowest ratio of $\dot{V}_E:\dot{V}CO_2$.

3.1.5 Statistical analyses

All statistical analyses were completed using the Statistical Package for the

Social Sciences (SPSS for Windows Version 25.0; IBM Corporation, Armonk,

NY) after confirming the assumptions met for parametric testing outlined in 2.11

of the General Methods chapter. The $\dot{V}O_{2peak}$ measures obtained from RI and

SE were assessed within each patient using unpaired Student's *t*-tests to

compare the 12-breath measures obtained at $\dot{V}O_{2peak}$. Even though this

comparison was made within each patient, unpaired tests were used because

the order of appearance in $\dot{V}O_2$ values is not paired. $\dot{V}O_{2max}$ was confirmed when

the RI and SE $\dot{V}O_{2peak}$ values did not differ significantly ($P > 0.05$). Where differences occurred ($P < 0.05$), the greater of the two $\dot{V}O_{2peak}$ values was reported with its 95% confidence interval (CI_{95}). This analysis resulted in the identification of two patient groups; one that confirmed $\dot{V}O_{2max}$ and the other that did not confirm $\dot{V}O_{2max}$. A two-way repeated measures ANOVA with group and phase (RI and SE) treated as the independent variables was used to examine differences in the peak pulmonary variables between the two patient cohorts across RI and SE, respectively. For variables that were only obtained on one phase of the test, unpaired Student's t-tests were used to evaluate differences between the two cohorts. Significance was accepted at $P < 0.05$ and all data are reported as mean ± SEM unless otherwise stated.

3.2 Results

3.2.1 Demographic, clinical and treatment variables

Table 3.1 illustrates the demographic, clinical and treatment variables for all 195 patients that completed the protocol. Of the 16 that were not included in the final analysis, 2 patients exhibited hyperventilation and the remaining 14 were only able to complete the RI phase of the test. Twelve of these patients felt unable to continue due to a combination of fatigue, dyspnoea, and chest pain whereas 2 patients were stopped due to safety concerns. Therefore 7% of the patients were unable to complete the RISE-95 protocol giving a 93% completion rate for the test.

Table 3.1. Demographic, physical, clinical and treatment characteristics of patients that completed the RISE-95 protocol. Data are expressed as mean ± SEM unless otherwise stated.

	$n = 195$
Male sex [% (n)]	85 (166)
Age (years)	65.2 ± 1.0
Weight (kg)	85.9 ± 1.2
Clinical factors	
NYHA functional class [% (n)]	
I	34 (66)
II	54 (106)
III	12 (23)
DM [% (n)]	23 (45)
Ischaemic aetiology [% (n)]	41 (80)
Mortality [% (n)]	20 (39)
Hospitalisations [% (n)]	41 (79)
LVEF (%)	29 ± 1
LVIDd (mm)	58 ± 1
Haemoglobin (g·L^{-1})	141.4 ± 1.1
Sodium (mmol·L^{-1})	139.7 ± 0.2
Potassium (mmol·L^{-1})	4.44 ± 0.03
Creatinine (µmol·mL^{-1})	97.1 ± 2.2
eGFR (mL·min^{-1}·1.73m^{-2})	69.8 ± 1.3
Treatment factors	
ACEi/ARB use [% (n)]	76 (149)
Ramipril equivalent dose (mg)	6.1 ± 0.8
Beta-blocker use [% (n)]	82 (159)
Bisoprolol equivalent dose (mg)	5.3 ± 0.3
Loop diuretic use [% (n)]	58 (113)
Furosemide equivalent dose (mg)	57.4 ± 4.4
ICD [% (n)]	7 (14)
CRT [% (n)]	12 (24)

NYHA, New York Heart Association; DM, type 2 diabetes mellitus; LVEF, left ventricular ejection fraction; LVIDd, left ventricular internal diameter at diastole; eGFR, estimated glomerular filtration rate; ACEi/ARB, angiotensin converting enzyme inhibitors/angiotensin receptor blockers; ICD, implantable cardioverter defibrillator; CRT, cardiac resynchronisation therapy.

The cardiopulmonary responses (n = 195) to the RI and SE phases of the RISE-95 protocol are outlined in Table 3.2. The SE phase was performed at a lower peak work rate and over a shorter duration (both $P < 0.0001$; Table 3.2). Importantly, however, mean group $\dot{V}O_{2peak}$ was significantly increased by 0.5 mL·kg^{-1}·min^{-1} (3%) in the SE phase compared to RI ($P < 0.0001$; Table 3.2). Furthermore the highest $\dot{V}O_{2peak}$ recorded over both phases of the test was 5.3% and 2.0% higher than RI and SE, respectively (both $P < 0.0001$; Table 3.2). Despite these findings, there were concomitant reductions in $\dot{V}CO_{2peak}$ and RER_{peak} in the SE phase by 6% and 9%, respectively (both $P < 0.0001$; Table 3.2). There was a small but significant 5% increase in breathing frequency in the SE phase ($P < 0.0001$), which was offset by a marginal 2% decrease in V_{Tpeak} ($P = 0.001$), both of which collectively yielded a trend for an increase in $\dot{V}_{Epeak}$ by 3% ($P = 0.09$; Table 3.2). Although SpO_2 (%) was decreased by 1% ($P = 0.006$), oxygen pulse increased by 0.7 mL·beat^{-1} (6 %) in the SE phase ($P < 0.0001$; Table 3.2).

Table 3.2. Cardiopulmonary responses to the RI and SE phases of the RISE-95 test in all 195 patients that completed the protocol. Data are expressed as mean ± SEM.

	RI			SE		
Ramp rate ($W \cdot min^{-1}$)	13.4	±	0.2			
Peak work rate (W)	97	±	3	92	±	3*
Duration (mins)	6.7	±	0.2	2.0	±	0.1*
$\dot{V}O_{2peak}$ ($L \cdot min^{-1}$)	1.23	±	0.03	1.26	±	0.04*
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	14.3	±	0.3	14.8	±	0.4*
Highest $\dot{V}O_{2peak}$, $mL \cdot kg^{-1} \cdot min^{-1}$ (CI_{95})	15.1 ± 0.3 (0.91 ± 0.06)*					
$\dot{V}CO_{2peak}$ ($L \cdot min^{-1}$)	1.40	±	0.04	1.31	±	0.04*
RER_{peak}	1.13	±	0.01	1.03	±	0.01*
$\dot{V}_{Epeak}$ ($L \cdot min^{-1}$)	49.3	±	1.2	50.7	±	1.4
B_f (breaths$\cdot min^{-1}$)	29.7	±	0.5	31.1	±	0.5*
V_{Tpeak} (L)	1.68	±	0.04	1.64	±	0.04*
LT ($L \cdot min^{-1}$)	0.78	±	0.02			
LT ($mL \cdot kg^{-1} \cdot min^{-1}$)	9.1	±	0.2			
LT (% $\dot{V}O_{2peak}$)	65	±	1			
$\dot{V}_E/\dot{V}CO_2$ nadir	34.3	±	0.5			
Peak HR (beats$\cdot min^{-1}$)	118	±	2	118	±	2
SpO_2 (%)	96	±	1	95	±	1*
Oxygen pulse ($mL \cdot beat^{-1}$)	11.4	±	0.6	12.1	±	0.7*

$\dot{V}O_{2peak}$, peak pulmonary oxygen uptake; CI_{95}, 95% confidence interval; $\dot{V}CO_{2peak}$, peak pulmonary carbon dioxide output; RER_{peak}, peak respiratory exchange ratio; $\dot{V}_{Epeak}$, peak ventilation; B_f, breathing frequency; V_{Tpeak}, peak tidal volume; LT, lactate threshold; $\dot{V}_E/\dot{V}CO_2$, ratio of ventilation to carbon dioxide output; HR, heart rate; SpO_2, arterial oxygen saturation. *$P < 0.01$ vs. RI phase within subjects.

3.2.2 Confirming $\dot{V}O_{2max}$ using the RISE-95 protocol

The cardiopulmonary responses (n = 195) to the RISE-95 protocol are presented in Table 3.3. Using a within-subject comparison to assess the 12 highest breaths recorded in the RI and SE phases, the RISE-95 protocol was able to statistically identify 74 patients (39%) that confirmed $\dot{V}O_{2max}$ without the need for secondary criteria (Table 3.3 and Figure 3.1). The RISE-95 protocol demonstrated that the remaining 121 patients (61%) failed to confirm $\dot{V}O_{2max}$. Collectively these 121 patients increased $\dot{V}O_{2peak}$ by a mean 0.7 $mL \cdot kg^{-1} \cdot min^{-1}$ (5%) between the RI

and SE phases (Table 3.3 and Figure 3.1); however, amongst these 121 patients, a small proportion (n = 32) actually had a decrease in $\dot{V}O_{2peak}$ between these phases. Nevertheless, these 121 patients that failed to confirm $\dot{V}O_{2peak}$ had a 1.7 mL·kg^{-1}·min^{-1} (11%) higher $\dot{V}O_{2peak}$ on the SE phase of the test compared to patients that confirmed $\dot{V}O_{2max}$. This was confirmed by a significant phase*group interaction in the two-way repeated measures ANOVA showing that the two cohorts had divergent $\dot{V}O_{2peak}$ responses between RI and SE (P = 0.006; Table 3.3). Furthermore, the unconfirmed cohort had a 2.0 mL·kg^{-1}·min^{-1} (14%) higher $\dot{V}O_{2peak}$ when comparing the highest $\dot{V}O_{2peak}$ achieved across both phases (P = 0.005; Table 3.3). Representative traces of the breath-by-breath pulmonary oxygen uptake ($\dot{V}O_2$) dynamics from a confirmed cohort patient and an unconfirmed cohort patient are displayed in Figure 3.2 to highlight the differences in $\dot{V}O_{2peak}$ responses between the RI and SE phases.

The two-way repeated measures ANOVAs confirmed that the two cohorts exhibited some divergent responses in several of the pulmonary and cardiac variables obtained from the two phases of the test. Firstly, there was a significant phase*group interaction (P = 0.009) for $\dot{V}CO_{2peak}$, which decreased by 0.14 L·min^{-1} (10%) in the confirmed cohort but only by 0.06 L·min^{-1} (4%) in the unconfirmed cohort (Table 3.3). There was also a significant phase*group interaction (P = 0.001) for peak ventilation, which decreased by 1.2 L·min^{-1} (2%) between RI and SE in the confirmed cohort but increased by 2.9 L·min^{-1} (6%) across the two phases in the unconfirmed cohort (Table 3.3). This discrepancy in peak ventilation may be attributed to a significant phase*group interaction (P = 0.033) for peak breathing frequency, which increased by 1.9 breaths·min^{-1}

(6%) in the unconfirmed cohort between RI and SE but only increased by 0.6 breaths·min^{-1} (2%) in the confirmed cohort (Table 3.3). Finally, there was also a significant phase*group interaction ($P = 0.007$) for peak HR, which decreased by 2 beats·min^{-1} (2%) between the two phases in the confirmed cohort but increased by 2 beats·min^{-1} (2%) in the unconfirmed cohort (Table 3.3). An additional difference to note was that the unconfirmed cohort reached lactate threshold at a $\dot{V}O_2$ that was 1.1 mL·kg^{-1}·min^{-1} (13%) higher than the confirmed cohort during the RI phase ($P = 0.005$; Table 3.3), suggesting that they had a delayed onset of anaerobic metabolism. In terms of clinical variables, the only differences between the two cohorts were a higher presence of ischaemia within the confirmed cohort (50% vs. 36%; $P = 0.031$) whilst the unconfirmed cohort were younger by an average of 4.4 years ($P = 0.018$).

Table 3.3. Cardiopulmonary responses to the RI and SE phases of the RISE-95 test in patients who either confirmed or failed to confirm $\dot{V}O_{2max}$. Data are expressed as mean ± SEM unless otherwise stated.

	$\dot{V}O_{2max}$					
	Confirmed			**Unconfirmed**		
	RI		**SE**	**RI**		**SE**
n (%)	74 (37.9)			121 (62.1)		
Peak work rate (W)	91 ± 4		87 ± 4	100 ± 4		95 ± 3
Duration (mins)	6.6 ± 0.3		1.8 ± 0.1	6.8 ± 0.2		2.1 ± 0.1
$\dot{V}O_{2peak}$ (L·min^{-1})*	1.18 ± 0.05		1.19 ± 0.05	1.26 ± 0.04		1.32 ± 0.05
$\dot{V}O_{2peak}$ (mL·kg^{-1}·min^{-1})*	13.7 ± 0.5		13.7 ± 0.5	14.7 ± 0.4		15.4 ± 0.5
Highest $\dot{V}O_{2peak}$, mL·kg^{-1}·min^{-1} (CI$_{95}$)	13.9 ± 0.5 (1.29 ± 0.11)			15.9 ± 0.5 (0.68 ± 0.05)†		
$\dot{V}CO_{2peak}$ (L·min^{-1})*	1.34 ± 0.06		1.20 ± 0.05	1.44 ± 0.05		1.38 ± 0.05
RER_{peak}	1.13 ± 0.01		1.02 ± 0.01	1.13 ± 0.01		1.04 ± 0.01
$\dot{V}_{Epeak}$ (L·min^{-1})*	48.9 ± 1.8		47.7 ± 1.9	49.6 ± 1.6		52.5 ± 1.9
B_f (breaths·min^{-1})*	30.0 ± 0.8		30.6 ± 0.8	29.5 ± 0.6		31.4 ± 0.7
V_{Tpeak} (L)	1.66 ± 0.05		1.58 ± 0.05	1.70 ± 0.05		1.68 ± 0.05
LT (L·min^{-1})	0.72 ± 0.03			0.82 ± 0.03†		
LT (mL·kg^{-1}·min^{-1})	8.4 ± 0.2			9.5 ± 0.3†		
LT (% $\dot{V}O_{2peak}$)	63 ± 2			66 ± 1		
$\dot{V}_E/\dot{V}CO_2$ nadir	35.6 ± 0.9			33.4 ± 0.6		
Peak HR (beats·min^{-1})*	114 ± 3		112 ± 3	120 ± 3		122 ± 3
SpO_2 (%)	95 ± 1		95 ± 2	96 ± 1		96 ± 1
Oxygen pulse (mL·beat^{-1})	11.1 ± 0.8		11.8 ± 1.1	11.6 ± 0.8		12.3 ± 0.9

$\dot{V}O_{2peak}$, peak pulmonary oxygen uptake; CI$_{95}$, 95% confidence interval; $\dot{V}CO_{2peak}$, peak pulmonary carbon dioxide output; RER_{peak}, peak respiratory exchange ratio; $\dot{V}_{Epeak}$, peak ventilation; B_f, breathing frequency; V_{Tpeak}, peak tidal volume; LT, lactate threshold; $\dot{V}_E/\dot{V}CO_2$, ratio of ventilation to carbon dioxide output; HR, heart rate; SpO_2, arterial oxygen saturation. *$P < 0.05$ using a mixed two-way repeated measures ANOVA with group and phase (RI and SE) as the independent variables. †$P < 0.05$ between groups.
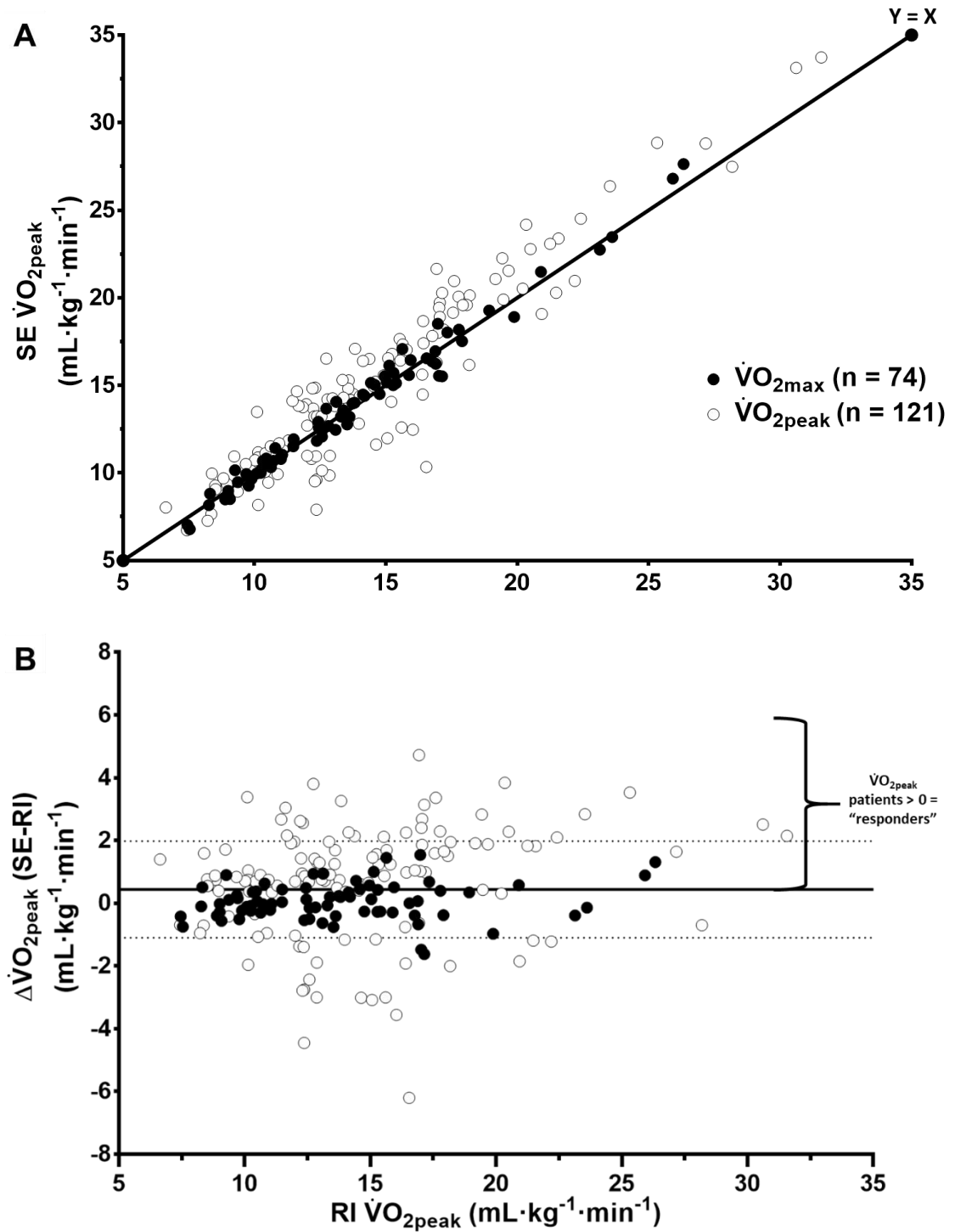
Figure 3.1. $\dot{V}O_{2peak}$ measures from the RISE-95 protocol. Relationship (A) and Bland-Altman plot (±SD) of the mean difference (Δ) (B) between the $\dot{V}O_{2peak}$ achieved during the RI and SE phases of the RISE-95 protocol in patients who either confirmed (n = 74; ●) or failed to confirm (n = 121; ○) $\dot{V}O_{2max}$.
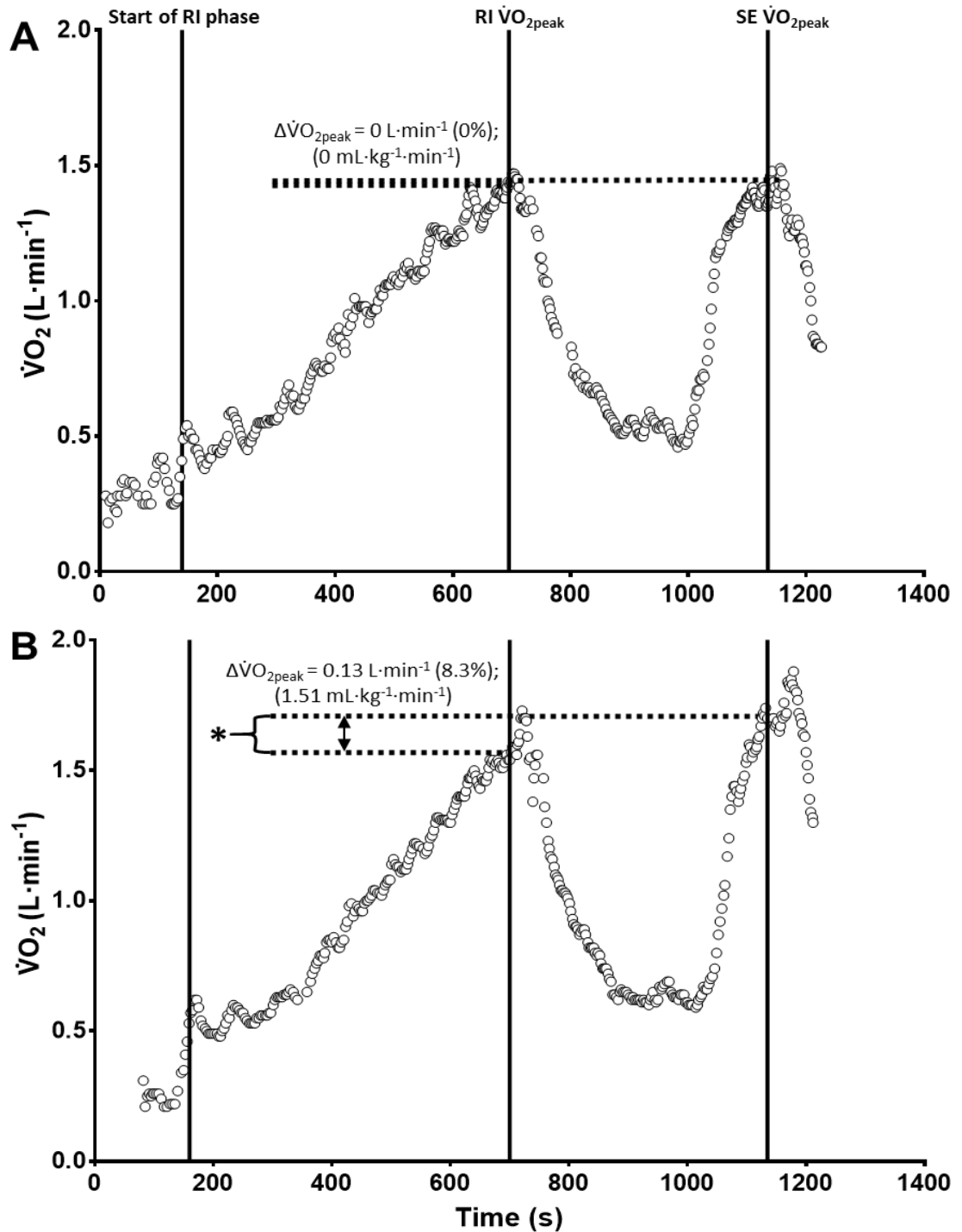
Figure 3.2. Representative traces of breath-by-breath $\dot{V}O_2$ dynamics from two patients that completed both phases of the RISE-95 protocol. In the top panel (A), the patient confirmed $\dot{V}O_{2max}$. The bottom panel (B) shows a patient that failed to achieve $\dot{V}O_{2max}$ as demonstrated by a significant increase in the $\dot{V}O_{2peak}$ recorded in the SE phase compared to the RI phase. Dotted lines correspond to the $\dot{V}O_{2peak}$ measures recorded in the RI and SE phases. *$P < 0.05$ between RI and SE.

3.2.3 Identifying patients that increased $\dot{V}O_{2peak}$ in the step-exercise phase of the protocol

Table 3.5 illustrates the cardiopulmonary responses to the RI and SE phases of the test between "responders" and "non-responders". Of the 121 patients that failed to confirm $\dot{V}O_{2max}$, 89 (46% of total cohort; Figures 3.1B and 3.3A) were defined as "responders" as they significantly increased their $\dot{V}O_{2peak}$ by a mean of 1.6 mL·kg^{-1}·min^{-1} (range 0.3-4.7 mL·kg^{-1}·min^{-1}) between the RI and SE phases (Figure 3.3B and Table 3.5). This corresponded to an 11.0% (range 1.5-33.5%) increase in $\dot{V}O_{2peak}$ (Figure 3.3C). Notably, out of these 89 patients, 38 increased their $\dot{V}O_{2peak}$ by > 10% in SE compared to RI. The remaining 106 (54%) patients, deemed as "non-responders", exhibited a mean difference (Δ) in $\dot{V}O_{2peak}$ of -0.5 mL·kg^{-1}·min^{-1} (range -6.2-1.5 mL·kg^{-1}·min^{-1}; Figure 3.3B and Table 3.5) between the RI and SE phases, which corresponded to a percentage difference (Δ) of -3.9% (range -37.5-9.7%; Figure 3.3C). These divergent responses were confirmed by a significant phase*group interaction in the two-way repeated measures ANOVA ($P < 0.0001$; Table 3.5). Similarly, "responders" had a 2.5 mL·kg^{-1}·min^{-1} (18%) higher $\dot{V}O_{2peak}$ compared to "non-responders" when assessing the highest $\dot{V}O_{2peak}$ achieved across both phases ($P < 0.0001$; Table 3.5).

The two cohorts also exhibited many divergent responses between the RI and SE phases as evidenced from several significant phase*group interactions in the two-way ANOVAs. Firstly, there was a trend for the two cohorts to differ in their exercise durations across the two protocols ($P = 0.0104$) with the "responders" exercising for 0.8 minutes (50%; $P < 0.0001$; Table 3.5) longer in the SE phase compared to "non-responders". There was a significant phase*group interaction

($P < 0.0001$) for $\dot{V}CO_{2peak}$, which decreased by 0.19 L·min^{-1} (14%) in "non-responders" but increased by 0.04 L·min^{-1} (3%) in "responders" (Table 3.5). Similarly, $\dot{V}_{Epeak}$ decreased by 3.1 L·min^{-1} (6%) between RI and SE in "non-responders" but increased by 6.6 L·min^{-1} (13%) in "responders" ($P < 0.0001$; Table 3.5). This response was matched by similar divergent responses in V_{Tpeak}, which decreased by 0.11 L (7%) in "non-responders" but increased by 0.04 L (2%) in "responders" ($P = 0.0301$; Table 3.5). Additionally, breathing frequency increased by 3.3 breaths·min^{-1} (11%) in "responders" whilst the "non-responders" had a decrease in breathing frequency of 0.1 breaths·min^{-1} (0.3%) ($P > 0.0001$; Table 3.5). These changes in pulmonary variables likely contributed to a significant phase*group interaction ($P < 0.0001$) for RER_{peak}, which decreased by 0.12 (11%) in the "non-responders" but only decreased by 0.08 (7%) in the "responders" (Table 3.5). These divergent pulmonary responses were matched by comparable cardiac responses such that the "non-responders" had a decrease in peak HR of 4 beats·min^{-1} (4%) between the two phases whilst "responders" increased their peak HR by 4 beats·min^{-1} (3%; $P < 0.0001$; Table 3.5). There were also significant between group differences during the RI phase alone, with "responders" achieving LT at a $\dot{V}O_2$ that was 1.0 mL·kg^{-1}·min^{-1} (12%) higher than "non-responders" ($P = 0.013$; Table 3.5). "Responders" also had a significantly lower $\dot{V}_E/\dot{V}CO_2$ nadir ($P = 0.047$; Table 3.5).

Table 3.4 presents the demographic, clinical and treatment variables for the patients identified as either "responders" or "non-responders". Of note, "responders" were younger by an average of 4.9 years ($P = 0.005$), were more likely to be NYHA class I (44% vs. 26%; $P = 0.023$), and were less likely to be

ischaemic (34% vs. 47%; $P = 0.05$). Interestingly, however, this cohort had more

hospitalisations in the follow-up period (48% vs. 34%; $P = 0.042$).
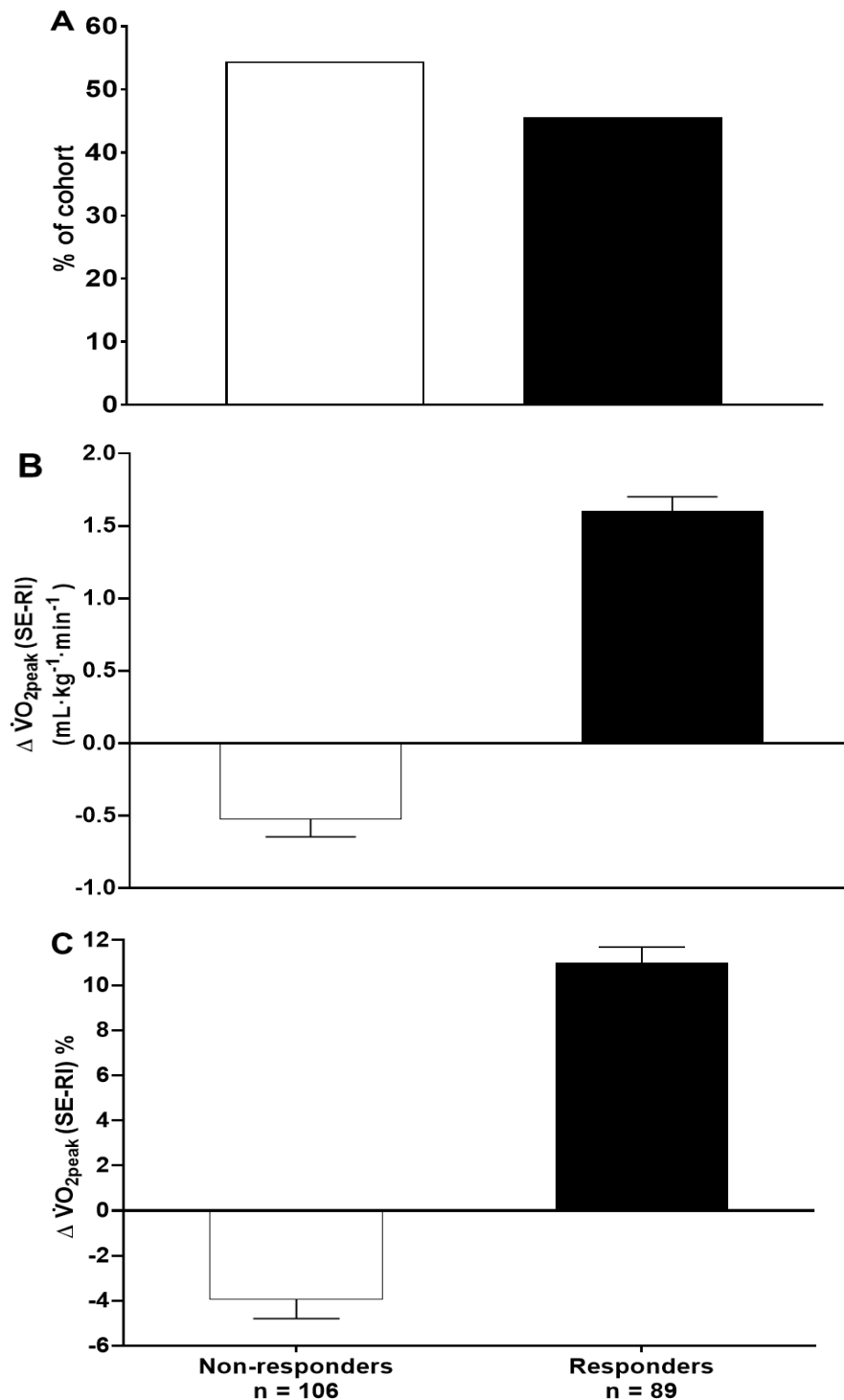
Figure 3.3. Comparing "responders" to "non-responders" from the RISE-95 test. Percentage of the total cohort that were defined as either "non-responders" (n = 106) or "responders" (n = 89) as determined by the difference (Δ) in $\dot{V}O_{2peak}$ between the RI and SE phases of the RISE-95 protocol (A). Group mean (± SEM) of the difference (Δ) in $\dot{V}O_{2peak}$ between the RI and SE phases of the protocol in "non-responders" vs. "responders" expressed as an absolute $\dot{V}O_{2peak}$ difference (Δ) (B) and a relative percentage difference (C).

Table 3.4. Demographic, physical, clinical and treatment characteristics of "responders" and "non-responders" as determined by the difference (Δ) in $\dot{V}O_{2peak}$ between the RI and SE phases of the RISE-95 protocol. Data are expressed as mean ± SEM unless otherwise stated. Significant differences ($P <$ 0.05) are shown in bold.

	Non-responders	Responders
n (%)	106 (54)	89 (46)
Male sex [% (n)]	86 (91)	84.3 (75)
Age (years)	67.4 ± 1.3	62.5 ± 1.4†
Weight (kg)	85.1 ± 1.6	86.9 ± 1.8
Clinical factors		
NYHA functional class [% (n)]		
I	26 (27)	44 (39)*
II	62 (66)	45 (40)
III	12 (13)	11 (10)
DM [% (n)]	21 (22)	26 (23)
Ischaemic aetiology [% (n)]	47 (50)	34 (30)*
Mortality [% (n)]	20 (21)	20 (18)
Hospitalisations [% (n)]	34 (36)	48 (43)*
LVEF (%)	30 ± 1	28 ± 1
LVIDd (mm)	56 ± 1	59 ± 1
Haemoglobin ($g \cdot L^{-1}$)	140.1 ± 0.2	143.0 ± 0.2
Sodium ($mmol \cdot L^{-1}$)	139.5 ± 0.3	139.9 ± 0.3
Potassium ($mmol \cdot L^{-1}$)	4.42 ± 0.05	4.47 ± 0.04
Creatinine ($\mu mol \cdot mL^{-1}$)	96.7 ± 2.7	97.6 ± 3.7
eGFR ($mL \cdot min^{-1} \cdot 1.73m^{-2}$)	69.4 ± 1.7	70.3 ± 2.0
Treatment factors		
ACEi/ARB use [% (n)]	75 (79)	79 (70)
Ramipril equivalent dose (mg)	5.9 ± 0.4	6.4 ± 0.4
Beta-blocker use [% (n)]	79 (84)	85 (76)
Bisoprolol equivalent dose (mg)	4.9 ± 0.3	5.7 ± 0.4
Loop diuretic use [% (n)]	58 (61)	58 (52)
Furosemide equivalent dose (mg)	55.3 ± 5.0	60.0 ± 7.9
ICD [% (n)]	5 (5)	10 (9)
CRT [% (n)]	12 (13)	12 (11)

NYHA, New York Heart Association; DM, type 2 diabetes mellitus; LVEF, left ventricular ejection fraction; LVIDd, left ventricular internal diameter at diastole; eGFR, estimated glomerular filtration rate; ACEi/ARB, angiotensin converting enzyme inhibitors/angiotensin receptor blockers; ICD, implantable cardioverter defibrillator; CRT, cardiac resynchronisation therapy. *$P <$ 0.05 vs. non-responders; †$P <$ 0.01 vs. non-responders.

Table 3.5. Cardiopulmonary responses to the RI and SE phases of the RISE-95 test in "responders" and "non-responders" as determined by the difference (Δ) in $\dot{V}O_{2peak}$ between the RI and SE phases of the protocol. Data are expressed as mean ± SEM unless otherwise stated.

	Non-Responders		Responders	
	RI	SE	RI	SE
n (%)	106 (54.4)		89 (45.6)	
Peak work rate (W)*	92 ± 4	87 ± 4	103 ± 4[†]	97 ± 4
Duration (mins)	6.6 ± 0.2	1.6 ± 0.1	6.8 ± 0.2	2.4 ± 0.1
$\dot{V}O_{2peak}$ (L·min^{-1})*	1.19 ± 0.05	1.15 ± 0.05	1.28 ± 0.05	1.42 ± 0.05
$\dot{V}O_{2peak}$ (mL·kg^{-1}·min^{-1})*	13.8 ± 0.4	13.3 ± 0.4	14.9 ± 0.5	16.5 ± 0.5
Highest $\dot{V}O_{2peak}$, mL·kg^{-1}·min^{-1} (CI$_{95}$)	14.0 ± 0.4 (1.11 ± 0.09)		16.5 ± 0.5 (0.67 ± 0.05)[†]	
$\dot{V}CO_{2peak}$ (L·min^{-1})*	1.34 ± 0.05	1.15 ± 0.05	1.47 ± 0.06	1.51 ± 0.06
RER$_{peak}$*	1.13 ± 0.01	1.01 ± 0.01	1.14 ± 0.01	1.06 ± 0.01
$\dot{V}_{Epeak}$ (L·min^{-1})*	48.4 ± 1.6	45.3 ± 1.6	50.5 ± 1.8	57.1 ± 2.2
B$_f$ (breaths·min^{-1})*	29.9 ± 0.6	29.8 ± 0.6	29.4 ± 0.7	32.7 ± 0.8
V$_{Tpeak}$ (L)*	1.64 ± 0.05	1.53 ± 0.05	1.73 ± 0.05	1.77 ± 0.05
LT (L·min^{-1})	0.74 ± 0.02		0.83 ± 0.03[†]	
LT (mL·kg^{-1}·min^{-1})	8.6 ± 0.2		9.6 ± 0.3[†]	
LT (% $\dot{V}O_{2peak}$)	64.2 ± 1.3		65.8 ± 1.3	
$\dot{V}_E/\dot{V}CO_2$ nadir	35.3 ± 0.7		33.1 ± 0.6[†]	
Peak HR (beats·min^{-1})*	114 ± 3	110 ± 3	123 ± 3[†]	127 ± 3
SpO$_2$ (%)	94 ± 1	95 ± 1	98 ± 1	96 ± 1
Oxygen pulse (mL·beat^{-1})	11.9 ± 1.0	12.3 ± 1.2	10.9 ± 0.3	11.8 ± 0.6

$\dot{V}O_{2peak}$, peak pulmonary oxygen uptake; CI$_{95}$, 95% confidence interval; $\dot{V}CO_{2peak}$, peak pulmonary carbon dioxide output; RER$_{peak}$, peak respiratory exchange ratio; $\dot{V}_{Epeak}$, peak ventilation; B$_f$, breathing frequency; V$_{Tpeak}$, peak tidal volume; LT, lactate threshold; $\dot{V}_E/\dot{V}CO_2$, ratio of ventilation to carbon dioxide output; HR, heart rate; SpO$_2$, arterial oxygen saturation. *$P < 0.05$ using a mixed two-way repeated measures ANOVA with group and phase (RI and SE) as the independent variables. †$P < 0.05$ between groups.

3.2.4 Responses to the RISE-95 protocol in D-HF patients

Of the 195 CHF patients, 45 (23%) had concomitant type 2 diabetes (D-HF). Compared to the 150 non-DM CHF patients, these 45 D-HF patients had higher mortality in follow-up (33% vs. 16%; $P = 0.011$), were less likely to be NYHA class I (18% vs. 39%; $P = 0.034$), were more ischaemic (56% vs. 38%; $P = 0.036$), were more likely to be receiving a loop diuretic (82% vs. 55%; $P = 0.001$) and were more likely to have an ICD (16% vs. 5%; $P = 0.016$) or CRT device (22% vs. 10%; $P = 0.027$). Additionally, D-HF patients were on average 9.3 kg (11%) heavier (93.0 ± 3.1 vs. 83.7 ± 1.3 kg; $P = 0.006$), and were receiving higher dosages of beta-blockers (6.3 ± 0.5 mg vs. 4.9 ± 0.3 mg; $P = 0.023$) and loop diuretics (75mg ± 11 mg vs. 49 ± 4 mg; $P = 0.038$).

Compared to CHF patients, D-HF patients had a 20% lower $\dot{V}O_{2peak}$ in both the RI and SE phases of the RISE-95 protocol (both $P < 0.0001$; Figure 3.4). Furthermore the highest $\dot{V}O_{2peak}$ measured across both phases was also 3.13 mL·kg^{-1}·min^{-1} (20%) lower in D-HF ($P < 0.0001$). The functional consequence of this exercise intolerance is demonstrated by comparably lower peak work rates in D-HF compared to CHF for both the RI (84 ± 5 W vs 100 ± 3 W; $P = 0.017$) and SE phases (80 ± 4 W vs 95 ± 3 W; $P = 0.017$). Compared to CHF, D-HF patients also exhibited a trend for a lower exercise duration over the RI phase only (6.0 ± 0.3 minutes vs. 6.9 ± 0.2 minutes; $P = 0.071$).
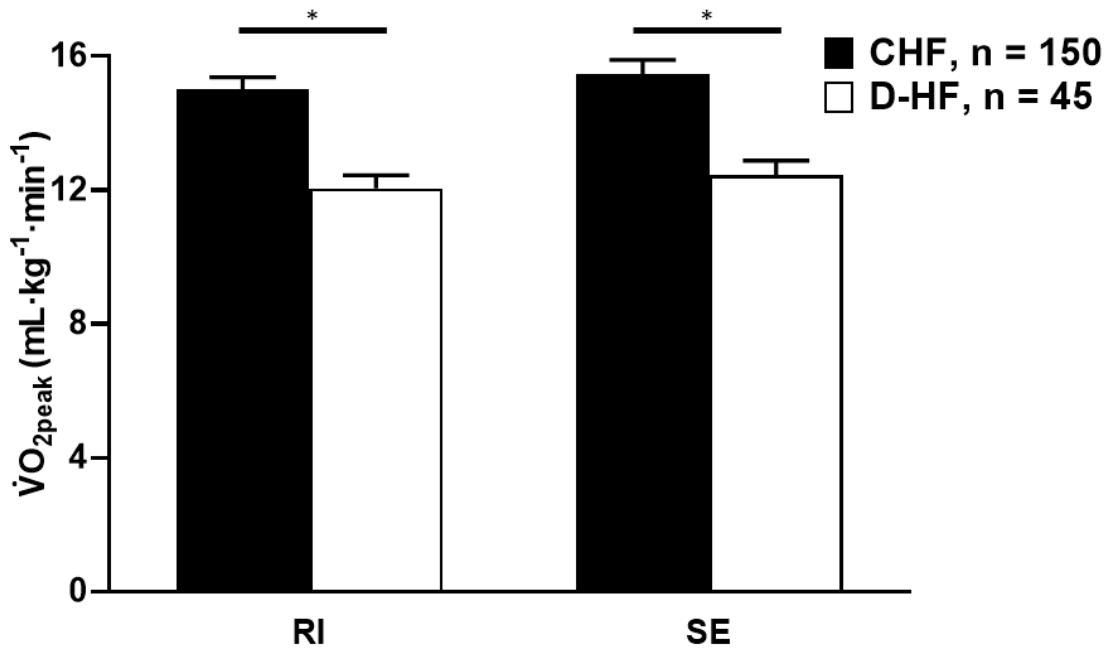
Figure 3.4. $\dot{V}O_{2peak}$ responses measured in the RI and SE phases of the RISE-95 protocol between CHF and D-HF patients. *$P < 0.0001$ between groups.

Within the D-HF cohort of 45 patients, 16 (36%) confirmed $\dot{V}O_{2max}$ and 29 (64%) failed to confirm $\dot{V}O_{2max}$. Additionally, 23 (51%) were defined as "responders" and 22 (49%) as "non-responders" (Figure 3.5A). Amongst the 150 CHF patients, 66 (44%) were "responders" and 84 (56%) were "non-responders" (Figure 3.5A). There were no significant differences in terms of the percentages of "non-responders" and "responders" between the CHF and D-HF cohorts ($P > 0.05$; Figure 3.5A). However, within the "responders" cohort, the absolute increase in $\dot{V}O_{2peak}$ was found to be 0.49 mL·kg^{-1}·min^{-1} (29%) lower in D-HF compared to CHF ($P = 0.028$; Figure 3.5B), suggesting that CHF patients may be able to access a greater absolute "aerobic reserve" compared to D-HF. Nevertheless, both the CHF and D-HF "responders" increased by a similar amount when expressed as a relative percentage difference (Δ) in $\dot{V}O_{2peak}$ between the two phases ($P > 0.05$; Figure 3.5C).
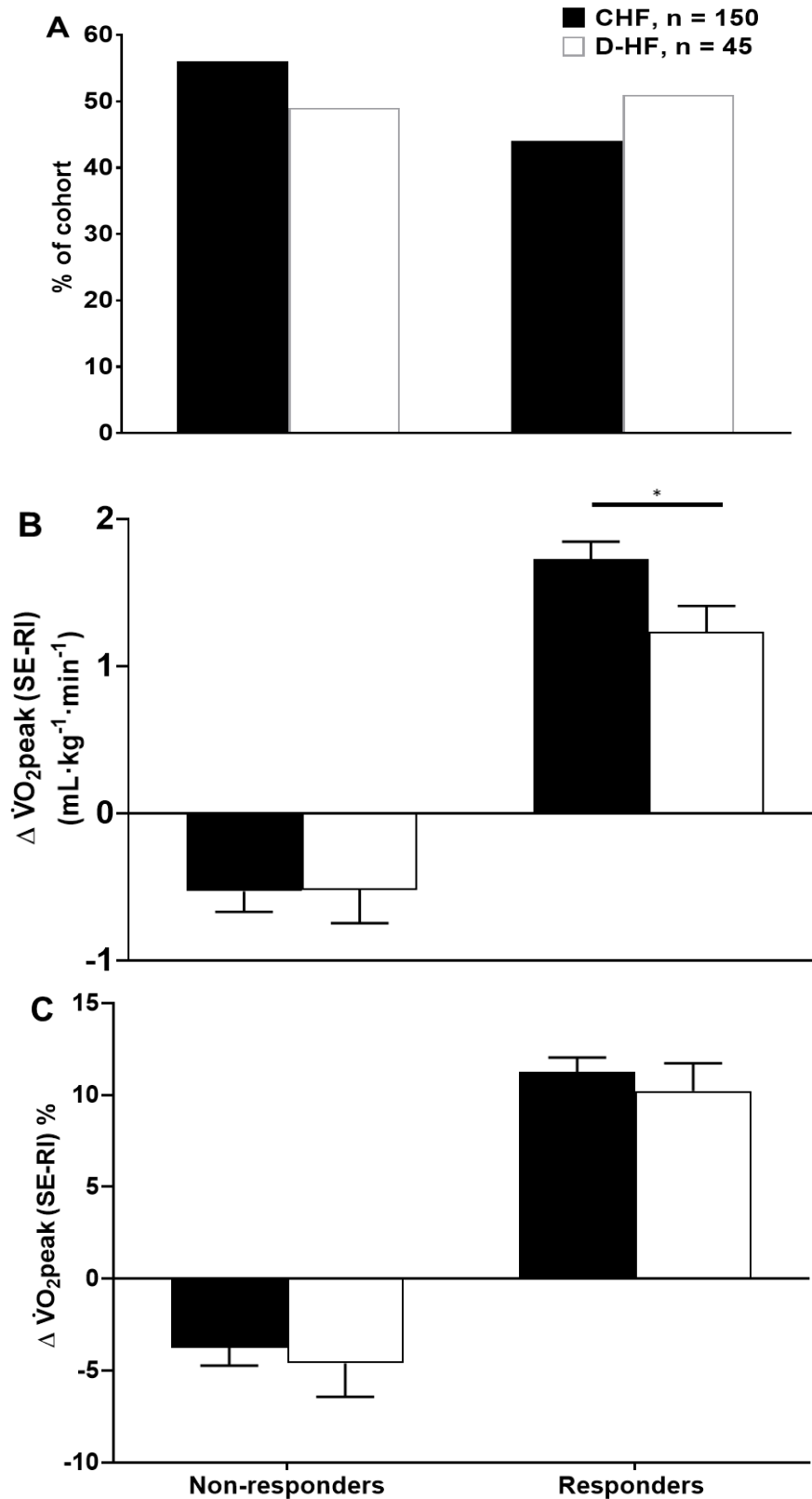
Figure 3.5. $\dot{V}O_{2peak}$ responses of "responders" and "non-responders" within CHF and D-HF patients . Percentage of the patients that were defined as either "non-responders" or "responders" within the CHF and D-HF patient cohorts (A). Group mean (± SEM) of the difference (Δ) in $\dot{V}O_{2peak}$ between the RI and SE phases of the protocol in "non-responders" vs. "responders" within the CHF and D-HF patient cohorts expressed as an absolute $\dot{V}O_{2peak}$ difference (Δ) (B) and a relative percentage difference (C).

Although D-HF "responders" were able to increase peak ventilation and breathing frequency between the RI and SE phases (both $P < 0.05$), there were no differences in $\dot{V}CO_{2peak}$, VT_{peak}, and peak HR (all $P > 0.05$), thus suggesting that peripheral factors may play a more significant role in improving $\dot{V}O_{2peak}$ in these patients. Indeed, oxygen pulse significantly increased from 10.9 ± 0.7 mL·beat^{-1} to 13.2 ± 2.0 mL·beat^{-1} ($P < 0.0001$) between RI and SE in the D-HF "responders". Although CHF "responders" also increased oxygen pulse between the two phases (10.9 ± 0.4 mL·beat^{-1} vs. 11.4 ± 0.4 mL·beat^{-1}; $P = 0.019$), the magnitude of difference was comparably attenuated. This was supported by a trend for a greater increase in oxygen pulse in the D-HF "responders" compared to CHF "responders" between the RI and SE phases (2.3 ± 1.6 mL·beat^{-1} vs. 0.5 ± 0.2 mL·beat^{-1}; $P = 0.067$). Therefore these D-HF "responders" appear to be characterised by greater peripheral limitations which are only overcome with prior exercise.

In terms of clinical and treatment variables, D-HF "responders" were less likely to be ischaemic (39% v 73%; $P = 0.023$) yet recorded more hospitalisations in the follow-up period compared to D-HF "non-responders" (61% v 27%; $P = 0.023$). This is consistent with the collective CHF cohort. Additionally, D-HF "responders" were also on a higher dosage of bisoprolol compared to the D-HF "non-responders" (7.7 ± 0.6 vs. 5.0 ± 0.7 mg; $P = 0.010$). Interestingly, however, these differences between "non-responders" and "responders" in terms of ischaemia and hospitalisations were not evident when assessing the CHF cohort without DM alone (both $P > 0.05$ between "non-responders" vs. "responders").

This suggests that the RISE-95 protocol is more sensitive for detecting these important clinical factors in D-HF patients. Nevertheless, this comparison did demonstrate that the RISE-95 protocol was more sensitive for determining NYHA classification in the CHF without DM cohort as 35 (53%) of the 66 "responders" were NYHA class I whereas only 23 (27%) of the 84 "non-responders" fell into this classification ($P = 0.002$). Thus, the RISE-95 protocol may be of clinical utility in terms of identifying and directing treatment in both CHF and D-HF patients.

3.3 Discussion

The primary novel findings from this current study were that 1) the RISE-95 protocol was well tolerated by a large cohort of CHF patients in a clinical setting with a 93% completion rate; 2) the test was sufficiently sensitive to confirm $\dot{V}O_{2max}$ using patient-specific criterion without resorting to secondary criteria; 3) $\dot{V}O_{2max}$ was only confirmed in 39% of CHF patients; 4) this test identified a large subpopulation of 89 patients (46% of the total cohort) termed "responders" that significantly increased their $\dot{V}O_{2peak}$ by an average of 1.6 mL·kg^{-1}·min^{-1} and also displayed distinct clinical characteristics; and 5) D-HF patients have a reduced exercise capacity and limited "aerobic reserve" (i.e. a blunted response to the SE phase of the test), which may explain the poorer prognosis seen within this subpopulation.

3.3.1 Application of the RISE-95 protocol in patients

One of the primary findings from this current chapter was that a large unselected cohort of CHF patients successfully tolerated this novel protocol without any adverse events. Indeed, nearly 200 patients completed the protocol safely with

only 14 (7%) unable to complete both phases of the test giving a completion rate of 93%. This is significant given that others have previously suggested that a verification phase following a RI test is unethical and unrealistic in clinical populations (van Breda *et al.*, 2017). The current study demonstrates that this was not the case in our cohort of CHF patients and therefore supports previous studies showing the safety and efficacy of such tests in different patient populations (de Groote *et al.*, 2004; Saynor *et al.*, 2013; Astorino *et al.*, 2018).

3.3.2 Confirming $\dot{V}O_{2max}$ in patients using the RISE-95 protocol

$\dot{V}O_{2max}$ is considered the gold standard measure of the maximal integrated functioning of the pulmonary, cardiovascular, and neuromuscular systems to uptake, transport, and utilize O_2. $\dot{V}O_{2max}$ is traditionally confirmed by a criterion plateau in $\dot{V}O_2$ despite an increase in work rate (Hill & Lupton, 1923). However, this plateau is rarely evident in CHF patients during continuous RI protocols (Piepoli *et al.*, 2006), which has led to the recommendation that a constant-load SE verification phase be completed following RI to satisfy this criterion (Rossiter *et al.*, 2006). Using patient-specific criterion to compare $\dot{V}O_{2peak}$ measured during RI and SE, the current RISE-95 protocol was sufficiently sensitive to confirm $\dot{V}O_{2max}$ in just 39% of all CHF patients without requiring secondary criteria. This contrasts with previous studies that have questionably rejected the necessity of verification phases based on the finding that the group mean "$\dot{V}O_{2max}$" is not statistically different between the two phases and therefore the $\dot{V}O_{2peak}$ from RI alone is sufficient to assume a plateau (Hawkins *et al.*, 2007; Murias *et al.*, 2018). However, as previously noted (Noakes, 2008), this logic is flawed as it dismisses any potential within-subject differences between the two phases. Indeed, by

statistically comparing the highest mean $\dot{V}O_2$ achieved across 12 consecutive breaths in RI and SE, the current study demonstrates that large within-subject differences can exist, at least within CHF patients (Figures 3.1 and 3.2). Thus, future studies and current guidelines should consider the inclusion of a verification phase, such as that used in the RISE-95 protocol, to unequivocally confirm $\dot{V}O_{2max}$ in the absence of a plateau.

Traditionally several corroborating secondary criteria measurements have been used to confirm $\dot{V}O_{2max}$ in the absence of the criterion plateau (Howley *et al.*, 1995). Of these secondary criteria, RER_{peak} is the most commonly used and recommended in CHF patients (Piepoli *et al.*, 2006), with several thresholds (e.g. 1.00 (Gibbons *et al.*, 1997); > 1.05 (Mehra *et al.*, 2016); ≥ 1.10 (Mezzani *et al.*, 2009)) being applied to "confirm" $\dot{V}O_{2max}$. However, the current study questions the validity of RER_{peak} for confirming (or refuting) $\dot{V}O_{2max}$ attainment as we found that RER_{peak} can significantly decrease even with a significant increase in $\dot{V}O_{2peak}$ (Table 3.2). This seemingly paradoxical finding is explained by a simultaneous decrease in $\dot{V}CO_{2peak}$, which was likely due to the 5 minute recovery phase allowing sufficient time for the lungs to fully expire CO_2 accumulated from the metabolic acidosis induced by the RI phase. Nevertheless, this finding compliments previous studies querying the validity of RER_{peak}, which is significantly influenced by the ramp rate in both healthy (Buchfuhrer *et al.*, 1983) and CHF (Bowen *et al.*, 2012a) populations. Thus RER_{peak} should not be used to confirm $\dot{V}O_{2max}$ in CHF patients.

3.3.3 Identifying "responders" to the RISE-95 protocol

Another interesting finding of the present study was that 89 (46%) patients statistically increased their $\dot{V}O_{2peak}$ in the SE phase of the test. This subpopulation of "responders" achieved a $\dot{V}O_{2peak}$ in the SE phase that was, on average, 1.6 mL·kg^{-1}·min^{-1} higher than RI, which corresponds to a remarkable 11% increase. Furthermore there was significant inter-individual variability even within these 89 patients with a range of differences from 0.3-4.7 mL·kg^{-1}·min^{-1} (1.5-33.5%). Notably, 38 of these 89 patients, which represents nearly 20% of the entire cohort, were able to increase their $\dot{V}O_{2peak}$ by > 10% in SE compared to RI. Importantly, these large individual differences may have been overlooked if only the group means were examined, as these only showed a modest increase of 0.5 mL·kg^{-1}·min^{-1} (3%) in $\dot{V}O_{2peak}$ between the RI and SE phases in all 195 patients (Table 3.2). Therefore, the significant advantage of performing the RISE-95 protocol, and employing patient-specific criterion that directly compare the 12-breath averages between the RI and SE phases, is the ability to identify those CHF patients who, for some yet unknown reason, are capable of increasing their $\dot{V}O_{2peak}$ further in the SE phase.

This increase of 1.6 mL·kg^{-1}·min^{-1} in $\dot{V}O_{2peak}$ in these "responders" is of substantial clinical significance given that every reduction of just 1 mL·kg^{-1}·min^{-1} increases all-cause mortality risk by ~16% in CHF patients (Keteyian *et al.*, 2016). The corresponding 11% increase in $\dot{V}O_{2peak}$ is also clinically significant given that a 6% increase in $\dot{V}O_{2peak}$ can reduce all-cause mortality by 7% in CHF patients (Swank *et al.*, 2012). Additionally, this acute increase in $\dot{V}O_{2peak}$ is also impressive when compared to studies that intentionally aimed to increase $\dot{V}O_{2peak}$ in CHF patients with long-term exercise interventions. For instance, this

increase of 1.6 mL·kg^{-1}·min^{-1} in $\dot{V}O_{2peak}$ is greater than the 1.3 mL·kg^{-1}·min^{-1} increase achieved after a 12-week high-intensity interval training program in CHF patients (Benda *et al.*, 2015). Furthermore, given that 10 years of regular exercise training in CHF patients has been shown to increase $\dot{V}O_{2peak}$ by just 3.6 mL·kg^{-1}·min^{-1} (Belardinelli *et al.*, 2012), the acute increase of 1.6 mL·kg^{-1}·min^{-1} seen with the RISE-95 protocol is rather remarkable.

Such improvements in $\dot{V}O_{2peak}$ are rarely seen in healthy subjects performing similar protocols (Day *et al.*, 2003; Rossiter *et al.*, 2006). This suggests that CHF patients may have an "aerobic reserve", which is alleviated by RI, allowing a higher $\dot{V}O_{2peak}$ in SE. The functional consequence of this increase in $\dot{V}O_{2peak}$ in terms of exercise tolerance was demonstrated by "responders" exercising at a 12% higher peak work rate and for 50% longer during the SE phase of the protocol compared to "non-responders" (Table 3.5).

To further elucidate the mechanisms which may be allowing this increase in aerobic capacity, we statistically compared the "non-responders" and "responders" in terms of exercise responses. This discrimination identified some divergent responses between the RI and SE phases of the protocol within these two cohorts. Firstly, $\dot{V}CO_{2peak}$ decreased in "non-responders" but increased in "responders", with this contributing towards a higher RER$_{peak}$ in the "responders" during the SE phase (Table 3.5). This difference was also reflected in a lower $\dot{V}_E/\dot{V}CO_2$ nadir and a higher lactate threshold in the "responders". Another significant divergent response was that $\dot{V}_{Epeak}$ decreased in "non-responders" but increased in "responders" as a result of comparable responses in V_{Tpeak} and

breathing frequency (Table 3.5). This difference was matched by similar cardiac responses as "non-responders" exhibited a decrease in peak HR whilst "responders" increased their peak HR between the two phases (Table 3.5). Oxygen pulse significantly increased by 0.9 mL·beat^{-1} in the SE phase in only the "responders" (Table 3.5). This suggests that, even in the presence of an increased peak heart rate, O_2 consumption is still increased, possibly as a result of increased extraction and utilization within the skeletal muscle mitochondria. Interestingly, even in all 195 patients oxygen pulse significantly increased by an average of 0.7 mL·beat^{-1}, despite no change in peak HR, and therefore may have contributed to the modest yet significant 0.5 mL·kg^{-1}·min^{-1} increase in $\dot{V}O_{2peak}$ in the SE phase (Table 3.2). Thus, these data suggest that CHF patients, particularly those that may be discriminated as "responders" using the RISE-95 protocol, may have an intramuscular limitation in O_2 extraction, possibly due to mitochondrial utilisation, which is overcome with prior exercise, as previously noted (Bowen *et al.*, 2012b).

In terms of clinical characteristics, it was observed that these "responders" were younger, more likely to be NYHA class I and less likely to be ischaemic. Interestingly, however, this cohort had more hospitalisations in the follow-up period (48% vs. 34%). Therefore use of the RISE-95 protocol should be considered in CHF patients to identify these "responders" and subsequently guide the appropriate clinical and therapeutic intervention(s). A better understanding of the mechanisms underpinning this "aerobic reserve", and an improved understanding of the characteristics defining these "responders", may prove fruitful for developing targeted therapeutic interventions that

pharmacologically increase $\dot{V}O_{2peak}$ in CHF patients and thus improve symptoms, prognosis and outcomes in this exercise intolerant population.

3.3.4 Application of the RISE-95 protocol in D-HF patients

Another aim of the present study was to determine if the RISE-95 protocol was sensitive to clinically significant differences in subpopulations by comparing D-HF patients with CHF patients. Consistent with previous studies (Cubbon *et al.*, 2013), we found that the D-HF cohort exhibited poorer symptoms and a worse prognosis characterised by an increased risk of all-cause mortality. Furthermore, D-HF patients also had a 20% lower $\dot{V}O_{2peak}$ across both phases of the protocol with comparable reductions in peak work rate, which is consistent with previous findings (Tibb *et al.*, 2005; Ingle *et al.*, 2006). However, it remains unclear what mechanisms contribute towards these differences in D-HF patients compared to the CHF population without DM.

By using the RISE-95 protocol, we were able to compare the "responder" patients within both the D-HF and CHF cohorts. This comparison highlighted that the absolute increase in $\dot{V}O_{2peak}$ is 29% lower in D-HF compared to CHF (Figure 3.5B), suggesting that D-HF patients have greater limitations within their "aerobic reserve". Indeed, this was supported by the fact that, unlike CHF "responders", D-HF "responders" were unable to significantly increase $\dot{V}CO_{2peak}$, VT_{peak}, and peak HR between the RI and SE phases. Therefore peripheral factors may play a more significant role in increasing $\dot{V}O_{2peak}$ in these patients. This postulation was supported by a trend for a greater increase in oxygen pulse in the D-HF "responders" compared to CHF "responders" between the RI and SE phases,

suggesting that these patients are able to improve skeletal muscle O_2 extraction to a greater extent with prior exercise. Thus, CHF "responders" appear to be able to increase $\dot{V}O_{2peak}$ predominantly through increases in peak HR and respiration whereas D-HF "responders" rely substantially more on increasing O_2 extraction within the skeletal muscles. This discrepancy also likely explains why D-HF "responders" have a lower "aerobic reserve" compared to CHF "responders". Future work is therefore required to uncover the mechanisms that contribute to the reduced $\dot{V}O_{2peak}$ and "aerobic reserve" in D-HF patients, with a particular emphasis on what factors may be limiting skeletal muscle O_2 extraction. A better understanding of the mechanism(s) which allow these limitations to be overcome (i.e. prior exercise in D-HF "responders") may help identify potential therapeutic targets by which $\dot{V}O_{2peak}$ can be pharmacologically increased in D-HF patients.

Further scrutiny of the clinical characteristics between CHF and D-HF patients defined as either "non-responders" and "responders" also yielded some interesting findings. D-HF "responders" were less likely to be ischaemic (39% v 73%) yet recorded more hospitalisations (61% v 27%) compared to D-HF "non-responders". Interestingly, these differences were not apparent when assessing the CHF cohort without DM alone. Thus D-HF, rather than CHF, patients appear to contribute substantially more to the higher incidence of hospitalisations seen in the "responders". However, the RISE-95 protocol was more sensitive for determining NYHA classification in the CHF without DM cohort. Therefore the RISE-95 protocol is capable of identifying significant clinical characteristics in both CHF and D-HF patients and may be used to direct treatment(s) and/or intervention(s).

3.3.5 Clinical implications

The current study proposes that RER_{peak} should no longer be used as a corroborating secondary criteria for confirming $\dot{V}O_{2max}$ and that the RISE-95 test should be used to confirm $\dot{V}O_{2max}$ attainment in CHF patients. The RISE-95 protocol could easily be incorporated within large clinical settings, and the method proposed in the current study of confirming $\dot{V}O_{2peak}$ by statistically comparing the final 12 breaths of exercise in the RI and SE phases, could also be installed within commercial software to provide a quick and accurate confirmation of $\dot{V}O_{2max}$ attainment. This study demonstrates that a SE phase at 95% peak work rate, rather than a supramaximal effort at 105% as has been proposed (Poole & Jones, 2017), is safe and sufficient to confirm $\dot{V}O_{2max}$ and may be more desirable in patient populations to encourage tolerability and avoid potentially adverse events.

3.3.6 Limitations

One of the potential limitations of this chapter is that the clinical application of cardiopulmonary exercise tests may not be as rigorous as ramp-incremental tests undertaken in a supervised laboratory environment, where patients are perhaps more likely encouraged to exercise to volitional exhaustion. However, as one of the primary outcomes of this study was to determine the clinical utility of the RISE-95 protocol in a large patient population, this drawback was known and accepted beforehand.

These assertions have significant implications for current clinical practice and how future CPX tests are conducted in clinical populations, such as CHF

patients. It is essential that accurate and reliable $\dot{V}O_{2max}$ measurements are obtained in such clinical populations so clinicians can be confident in the accuracy of such measures to discriminate clinically meaningful differences resulting from disease progression and/or therapeutic/rehabilitation intervention(s) (Williams *et al.*, 2017). Such knowledge can inform on the prognosis/symptomatic status of the patient and thus guide future therapeutic treatment(s) and/or clinical intervention(s). Indeed, it is conceivably plausible that incorrect measurements of $\dot{V}O_{2max}$ may result in misguided clinical evaluations and, consequentially, misdirected therapeutic treatments and interventions.

3.4 Conclusions

The present study demonstrates that the RISE-95 exercise protocol is well tolerated by a large cohort of CHF patients and is sufficiently sensitive to confirm $\dot{V}O_{2max}$ using patient-specific criterion without requiring secondary criteria. Therefore this protocol should be incorporated within standard clinical practice. Additionally, this protocol identified a large subpopulation of CHF patients capable of significantly increasing their $\dot{V}O_{2peak}$ between the RI and SE phases of the test. This cohort have a distinct clinical phenotype characterised by an increased risk of hospitalisation despite being younger and less symptomatic. Finally, this study identified that the reduced $\dot{V}O_{2peak}$ and "aerobic reserve" observed in D-HF patients may be due to impaired skeletal muscle O_2 extraction. Therefore future research is required to better understand the mechanism(s) contributing towards this limitation to help identify potential therapeutic targets by which $\dot{V}O_{2peak}$ may be pharmacologically increased.

Chapter 4 Results II

Skeletal muscle mitochondrial dysfunction from upper body biopsies in chronic heart failure and type 2 diabetes

4.1 Introduction

Chronic heart failure (CHF) is characterised by exercise intolerance in the presence of cardiac dysfunction. However CHF patients with type 2 diabetes mellitus (DM) as a co-morbidity (diabetic heart failure; D-HF) have even greater impairments of exercise tolerance by ~20 % in terms of peak pulmonary O_2 uptake ($\dot{V}O_{2peak}$) (Results I) (Guazzi *et al.*, 2003; Tibb *et al.*, 2005) and 6-minute walk test (6-MWT) distance (Ingle *et al.*, 2006). This reduced exercise capacity is associated with greater mortality (Myers *et al.*, 2002) and a worse prognosis (de Groote *et al.*, 2004; From *et al.*, 2006; MacDonald *et al.*, 2008a) even with current optimal therapies (Cubbon *et al.*, 2013). However the mechanisms underpinning the poorer exercise tolerance in D-HF compared to CHF remain poorly resolved. One key mechanism may be related to exacerbated cardiac dysfunction in D-HF, yet studies have shown that D-HF patients have similar measures of cardiac function compared with CHF patients (MacDonald *et al.*, 2008b; Cubbon *et al.*, 2013). Whether global measures of left ventricular performance provide the most appropriate assessment of cardiac function between cohorts remains to be established. However, at least for now, evidence suggests exercise intolerance in D-HF is at least partially exacerbated by peripheral mechanisms.

The most common assessment of exercise intolerance in CHF patients is $\dot{V}O_{2peak}$, which is dictated by the products of the Fick equation ($\dot{Q}$ and O_2 extraction). As such, reduced skeletal muscle O_2 extraction, possibly due to mitochondrial impairments, could be a key determinant underlying the reduced $\dot{V}O_{2peak}$ in D-HF patients. Indeed, patients with CHF demonstrate skeletal muscle impairments that closely correlate to symptoms and exercise tolerance independent of cardiac function (Wilson *et al.*, 1985; Massie *et al.*, 1987a; Lipkin *et al.*, 1988; Mancini *et al.*, 1989; Mancini *et al.*, 1992; Schaufelberger *et al.*, 1996; Okita *et al.*, 1998). In particular, mitochondrial impairments have been reported in patients with CHF (Drexler *et al.*, 1992; Massie *et al.*, 1996; Guzman Mentesana *et al.*, 2014), both in terms of content and function, which would limit the ability of muscle to utilize O_2 to support energetic demands. However, whether D-HF exacerbates mitochondrial impairments beyond that observed in patients with CHF, or even DM, remains to be determined. As CHF and DM are associated with many factors that have been suggested to induce skeletal muscle abnormalities, such as fibre-type shifts, mitochondrial dysfunction, elevated inflammatory cytokines, increased ROS, accumulation of intracellular lipids, and detraining, it is likely that D-HF patients may suffer even greater deficits to mitochondria.

At present, there are limited data available regarding mitochondrial alterations in D-HF patients. Despite the absence of a control group, one study observed that D-HF patients have low mitochondrial protein expression as well as low mitochondrial cristae abundance and volume density in leg muscle biopsies in 10 people on optimal therapy (Taub *et al.*, 2012). Interestingly, studies in CHF patients have largely shown that mitochondrial function, determined as the

activity of the individual enzyme complexes, remains normal whereas content is lower compared to controls (Drexler *et al.*, 1992; Mettauer *et al.*, 2001; Toth *et al.*, 2012). This indicates that quantitative rather than qualitative mitochondrial impairments might form the most important factor for exercise intolerance in CHF patients. This has also been reported in DM patients (i.e., mitochondrial respiration is normal after accounting for mitochondrial content) (Kelley *et al.*, 2002; Morino *et al.*, 2005; Ritov *et al.*, 2005; Boushel *et al.*, 2007). In subjects matched for physical activity, previous studies have found very few differences in mitochondrial measures (Mettauer *et al.*, 2001; Williams *et al.*, 2004; Toth *et al.*, 2012; Middlekauff *et al.*, 2013). However, these findings may have been confounded by detraining of the lower limbs. Indeed, while the lower limbs can be impacted significantly in disease due to associated detraining, the upper limbs may provide a more appropriate sampling site as they are more often recruited during daily activities (Rabøl *et al.*, 2010).

The present study, therefore, took advantage of routine pacemaker implantation surgery performed in the upper limbs of age-matched control, DM, CHF, and D-HF patients, to sample the *pectoralis major* in order to examine whether D-HF induces significant impairments to mitochondrial function and content in isolation from the changes induced by detraining. We hypothesized that patients with D-HF would demonstrate greater decrements in mitochondrial respiration compared to DM and CHF patients with these functional differences being negated once normalized to mitochondrial content.

4.2 Methods

4.2.1 Patients

Eligible and consecutive patients (n = 130) undergoing routine pacemaker therapy at Leeds General Infirmary volunteered to participate in the present study. All patients provided written informed consent prior to the study (Appendix D) after reading the participant information sheet (Appendix C). The study was approved by the Leeds West Research Ethics Committee (11/YH/0291) and the Leeds Teaching Hospitals R+D committee (CD11/10015) and all procedures were conducted in accordance with the Declaration of Helsinki (Appendices A and B). Patients were grouped into four cohorts based on the criteria for CHF and DM outlined in 2.1 in the General Methods chapter.

4.2.2 Muscle sampling and high-resolution respiroemtry

Skeletal muscle biopsies from *pectoralis major* were obtained from all patients during pacemaker surgery as outlined in 2.5.1 in the General Methods chapter. Samples (~50mg) were immediately placed in 1 mL of ice-cold BIOPS (Table 2.1) (Fontana-Ayoub *et al.*, 2014) and transported on ice back to the laboratory. Samples then underwent the appropriate mechanical and chemical permeabilization steps as outlined in 2.6.2 of the General Methods chapter.

Samples were then dried, weighed (wet weight, W_W) to ~2-6 mg (Kuznetsov *et al.*, 2008) and placed within the two sealed chambers of the Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria), each of which contained 2 mL Mir05 (Figure 2.6B) and 5 µM blebbistatin (13186, Cayman Chemical, Ann Arbor, MI). Thereafter a standard SUIT protocol (outlined in 2.6.4 of the General Methods

chapter) was followed on the high-resolution respirometer. All solutions used in respirometry, including stock and final concentrations, injection volume and dilution, plus manufacturer's names and product codes are detailed in Table 2.3. Following the SUIT protocol, the complex IV assay described in 2.6.5 was included to normalise the data to a marker of mitochondrial content. To confirm the validity of this method (Figure 2.8), mitochondrial content was quantified in a selection of samples (n = 27) using the citrate synthase assay given in 2.7. The RCR and FCRs were calculated according to the formulae outlined in 2.6.6.

In 7 samples there was a complete failure to respond to any of the titrations. These samples were not included in the final analysis, which resulted in a total of 15 excluded samples (n = 8 mitochondrial membrane damage and n = 7 non-responders). Overall, therefore, 115 patients were included in the final analysis.

4.2.3 Statistical analyses

All statistical analyses were completed using the Statistical Package for the Social Sciences (SPSS for Windows Version 25.0; IBM Corporation, Armonk, NY) after confirming the assumptions met for parametric testing and removing outliers as detailed in 2.11 of the General Methods chapter. Continuous variables were compared between groups using unpaired Student's t-tests and separate one-way (one-factor) 1 × 4 ANOVA tests with post hoc analyses using Tukey's multiple comparisons test. Categorical variables were compared using the two-sided Pearson's χ^2 test. Categorical variables are presented as a percentage (%) of the cohort and the number (n). Continuous variables are expressed as mean

± SEM unless otherwise stated. Statistical significance was accepted as $P <$ 0.05.

4.3 Results

4.3.1 Demographic and clinical variables

The demographic and physical characteristics of the 115 patients included in the final analysis are presented in Table 4.1. There were between group differences in body weight and renal function.

Table 4.1. Demographic, physical, and clinical characteristics of patients included in the final analysis grouped by their respective patient cohorts. Data are expressed as mean ± SEM unless otherwise stated. Significant differences ($P < 0.05$) are shown in bold.

	CON	DM	CHF	D-HF
n (%)	25 (22)	10 (9)	52 (45)	28 (24)
Male sex [% (n)]	64.0 (16)	90.0 (9)	82.7 (43)	85.7 (24)
Age (years)	72.2 ± 2.0	74.5 ± 1.9	71.6 ± 1.6	71.4 ± 1.9
Weight (kg)	81.0 ± 3.4	105.6 ± 8.9**	81.0 ± 2.6††	88.6 ± 3.3†
$\dot{V}O_{2peak}$ (mL·kg^{-1}·min^{-1})			15.3 ± 0.9	13.0 ± 0.6
Clinical Factors				
NYHA functional class [% (n)]				
I			7.7 (4)	3.6 (1)
II			55.8 (29)	50.0 (14)
III			36.5 (19)	46.4 (13)
Ischaemic aetiology [% (n)]			61.5 (32)	64.3 (18)
DCM aetiology [% (n)]			25.0 (13)	25.0 (7)
AF [% (n)]	48.0 (12)	30.0 (3)	17.3 (9)	28.6 (8)
CABG [% (n)]	28.0 (7)	10.0 (1)	21.2 (11)	25.0 (7)
Hypertension [% (n)]	36.0 (9)	60.0 (6)	32.7 (17)	57.1 (16)
LVEF (%)			24.8 ± 1.9	30.2 ± 2.2
LVIDd (mm)			58.5 ± 1.4	58.4 ± 1.9
Haemoglobin (g·L^{-1})	134.1 ± 3.7	140.8 ± 4.8	138.8 ± 2.2	128.1 ± 4.6
Sodium (mmol·L^{-1})	138.9 ± 0.8	136.3 ± 1.4	139.4 ± 0.7	132.8 ± 5.5
Potassium (mmol·L^{-1})	4.7 ± 0.1	4.6 ± 0.1	4.5 ± 0.1	4.6 ± 0.1
Creatinine (μmol·mL^{-1})	86.9 ± 4.0	106.7 ± 9.3	101.1 ± 5.9	106.3 ± 9.4
eGFR (mL·min^{-1}·1.73 m^{-2})	69.4 ± 3.2	51.8 ± 5.5**	60.8 ± 2.6*	55.4 ± 3.6**
Plasma Glucose (mmol·L^{-1})		9.3 ± 1.9		8.1 ± 0.6
HbA1c (mmol·mol^{-1})		50.5 ± 6.9		62.3 ± 3.3

$\dot{V}O_{2peak}$, peak pulmonary oxygen uptake; NYHA, New York Heart Association; DCM, dilated cardiomyopathy; AF, atrial fibrillation; CABG, coronary artery bypass graft; LVEF, left ventricular ejection fraction; LVIDd, left ventricular internal diameter at diastole; eGFR, estimated glomerular filtration rate; HbA1c, glycated haemoglobin. *$P < 0.05$ vs. CON; **$P < 0.01$ vs. CON; †$P < 0.05$ vs. DM; ††$P < 0.01$ vs. DM.

4.3.2 Drug and device therapy

The use of pharmacological treatments and device therapies by each group are shown in Table 4.2 alongside equivalent daily dosages (mg) where appropriate. Patients in the two CHF cohorts were more frequently taking beta-blockers and at a higher dosage compared to the two non-CHF cohorts, with the D-HF cohort receiving a higher dose than CHF ($P = 0.031$). Loop diuretic use was highest in the two CHF cohorts with D-HF patients more likely to be taking a loop diuretic and also at higher equivalent dose compared with CHF alone. This is in agreement with the impaired renal function and worse symptoms seen in these patients. There was a trend for aldosterone antagonist use to be higher in the D-HF cohort compared to CHF. Aspirin use was higher in the two CHF cohorts.

Table 4.2. Pharmacological treatments and device therapy. Data are expressed as mean ± SEM unless otherwise stated. Significant differences ($P < 0.05$) are shown in bold.

	CON	DM	CHF	D-HF
Pharmacological treatments				
ACEi use [% (n)]	36.0 (9)	50.0 (5)	61.5 (32)	53.6 (15)
Ramipril equivalent dose (mg)	4.3 ± 1.1	8.3 ± 1.7	6.8 ± 0.9	7.0 ± 0.8
Beta-blocker use [% (n)]	32.0 (8)	60.0 (6)	86.5 (45)**†	85.7 (24)**†
Bisoprolol equivalent dose	3.0 ± 0.6	2.9 ± 0.6	5.2 ± 0.5*†	7.6 ± 0.7**††
Loop diuretic use [% (n)]	16.0 (4)	30.0 (3)	48.1 (25)*	64.3 (18)**†
Furosemide equivalent dose	55 ± 15	33 ± 7	45 ± 4	100 ± 20*†‡
ARB use [% (n)]			21.2 (11)	32.1 (9)
Candesartan equivalent dose			15.3 ± 4.2	16.6 ± 3.1
Aldosterone antagonist use [%			44.2 (23)	57.1 (16)
Aldosterone antagonist dose			26.1 ± 1.1	29.7 ± 5.1
Statin use [% (n)]	48.0 (12)	90.0 (9)	63.5 (33)	67.9 (19)
Statin dose (mg)	35.0 ± 6.9	38.8 ± 7.2	46.4 ± 4.1	44.2 ± 6.2
Aspirin use [% (n)]	20.0 (5)	10.0 (1)	46.2 (24)*†	46.4 (13)*†
Metformin use [% (n)]		50.0 (5)		46.4 (13)
Insulin use [% (n)]		20.0 (2)		10.7 (3)
Device therapy				
PPM [% (n)]	96.0 (24)	90.0 (9)		
ICD [% (n)]	4.0 (1)	10.0 (1)	26.9 (14)	3.6 (1)
CRT [% (n)]			73.1 (38)	96.4 (27)

ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; PPM, permanent pacemaker; ICD, implantable cardioverter defibrillator; CRT, cardiac resynchronisation therapy. *$P < 0.05$ vs. CON; **$P < 0.01$ vs. CON; †$P < 0.05$ vs. DM; ††$P < 0.01$ vs. DM; ‡$P < 0.5$ vs. CHF.

4.3.3 High-resolution respirometry representative traces

Figure 4.1 shows four representative O_2 flux traces obtained from standard high-resolution respirometry experiments using the SUIT protocol outlined above. Specific titrations of certain substrates, uncouplers and inhibitors are highlighted on the figure. Of note the control patient has a higher response to ADP than the other three patients, which was blunted most significantly in D-HF patients.
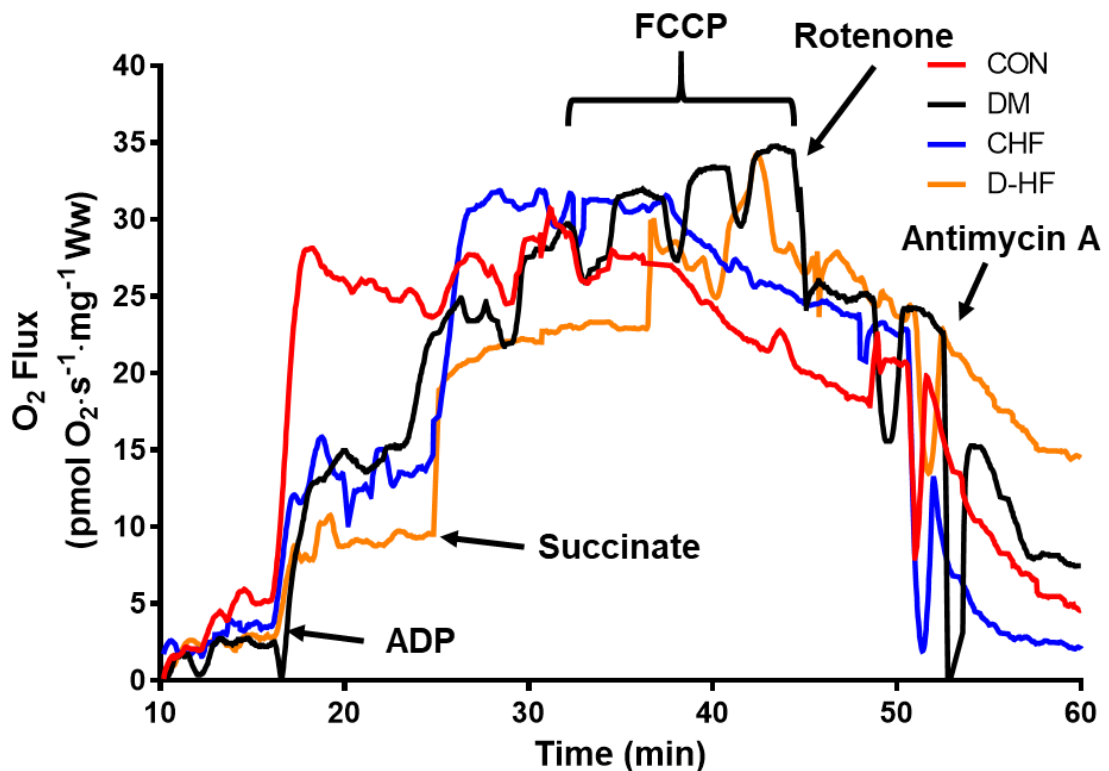
Figure 4.1. Representative O_2 flux traces for patients from each cohort time-aligned to match titrations. Arrows indicate where individual titrations were made throughout the protocol.

4.3.4 Mitochondrial oxygen flux

Mean group O_2 flux results from each of the respiratory states are presented in Figure 4.2. There were significant between group differences for each respiratory state measured. Follow-up multiple comparisons identified a lower complex I Leak respiration in D-HF compared to both CON ($P < 0.0001$; Figure 4.2) and CHF ($P = 0.011$; Figure 4.2). CHF was also lower compared to CON for complex

I Leak ($P = 0.042$; Figure 4.2). DM, CHF, and D-HF had lower measures than CON for complex I OXPHOS, by 33% ($P = 0.002$), 26% ($P < 0.0001$) and 62% ($P < 0.0001$), respectively (Figure 4.2). Additionally, complex I OXPHOS, in D-HF was 43% ($P = 0.008$; Figure 4.2) and 49% ($P < 0.0001$; Figure 4.2) lower than DM and CHF, respectively. For complex I+II OXPHOS, the only differences were seen in D-HF ($P < 0.0001$ vs. CON; $P = 0.007$ vs. DM; $P = 0.001$ vs. CHF; Figure 4.2). This same trend was seen for uncoupled complex I+II respiration with D-HF being lower than each respective cohort ($P = 0.003$ vs. CON; $P = 0.016$ vs. DM; $P = 0.004$ vs. CHF; Figure 4.2). For complex II-supported ETS capacity, D-HF was lower than both CON ($P = 0.003$; Figure 4.2) and DM ($P = 0.011$; Figure 4.2).
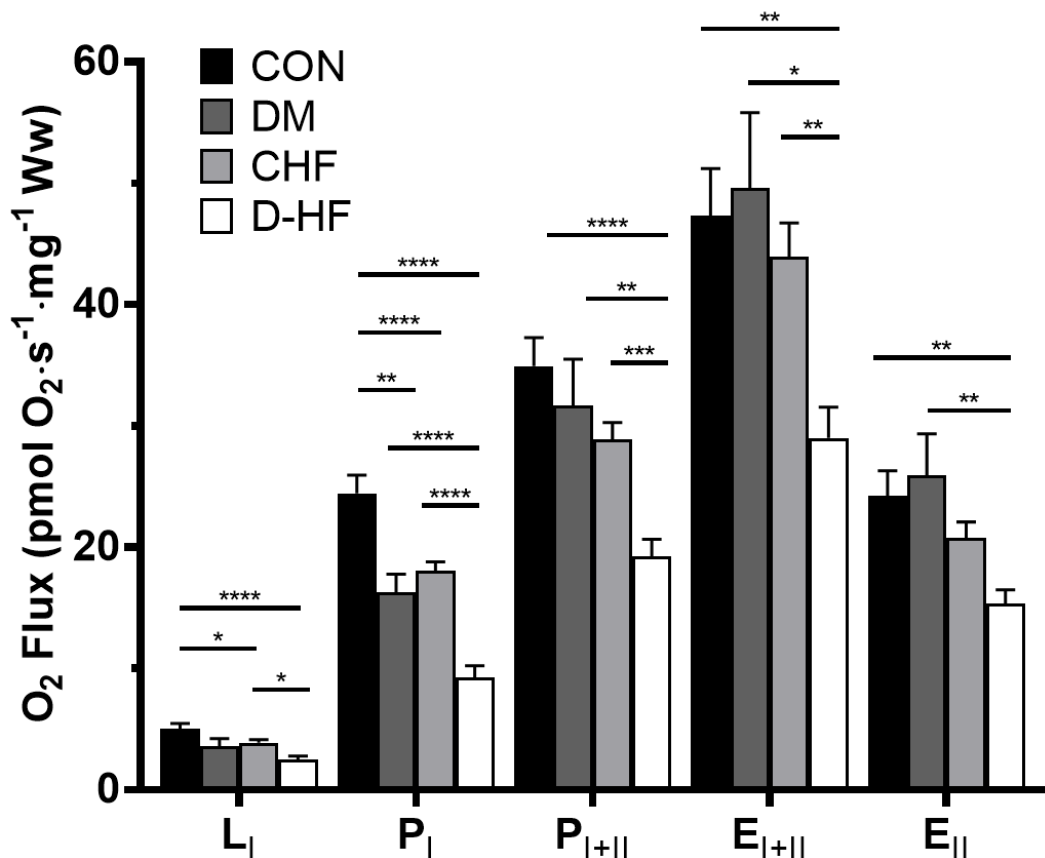
Figure 4.2. D-HF patients have lower mitochondrial respiration in all respiratory states compared to CON. The data displayed above are given as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ using Tukey's multiple comparisons post-hoc tests following significant one-way ANOVAs.

4.3.5 Respiratory control ratio

The RCR, a ratio of P_I to L_I respiration, for each group is presented in Figure 4.3. A main effect ($P = 0.024$) was detected between groups, with post-hoc tests revealing a 28% reduction in D-HF compared to CHF (Figure 4.3) with similar trends when compared to CON ($P = 0.070$) and DM ($P = 0.175$).
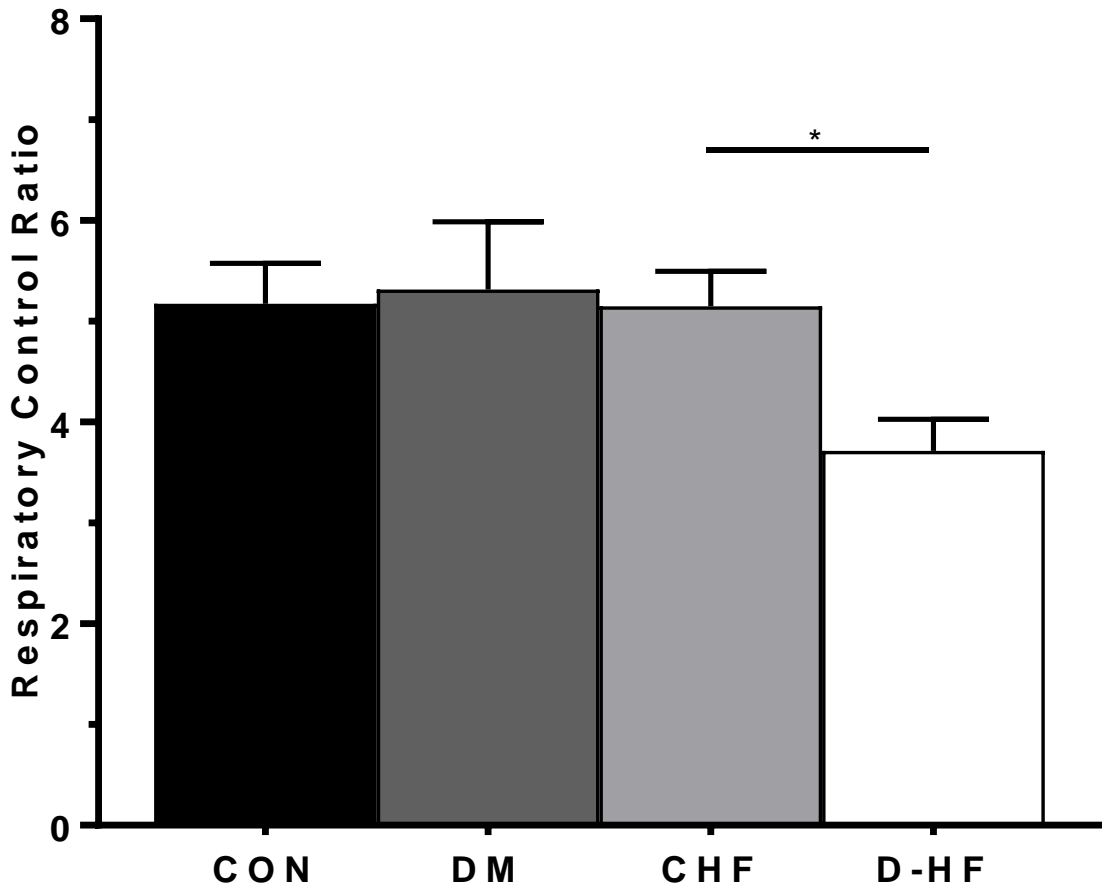
Figure 4.3. D-HF patients have a lower respiratory control ratio (RCR) compared to CHF. The RCR is the ratio between O_2 flux measured with complex I substrates in the Leak and OXPHOS respiratory states. The data displayed above are given as mean ± SEM. *$P < 0.05$ compared to CHF using Tukey's multiple comparisons post-hoc test following a significant one-way ANOVA.

4.3.6 Flux control ratios

Figure 4.4 shows the group mean flux control ratios for each respiratory state. For complex I OXPHOS, DM, CHF, and D-HF were lower by 36% ($P = 0.002$), 22% ($P = 0.005$), and 40% ($P < 0.0001$) compared to CON, respectively (Figure 4.4). D-HF was also 23% lower than CHF ($P = 0.0208$; Figure 4.4). For complex I+II OXPHOS, only CHF was lower than CON ($P = 0.0483$; Figure 4.4), while D-HF were higher than CHF for complex II-supported ETS capacity ($P = 0.020$; Figure 4.4).
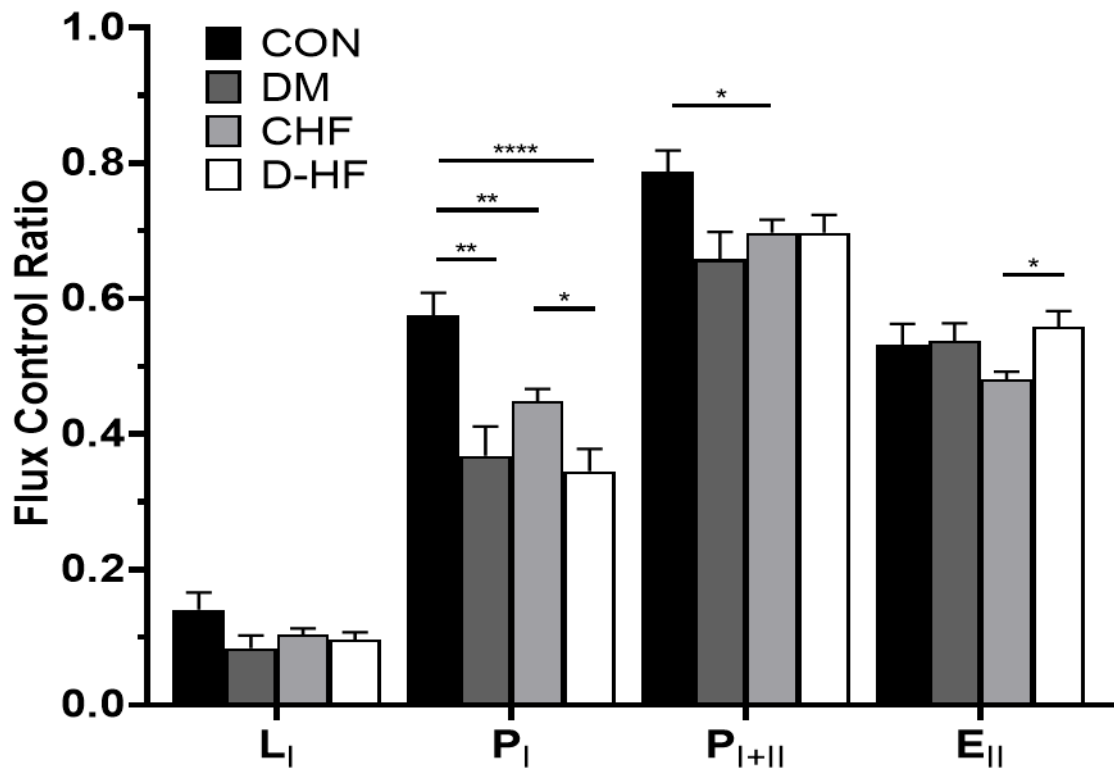
Figure 4.4. D-HF patients have a lower complex I flux control ratio. The data displayed above are given as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$ using Tukey's multiple comparisons post-hoc tests following significant one-way ANOVAs.

4.3.7 Complex IV assay for mitochondrial content

The O_2 flux measured with the artificial complex IV electron donors TMPD and ascorbate is presented in Figure 4.5. A main effect ($P = 0.003$) was detected, with post hoc tests revealing that O_2 flux was 33% lower in D-HF than in CON ($P = 0.001$; Figure 4.5). These findings were supported by measures of citrate synthase activity, which was 46% lower in D-HF compared to CON ($P = 0.010$; data not shown). The validity of the complex IV protocol was demonstrated by a significant correlation between these measures and citrate synthase activity in the 27 samples (Figure 2.8).
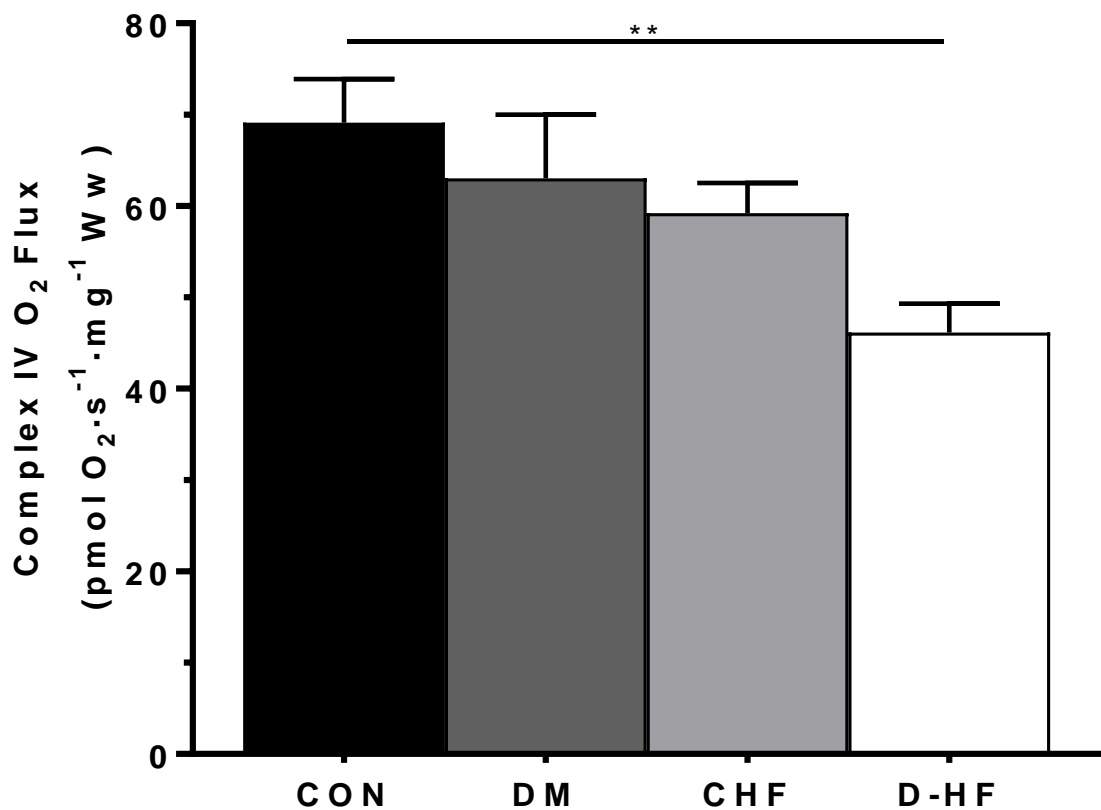
Figure 4.5. Mitochondrial content is lower in D-HF patients. Complex IV O_2 flux, as a marker of mitochondrial content, is significantly lower in D-HF compared to CON. The data displayed above are given as mean ± SEM. **$P < 0.01$ compared to CON using Tukey's multiple comparisons test following a significant ($P < 0.05$) one-way ANOVA.

4.3.8 Corrected mitochondrial respiration

To account for differences in mitochondrial content, the raw O_2 flux measures for each respiratory state were normalized to the O_2 flux recorded from the complex IV activity assay (Figure 4.6). After adjusting the data to this measure of mitochondrial content, complex I OXPHOS was the only respiratory state that remained significantly different between the 4 cohorts ($P < 0.0001$; Figure 4.6). For this measure, D-HF was 43% and 36% lower than CON and CHF, respectively (both $P < 0.0001$; Figure 4.6).
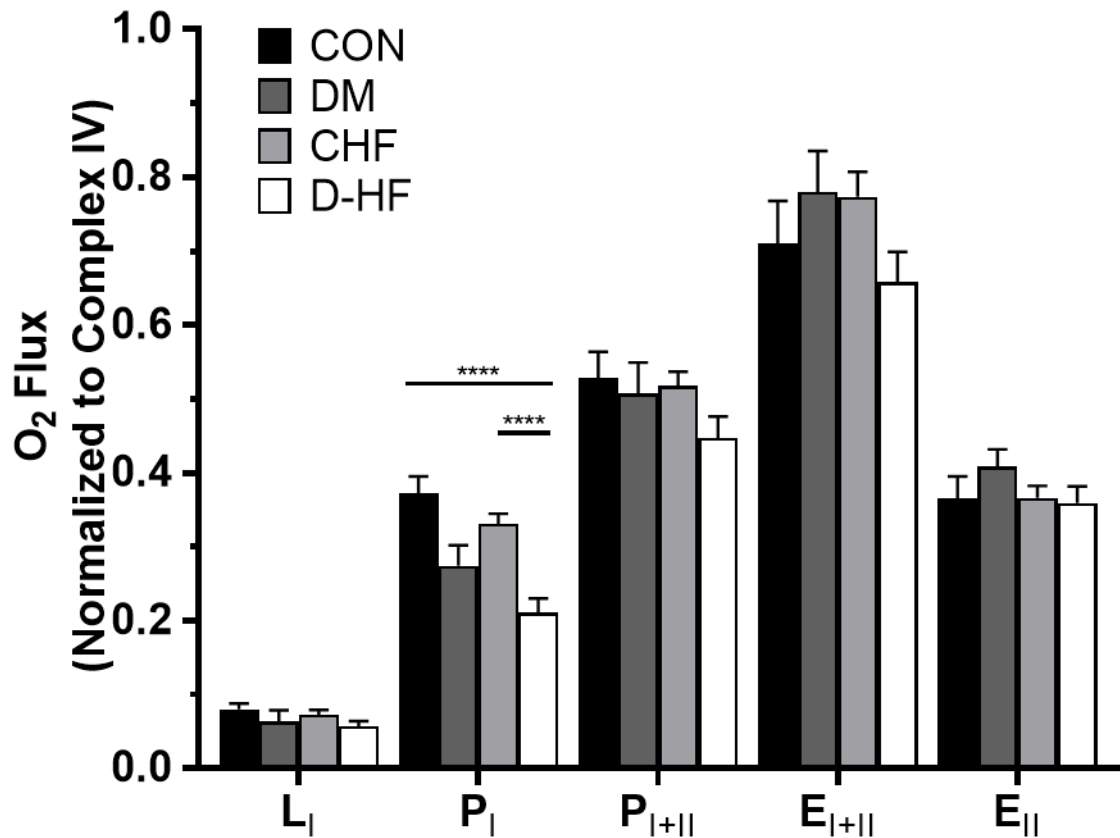
Figure 4.6. Impaired complex I function in D-HF patients. After correcting for mitochondrial content, only complex I OXPHOS remained significantly different between groups. The data displayed above are given as mean ± SEM. ****$P <$ 0.0001 using Tukey's multiple comparisons test following a significant ($P < 0.05$) one-way ANOVA.

4.3.9 Correlation between mitochondrial function and $\dot{V}O_{2peak}$

Exploratory analysis revealed that only O_2 flux with complex I OXPHOS substrates correlated with $\dot{V}O_{2peak}$ (Figure 4.7).
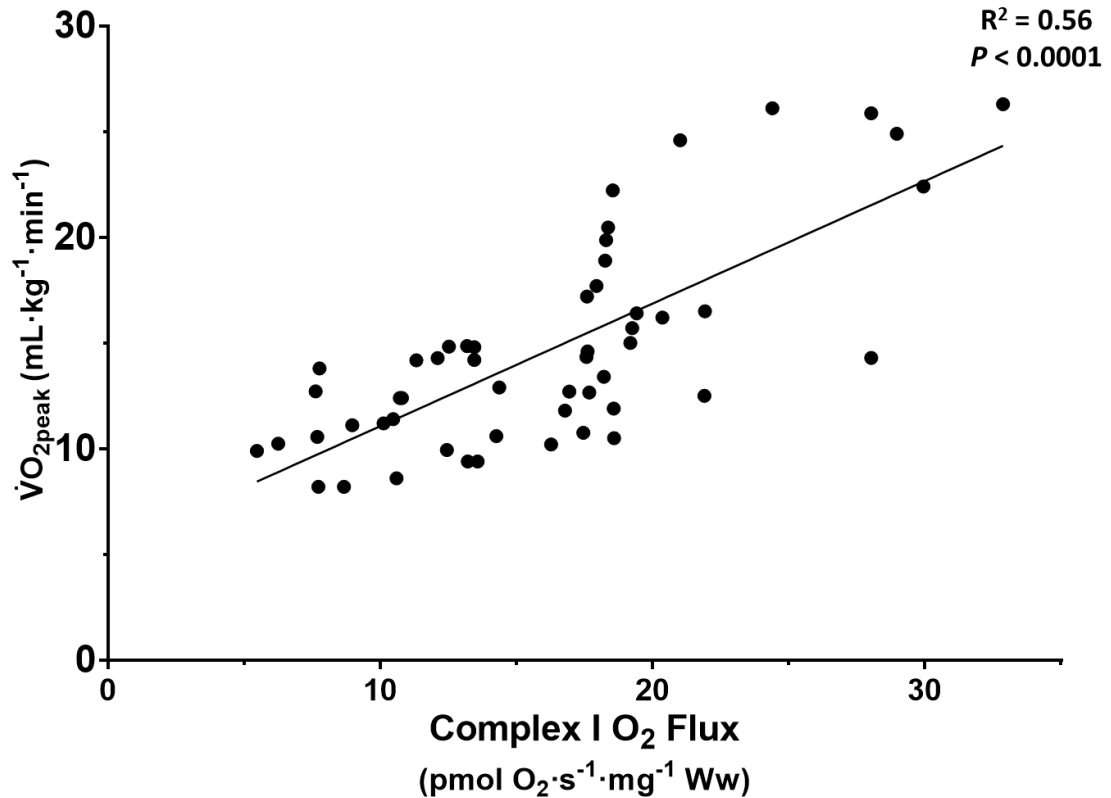
Figure 4.7. Complex I O_2 flux correlates with $\dot{V}O_{2peak}$. Oxygen flux with substrates for complex I OXPHOS significantly correlated with $\dot{V}O_{2peak}$ across all patients.

4.4 Discussion

The present study aimed to explore whether D-HF patients demonstrate greater deficits to mitochondrial function and content than CHF, which would help provide a novel mechanism for why this patient cohort present with exacerbated exercise intolerance despite similar cardiac function. The major finding from this study is that D-HF patients demonstrate more severe reductions in skeletal muscle mitochondrial respiration compared to either CHF or DM patients, with the greatest impact residing at complex I. Mitochondrial complex I dysfunction induced by D-HF was present even after normalizing for mitochondrial content, suggesting D-HF induces both qualitative as well as quantitative impairments –

a finding that was not observed in CHF and DM patients. Furthermore mitochondrial coupling, as assessed by the RCR, was further impaired in D-HF compared to CHF and DM, which implicates a limitation linking respiration and phosphorylation. Functional complex I impairments were closely correlated to $\dot{V}O_{2peak}$, which provides strong support of a new mechanism that mediates exercise intolerance in patients with D-HF. Collectively, therefore, this study indicates that patients with D-HF are characterised by severe skeletal muscle mitochondrial dysfunction, which resides at complex I, and is closely correlated to exercise intolerance.

4.4.1 Effects of CHF and DM on mitochondrial function

Traditionally, CHF has been associated with skeletal muscle mitochondrial dysfunction, which is closely associated with exercise intolerance (Drexler *et al.*, 1992; Massie *et al.*, 1996). However, most of these seminal early studies were in patients not on modern optimized medical therapies, such as beta-blockers, while mitochondrial alterations were inferred from standard measures such as assaying mitochondrial content (e.g. citrate synthase and succinate dehydrogenase enzyme activity levels) or isolating mitochondria for respiration. Both of these approaches, therefore, have limited our ability to draw inferences on the true effect of mitochondrial dysfunction in contemporary patients. Perhaps not surprisingly therefore, more recent studies on CHF patients with optimized drug therapy have found no significant differences in terms of *in situ* mitochondrial respiration when compared to controls matched for physical activity levels (Mettauer *et al.*, 2001; Williams *et al.*, 2004; Toth *et al.*, 2012; Middlekauff *et al.*, 2013). Similarly, many other mitochondrial measures in CHF

patients, with exception to a reduction in citrate synthase activity, have shown no differences for oxidative capacity and key mitochondrial enzymes, including complex I, of the *vastus lateralis* muscle between CHF patients and sedentary controls (Garnier *et al.*, 2005).

The current study used high resolution respirometry as assessed in permeabilized fibres and in a large cohort, to provide more rigorous insights into mitochondrial respiration across various complexes in CHF. The current study found that mitochondrial O_2 flux is lower in D-HF patients and to a certain extent in CHF and DM patients independently. However, we also observed that mitochondrial content was lower in D-HF patients. Once all the data were corrected for mitochondrial content the majority of differences were negated suggesting that quantitative mitochondrial differences play a key role. However, a novel finding was that D-HF patients still had lower complex I OXPHOS respiration compared to both control and CHF patients that persisted even after correcting for mitochondrial content. Any differences between previous studies and this current study may be explained by controls being matched for physical activity levels as the biopsy was from the *vastus lateralis*. In the current study we sampled from the *pectoralis major* muscle. This represents an alternative approach to investigating skeletal muscle in CHF, but may be beneficial since the upper limbs do not undergo such extensive non-specific detraining that frequently occurs in the locomotor muscles of the lower limbs as a result of a sedentary lifestyle.

In contrast to human studies in CHF patients, in which subjects have many confounding factors, several well-control animal studies have described specific skeletal muscle mitochondrial alterations as a result of CHF (De Sousa *et al.*, 2000; Garnier *et al.*, 2003; Zoll *et al.*, 2006). The hind limb skeletal muscles of rats with heart failure induced by aortic banding show that CHF induces a reduction in mitochondrial respiration and oxidative enzyme activity of ~40% (De Sousa *et al.*, 2000). Using a similar animal model, it was demonstrated that CHF decreased oxidative capacity and mitochondrial enzyme activities with concomitant reductions in the mRNA expression of mitochondrial proteins and transcription factors (Garnier *et al.*, 2003). Similarly, O_2 flux was reduced by ~30% in saponin-permeabilized fibres of the gastrocnemius muscle in rats 4 months after inducing a myocardial infarction via ligation of the left coronary artery (Zoll *et al.*, 2006). Specifically, they noted that these reductions were apparent with substrates for complex I. However, even though they observed comparable reductions in mitochondrial content measures, the authors failed to correct the O_2 flux measures to these and therefore failed to observe any potential qualitative impairments within complex I. Nevertheless there were no differences in the respiratory control ratio which may suggest that complex I was not specifically effected by this model. The discrepancies between these animal studies and the human studies may be explained by the large variability inherent with human studies coupled with the relatively small sample sizes studied.

Similar to CHF, studies in DM patients have shown skeletal muscle mitochondrial impairments when compared to age-matched healthy controls, including lower complex I respiration and citrate synthase activity (Kelley *et al.*, 2002; Boushel *et al.*, 2007; Mogensen *et al.*, 2007). However, these authors failed to normalise

complex I activity to mitochondrial content. Some studies have shown that complex I respiration is decreased in the skeletal muscle of DM patients although in this case, the authors concluded that DM patients have normal mitochondrial function since no differences between patients and control subjects remained after normalising the data to both citrate synthase activity and mitochondrial DNA, which are both markers for mitochondrial content (Boushel *et al.*, 2007). Nevertheless, others have highlighted evidence of skeletal muscle mitochondrial dysfunction in terms of function per mitochondrial mass. Specifically, one study demonstrated that O_2 flux in isolated mitochondria from the *vastus lateralis* muscle was lower in 10 obese DM patients compared to an age- and BMI-matched group of 8 non-DM controls (Mogensen *et al.*, 2007). Interestingly, though, this study showed that respiration was decreased in the presence of the complex I substrates malate and pyruvate, even after normalizing to citrate synthase activity as a marker of mitochondrial content. Collectively, these studies as well as the current study all describe mitochondrial impairments specific to complex I. However, discrepancies exist between the studies as to whether there are qualitative impairments within complex I after correcting for mitochondrial content. The current study shows that normalising to mitochondrial content negated the differences in complex I O_2 flux between the control and DM groups. This result suggests that quantitative differences probably play a bigger role than qualitative changes with regards to DM. Therefore, whilst DM alone appears to exert a small effect on mitochondrial function, the majority of evidence (and including ours here) illustrates that DM exerts its greatest impact on mitochondrial content.

4.4.2 Maladaptations of mitochondria in D-HF patients

While many studies have investigated mitochondrial function in either CHF or DM patients, the combined impact of D-HF remains largely unknown. This may have important implications, as D-HF patients have poorer outcomes compared to either CHF or DM alone, in terms of exercise intolerance, quality of life, and mortality (Guazzi *et al.*, 2002; Tibb *et al.*, 2005; Ingle *et al.*, 2006; MacDonald *et al.*, 2008a; Cubbon *et al.*, 2013). The few studies investigating D-HF have generally shown that patients present with several different mitochondrial perturbations, including lower mitochondrial protein expression, fewer mitochondrial cristae (Taub *et al.*, 2012), and lower mitochondrial antioxidant defences (Ramirez-Sanchez *et al.*, 2013). However these studies have lacked control, DM, and CHF groups to make observational comparisons.

In the present study one major key finding was that D-HF patients demonstrated the most severe impairments in mitochondrial respiration when compared to both the CHF and DM patient cohorts, which was specific to complex I. This suggests that D-HF patients have a reduced mitochondrial capacity per unit muscle mass, which would be predicted to contribute to a reduced $\dot{V}O_{2peak}$. Importantly, this effect would become even greater if D-HF patients demonstrate muscle atrophy, meaning $\dot{V}O_{2peak}$ would be lowered by both the loss of mass and the mitochondrial content in the remaining mass. This reduction in mitochondrial respiration per unit muscle mass is probably explained in part by a lower mitochondrial content, which was lowest in D-HF. The current method of measuring complex IV activity has the advantage of being easily incorporated in to respirometry studies and has been previously validated against the aforementioned established methods of assessing mitochondrial content

(Larsen *et al.*, 2012a) and our close correlation between these measures supports its utility. This lower mitochondrial content in D-HF is mirrored by that seen in previous studies of CHF and DM, which have both demonstrated lower mitochondrial content (Drexler *et al.*, 1992; Kelley *et al.*, 2002; Morino *et al.*, 2005; Boushel *et al.*, 2007; Toth *et al.*, 2012; Guzman Mentesana *et al.*, 2014) compared to matched controls, as assessed by electron microscopy, mitochondrial DNA content, and citrate synthase activity.

A major finding of the current data is that even after normalizing for mitochondrial content, D-HF patients still demonstrated substantial impairments to complex I function – a trend that was not observed in either the CHF or DM cohorts when compared to controls. This has important implications for our understanding of mitochondrial dysfunction in D-HF, as it suggests that while quantitative reductions underpin the lower respiration in CHF or DM, it seems that qualitative impairments are also playing a key role in D-HF. In particular, our data indicate a complex I impairment that is specific to only D-HF patients, which may offer a novel therapeutic target in this patient cohort. In addition, the qualitative measure of RCR also showed an impairment in D-HF patients that was not present in either CHF or DM alone. The RCR, which quantifies the capacity for complex I-supported OXPHOS, suggests that D-HF impairs the ability of the mitochondria to increase respiration at complex I following the addition of ADP. This finding is supported by comparable results in the FCR data, which showed that the complex I FCR was lower in all 3 patient groups compared to the control cohort. Furthermore, D-HF was significantly lower than CHF, thereby suggesting that DM and CHF may independently impact upon the coupling efficiency of complex I, which is therefore exacerbated in D-HF patients.

4.4.3 Complex I and exercise intolerance

Another observation of the present study was that complex I O_2 flux strongly correlated with exercise capacity, measured as $\dot{V}O_{2peak}$. Therefore complex I dysfunction may provide a mechanism by which mitochondrial O_2 uptake is impaired, which would then subsequently limit $\dot{V}O_{2peak}$ as defined by the muscle O_2 extraction component of the Fick equation. Thus, complex I may be considered a potential therapeutic target by which the exacerbated exercise intolerance seen in D-HF patients may be ameliorated. This study clearly indicates that D-HF induces specific impairment to mitochondrial function that likely underlies, in part, the severe reduction in exercise intolerance. While this current study did not explore the mechanisms that may induce mitochondrial dysfunction in D-HF patients, it may be related to a combination of mechanisms suggested to play a role in CHF and/or DM, such as fibre-type shifts, insulin resistance, chronic hyperglycaemia, elevated inflammatory cytokines, increased ROS, substrate overload, and accumulation of intracellular lipids.

4.4.4 Limitations

One of the drawbacks of high-resolution respirometry is that mitochondrial O_2 consumption is measured in an artificial hyperoxygenated environment that is not reflective of the *in vivo* tissue O_2 concentration, which is dictated by several factors, including tissue capillarity and the diffusion gradient of oxygen across the cell membrane. Nevertheless, given that all experiments were conducted under the same conditions, the finding that O_2 flux is reduced with complex I substrates is still novel and of clinical relevance. Another disadvantage is that this method relies on using fresh human skeletal muscle samples, as frozen

samples result in a reduction in mitochondrial oxygen consumption likely as a result of the mitochondrial membranes being damaged in the freeze-thawing process. Consequently, this limited the number of samples that could have potentially been taken and analysed, particularly from DM patients.

Aside from these methodological limitations, the current SUIT protocol employed in this study did not directly assess the enzymatic activities of complexes III, IV and V, which is possible in homogenised muscle tissue. However, as O_2 accepts electrons at complex IV, the O_2 flux measured in these SUIT protocols do provide an indirect *ex vivo* measure of the capacity of complexes III and IV as well as the electron carriers, ubiquinone and cytochrome *c*, to support electron flow to complex IV. It may even be argued that these *ex vivo* measures are more functionally relevant than isolated measures of individual complexes. Indeed, the observation that O_2 flux was only reduced in the presence of complex I substrates rather than with the complex II substrate succinate implies that D-HF patients have an impairment specifically within complex I and not complexes II, III or IV, respectively. An additional limitation is that this study did not directly measure ATP production or complex V activity, which would give a more accurate functional measurement of mitochondrial ATP resynthesis. Nevertheless, the reduction in O_2 flux with complex I substrates observed in the current study suggests that electron flow to complex IV is limited, which would decrease proton pumping across the inner mitochondrial membrane, thus reducing the mitochondrial membrane potential and the capacity for ATP resynthesis at complex V.

Additionally, the correlations between mitochondrial function and exercise capacity were determined from $\dot{V}O_{2peak}$ measurements taken from standard clinical cardiopulmonary ramp-incremental exercise tests, which, according to Results I, likely underestimated $\dot{V}O_{2peak}$. An intriguing hypothesis may be that the "responders" identified in Results I may be those individuals with a complex I impairment, which is overcome with the initial ramp-incremental component of the RISE-95 test thereby allowing them to achieve a higher $\dot{V}O_{2peak}$ during the step-exercise phase. Therefore future studies may wish to sample muscle biopsies from CHF and D-HF patients that also completed the RISE-95 protocol.

4.5 Conclusions

This study has shown for the first time that D-HF patients are characterized by skeletal muscle mitochondrial dysfunction and this is more severe when compared to CHF or DM patients. This dysfunction is the result of both qualitative and quantitative alterations at mitochondrial complex I, which was well correlated to exercise intolerance. As such, mitochondrial complex I specific dysfunction may represent a novel therapeutic target for treating exercise intolerance in patients with D-HF.

Chapter 5 Results III

Systemic skeletal muscle mitochondrial dysfunction in diabetic heart failure patients

5.1 Introduction

Patients with diabetic heart failure (D-HF) demonstrate more severe reductions in exercise intolerance compared to CHF without DM (Guazzi *et al.*, 2002; Tibb *et al.*, 2005; Ingle *et al.*, 2006), which cannot be simply explained by central factors such as lower left ventricular ejection fraction (MacDonald *et al.*, 2008a; Cubbon *et al.*, 2013). Peripheral skeletal muscle alterations are known to exacerbate symptoms of exercise intolerance in CHF and DM patients, due to mitochondrial abnormalities (Drexler *et al.*, 1992; Hambrecht *et al.*, 1995; Kelley *et al.*, 2002), an oxidative to glycolytic fibre type switch (Schaufelberger *et al.*, 1995; Mogensen *et al.*, 2007), and impaired microvasculature (Williams *et al.*, 2004; Groen *et al.*, 2014). As such, further understanding of the pathophysiology underlying these changes in D-HF patients may be particularly useful for developing potential therapeutic targets to reduce the symptoms of exercise intolerance.

Previous studies in CHF or DM patients have generally used thigh biopsies extracted from the *vastus lateralis* to investigate skeletal muscle (mitochondrial) alterations (Drexler *et al.*, 1992; Mettauer *et al.*, 2001; Kelley *et al.*, 2002; Williams *et al.*, 2004; Garnier *et al.*, 2005; Morino *et al.*, 2005; Ritov *et al.*, 2005; Boushel *et al.*, 2007; Mogensen *et al.*, 2007; Toth *et al.*, 2012). While it is generally agreed mitochondrial content is lower in both CHF and DM patients

when measured in the *vastus lateralis* (Drexler *et al.*, 1992; Mettauer *et al.*, 2001; Kelley *et al.*, 2002; Williams *et al.*, 2004; Garnier *et al.*, 2005; Morino *et al.*, 2005; Ritov *et al.*, 2005; Boushel *et al.*, 2007; Mogensen *et al.*, 2007), there is more controversy with regards to functional measures (normalized per muscle mass or mitochondria) with some (Ritov *et al.*, 2005; Mogensen *et al.*, 2007) but not all reporting impairments (Mettauer *et al.*, 2001; Garnier *et al.*, 2005; Boushel *et al.*, 2007). One explanation may be that the leg muscle is known to be considerably susceptible to detraining in disease (Olsen *et al.*, 2005; Reynolds *et al.*, 2007; Rehn *et al.*, 2012). In contrast to the lower limbs, however, the upper limbs may represent an alternative sampling site less impacted by detraining (i.e. due to constant recruitment during activities of daily living). Despite this, Results II confirmed that the *pectoralis major* demonstrates greater reductions in mitochondrial function in patients with D-HF when compared to CHF or DM, which was due to both quantitative and qualitative impairments residing at complex I. Nevertheless, the *pectoralis major* has not been historically studied and our findings are difficult to generalize to the current literature and require further validation. We argued that if the proportion of mitochondrial dysfunction induced by D-HF is similar between the upper and lower limb extremities, then it would: 1) validate the *pectoralis major* as an alternative research site for investigating muscle alterations; and 2) suggest a systemic whole-body pathology is acting to limit mitochondrial energetics.

This study, therefore, aimed to determine the impact of D-HF on mitochondrial function in the *vastus lateralis* muscle, and subsequently compare the degree of mitochondrial dysfunction demonstrated between upper (chest) and lower (leg) limb muscle groups. We hypothesized that mitochondrial alterations in the *vastus*

lateralis would be apparent, and of a similar proportion to that of the *pectoralis major*, which would indicate a systemic myopathy is present in D-HF, thus validating the *pectoralis major* as a viable alternative sampling site for assessing muscle alterations in disease.

5.2 Methods

5.2.1 Patients

Fifty eligible and consecutive patients undergoing routine pacemaker therapy at Leeds General Infirmary volunteered to participate in the present study. All patients provided written informed consent prior to the study (Appendix D) after reading through the relevant participant information sheet (Appendix C), which included a detailed outline regarding the addition of the *vastus lateralis* biopsy. The study was approved by the Leeds West Research Ethics Committee (11/YH/0291) and the Leeds Teaching Hospitals R+D committee (CD11/10015) and all procedures were conducted in accordance with the Declaration of Helsinki (Appendices A and B). Patients were grouped into four cohorts based on the criteria for CHF and DM outlined in 2.1 in the General Methods chapter. All patients consenting to the thigh biopsy were advised to stop taking any anti-coagulation medicine for 3 days prior to the procedure. Patients with known bleeding disorders were excluded from the study. One CHF patient was excluded from the study after consenting and performing an incremental cycle ergometer exercise test prior to the procedure that failed to show the presence of exercise intolerance ($\dot{V}O_{2peak}$ = 25.87 mL·kg^{-1}·min^{-1}). As only a $\dot{V}O_{2peak}$ < 20 mL·kg^{-1}·min^{-1} is associated with a poor prognosis in CHF (Mancini *et al.*, 1991; Myers *et al.*, 2002), this patient was considered to be unrepresentative of the exercise intolerant CHF population that this study was primarily interested in.

5.2.2 Muscle sampling and high-resolution respirometry

Immediately prior to the pacemaker procedure, percutaneous skeletal muscle biopsies from the *vastus lateralis* muscle were obtained from patients according to the experimental methods outlined in 2.5.2. Following the thigh muscle biopsy procedure, skeletal muscle biopsies from *pectoralis major* were obtained from all patients during their pacemaker operation as described in 2.5.1. Both samples (~20mg) were immediately placed in to separate labelled Eppendorf tubes each containing 1 mL of ice-cold BIOPS (Table 2.1) (Fontana-Ayoub *et al.*, 2014) and transported back to the laboratory on ice to be used for high-resolution respirometry analysis on the same day. The remaining muscle tissue from both samples was processed for histochemistry (~30 mg) or frozen in liquid nitrogen (~50 mg) and stored at -80 °C for later analyses. Following the procedure, patients received detailed verbal and written advice on how to look after the thigh biopsy site and were contacted several days later to ensure they had not experienced any adverse effects as a result of the procedure. The majority of patients reported that they had no discomfort with only 3 patients reporting minor discomfort the following day. Only one patient had an adverse response, which was remedied by stitching the incision site the following day to stop bleeding.

High-resolution respirometry was performed on 46 of the 49 patients that provided *vastus lateralis* samples. Three samples were omitted from the analysis as these muscle biopsy specimens did not contain sufficient mitochondria to perform respirometry analysis. Of these samples, one appeared to be predominantly subcutaneous adipose tissue and another was mostly blood with

little visible skeletal muscle tissue. These were only detected after inspecting the samples under a light microscope and could not have been noticed when performing the biopsy procedure. The third sample had become necrotic between taking the sample and transporting back to the laboratory and did not respond to any of the subsequent substrate titrations included in the protocol so the experiment was terminated.

All samples were transported back to the laboratory, typically within a few hours of sampling, and simultaneously underwent the mechanical and chemical permeabilization steps detailed in 2.6.2. *Vastus lateralis* and *pectoralis major* samples from the same patient were then measured in duplicate simultaneously on two separate Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria) machines. Both samples were then subjected to the SUIT protocol detailed in 2.6.4 as well as the complex IV activity assay given in 2.6.5. In all 46 samples, the recorded measures from each duplicate simultaneous experiment were averaged after correcting for ROX and the RCR and FCRs were calculated according to the formulae detailed in 2.6.6. The raw O_2 flux results for each respiratory state were also corrected to complex IV activity, as a marker of mitochondrial content.

5.2.3 Statistical analyses

Data were analysed using the Statistical Package for the Social Sciences (SPSS for Windows Version 25.0; IBM Corporation, Armonk, NY) after confirming the assumptions met for parametric testing and removing outliers as detailed in 2.11 of the General Methods chapter. Separate one-way (one-factor) 1 × 4 ANOVA

tests with appropriate post hoc tests were used to compare demographical variables, blood measurements, pharmacotherapy dosages and respirometry measurements between the four patient cohorts. Unpaired Student's t-tests were also employed when comparing just two cohorts. Categorical variables were compared using the two-sided Pearson's χ^2 test. Pearson correlations were included to examine the relationships between the O_2 flux measurements from the two muscle samples and to also explore the association between these measures and $\dot{V}O_{2peak}$. Categorical variables are presented as a percentage (%) of the cohort and the number (n). Continuous variables are expressed as mean ± SEM unless otherwise stated. Statistical significance was accepted as $P <$ 0.05.

5.3 Results

5.3.1 Demographic and clinical variables

Table 5.1 illustrates the demographic, physical and clinical characteristics for the 46 patients on whom respirometry was performed. The four cohorts were well matched for age, weight and sex.

Table 5.1. Demographic, physical and clinical characteristics of patients that provided *vastus lateralis* muscle biopsies grouped by their respective cohorts. Data are expressed as mean ± SEM unless otherwise stated. Significant differences ($P < 0.05$) are shown in bold.

	CON	DM	CHF	D-HF
n (%)	11 (24)	5 (11)	18 (39)	12 (26)
Male sex [% (n)]	90.9 (10)	80.0 (4)	88.9 (16)	100 (12)
Age (years)	74.0 ± 1.4	77.0 ± 3.9	68.8 ± 3.3	67.6 ± 2.9
Weight (kg)	84.4 ± 3.3	94.1 ± 15.0	85.0 ± 5.1	88.7 ± 4.0
$\dot{V}O_{2peak}$ (mL·kg^{-1}·min^{-1})			16.1 ± 1.3	13.6 ± 0.9
Clinical Factors				
NYHA functional class [% (n)]				
I			16.7 (3)	0 (0)
II			61.1 (11)	58.3 (7)
III			22.2 (4)	41.7 (5)
Ischaemic aetiology [% (n)]			55.6 (10)	66.7(8)
DCM aetiology [% (n)]			27.8 (5)	33.3 (4)
AF [% (n)]	27.3 (3)	0 (0)	33.3 (6)	41.7 (5)
CABG [% (n)]	0 (0)	0 (0)	22.2 (4)	16.7 (2)
Hypertension [% (n)]	27.3 (3)	80.0 (4)	38.9 (7)	41.7 (5)
LVEF (%)		> 50	23.4 ± 1.9	27.7 ± 2.2
LVIDd (mm)			62.2 ± 1.5	59.1 ± 2.3
Haemoglobin (g·L^{-1})	144.1 ± 3.8	135.0 ± 5.2	141.7 ± 3.7	134.0 ± 5.5
Sodium (mmol·L^{-1})	140.7 ± 0.8	139.0 ± 1.6	140.1 ± 0.8	137.5 ± 1.0
Potassium (mmol·L^{-1})	4.4 ± 0.2	4.7 ± 0.2	4.5 ± 0.1	4.7 ± 0.1
Creatinine (μmol·mL^{-1})	82.7 ± 5.8	82.6 ± 6.9	97.4 ± 11.4	116.5 ± 10.7
eGFR (mL·min^{-1}·1.73 m^{-2})	77.4 ± 4.4	75.4 ± 4.9	67.4 ± 4.8	58.4 ± 5.5
Plasma Glucose (mmol·L^{-1})		7.4 ± 1.1		10.2 ± 2.0
HbA1c (mmol·mol^{-1})		53.4 ± 7.5		63.8 ± 4.2

$\dot{V}O_{2peak}$, peak pulmonary oxygen uptake; NYHA, New York Heart Association; DCM, dilated cardiomyopathy; AF, atrial fibrillation; CABG, coronary artery bypass graft; LVEF, left ventricular ejection fraction; LVIDd, left ventricular internal diameter at diastole; eGFR, estimated glomerular filtration rate; HbA1c, glycated haemoglobin.

5.3.2 Drug and device therapy

The use of pharmacological treatments and device therapy by the 46 patients from each of the four cohorts are shown in Table 5.2. Equivalent daily dosages (mg) are given where appropriate. The two CHF cohorts received a higher frequency of angiotensin-converting enzyme inhibitors, beta-blockers, and loop diuretics treatment, while furosemide dose was highest in D-HF patients. Aspirin use was higher in the three patient cohorts compared to CON, while only D-HF patients received metformin and insulin treatment.

Table 5.2. Pharmacological treatments and device therapy. Data are expressed as mean ± SEM unless otherwise stated. Significant differences ($P < 0.05$) are shown in bold.

	CON	DM	CHF	D-HF
Pharmacological treatments				
ACEi use [% (n)]	9.1 (1)	60.0 (3)	77.8 (14)**†	75.0 (9)**†
Ramipril equivalent dose (mg)	10.0	7.5 ± 2.5	7.1 ± 1.2	8.9 ± 0.7
Beta-blocker use [% (n)]	0 (0)	40.0 (2)	100.0 (18)**†	100.0 (12)**†
Bisoprolol equivalent dose		2.8 ± 0.3	5.8 ± 0.9	7.6 ± 1.0
Loop diuretic use [% (n)]	0 (0)	20.0 (1)	66.7 (12)**†	75.0 (9)**††
Furosemide equivalent dose		20	47 ± 5†	113 ± 54††
ARB use [% (n)]				16.7 (2)
Candesartan equivalent dose				12.0 ± 4.0
Aldosterone antagonist use [%			55.6 (10)	83.3 (10)
Aldosterone antagonist dose			23.8 ± 1.3	23.8 ± 4.0
Statin use [% (n)]	72.7 (8)	100.0 (5)	55.6 (10)	75.0 (9)
Statin dose (mg)	35.0 ± 8.0	44.0 ± 9.9	52.7 ± 7.1	52.2 ± 9.4
Aspirin use [% (n)]	9.1 (1)	40.0 (2)*	44.4 (8)*	33.3 (4)*
Metformin use [% (n)]				75.0 (9)
Insulin use [% (n)]				16.7 (2)
Device therapy				
PPM [% (n)]	100.0 (11)	100.0 (5)	5.6 (1)	0 (0)
ICD [% (n)]	0 (0)	0 (0)	33.3 (6)	16.7 (2)
CRT [% (n)]			61.1 (11)	96.4 (10)

ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; PPM, permanent pacemaker; ICD, implantable cardioverter defibrillator; CRT, cardiac resynchronisation therapy. *$P < 0.05$ vs. CON; **$P < 0.01$ vs. CON; †$P < 0.05$ vs. DM; ††$P < 0.01$ vs. DM.

5.3.3 High-resolution respirometry representative traces from leg and chest samples
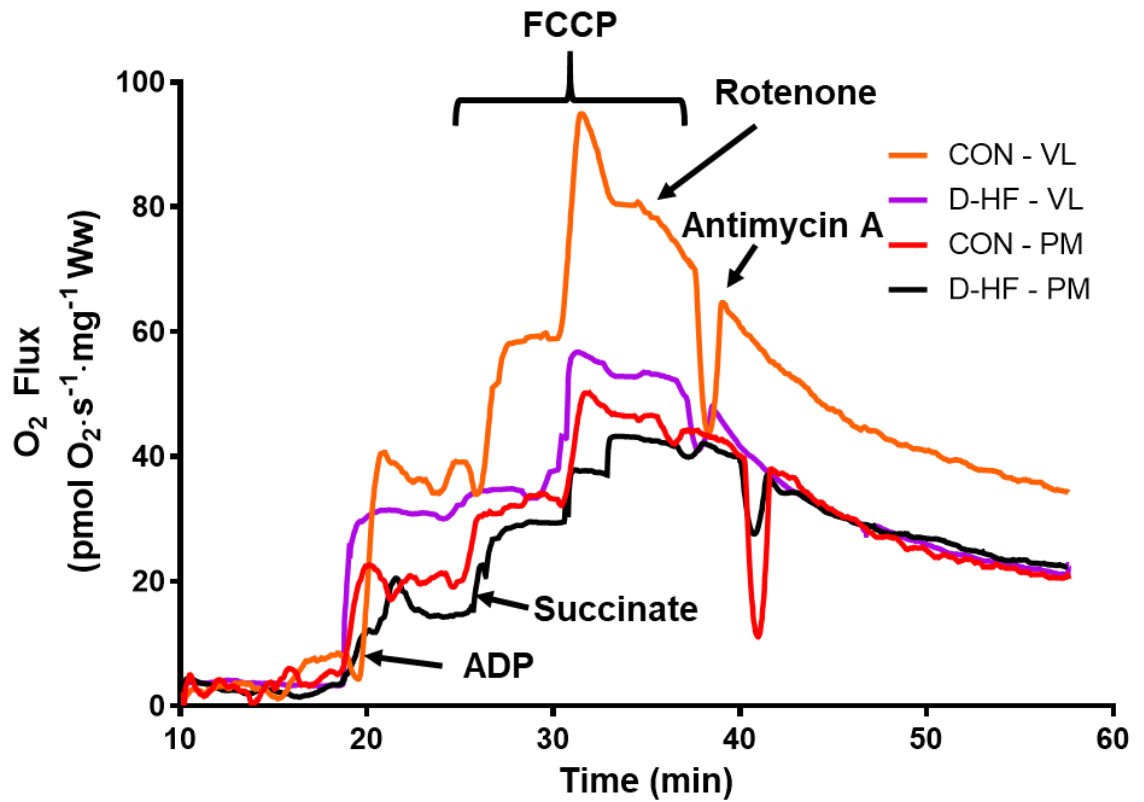
Figure 5.1. Representative O_2 flux traces of *vastus lateralis* (VL) and *pectoralis major* (PM) samples. Arrows indicate where individual titrations were made throughout the protocol.

5.3.4 Effects of D-HF on mitochondrial oxygen flux in leg muscle

Figure 5.2 illustrates the group mean O_2 flux results for the *vastus lateralis* samples. Complex I Leak respiration (L_I) was 54% lower in the D-HF cohort compared to CON ($P = 0.003$) and tended to be 43% lower compared to CHF ($P = 0.053$; Figure 5.2). Complex I OXPHOS was 52% ($P < 0.001$) and 40% ($P = 0.010$) lower in D-HF compared to CON and CHF, respectively, with a similar trend also observed when compared to DM ($P = 0.054$; Figure 5.2). Complex I+II OXPHOS was 39% lower in D-HF compared to CON ($P = 0.016$; Figure 5.2). Compared to CON, complex IV activity (as an index of mitochondrial content) was 30% and 36% lower in CHF ($P = 0.040$), and D-HF ($P = 0.020$) patients, respectively (Figure 5.2). There was also a trend for DM patients to be 33% lower than CON for complex IV activity ($P = 0.139$).
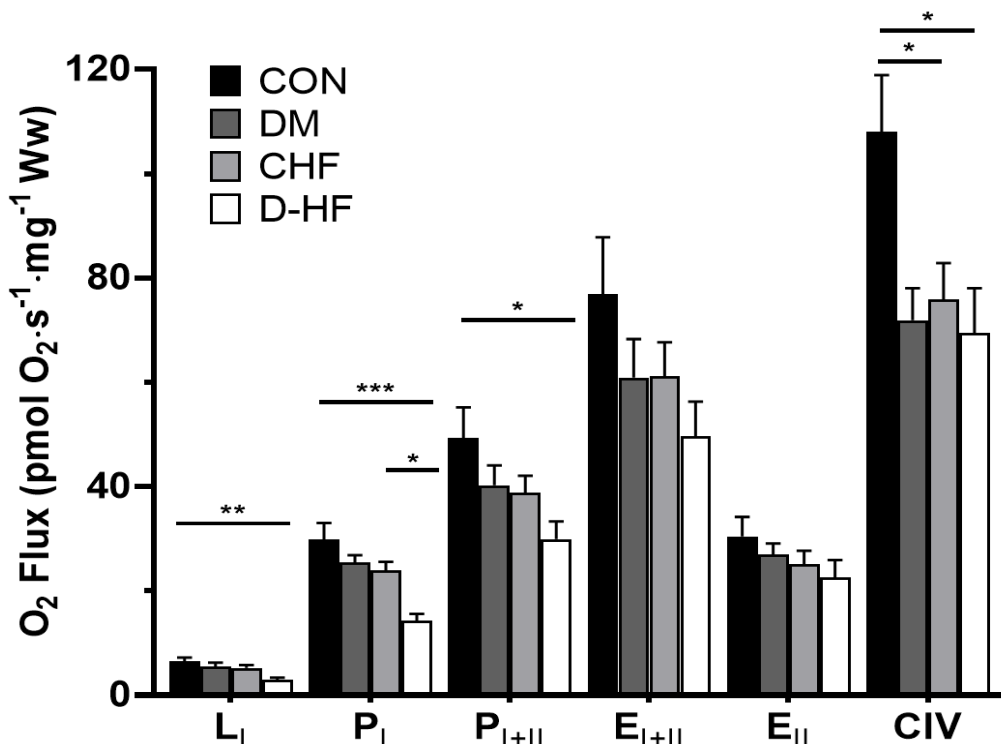
Figure 5.2. D-HF patients have lower mitochondrial respiration in *vastus lateralis* muscle samples. The data displayed above are given as mean ± SEM.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ using Tukey's multiple comparisons post-hoc tests following significant one-way ANOVAs.

Figure 5.3 displays the O_2 flux data after normalizing to complex IV activity as a marker of mitochondrial content. Although there was a main effect for complex I Leak ($P = 0.045$), pair-wise comparisons revealed no between group differences. Complex I OXPHOS was different between groups ($P = 0.005$), with D-HF being 35% and 30% lower compared to DM ($P = 0.009$) and CHF ($P = 0.025$), respectively (Figure 5.3).
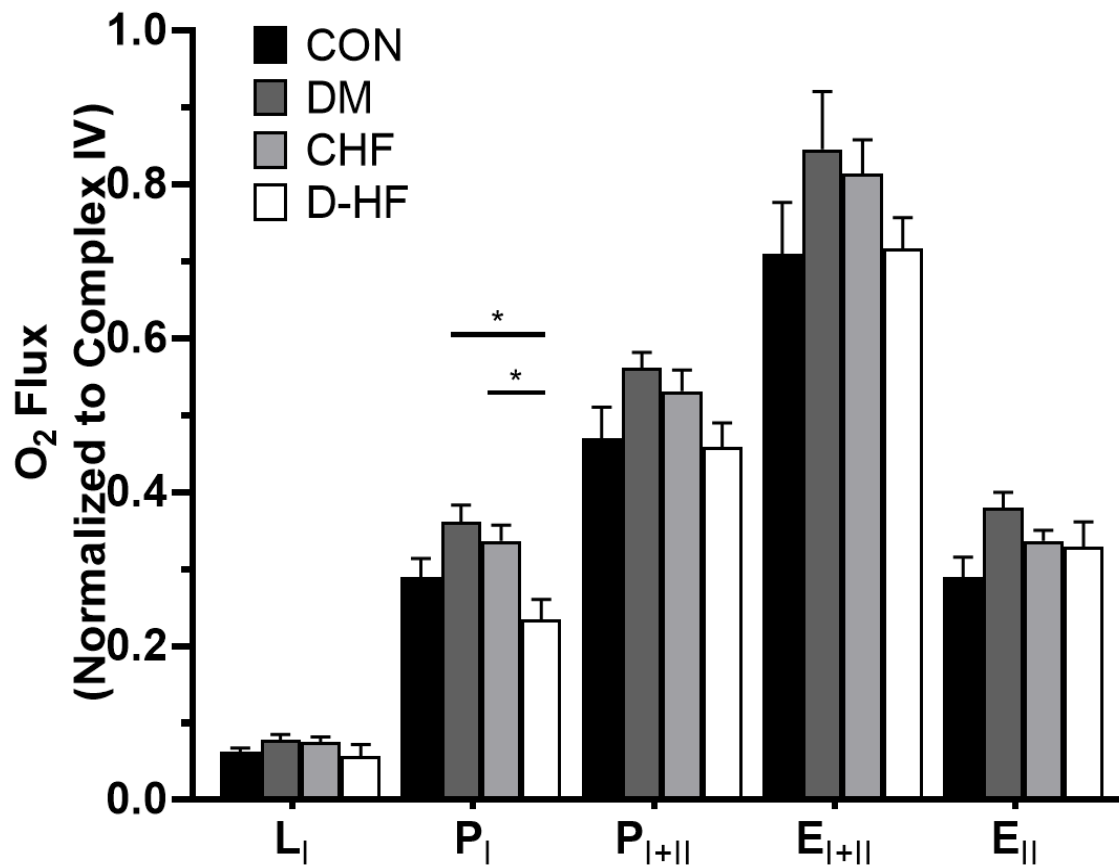
Figure 5.3. Impaired complex I function in leg muscle of D-HF patients. After correcting for mitochondrial content, only complex I OXPHOS remained significantly different between groups. The data displayed above are given as mean ± SEM. *$P < 0.05$ using Tukey's multiple comparisons test following a significant ($P < 0.05$) one-way ANOVA.

The RCR (a measure of respiratory coupling capacity) was not different between groups (P = 0.837; Figure 5.4A). Similarly, the flux control ratios did not differ between groups across all respiratory states (all $P > 0.05$; Figure 5.4B).
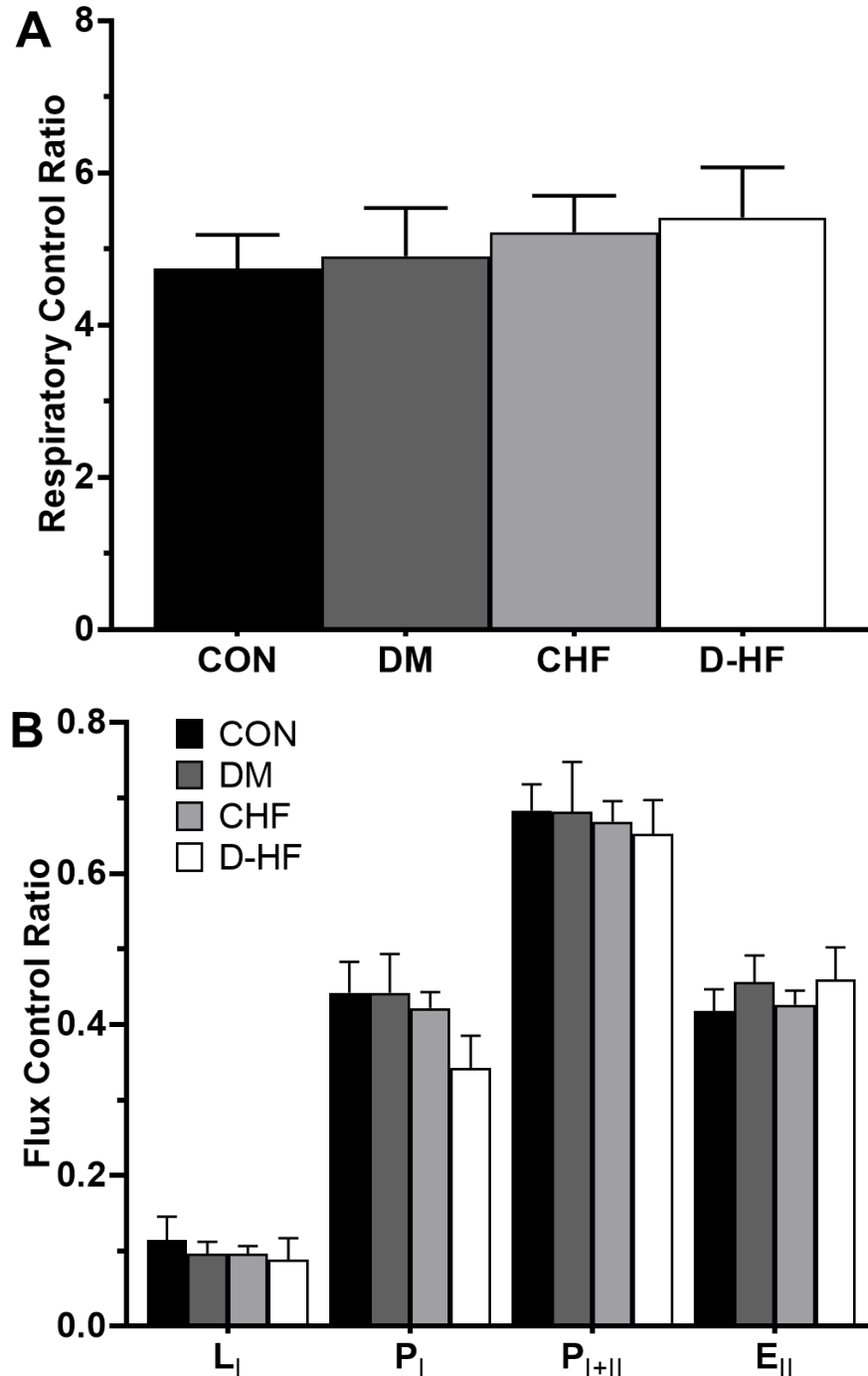
Figure 5.4. Respiratory control ratios and flux control ratios for leg muscle samples. Respiratory control ratios for *vastus lateralis* samples did not differ significantly between cohorts (A). The flux control ratios did not significantly differ between cohorts for all respiratory states (B). The data displayed above are given as mean ± SEM.

5.3.5 Correlation between mitochondrial function and exercise intolerance

To investigate the relationship between exercise intolerance and mitochondrial function, $\dot{V}O_{2peak}$ measures from an incremental cycle ergometer exercise test were compared with O_2 flux measures from the two muscle biopsy sites (Figure 5.5). There were correlations between $\dot{V}O_{2peak}$ with both complex I OXPHOS and complex IV (as a marker of mitochondrial content) O_2 fluxes in both the *vastus lateralis* and *pectoralis major* muscle biopsies (all $P < 0.05$; R^2: 0.29-0.61; Figure 5.5).
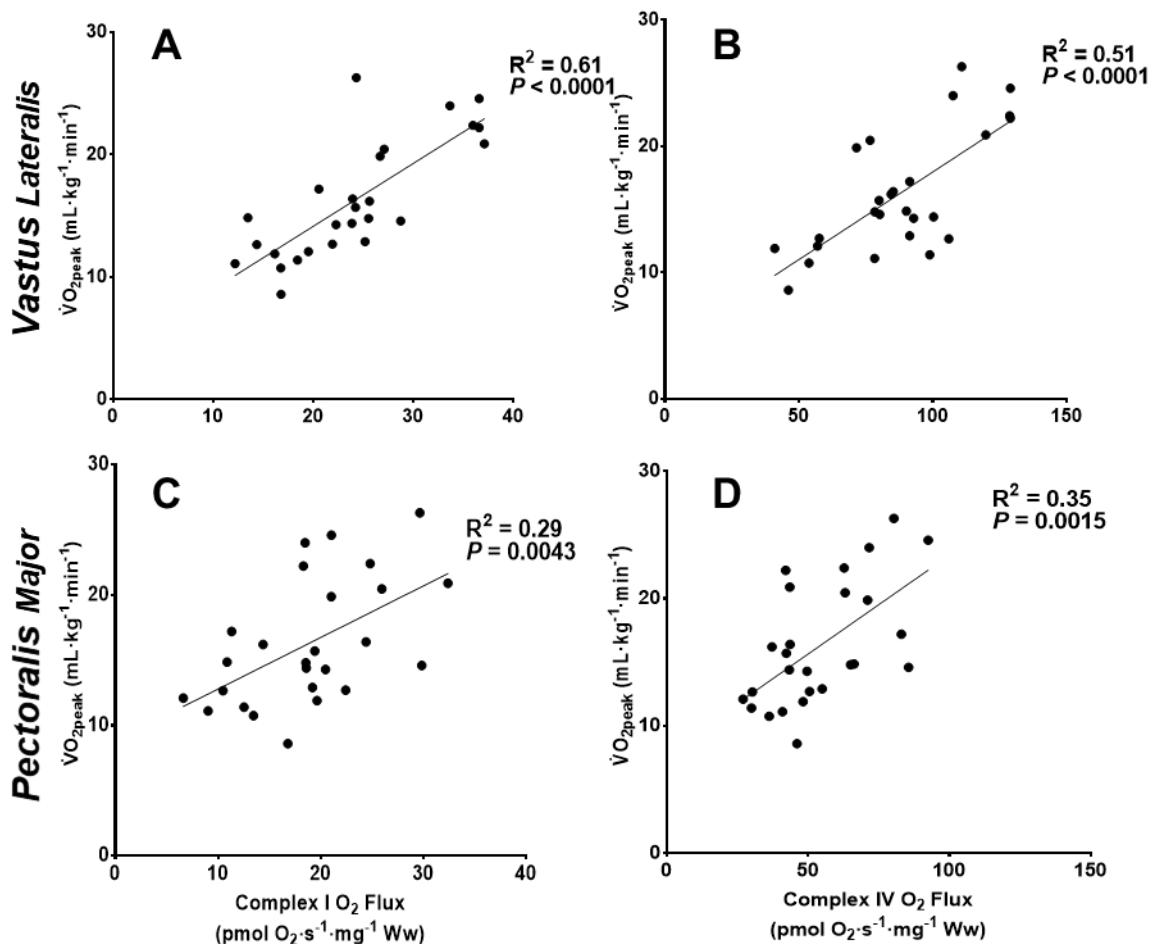
Figure 5.5. Muscle mitochondrial function correlates with $\dot{V}O_{2peak}$. Complex I and complex IV O_2 flux measurements were both significantly correlated with $\dot{V}O_{2peak}$ in both the *vastus lateralis* (A & B) and *pectoralis major* muscle samples (C & D).

5.3.6 Comparison of mitochondrial oxygen flux between leg and chest muscles

Figure 5.6 displays the O_2 flux data, both in absolute and relative terms, as well as the respiratory control ratio and flux control ratios for the *pectoralis major* samples taken from the 46 patients that were included in the *vastus lateralis* analysis above. These data showed comparable results to the *pectoralis major* data obtained from the larger cohort of participants that were included in Results II. There were between group differences for the O_2 flux measurements (Figure 5.6A). Firstly, complex I OXPHOS was 26% and 54% lower in CHF ($P = 0.030$) and D-HF ($P < 0.0001$) compared to CON, respectively (Figure 5.6A). Furthermore D-HF was also 47% and 38% lower compared to both DM ($P = 0.008$) and CHF ($P = 0.013$) for complex I OXPHOS (Figure 5.6A). Similarly, both CHF and D-HF were 25% ($P = 0.049$) and 47% ($P = 0.0002$) lower than CON for complex I+II OXPHOS (Figure 5.6A). D-HF were also 40% ($P = 0.030$) lower than DM for this measure with a similar trend seen when compared to CHF ($P = 0.080$; Figure 5.6A). D-HF were also 37% ($P = 0.030$) lower than CON for complex II-supported ETS capacity (Figure 5.6A). The RCR tended to be ~35% lower in D-HF compared to the 3 other groups ($P = 0.203$; Figure 5.6B). After normalizing the data to complex IV O_2 flux, as a marker of mitochondrial content, only D-HF remained significantly lower than the other cohorts (Figure 5.6C). Specifically, for complex I OXPHOS, D-HF were 29% ($P = 0.008$), 36% ($P = 0.003$), and 28% ($P = 0.004$) lower than CON, DM, and CHF, respectively (Figure 5.6C). Additionally, for complex I+II OXPHOS, D-HF were 19% ($P = 0.046$) and 27% ($P = 0.007$) lower than CON and DM, with a similar trend observed when compared to CHF ($P = 0.068$; Figure 5.6C). There were similar findings when the data were expressed as flux control ratios (Figure 5.6D). For complex I

OXPHOS, D-HF were 34% ($P = 0.0003$), 31% ($P = 0.012$), and 30% ($P = 0.001$) lower than CON, DM, and CHF, respectively (Figure 5.6D). Additionally, D-HF were 24% ($P = 0.001$) lower than CON for complex I+II OXPHOS (Figure 5.6D).
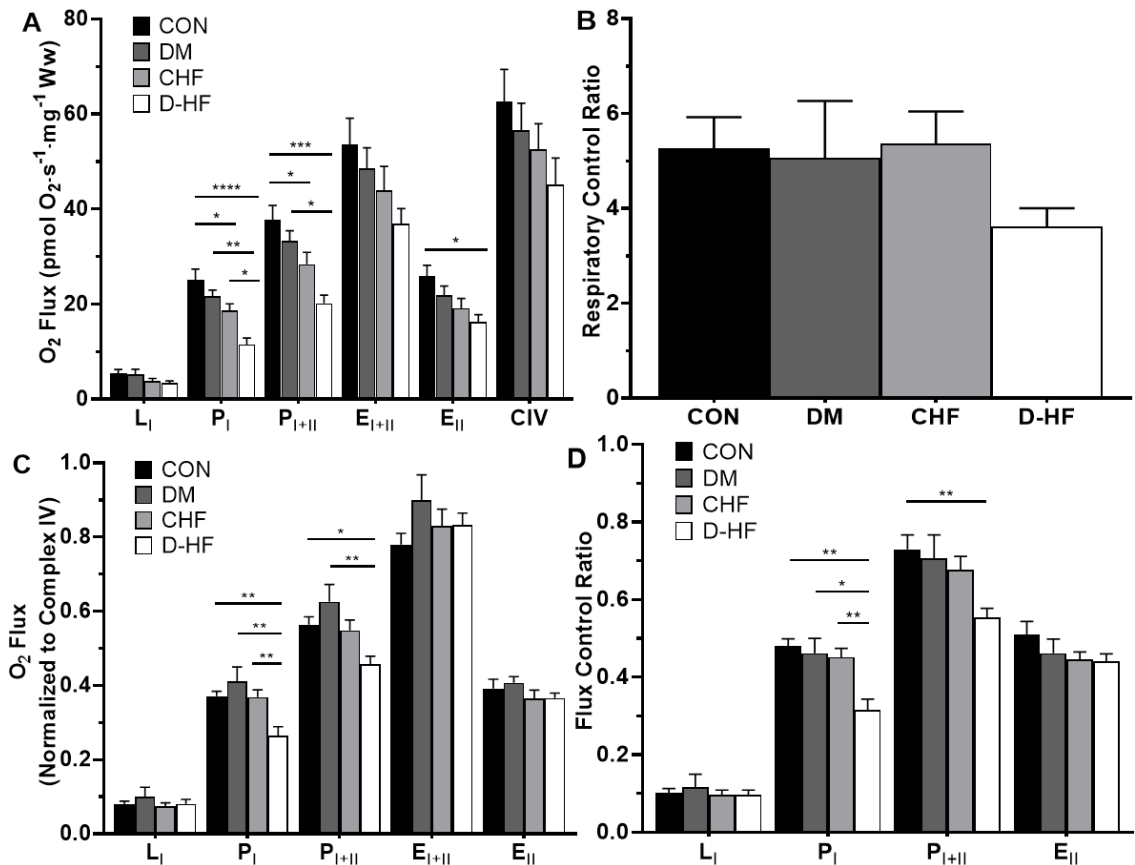
Figure 5.6. D-HF patients that provided *vastus lateralis* samples also exhibit impaired mitochondrial function in *pectoralis major* thereby confirming the systemic effect of this condition. D-HF patients have lower skeletal muscle mitochondrial respiration in *pectoralis major* (A). There was a trend for the respiratory control ratio to be lower in D-HF compared to the other cohorts in *pectoralis major* samples (B). After correcting for mitochondrial content, complex I OXPHOS and complex I+II OXPHOS remained significantly lower in D-HF patients in *pectoralis major* (C). The flux control ratios for both complex I OXPHOS and complex I+II OXPHOS were also lower in *pectoralis major* samples from D-HF patients (D). The data displayed above are given as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ using Tukey's multiple comparisons post-hoc tests following significant one-way ANOVAs.

To investigate the mitochondrial responses between leg and chest muscle, data collected from these two sampling sites were compared across the different respiratory states (Figure 5.7). Absolute values of mitochondrial O_2 flux were well correlated between *vastus lateralis* and *pectoralis major* for all respiratory states

as well as complex IV activity (all $P < 0.01$; R^2: 0.17-0.49; Figure 5.7). Interestingly, once normalized to complex IV activity, only complex I Leak and OXPHOS correlated across the two muscles (both $P < 0.05$; R^2: 0.10-0.17; Figures 5.8A and 5.8B). Similarly, when data were expressed as flux control ratios, only complex I Leak and OXPHOS correlated between the two muscles (both $P < 0.05$; R^2: 0.15-0.23; Figures 5.8C and 5.8D). There was also a trend ($P = 0.056$) for a correlation between the two muscles when expressed as the RCR (Data not shown).
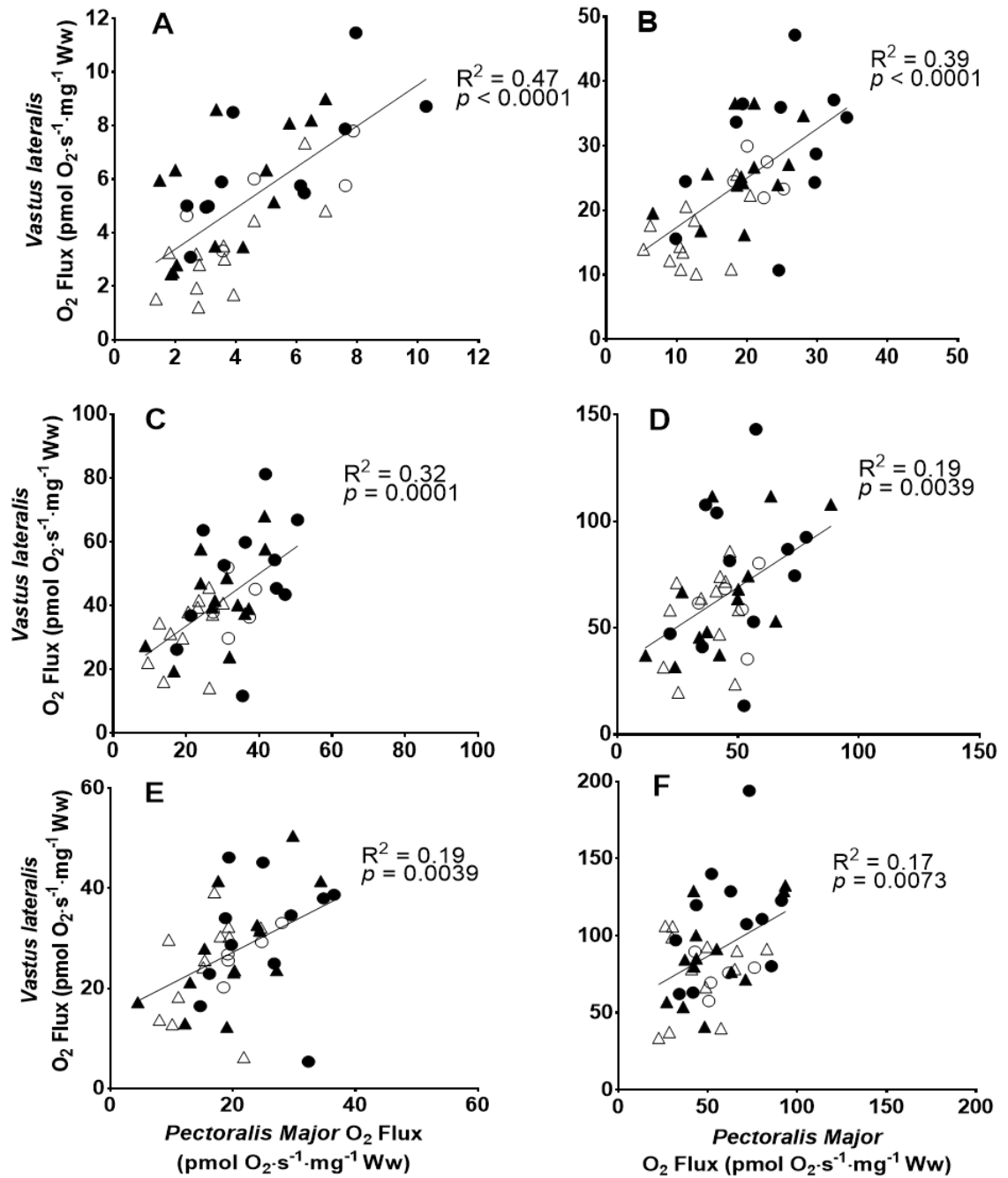
Figure 5.7. Mitochondrial oxygen flux is well correlated between leg and chest samples for complex I Leak (A), complex I OXPHOS (B), complex I+II OXPHOS (C), complex I+II ETS (D), complex II ETS (E), and complex IV activity (F). The Pearson R^2 value and P value obtained for each measure are displayed on the relevant panel. Filled circles are CON (n = 11), open circles are DM (n = 5), filled triangles are CHF (n = 15), and open triangles are D-HF (n = 10).
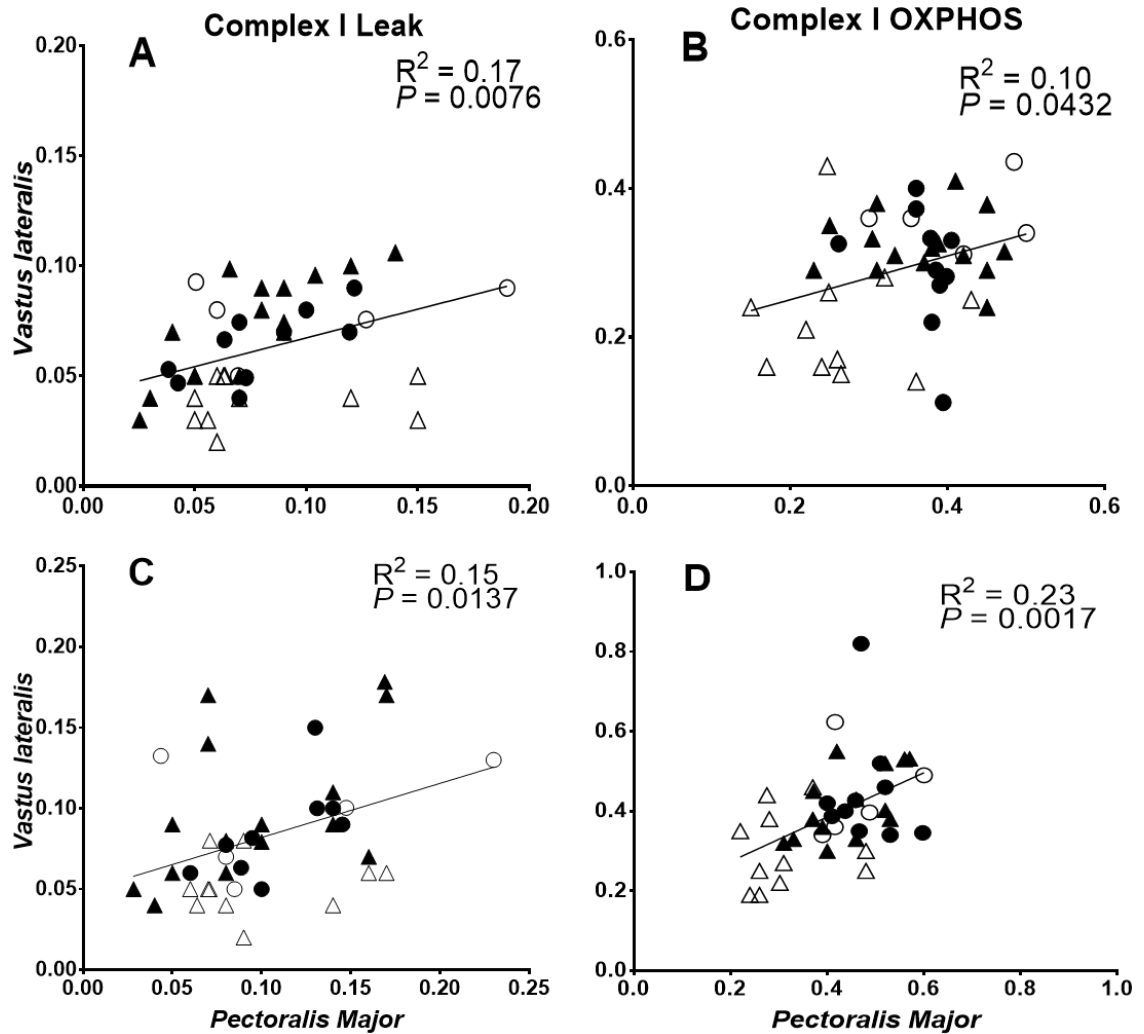
Figure 5.8. Complex I function is well correlated between leg and chest samples. After correcting for complex IV O_2 flux, as a marker of mitochondrial content, complex I Leak (A) and complex I OXPHOS (B) remained significantly correlated between the two muscle samples. Similarly, when expressed as a flux control ratio, complex I Leak (C) and complex I OXPHOS (D) remained significantly correlated. The Pearson R^2 value and P value obtained for each measure are displayed on the relevant panel. Filled circles are CON (n = 11), open circles are DM (n = 5), filled triangles are CHF (n = 15), and open triangles are D-HF (n = 10).

5.3.7 Mitochondrial function between leg and chest muscle

To further characterise the relationship between *vastus lateralis* and *pectoralis major* O_2 flux, we pooled data from all participants for each muscle group in order to provide global comparisons across respiratory states independent of disease state (Figure 5.9). In general, *vastus lateralis* tissue had higher absolute O_2 flux compared to the *pectoralis major* samples across all respiratory states ranging

from 16% for complex I OXPHOS to 36% for complex IV activity (Figure 5.9A). However, after normalizing the data to complex IV O_2 flux as a marker of mitochondrial content, the chest samples were higher than the leg muscle: 24% for complex I Leak ($P = 0.006$), 15% for complex I OXPHOS ($P = 0.003$), and 13% for complex II ETS ($P = 0.024$; Figure 5.9B). The RCR did not differ between the two muscle samples (Figure 5.9C; $P = 0.138$), while complex I Leak for FCR was 17 % higher in *pectoralis major* compared to *vastus lateralis* ($P = 0.006$; Figure 5.9D). Therefore, this suggests that while mitochondrial capacity is higher per muscle mass in the leg, mitochondrial O_2 flux normalised per mitochondrial content is higher in the chest muscle.
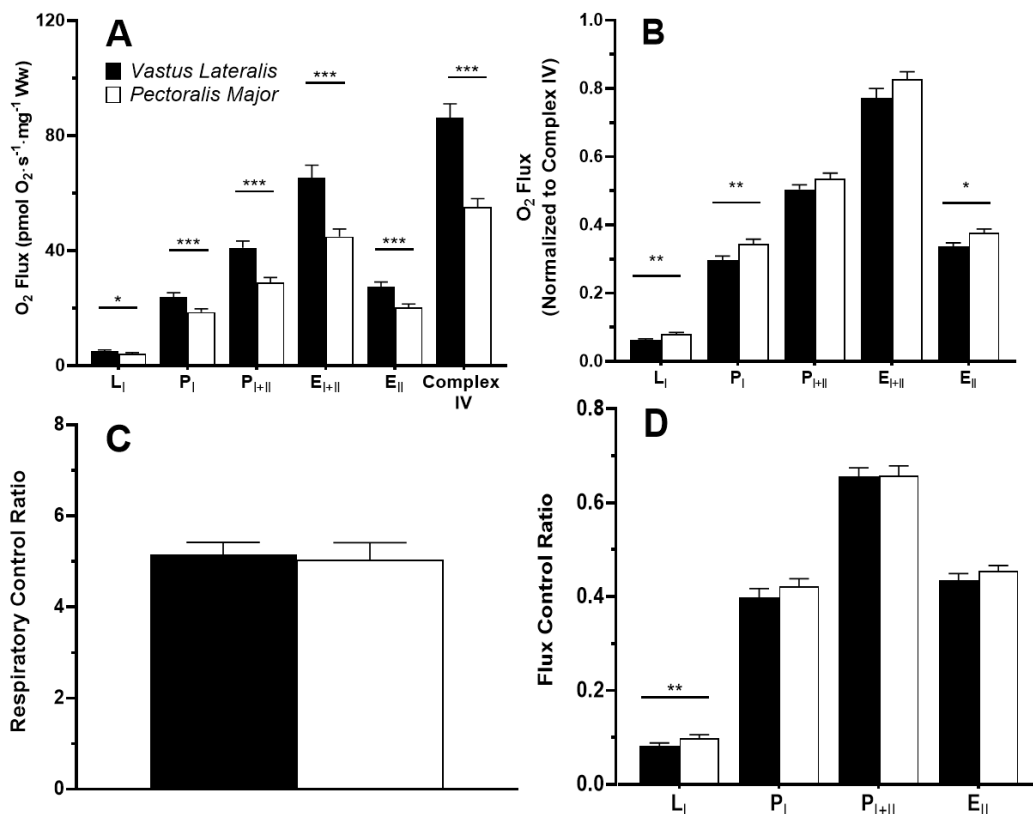
Figure 5.9. Comparison of mitochondrial function between chest and leg samples. Oxygen flux per milligram of muscle tissue is consistently higher in *vastus lateralis* samples compared to *pectoralis major* (A). This trend is reversed after normalizing the data to complex IV activity (B). The respiratory control ratio did not differ significantly between the two tissue samples (C). Only complex I Leak respiration was significantly higher in the *pectoralis major* muscle when expressed as a flux control ratio (D).

5.4 Discussion

The novel finding of the present study is that patients with D-HF demonstrate impairments to absolute mitochondrial respiration per unit muscle mass in the leg when compared to CHF, DM, and control patients. In particular, mitochondrial deficits at complex I remained in the *vastus lateralis* even after being normalized to mitochondrial content, which supports both qualitative and quantitative limitations to mitochondrial function. As mitochondrial impairments observed in D-HF were of a similar degree and well correlated to those reported from the chest muscle (Results II), a systemic mechanism may be acting to induce a mitochondrial myopathy to limit whole-body exercise tolerance. Overall, these data provide strong validation that the *pectoralis major* may be used as an alternative sampling site for investigating mitochondrial dysfunction in patients.

5.4.1 Effects of D-HF on mitochondrial function in leg muscle

Previous studies have examined biopsies from the *vastus lateralis* muscle in patients with both CHF (Drexler *et al.*, 1992; Mettauer *et al.*, 2001; Williams *et al.*, 2004; Garnier *et al.*, 2005; Brassard *et al.*, 2006; Toth *et al.*, 2012; Middlekauff *et al.*, 2013) and DM (Kelley *et al.*, 2002; Ritov *et al.*, 2005; Boushel *et al.*, 2007; Mogensen *et al.*, 2007; Phielix *et al.*, 2008; Rabøl *et al.*, 2010; Ritov *et al.*, 2010; Larsen *et al.*, 2011). However, no previous studies have characterized *in situ* mitochondrial function in leg muscle from D-HF patients as compared to age-matched CHF or DM controls. While previous studies have taken thigh muscle biopsies from D-HF patients (Taub *et al.*, 2012; Ramirez-Sanchez *et al.*, 2013), these pilot studies assessed only 5 patients and lacked relevant control groups of DM or CHF patients. Furthermore they did not

measure mitochondrial function *in situ* using high-resolution respirometry so were unable to specifically identify dysfunction within the individual complexes.

Previous studies have failed to detect significant differences between CHF patients and sedentary age-matched controls when using respirometry methods to measure *in situ* mitochondrial O_2 flux of saponin-permeabilized muscle fibres from the *vastus lateralis* (Mettauer *et al.*, 2001; Garnier *et al.*, 2005). These findings are corroborated by studies demonstrating no significant differences in mitochondrial ATP production rate and oxidative enzyme activity in leg muscle from CHF patients (Williams *et al.*, 2004; Toth *et al.*, 2012; Middlekauff *et al.*, 2013). Whilst these findings are in agreement with the present investigation, we did observe a trend for mitochondrial O_2 flux to be 17-30% lower in in all of the respiratory states measured between CHF patients and controls (Figure 5.2). Potential explanations for any discrepancies may reside in the different methods employed (different substrate protocols employed) or the age of the CHF patients (i.e., mean age of CHF patients in the current study was 69 years while previous studies the age averaged 55 years (Mettauer *et al.*, 2001; Garnier *et al.*, 2005; Middlekauff *et al.*, 2013)). Thus, any reductions in mitochondrial function observed in CHF may take place over a prolonged period of time with concomitant effects of the ageing process on mitochondrial function (Johnson *et al.*, 2013). Interestingly, after normalizing for mitochondrial content such differences were no longer apparent, which would support other studies that have shown CHF lowers mitochondrial content by ~50 % even when compared to sedentary activity-matched controls (Mettauer *et al.*, 2001) or by seminal studies showing a 20% reduction in the surface and volume density of mitochondrial cristae (Drexler *et al.*, 1992). Overall, therefore, the present data

together with previous studies strongly suggest that a reduction in leg mitochondrial content is likely playing a leading role to impair mitochondrial bioenergetics, and thus exercise intolerance, in CHF patients.

Oxygen flux was not significantly lower in DM patients compared to controls across the respiratory states. Several previous studies have reported mitochondrial respiration per unit muscle mass in *vastus lateralis* biopsies from DM patients using the same or very similar methods to those used in the present study. Collectively, these studies have identified reductions in O_2 flux or enzyme activity in complex I, complex I+II, and uncoupled respiration (ETS) (Kelley *et al.*, 2002; Ritov *et al.*, 2005; Boushel *et al.*, 2007; Mogensen *et al.*, 2007; Phielix *et al.*, 2008; Rabøl *et al.*, 2009; Ritov *et al.*, 2010; Larsen *et al.*, 2011). Interestingly, however, in some (Boushel *et al.*, 2007; Rabøl *et al.*, 2009; Larsen *et al.*, 2011), but not all (Ritov *et al.*, 2005; Mogensen *et al.*, 2007; Phielix *et al.*, 2008; Ritov *et al.*, 2010), of these studies these differences were eradicated after adjusting the data to a marker of mitochondrial content, such as citrate synthase activity. Using the same substrate combinations as the current study, previous studies have reported a 24% and 17% reduction in complex I and complex I+II OXPHOS, respectively (Rabøl *et al.*, 2009). Similarly, others have reported differences of 18% and 27% for complex I Leak and complex I OXPHOS (Boushel *et al.*, 2007). These findings are not too dissimilar to the 16%, 15% and 18% differences observed in the present study between DM and control for complex I Leak, complex I OXPHOS and complex I+II OXPHOS, respectively. Similarly, these two studies also found that these differences were negated after normalising the data to citrate synthase activity (Boushel *et al.*, 2007; Rabøl *et al.*, 2009), which is also in agreement with the current study, thereby suggesting that lower

mitochondrial content is the primary driver for mitochondrial deficits in DM patients.

In relation to D-HF patients, the present study noted a significant 36% reduction in mitochondrial content compared to controls, as evidenced by lower complex IV activity, which was also lower in both the CHF and DM patients. Consistent with previous studies in CHF or DM, it seems D-HF induces a deficit in mitochondrial content. Importantly, however, the current study still found respiratory impairments were present in D-HF patients after normalizing the data to mitochondrial content. This was not apparent in either the CHF or DM patients alone. This would support the hypothesis that the D-HF phenotype *per se* induces qualitative as well as quantitative mitochondrial impairments, which seem to primarily reside at complex I. Collectively, therefore, these findings build upon the hypothesis we drew from the *pectoralis major* muscle in that CHF and DM independently lower mitochondrial content, but also that D-HF leads to a specific complex I dysfunction. Whether this complex I impairment provides a potential mechanism for the lower exercise capacity in D-HF remains unclear, but our correlative data support this hypothesis.

5.4.2 Mitochondrial function is comparable across upper and lower limbs

An additional aim of the current study was to examine if mitochondrial function yielded comparable results between *vastus lateralis* and *pectoralis major* muscles, in order to validate our novel chest sampling method. We found O_2 flux measurements across respiratory states were well correlated between both leg

and chest muscle samples (Figure 5.7), which provides initial evidence that *pectoralis major* sampling may be considered an alternative surrogate for investigating mitochondrial dysfunction in both health and disease.

This is the first study to demonstrate parallel upper and lower limb mitochondrial function in both CHF and DM populations. Previous studies have compared leg with shoulder biopsies in DM patients and age-matched obese and lean controls (Larsen *et al.*, 2009; Rabøl *et al.*, 2010). Similar to the current study, these two studies found that O_2 flux per milligram muscle tissue are consistently higher in the *vastus lateralis* muscle (Larsen *et al.*, 2009; Rabøl *et al.*, 2010), yet they differed after normalising data to citrate synthase activity. For example, one study found respiration per mitochondria content in the *musculus deltoideus* was higher than vastus lateralis (Rabøl *et al.*, 2010), which is similar to our data here where *pectoralis major* had higher respiratory function normalized for mitochondrial content. This finding suggests that intrinsic qualitative function for this muscle is higher than that seen in *vastus lateralis*, which, in contrast, is characterised by an increased mitochondrial content. Although studies have shown arm and leg muscle have divergent adaptive responses to exercise training (Turner *et al.*, 1997; Nordsborg *et al.*, 2015), the exact mechanisms behind this higher intrinsic function in *pectoralis major* remain to be fully elucidated. It is likely that the higher O_2 fluxes and mitochondrial content seen in *vastus lateralis* muscle are localised effects driven by the higher metabolic requirements placed upon this muscle in daily life, which would promote a shift in muscle phenotype to predominantly type I oxidative fibres and a higher mitochondrial content (Holloszy & Booth, 1976). While mitochondrial O_2 flux has yet to be investigated in two separate muscle tissues from CHF patients,

previous studies have shown that CHF patients present with comparable abnormalities across both upper and lower limbs, including a higher activity of the metaboreflex system during exercise (Scott *et al.*, 2002) and earlier decreases in muscle PCr and pH during submaximal exercise (Nagai *et al.*, 2004).

One major finding of the current study is that D-HF causes mitochondrial complex I dysfunction when normalized to either muscle mass or mitochondrial content in both leg and chest muscle – a finding that neither we nor others have reported in CHF or DM patients previously (Mettauer *et al.*, 2001; Garnier *et al.*, 2005; Boushel *et al.*, 2007). Given these effects were seen in both the lower and upper limbs in D-HF only, it is intriguing to speculate that a systemic mechanism(s) may be driving a potential mitochondrial myopathy. Interestingly, RCR values were comparable across all groups when assessed in leg muscle, which is in contrast to that observed in the chest muscle where D-HF patients showed greater deficits. While previous studies have reported no significant differences in the RCR in the *vastus lateralis* muscle between controls and DM (Boushel *et al.*, 2007; Larsen *et al.*, 2011) or CHF patients (Mettauer *et al.*, 2001), our findings suggest mitochondria in D-HF show greater impairments to respond to an influx of ADP in the upper compared to the lower limbs. While the underlying mechanisms causing this potential systemic mitochondrial myopathy in D-HF are yet to be determined, likely factors include chronic hyperglycaemia, insulin resistance, circulating inflammatory cytokines, and ROS. Importantly, as similar impairments were largely seen in D-HF between chest and leg mitochondrial function, it would imply a detraining effect is playing a limited role as this would be expected to impact the leg muscles to a greater extent.

5.4.3 Limitations

The current study did not investigate any differences in fibre type composition between *pectoralis major* and *vastus lateralis*. Therefore it is unknown whether the observed differences between these two muscles in terms of mitochondrial function may be attributed to differences in muscle fibre types e.g. a higher proportion of type I fibres may explain the higher mitochondrial O_2 flux observed in *vastus lateralis*. Moreover, it is also unknown how each of the clinical populations included in this study may differ in terms of fibre type composition within each of these muscles. Therefore future studies may wish to further explore fibre type composition within two different muscles.

5.5 Conclusions

This study demonstrated that skeletal muscle mitochondrial dysfunction in D-HF patients is not isolated to the *pectoralis major* by showing comparable defects in the *vastus lateralis*. This dysfunction appears to be, at least in part, due to a reduction in mitochondrial content, which has been extensively reported in CHF and DM populations alone. However, and similar to the *pectoralis major*, a qualitative defect to mitochondrial complex I function specific to D-HF patients was apparent even after adjusting for mitochondrial content. This study further demonstrated O_2 flux measurements measured from chest and leg muscle are well correlated, which provides validation for using the *pectoralis major* as an appropriate sampling site for investigating mitochondrial dysfunction in patients with chronic disease when more viable than invasive leg biopsies (e.g., during routine pacemaker surgery

Chapter 6 Results IV

Identification of the mechanisms contributing to skeletal muscle mitochondrial dysfunction in diabetic heart failure

6.1 Introduction

Diabetic heart failure (D-HF) patients are characterised by a systemic skeletal muscle mitochondrial dysfunction, with both quantitative and qualitative impairments being revealed in the last few chapters. Mitochondrial dysfunction may contribute, at least in part, to the poorer exercise tolerance seen in D-HF patients, as it would be expected to decrease the capacity of the muscle to extract and utilise O_2. Together with O_2 delivery, the latter is a major component of the Fick equation and thus a key determinant of peak pulmonary oxygen uptake ($\dot{V}O_{2peak}$). As such, a better understanding of the mechanisms contributing to mitochondrial dysfunction in D-HF patients may uncover novel therapeutic targets to treat symptoms of exercise intolerance, and thus improve prognosis.

Imbalance in cellular gene expression is implicated in many pathological conditions. In this regard, seminal studies have identified a lower expression in the genes encoding for mitochondrial OXPHOS proteins, including PPARγ coactivator-1α (PGC-1α) in DM and insulin-resistant skeletal muscle (Sreekumar *et al.*, 2002; Yang *et al.*, 2002; Mootha *et al.*, 2003; Patti *et al.*, 2003). Interestingly, similar results have been observed in animal models of CHF (Garnier *et al.*, 2003; Zoll *et al.*, 2006; Seiler *et al.*, 2016); however patient data

remains inconclusive (Garnier *et al.*, 2003; Toth *et al.*, 2012; Forman *et al.*, 2014). Additionally, studies in DM patients have shown that several genes encoding for the core subunits of complex I, such as NDUFS1, are downregulated in skeletal muscle (Wu *et al.*, 2017). One key mechanism modulating mitochondrial gene and protein expression is increased mitochondrial ROS production (Kowaltowski *et al.*, 2009). Animal models (Coirault *et al.*, 2007; Bowen *et al.*, 2015a; Martinez *et al.*, 2015; Mangner *et al.*, 2016) and patient studies (Linke *et al.*, 2005; Guzman Mentesana *et al.*, 2014) with CHF have shown enhanced skeletal muscle ROS production and oxidative stress. However, whether D-HF muscle generates elevated mitochondrial ROS remains unknown.

In addition, reductions in muscle mitochondrial function in D-HF may be linked to fiber atrophy and isoform shifts (Schiaffino & Reggiani, 2011), with several studies demonstrating these effects in CHF and DM patients. Specifically, these studies demonstrate that both CHF and DM induce a similar fibre type shift from type I oxidative to type II glycolytic fibres (Sullivan *et al.*, 1990; Schaufelberger *et al.*, 1995; Massie *et al.*, 1996; Schaufelberger *et al.*, 1997; Sullivan *et al.*, 1997; Oberbach *et al.*, 2006; Mogensen *et al.*, 2007). This fibre type shift is associated with atrophy, which is more apparent in type II fibres in CHF and DM (Li *et al.*, 2007; Yu *et al.*, 2008; Kemp *et al.*, 2009). Furthermore, impairment in O_2 delivery may also predispose to mitochondrial alterations and exercise intolerance. Several studies have shown that CHF and DM are both characterised by impairments in skeletal muscle capillarisation (Marin *et al.*, 1994; Schaufelberger *et al.*, 1995; Schaufelberger *et al.*, 1997; Duscha *et al.*, 1999; Nusz *et al.*, 2003; Williams *et al.*, 2004; Groen *et al.*, 2014); however the impact in D-HF patients remains unknown.

The current chapter, therefore, aimed to characterise in detail the molecular, cellular, and structural alterations that occur in D-HF skeletal muscle, in order to identify mechanisms that may be potentially underlying the severe mitochondrial dysfunction in these patients. We were particularly interested in determining whether differences existed between D-HF relative to both the DM and CHF patients in relation to measures of muscle gene expression, mitochondrial ROS production, fibre properties, and capillarity.

6.2 Methods

6.2.1 Patients

Seventy-seven eligible and consecutive patients undergoing routine pacemaker therapy at Leeds General Infirmary volunteered to participate in the present study. All patients provided written informed consent (Appendix D) prior to the study after reading through the relevant participant information sheet (Appendix C). The study was approved by the Leeds West Research Ethics Committee (11/YH/0291) and the Leeds Teaching Hospitals R+D committee (CD11/10015) and all procedures were conducted in accordance with the Declaration of Helsinki (Appendices A and B). Patients were grouped into four cohorts based on the criteria for CHF and DM outlined in 2.1 in the General Methods chapter.

6.2.2 *Pectoralis major* muscle sampling and tissue processing

Skeletal muscle biopsies from *pectoralis major* were obtained from all patients during their pacemaker operation as described in 2.5.1 of the General Methods

chapter. Immediately following the biopsy, one sample was immediately snap-frozen in liquid nitrogen and stored at -80 °C for gene expression analyses as detailed in 2.10. A second sample (~20mg) was placed in to a labelled 1.5 mL Eppendorf tube containing 1 mL of ice-cold BIOPS (Table 2.1) (Fontana-Ayoub *et al.*, 2014) to be used for simultaneous high-resolution respirometry with H_2O_2 production measurements as described in 2.8. A third sample was mounted on a pinned cork disc in optimum cutting temperature (Thermo Fisher Scientific, Waltham, MA) embedding medium (Figure 2.5). This sample was snap frozen in isopentane (2-Methylbutane; Sigma-Aldrich, St Louis, MO) cooled in liquid nitrogen and stored at -80 °C until later immunohistochemical analyses, which are outlined in 2.9.

6.2.3 Statistical analyses

Data were analysed using the Statistical Package for the Social Sciences (SPSS for Windows Version 25.0; IBM Corporation, Armonk, NY) after confirming the assumptions met for parametric testing and removing outliers as detailed in 2.11 of the General Methods chapter. Independent samples Student *t*-tests and separate one-way (one-factor) 1 × 4 ANOVA tests with Tukey's post hoc test were used to compare differences in continuous variables between the four patient cohorts. Categorical variables were compared using the two-sided Pearson's χ^2 test. Categorical variables are presented as a percentage (%) of the cohort and the number (n). Continuous variables are expressed as mean ± SEM unless otherwise stated. Statistical significance was accepted as $P < 0.05$.

6.3 Results

6.3.1 Demographic and clinical variables

Table 6.1 illustrates the demographic, physical and clinical characteristics for the 77 patients included in these analyses. The four cohorts were well matched for weight and sex with a trend for the D-HF cohort to be younger. In agreement with previous studies, D-HF had a lower $\dot{V}O_{2peak}$ compared to CHF. Despite this, D-HF had better cardiac function, as evidenced by a higher LVEF compared to CHF. In agreement with the previous chapters, DM and D-HF also had greater renal impairments, as indicated by a lower eGFR.

6.3.2 Drug and device therapy

The use of pharmacological treatments and device therapy by the 77 patients are shown in Table 6.2. Equivalent daily dosages (mg) are given where appropriate. The two CHF cohorts received a higher frequency of angiotensin-converting enzyme inhibitors, beta-blockers, and loop diuretics, with dosages for these tending to be higher in the D-HF patients.

Table 6.1. Demographic, physical, and clinical characteristics of patients grouped by their respective patient cohorts. Data are expressed as mean ± SEM unless otherwise stated. Significant differences ($P < 0.05$) are shown in bold.

	CON	DM	CHF	D-HF
n (%)	20 (26.0)	17 (22.0)	20 (26.0)	20 (26.0)
Male sex [% (n)]	75.0 (15)	88.2 (15)	90.0 (18)	90.0 (18)
Age (years)	74.2 ± 1.6	77.6 ± 1.6	72.4 ± 2.5	69.1 ± 2.3
Weight (kg)	85.5 ± 3.7	94.8 ± 6.4	82.0 ± 3.4	82.4 ± 3.4
$\dot{V}O_{2peak}$ (mL·kg^{-1}·min^{-1})			15.3 ± 1.4	12.1 ± 0.5‡
Clinical Factors				
NYHA functional class [% (n)]				
I			10.0 (2)	5.0 (1)
II			65.0 (13)	50.0 (10)
III			25.0 (5)	45.0 (9)
Ischaemic aetiology [% (n)]			60.0 (12)	65.0 (13)
DCM aetiology [% (n)]			30.0 (6)	35.0 (7)
AF [% (n)]	40.0 (8)	17.6 (3)	30.0 (6)	40.0 (8)
CABG [% (n)]	20.0 (4)	11.8 (2)	30.0 (6)	40.0 (8)
Hypertension [% (n)]	35.0 (7)	47.1 (8)	40.0 (8)	55.0 (11)
LVEF (%)		>50	22.6 ± 2.6	30.0 ± 2.2‡
LVIDd (mm)			59.8 ± 1.7	59.3 ± 1.8
Haemoglobin (g·L^{-1})	136.4 ± 4.6	132.9 ± 4.3	141.2 ± 3.0	135.4 ± 4.4
Sodium (mmol·L^{-1})	139.3 ± 0.9	137.0 ± 1.2	139.9 ± 0.8	132.7 ± 6.7
Potassium (mmol·L^{-1})	4.4 ± 0.1	4.7 ± 0.1	4.6 ± 0.1	4.6 ± 0.1
Creatinine (μmol·mL^{-1})	87.9 ± 4.1	104.3 ± 7.2	107.8 ± 10.4	114.4 ± 10.5
eGFR (mL·min^{-1}·1.73 m^{-2})	69.6 ± 3.6	57.2 ± 4.2*	61.8 ± 4.0	52.5 ± 4.1*
Plasma Glucose (mmol·L^{-1})		9.1 ± 1.3		8.8 ± 0.8
HbA1c (mmol·mol^{-1})		47.6 ± 4.8		62.6 ± 4.2

$\dot{V}O_{2peak}$, peak pulmonary oxygen uptake; NYHA, New York Heart Association; DCM, dilated cardiomyopathy; AF, atrial fibrillation; CABG, coronary artery bypass graft; LVEF, left ventricular ejection fraction; LVIDd, left ventricular internal diameter at diastole; eGFR, estimated glomerular filtration rate; HbA1c, glycated haemoglobin. *$P < 0.05$ vs. CON; ‡$P < 0.05$ vs. CHF.

Table 6.2. Pharmacological treatments and device therapy. Data are expressed as mean ± SEM unless otherwise stated. Significant differences ($P < 0.05$) are shown in bold.

	CON	DM	CHF	D-HF
Pharmacological treatments				
ACEi use [% (n)]	30.0 (6)	35.3 (6)	75.0 (15)**††	65.0 (13)**††
Ramipril equivalent dose (mg)	4.8 ± 1.2	8.8 ± 1.3	5.7 ± 0.8	7.7 ± 0.9
Beta-blocker use [% (n)]	25.0 (5)	41.2 (7)	95.0 (19)**††	85.0 (17)**†
Bisoprolol equivalent dose	3.8 ± 0.8	2.9 ± 0.6	5.2 ± 0.7	6.8 ± 0.9
Loop diuretic use [% (n)]	15.0 (3)	17.6 (3)	45.0 (9)*†	75.0 (15)**††
Furosemide equivalent dose	66.7 ± 13.3	33.3 ± 6.7	44.4 ± 6.5	82.9 ± 13.2
ARB use [% (n)]			10.0 (2)	15.0 (3)
Candesartan equivalent dose			6.0 ± 2.0	17.3 ± 8.1
Aldosterone antagonist use [%			45.0 (9)	60.0 (12)
Aldosterone antagonist dose			23.6 ± 1.4	31.8 ± 7.4
Statin use [% (n)]	65.0 (13)	(13)	75.0 (15)	65.0 (13)
Statin dose (mg)	35.0 ± 10.4	34.6 ± 5.9	39.2 ± 4.6	50.0 ± 7.7
Aspirin use [% (n)]	30.0 (6)	11.8 (2)	45.0 (9)	30.0 (6)
Metformin use [% (n)]		29.4 (5)		45.0 (9)
Insulin use [% (n)]		17.6 (3)		10.0 (2)
Device therapy				
PPM [% (n)]	95.0 (19)	94.1 (16)		
ICD [% (n)]	5.0 (1)	5.9 (1)	20.0 (4)	5.0 (1)
CRT [% (n)]			80. (16)	95.0 (19)

ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; PPM, permanent pacemaker; ICD, implantable cardioverter defibrillator; CRT, cardiac resynchronisation therapy. *$P < 0.05$ vs. CON; **$P < 0.01$ vs. CON; †$P < 0.05$ vs. DM; ††$P < 0.01$ vs. DM.

6.3.3 Gene expression

Figure 6.1 illustrates the relative (to the control cohort) mRNA expression of the 6 genes of interest included in this study. PGC-1α expression was 31%, 13% and 24% lower in DM, CHF, and D-HF compared to CON, respectively, but did not reach significance ($P > 0.05$). Compared to CON, SOD2 expression was not different in DM and CHF ($P > 0.05$); however, it was reduced by 42% in D-HF ($P = 0.036$; Figure 6.1B) and tended to be 36% lower compared to CHF ($P = 0.087$). NDUFS1 expression was lower by 55% ($P = 0.031$) and 49% ($P = 0.037$; Figure 6.1C) in D-HF compared to CON and CHF, respectively. NDUFS1 expression also tended to be 49% lower in D-HF compared to DM ($P = 0.072$). In contrast, no differences were found between groups for the gene expression of NDUFS3, FIS1or OPA1 (all $P > 0.05$; Figure 6.1E-F).
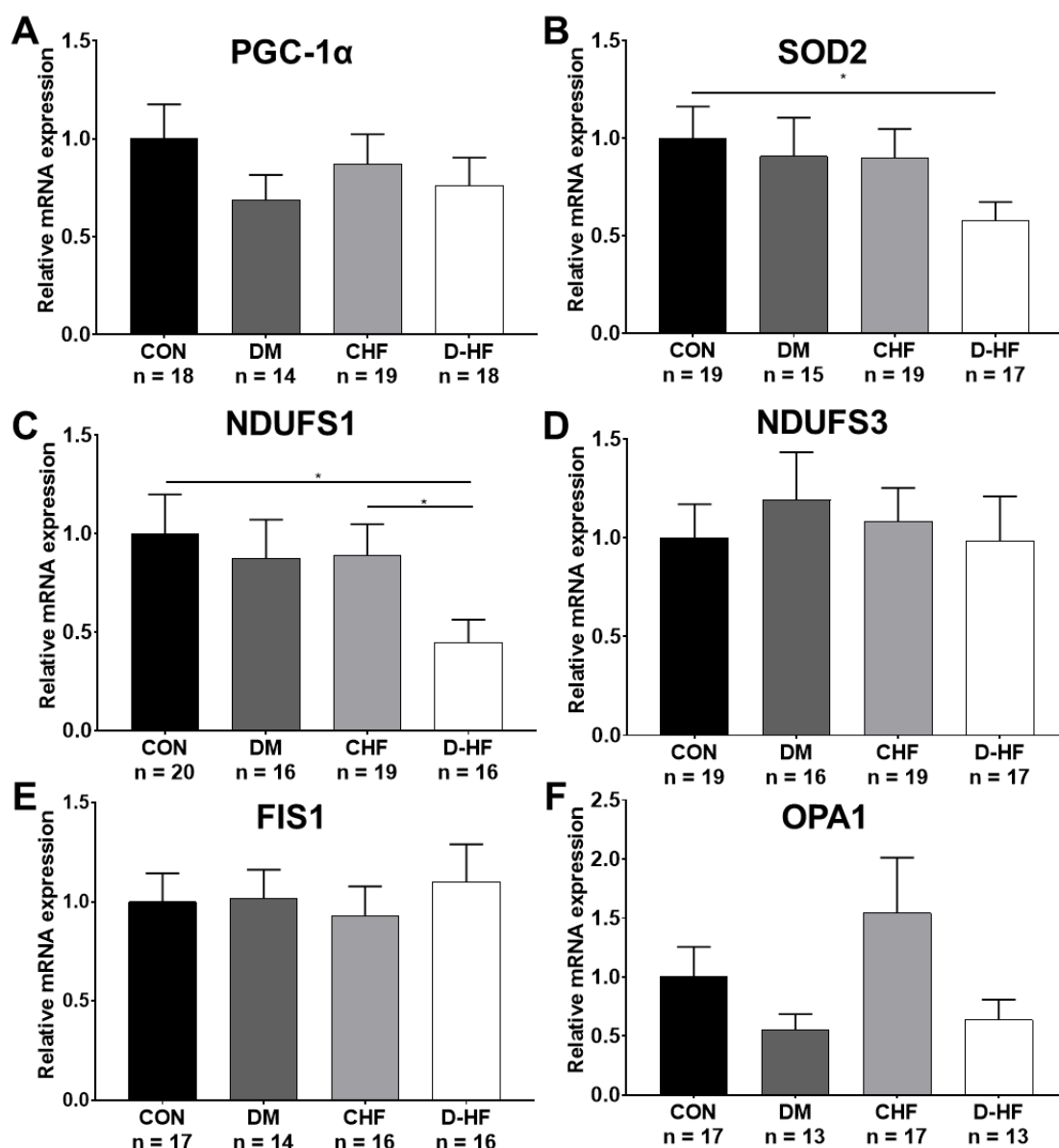
Figure 6.1. Relative mRNA expression of key mitochondrial genes. SOD2 and NDUFS1 were both lower in D-HF patients. The data displayed above are given as mean ± SEM. *$P < 0.05$ using independent samples Student t-tests.

6.3.4 High-resolution respirometry with H_2O_2 production

Figure 6.2 illustrates the relative H_2O_2 production across the different respiratory states. D-HF resulted in an ~8-fold and ~7-fold higher H_2O_2 production with complex I substrates in the Leak state (L_I) compared to CON ($P = 0.007$) and DM ($P = 0.011$), respectively (Figure 6.2). During complex I OXPHOS (P_I), H_2O_2 production was ~14-fold, ~12-fold, and ~4-fold higher compared to CON ($P < 0.0001$), DM ($P = 0.0001$), and CHF ($P = 0.0009$), respectively (Figure 6.2).

During simultaneous electron flow with complex I and II substrates (P_{I+II}), H_2O_2 production was ~6-fold and ~4-fold higher in D-HF compared to CON ($P = 0.016$) and DM ($P = 0.041$), respectively (Figure 6.2). Following uncoupling with these substrates still present (E_{I+II}), H_2O_2 production was ~7-fold, ~6-fold and ~3-fold higher in D-HF compared to CON ($P = 0.0002$), DM ($P = 0.0005$), and CHF ($P = 0.0018$), respectively (Figure 6.2). This same trend continued even after inhibiting complex I (E_{II}) with H_2O_2 production being ~8-fold, ~11-fold and ~5-fold higher in D-HF compared to CON, DM, and CHF, respectively (All $P < 0.0001$; Figure 6.2). Figure 6.3 displays the relationship between complex I H_2O_2 production and complex I function.
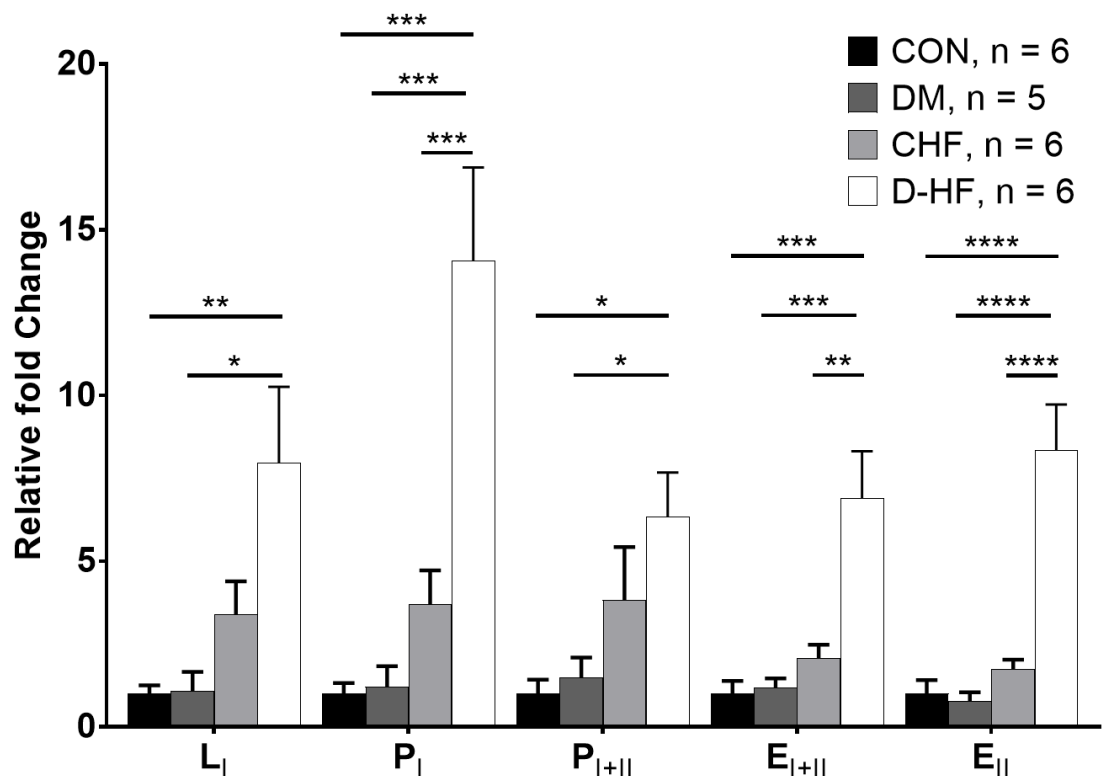
Figure 6.2. Relative fold changes in mitochondrial H_2O_2-emission across different respiratory states during simultaneous high-resolution respirometry. The data displayed above are given as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ using Tukey's multiple comparisons post-hoc tests following significant one-way ANOVAs.
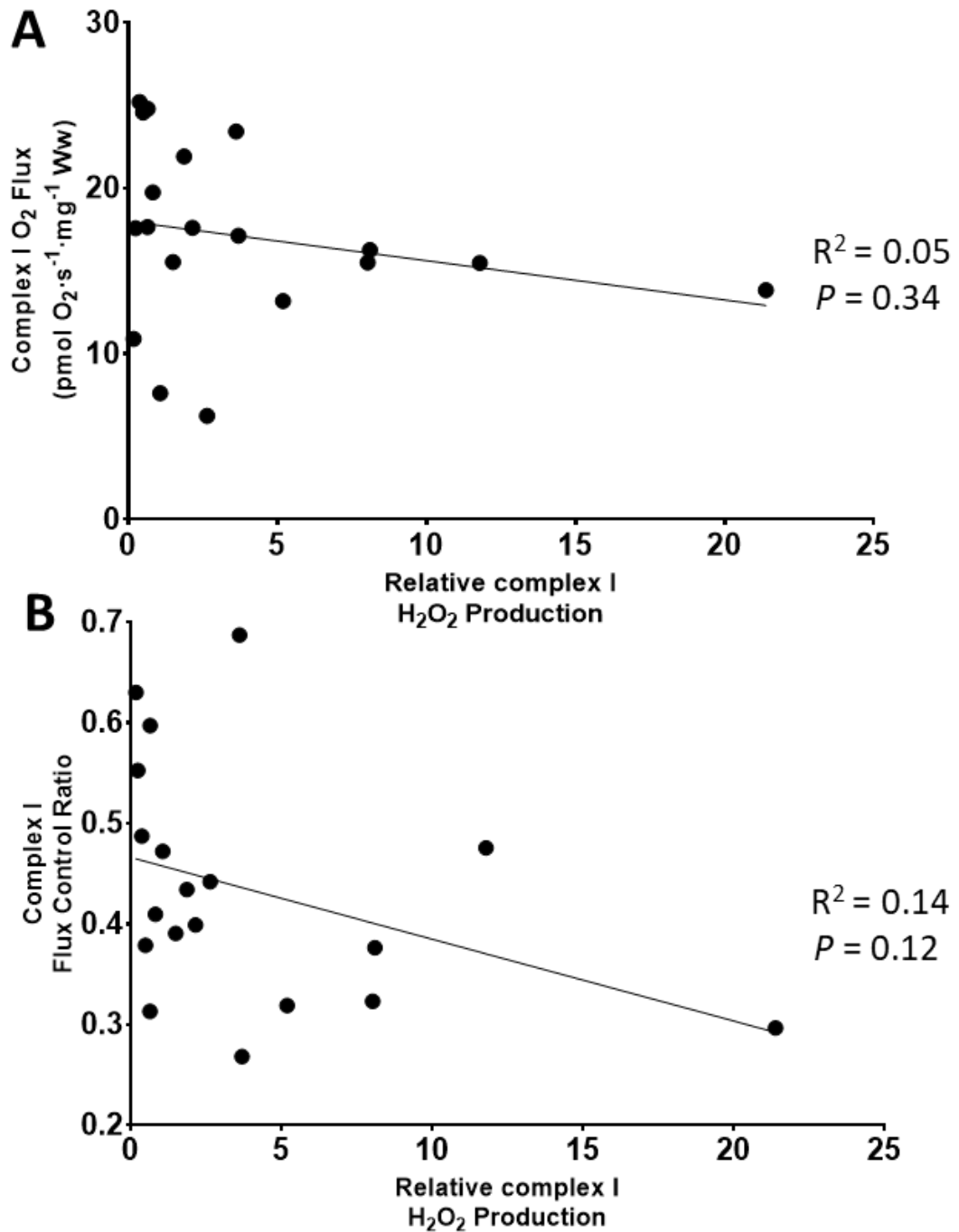
Figure 6.3. The relationship between complex I H_2O_2 production and complex I function in terms of O_2 flux (A) and the flux control ratio (B).

6.3.5 Muscle isoform composition and fibre cross-sectional area

Figure 6.4 shows the mean fibre-specific cross-sectional area (i.e., FCSA). Mean

FCSA in D-HF patients was 44% and 30% smaller than CON (P = 0.001) and

CHF (P = 0.044), respectively (Figure 6.4). Type I FCSA did not differ between

groups; however, type IIa FCSA was 39% smaller in DM compared to CON ($P =$ 0.009; Figure 6.4) and there was a trend for CHF to have smaller type IIa fibres compared to CON ($P = 0.115$). Type IIa FCSA was also 53% and 39% smaller in D-HF compared to CON ($P < 0.0001$) and CHF ($P = 0.001$), respectively (Figure 6.4). Type IIx fibres were 50% smaller in DM compared to CON ($P = 0.015$; Figure 6.4). Furthermore, type IIx FCSA was 65% and 52% smaller in D-HF compared to CON ($P = 0.0003$) and CHF ($P = 0.014$), respectively (Figure 6.4).
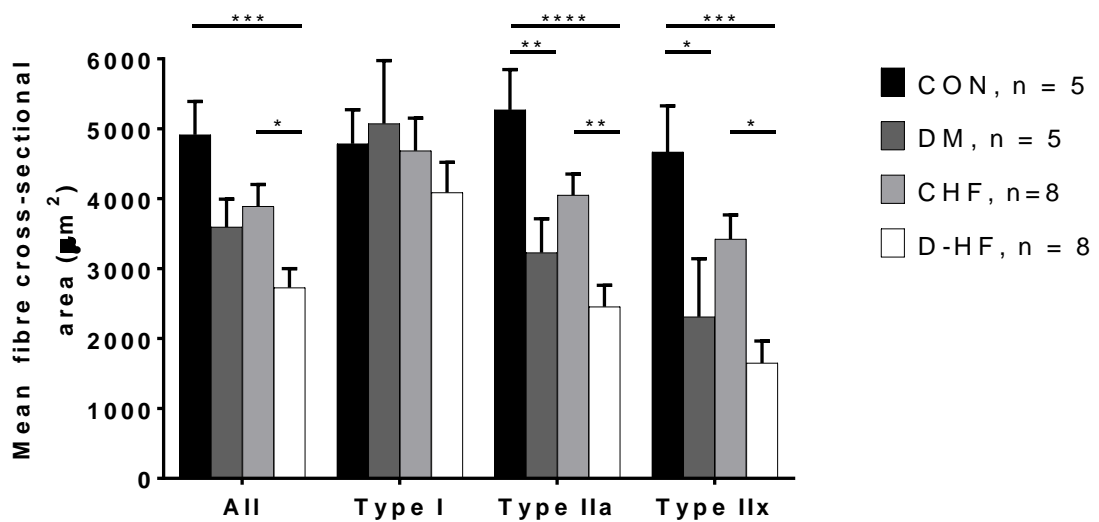
Figure 6.4. Mean fibre cross-sectional area (μm^2) across each cohort according to fibre type. The data displayed above are given as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ using Tukey's multiple comparisons post-hoc tests following significant one-way ANOVAs.

Figure 6.5 shows the numerical and areal densities for each fibre type within each of the cohorts. CHF had a higher areal density of type IIx fibres compared to DM ($P = 0.040$) and D-HF ($P = 0.035$), respectively (Figure 6.5). D-HF also had a higher areal density of type I fibres compared to CHF ($P = 0.014$; Figure 6.5).
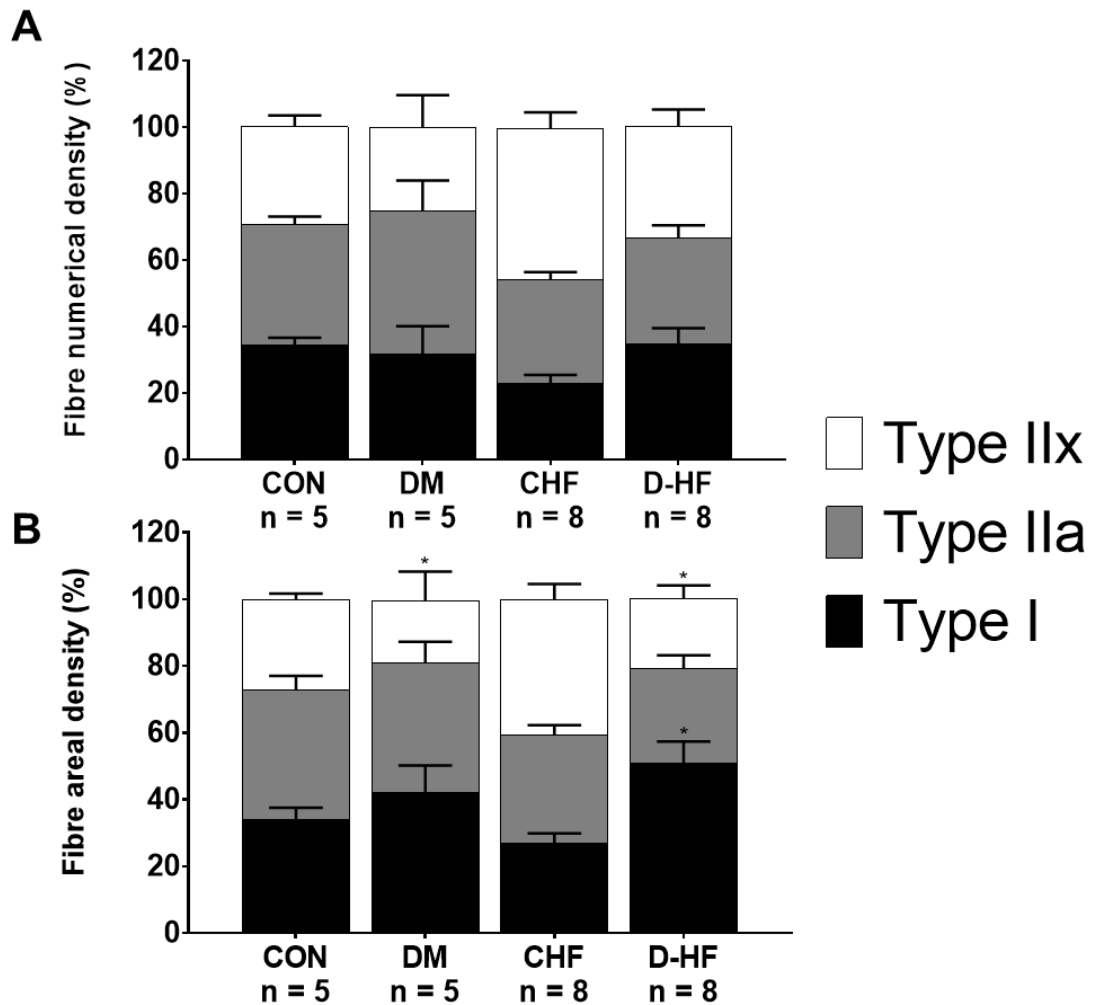
Figure 6.5. Fibre numerical and areal densities for each fibre type according to the respective cohorts. The data displayed above are given as mean ± SEM. *P < 0.05 vs. CHF for corresponding fibre type using Tukey's multiple comparisons post-hoc tests following significant one-way ANOVAs.

Figure 6.6 shows the individual (A-C) and composite (D) images obtained from a serial transverse cryo-section stained for capillaries, basal membrane, and type I MHC isoform, while representative composite images from each patient cohort are presented in Figure 6.7.
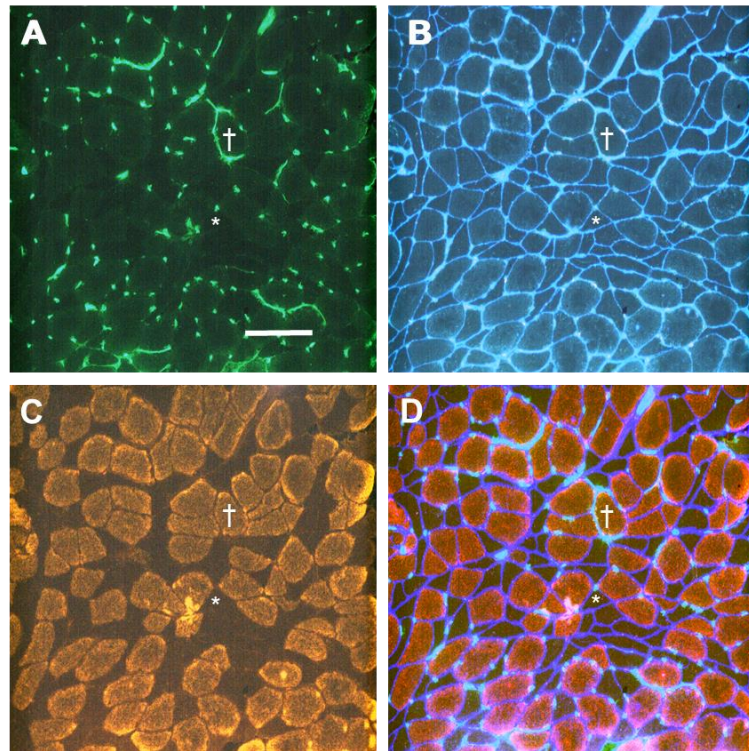
Figure 6.6. Staining protocol for determining capillaries and fibre types. Individual (A-C) and composite (D) images obtained from a serial transverse cryo-section stained for capillaries (A), basal membrane (B), and type I MHC isoform (C). †Shows corresponding type I fibres in each image. *Shows corresponding type II fibres in each image. Scale bar represents 200 µm.
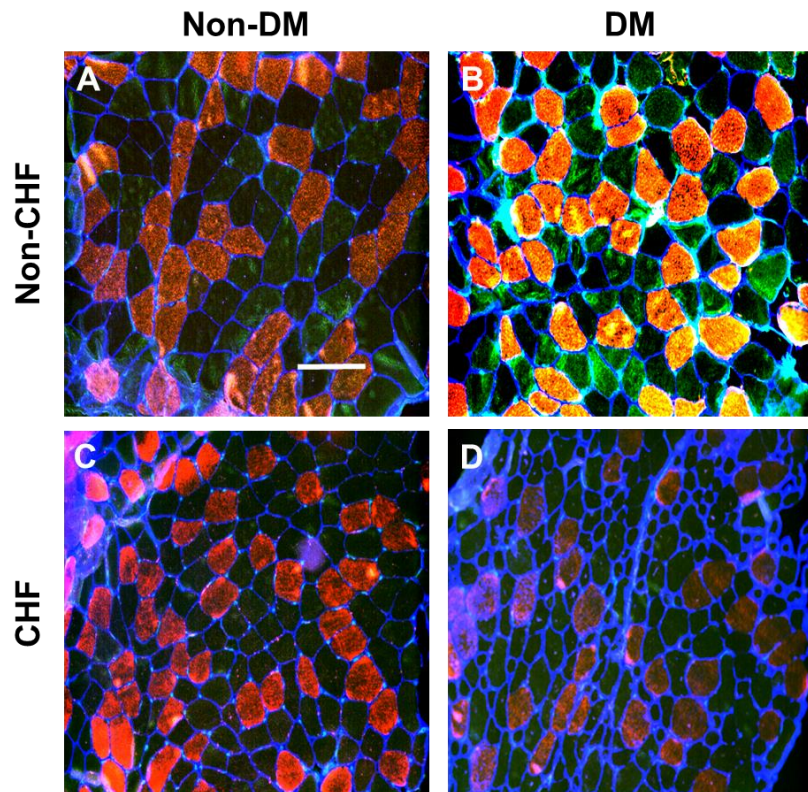
Figure 6.7. Representative composite images of histological staining obtained from CON (A), DM (B), CHF (C), and D-HF patients (D). Type I MHC fibres are stained red; type II MHC fibres are stained green; the basal membrane is stained blue. Scale bar represents 200 µm.

6.3.6 Skeletal muscle capillarisation

Figure 6.8 displays both the global and localised (fibre type-specific) indices of capillarity as well as the capillary domain area and heterogeneity of capillary distribution. Although there were no significant differences in the capillary-to-fibre ratio, the LCFR was significantly lower in D-HF patients in both IIa and IIx fibres, with LCFR for IIa fibres 34% lower in D-HF compared to CON ($P = 0.011$; Figure 6.8C). Additionally, the LCFRs for IIx fibres were 40% and 55% lower in DM ($P = 0.023$) and D-HF ($P = 0.0003$) compared to CON (Figure 6.8C). Conversely, capillary density and LCD were both higher in D-HF fibres compared to CON and CHF. Capillary density was 63% and 37% higher in D-HF compared to CON ($P = 0.007$) and CHF ($P = 0.034$), respectively (Figure 6.8B). The LCD was 46% ($P = 0.023$), 62% ($P = 0.021$), and 49% ($P = 0.029$) higher in D-HF compared to CON for type I, type IIa and type IIx fibres, respectively (Figure 6.8D). Furthermore, the LCD was 41% ($P = 0.0499$) and 45% ($P = 0.0174$) higher in D-HF compared to CHF for type IIa and type IIx fibres, respectively (Figure 6.8D). These differences in LCD and CD likely reflect the previous findings of atrophy in these corresponding fibre types (Figure 6.4). The capillary domain area was also lower across the cohorts according to the one-way ANOVA ($P = 0.040$) but there were no significant pairwise comparisons in the post-hoc tests with a trend for a lower capillary domain area in D-HF compared to CON ($P = 0.052$; Figure 6.8E). There were no differences in the heterogeneity of capillary distribution between cohorts (Figure 6.8F).
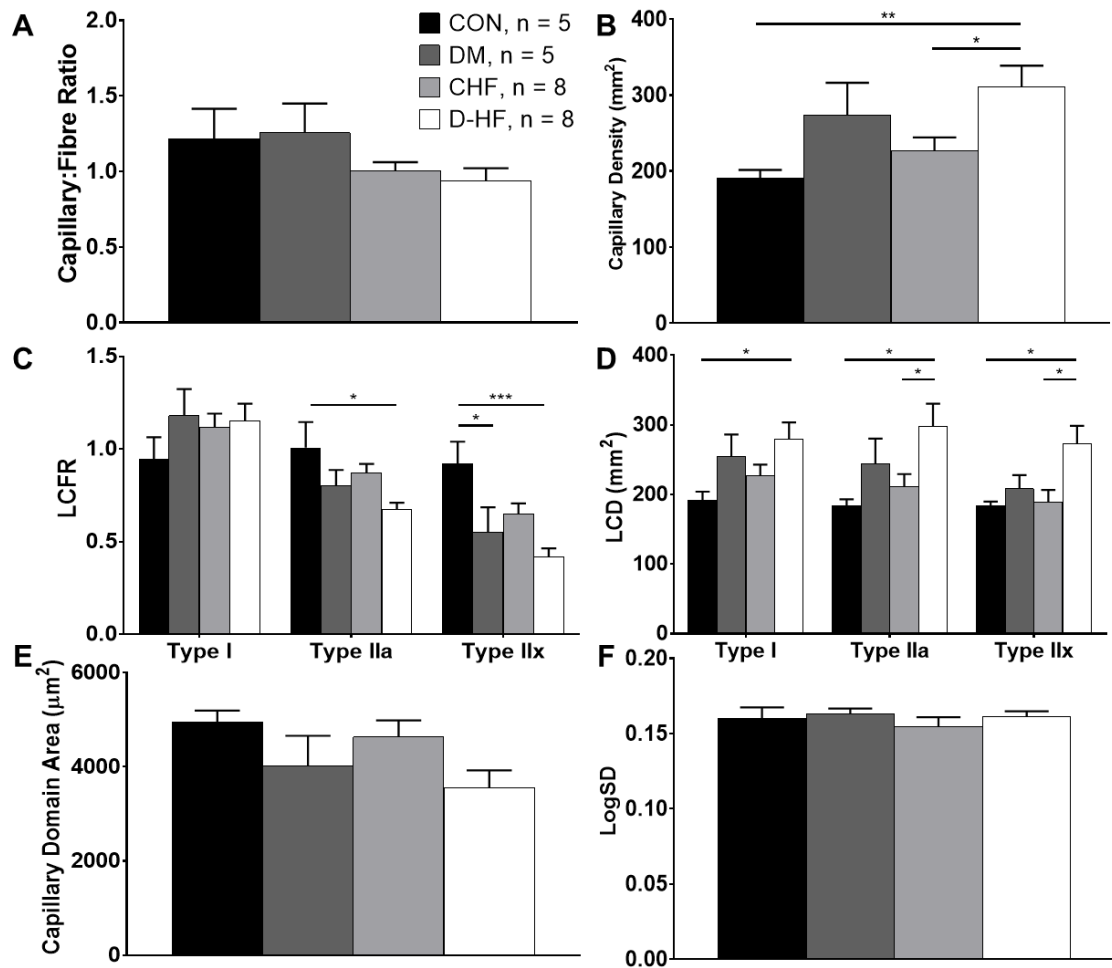
Figure 6.8. Global and localised indices of capillarisation. D-HF was associated with a lower LCFR yet higher capillary density and LCD. The data displayed above are given as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ using Tukey's multiple comparisons post-hoc tests following significant one-way ANOVAs.

6.4 Discussion

The primary findings of this study are that in skeletal muscle from D-HF patients there is: 1) a lower expression of the key mitochondrial regulatory genes SOD2 and NDUFS1; 2) a substantial increase in mitochondrial-derived ROS; 3) significant fibre atrophy; and 4) impairments to capillarity including a lower local capillary-to-fibre ratio (LCFR). Overall, therefore, these findings provide novel data on various mechanisms that may contribute to the severe skeletal muscle mitochondrial pathology (and exercise intolerance) demonstrated by D-HF patients.

6.4.1 Mitochondrial related genes

The current study observed that both the SOD2 and NDUFS1 genes were downregulated in skeletal muscle tissue from D-HF patients. Whether these represent potential therapeutic targets in D-HF to treat mitochondrial dysfunction, remains an exciting future avenue. The SOD2 gene encodes for the MnSOD/SOD2 enzyme, which is the SOD isoform residing within the mitochondrial matrix and is a primary antioxidant combating mitochondrial ROS (Murphy, 2009). SOD2 dismutates $O_2^{\cdot-}$, produced primarily at complexes I and III, to generate H_2O_2. Previous studies have shown that CHF and DM are associated with a lower SOD and SOD2 skeletal muscle gene expression, protein content and enzyme activity levels (Linke *et al.*, 2005; Rush *et al.*, 2005; Holmstrom *et al.*, 2012; Ramirez-Sanchez *et al.*, 2013; Mangner *et al.*, 2015; Martinez *et al.*, 2015). Further evidence comes from murine studies, where SOD2 genetic deletion confirmed its causal role in the development of mitochondrial dysfunction and subsequent CHF pathology (Nojiri *et al.*, 2006), while SOD2 overexpression prevented high-fat diet induced insulin resistance (Boden *et al.*, 2012). Collectively, these data support the inference that a low expression of SOD2 could contribute to the severe mitochondrial pathology and disease severity observed in D-HF patients. Further research is therefore required to determine the exact role of SOD2 in D-HF.

Few studies in either CHF or DM have assessed the expression of genes encoding for core mitochondrial subunits of complex I, such as NDUFS1 and NDUFS3. NDUFS1 is the largest (75 kDa) subunit of complex I and forms a component of the 8 iron-sulphur chains involved in transferring electrons from NADH oxidation at the flavin mononucleotide to the ubiquinone binding site

where the electron acceptor, ubiquinone, is reduced to ubiquinol (Hirst, 2013; Zhu *et al.*, 2016). In line with the current study, one previous study has shown that several genes encoding for the subunits of complex I, including NDUFS1, are downregulated in skeletal muscle samples from DM patients (Wu *et al.*, 2017). Thus it remains plausible that a lower expression of the NDUFS1 gene could impact NDUFS1 protein expression or function to consequently impair complex I respiration (and even increase the formation of $O_2^{\cdot-}$). Further research is thus required to confirm whether low NDUFS1 expression impacts mitochondrial function in D-HF.

6.4.2 Mitochondrial ROS

To further characterise the functional consequences of the lower SOD2 and NDUFS1 gene expression, we determined mitochondrial H_2O_2 production simultaneously with O_2 flux measurements in order to provide a marker of mitochondrial-derived ROS (Krumschnabel *et al.*, 2015b; Makrecka-Kuka *et al.*, 2015). This method highlighted that permeabilized skeletal muscle fibres from D-HF patients have a substantially higher mitochondrial ROS production compared to CON, DM and CHF patients, and in particularly at complex I. This novel finding supports previous data where both CHF and DM have been associated with increased global markers of ROS in skeletal muscle (Tsutsui *et al.*, 2001; Rush *et al.*, 2005; Coirault *et al.*, 2007; Bonnard *et al.*, 2008; Anderson *et al.*, 2009). The mechanisms underlying this elevated mitochondrial ROS in D-HF remain unclear, but it could be related to hyperlipidaemia and hyperglycaemia, as these conditions have been shown to be associated with increased mitochondrial ROS production that result in mitochondrial dysfunction (Bonnard *et al.*, 2008;

Anderson *et al.*, 2009; Yokota *et al.*, 2009; Bravard *et al.*, 2011). Likewise, animal models of CHF (Tsutsui *et al.*, 2001; Rush *et al.*, 2005; Coirault *et al.*, 2007; Supinski & Callahan, 2007; Martinez *et al.*, 2015; Laitano *et al.*, 2016; Mangner *et al.*, 2016) and CHF patients (Guzman Mentesana *et al.*, 2014) also exhibit enhanced skeletal muscle ROS production and oxidative stress (Ohta *et al.*, 2011). Several studies have shown that treatment with mitochondrial-specific antioxidants, including SOD2, in addition to exercise training, are able to ameliorate oxidative stress in skeletal muscle and this is linked to improved mitochondrial function and insulin sensitivity in both CHF (Ennezat *et al.*, 2001; Gielen *et al.*, 2005; Linke *et al.*, 2005; Supinski & Callahan, 2005; Okutsu *et al.*, 2014; Gomes *et al.*, 2016; Mangner *et al.*, 2016) and DM (Yokota *et al.*, 2009; Hey-Mogensen *et al.*, 2010; Brinkmann *et al.*, 2012; de Oliveira *et al.*, 2012; Barbosa *et al.*, 2013; Takada *et al.*, 2015; Mason *et al.*, 2016). Overall, these data provide strong support for mitochondrial-derived ROS playing a key role in the pathogenesis of the reduced muscle mitochondrial function in D-HF patients.

6.4.3 Skeletal muscle isoform and atrophy

Given that previous studies have documented a fibre type shift from type I to type IIx fibres in both CHF (Sullivan *et al.*, 1990; Schaufelberger *et al.*, 1995; Simonini *et al.*, 1996; Schaufelberger *et al.*, 1997; Sullivan *et al.*, 1997; Middlekauff *et al.*, 2013) and DM (Marin *et al.*, 1994; Nyholm *et al.*, 1997; Oberbach *et al.*, 2006), and this isoform is associated with an increase in mitochondrial H_2O_2 production (Anderson & Neufer, 2006), we decided to determine if D-HF patients exhibit a fibre type shift. However, we did not find any differences between patient cohorts and controls in terms of numerical fibre density. Nevertheless, we did observe

differences in fibre areal densities between CHF patients and the two DM cohorts. However, as areal density is influenced by changes in FCSA (Egginton, 1990a), these differences were likely driven by the observation of a greater atrophy of both type IIa and IIx fibres in DM and D-HF patients in the presence of a preserved FCSA of type I fibres in all patient cohorts.

We also observed a trend for type IIa fibres to atrophy in CHF patients. These findings support previous studies indicating fibre type-specific atrophy of glycolytic type II fibres in CHF (Sullivan *et al.*, 1990; Xu *et al.*, 1998; Li *et al.*, 2007; Yu *et al.*, 2008), DM (Almond & Enser, 1984; Kemp *et al.*, 2009), and ageing skeletal muscle (Deschenes, 2004; Brunner *et al.*, 2007; Picard *et al.*, 2011a; Nilwik *et al.*, 2013; Murgia *et al.*, 2017). The functional consequence of this fibre atrophy includes reduced muscle mass, which in turn decreases muscle strength (dynapenia), thereby contributing to a vicious cycle of inactivity and exercise intolerance that ultimately increases mortality risk in CHF and DM (Anker *et al.*, 1997; Park *et al.*, 2006; Park *et al.*, 2009; Kim *et al.*, 2010; Leenders *et al.*, 2013; Kim *et al.*, 2014). While the exact mechanisms mediating this fibre-type specific atrophy are still being uncovered, it is believed that increased inflammation, oxidative stress, and angiotensin II concentrations as well as reduced Akt (protein kinase b) activation and PGC-1α expression all can promote protein degradation while inhibiting protein synthesis in CHF and DM (Li *et al.*, 1998; Li *et al.*, 2003b; Cai *et al.*, 2004; Wang *et al.*, 2006; Geng *et al.*, 2011; Ostler *et al.*, 2014; Du Bois *et al.*, 2015). As such, in the current study our D-HF group may be characterized by elevated proteolysis (i.e. increased activation of ubiquitin-proteasome, autophagy-lysosomal, and caspase-3 pathways) and/or a down-regulation in anabolic signalling, such as the IGF1-Akt-mTOR pathway

(Perry *et al.*, 2016). Interestingly, elevated mitochondrial ROS has been shown to directly induce atrophy in animal models of wasting (Fulle *et al.*, 2004). As such, further work is therefore warranted to confirm whether impairments in protein homeostasis contribute to the greater fibre atrophy in D-HF and whether increased mitochondrial ROS is playing a causal role.

6.4.4 Skeletal muscle capillarisation

To provide greater insight into potential O_2 delivery limitations in D-HF, the current study also employed state-of-the-art computational capillarity modelling (Al-Shammari *et al.*, 2014). Impairments in capillarity can reduce convective and diffusive O_2 delivery to mitochondria, thus limiting mitochondrial function. Interestingly, we observed an increase in capillary density (CD) in D-HF patients, which was matched by comparable increases in the fibre-type specific measure of LCD. However, using the scale-independent measure of LCFR, we were able to detect significant fibre-type specific capillary rarefaction in both type II fibres in D-HF patients as well as type IIx fibres in DM patients. It is interesting to note that these differences were not evident when the global measure of capillary-to-fibre ratio (CF) was quantified. Thus the increased sensitivity of the LCFR method was sufficient to identify significant differences that may have otherwise been undetected. Although an increase in CD has also been previously observed in both CHF (Mancini *et al.*, 1989) and insulin-resistant subjects (Eriksson *et al.*, 1994), the majority of studies have shown a decrease in CD and C:F (Marin *et al.*, 1994; Schaufelberger *et al.*, 1995; Schaufelberger *et al.*, 1997; Duscha *et al.*, 1999; Nusz *et al.*, 2003; Williams *et al.*, 2004; Groen *et al.*, 2014). Our divergent responses may partly be explained by the greater atrophy of type II fibres in D-

HF patients. Thus, even with an increased CD within a particular region of interest, the LCFR is still decreased due to a higher number of fibres within the region.

Previous studies measuring LCFR, have shown that it is reduced in both fibre types in DM patients (Groen *et al.*, 2014). Similarly, a decreased number of capillaries contacting both type I and IIx fibres has been reported in CHF patients (Williams *et al.*, 2004). However, some studies have shown that CHF reduces the capillary-to-fibre ratio specifically in type II muscle fibres (Xu *et al.*, 1998). Although we did not explore the exact mechanisms contributing to the fibre type-specific reduction in LCFR in D-HF patients, it is possible to speculate on some. In the context of insulin resistance, it has been shown that a decreased expression of vascular endothelial growth factor induces capillary rarefaction in skeletal muscle (Bonner *et al.*, 2013). Similarly, intramuscular VEGF overexpression has been shown to increase capillarity in an animal model of CHF (Zisa *et al.*, 2009). Thus VEGF may be considered a potential therapeutic target for ameliorating capillary rarefaction in D-HF patients. A reduced LCFR may even decrease O_2 delivery to individual fibres such that a hypoxic state is induced. Therefore it seems reasonable to speculate that severe impairments to microvascular O_2 delivery in D-HF patient may render some areas of the muscle hypoxic, which is well known to promote complex I deactivation (Galkin *et al.*, 2009). As such, this may be another mechanism contributing to the low mitochondrial function in D-HF patients. This may even be exacerbated by endothelial dysfunction, which is known to occur in DM (Bauer *et al.*, 2007; Muniyappa & Sowers, 2013) and CHF (Hambrecht *et al.*, 1999b).

6.4.5 Limitations

One possible limitation of the current study was the limited analysis of gene expression aside from genes encoding for mitochondrial complex I proteins. However, given that gene expression analyses are time-consuming and expensive, the current study had to be selective in the gene targets included. Therefore, given that the previous two chapters had identified complex I as a dysfunctional mitochondrial protein in D-HF patients, the current chapter decided to focus on genes encoding for this enzyme. An additional drawback is the inability to confidently exclude other potential sources of superoxide anion production within the cell which may have contributed to the elevated H_2O_2 production levels observed in D-HF patients. Indeed, the activity and expression of NADPH oxidase, a membrane-bound enzyme which produces superoxide anion in the process of regenerating the $NADP^+$ pool, is upregulated with hyperglycaemia and angiotensin II overactivation, both of which characterise the D-HF phenotype. Therefore future studies may wish to isolate the effects of mitochondrial and other cellular sources of superoxide anion production. Finally, this study also draws into question the utility of the capillary:fibre ratio as a useful marker of morphological adaptations within the muscle. As this global measure only quantifies the ratio between these two indices, it does not provide an indication of how each of these variables are changing alone and therefore often lacks the sensitivity to identify localised changes within the redistribution of capillaries around individual fibres of different isoforms. Therefore future studies may wish to incorporate the LCFR, as in the current chapter, to better identify changes within the redistribution of capillaries around individual fibres. Finally, this study also failed to measure any plasma concentrations of pro-inflammatory

cytokines or angiotensin II as possible markers mediating the systemic complex I dysfunction observed in Results III. Therefore future studies should include blood samples to measures these variables and provide a more conclusive understanding of the potential mechanisms mediating complex I dysfunction.

6.5 Conclusions

In summary, the current study elucidated several novel mechanisms that may contribute to the severe skeletal muscle mitochondrial dysfunction and exercise intolerance demonstrated in D-HF patients. Evidence included low expression of key mitochondrial genes (i.e. SOD2 and NDUFS1), elevated mitochondrial-derived ROS, fibre atrophy, and impaired capillarity. As such, these may represent viable therapeutic targets for attenuating muscle mitochondrial impairments in patients with D-HF.

Chapter 7 General discussion

Chronic heart failure (CHF) and type 2 diabetes mellitus (DM) remain two major primary causes of mortality and morbidity (Kannel & McGee, 1979; Metra & Teerlink, 2017). Importantly, recent evidence shows a disturbing trend indicating an increased prevalence of both conditions in combination, which is termed diabetic heart failure (D-HF) (Lehrke & Marx, 2017). Estimates indicate that ~25-30% of CHF patients have DM (MacDonald *et al.*, 2008a; MacDonald *et al.*, 2008b; Cubbon *et al.*, 2013; van Deursen *et al.*, 2014; Dei Cas *et al.*, 2015; Seferovic *et al.*, 2018), yet despite this large, and increasingly growing, population there are no targeted therapeutic treatments for D-HF patients, which is somewhat surprising given that these patients have a significantly worse prognosis than CHF alone (Shindler *et al.*, 1996; de Groote *et al.*, 2004; Gustafsson *et al.*, 2004; Cubbon *et al.*, 2013; Dauriz *et al.*, 2017; Seferovic *et al.*, 2018). We hypothesised that, as mortality is strongly determined by exercise capacity (Myers *et al.*, 2002) and D-HF patients have a worse exercise intolerance than CHF (Guazzi *et al.*, 2002; Tibb *et al.*, 2005; Ingle *et al.*, 2006), that an improved understanding of the mechanisms limiting exercise capacity in D-HF may uncover potential therapeutic targets to ameliorate symptoms and improve outcomes in this population. To this end, the present thesis focused on improving our understanding of exercise intolerance, and in particular skeletal muscle alterations, in D-HF patients.

7.1 Summary of key findings

The first study (Results I) of this thesis employed a novel CPX test (termed the RISE-95) in a clinical hospital to better evaluate exercise intolerance in the

general CHF population, but with an added focus on the response of D-HF patients. This study confirmed previous clinical findings indicating that $\dot{V}O_{2peak}$ and exercise capacity are reduced by ~20% in D-HF patients compared to typical CHF patients (Guazzi *et al.*, 2002; Tibb *et al.*, 2005; Ingle *et al.*, 2006). Furthermore this chapter also extended the findings of a previous pilot study performed in the research laboratory, showing that around 20% of CHF patients can access an "aerobic reserve" that allows them to increase $\dot{V}O_{2peak}$ by a clinically-important threshold (Bowen *et al.*, 2012a). Interestingly, this "aerobic reserve" was still evident in the D-HF population.

To further elucidate the potential mechanisms contributing to exercise intolerance in D-HF patients, the second study (Results II) investigated skeletal muscle mitochondrial dysfunction in age-matched control, DM, CHF and D-HF patients. This study exploited the opportunity to extract muscle biopsies from the *pectoralis major* muscle of patients undergoing routine pacemaker and ICD/CRT device implantations. The major finding from this study was that D-HF patients demonstrated more severe reductions in skeletal muscle mitochondrial respiration compared to either CHF or DM patients. Interestingly, after normalizing the data to account for differences in mitochondrial content, we identified a specific complex I myopathy. Therefore it was concluded that D-HF induces both qualitative as well as quantitative impairments to mitochondria. To corroborate these findings and validate chest muscle sampling, the next study (Results III) examined skeletal muscle mitochondrial dysfunction in leg *vastus lateralis* biopsies, obtained from the same patients. Again, this study confirmed the previous observation that D-HF induces both quantitative and qualitative mitochondrial impairments, with the latter residing at complex I. That these

alterations were present in both upper and lower limb muscles suggest a systemic effect is acting in D-HF to induce mitochondrial deficits.

The final study of this thesis (Results IV) aimed to further characterise the potential mechanisms contributing to skeletal muscle mitochondrial impairments in D-HF, and uncovered several contributory factors likely involved, such as an increased mitochondrial ROS production and downregulated expression of key mitochondrial genes. Furthermore this study revealed that D-HF patients exhibit a type II fibre-specific atrophy and capillary rarefaction, which may also contribute to mitochondrial impairments. Collectively, these findings expand our current knowledge regarding how D-HF impacts skeletal muscle and identifies potential therapeutic targets (i.e. the mitochondria) by which exercise intolerance may be ameliorated in these patients.

7.2 Why is exercise intolerance exacerbated in D-HF patients?

The Results I chapter of this thesis confirmed several previous studies (Guazzi *et al.*, 2002; Tibb *et al.*, 2005; Ingle *et al.*, 2006), which indicated that exercise intolerance (i.e. $\dot{V}O_{2peak}$), is reduced in D-HF compared to CHF. Notably, the novel finding from this study was that around half of both CHF and D-HF patients exhibit an "aerobic reserve" that permits them to increase $\dot{V}O_{2peak}$ in the step-exercise (SE) phase of the RISE-95 protocol. Collectively, 46% of patients statistically increased their $\dot{V}O_{2peak}$ in the SE phase of the test by an average of 1.6 $mL \cdot kg^{-1} \cdot min^{-1}$, or 11%, as compared to the "gold-standard" ramp-incremental protocol (Table 3.5). In comparison to the other 54% of patients who did not display this "aerobic reserve", this 11% increase in $\dot{V}O_{2peak}$ in the SE

phase translated to a higher peak work rate and exercise duration, respectively. While these findings may be somewhat surprising considering the only previous study in CHF patients using the RISE-95 protocol found that only ~20% of patients increased $\dot{V}O_{2peak}$ by an average of 1.3 mL·kg^{-1}·min^{-1} (Bowen *et al.*, 2012a), this study was performed under research laboratory settings from a small sample size (n = 24). The substantial improvements in exercise tolerance and functional capacity seen in the present study are clinically significant, given that a 6% higher $\dot{V}O_{2peak}$ is associated with a 7% lower risk of all-cause mortality in CHF patients (Swank *et al.*, 2012). As this response is rarely seen in healthy subjects performing similar exercise protocols (Day *et al.*, 2003; Rossiter *et al.*, 2006), this raises two intriguing questions that remain to be addressed: 1) Is it possible to better direct treatment and predict prognosis in these individuals that exhibit (or not) an "aerobic reserve"?; and 2) What physiological mechanisms exist in some, but not all, CHF patients that allow them to access this "aerobic reserve"? (Azevedo *et al.*, 2018).

To address the first question, whether improved findings from the RISE-95 can better direct treatment and predict prognosis in individuals that exhibit an "aerobic reserve", we compared the clinical characteristics of "responders" to "non-responders" from the RISE-95 test. Despite being younger and having less severe symptoms, as evidenced by a lower NYHA classification, "responders", who exhibited an "aerobic reserve", had *more* hospitalisations in the follow-up period. Therefore it would appear paradoxically that this "aerobic reserve" is actually predictive of a poorer prognosis. As such, these initial data would indicate "responder" patients to the RISE-95 test would require more urgent and aggressive clinical treatment and management.

To help answer the second question, we can simply probe the question whether some CHF and D-HF patients exhibit a limitation to exercise that may be acutely alleviated. This limitation may be of central origin (thereby reducing convective O_2 delivery), or a peripheral mechanism (thus reducing microvascular O_2 diffusion and/or mitochondria O_2 utilization). Therefore a better understanding of the mechanisms underpinning this limitation could potentially yield novel therapeutic targets to treat exercise intolerance in these patients. To provide insight into this, the RISE-95 test showed that "responder" and "non-responder" patients exhibited several divergent cardiopulmonary responses between the two exercise phases. In particular, the "responders" increased both peak HR and O_2 pulse in the SE phase of the protocol (Table 3.5), which provides evidence that these patients probably increase both skeletal muscle O_2 delivery and extraction. The increase in O_2 pulse indicates that, even with an increased peak HR, O_2 consumption for each heartbeat also increased, possibly due to increased stroke volume, improved convective O_2 delivery and/or increased mitochondrial O_2 extraction and utilisation. Interestingly, even when all patients were assessed, there was a significant increase in O_2 pulse between the two phases of the test despite no change in peak HR, and this may have contributed to a modest yet significant 0.5 mL·kg^{-1}·min^{-1} increase in $\dot{V}O_{2peak}$ (Table 3.2). Hence, these data support the hypothesis that exercise intolerance in CHF patients may be limited by varying mechanisms, including an intramuscular limitation in O_2 extraction (Bowen *et al.*, 2012b).

To further investigate an intramuscular limitation to O_2 extraction in some patients, we compared 12 patients that had completed the RISE-95 test where we had obtained *pectoralis major* muscle biopsies. Interestingly, our findings revealed that patients with an "aerobic reserve" tended to exhibit mitochondrial dysfunction, as evidenced by a lower complex I-supported O_2 flux (Figure 7.1). While speculative, these data provide initial evidence that a mitochondrial limitation residing at complex I may be overcome following the first RI exercise phase, thus allowing patients to access their "aerobic reserve" and achieve a higher $\dot{V}O_{2peak}$ during the second SE bout. Therefore an improved understanding of the physiological mechanisms allowing access to this "aerobic reserve" in some patients would likely reveal therapeutic targets to reduce exercise intolerance.
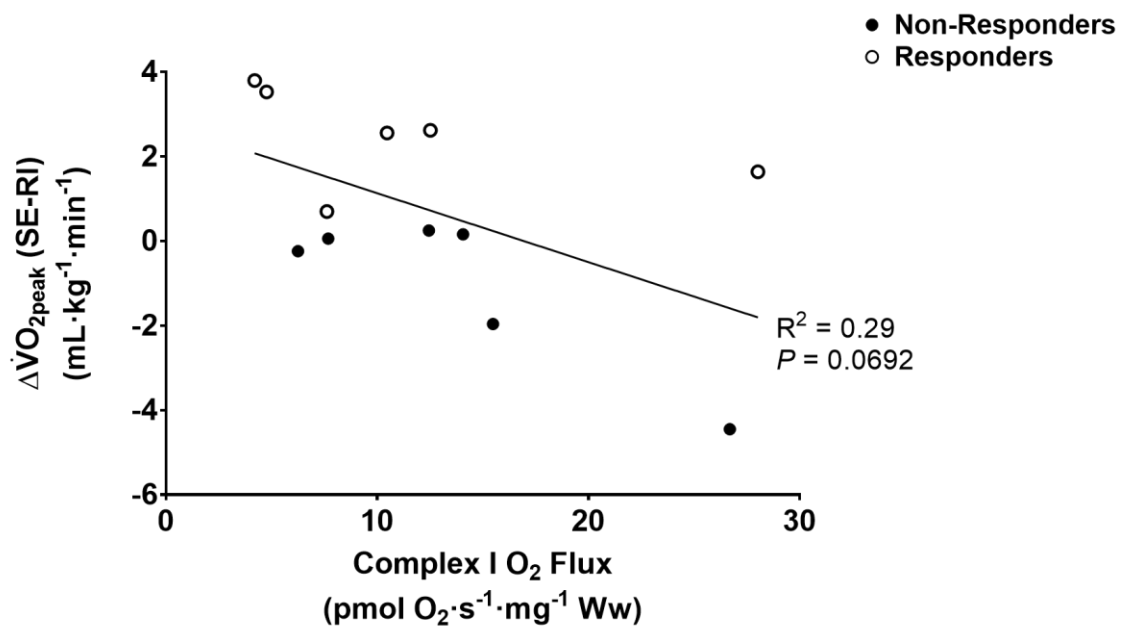
Figure 7.1. The relationship between complex I O_2 flux and $\Delta\dot{V}O_{2peak}$ between the SE and RI phases of the RISE-95 test tended to be inversely correlated, thereby suggesting that patients with an "aerobic reserve" may be limited by a complex I-specific impairment.

7.3 What mechanisms contribute to skeletal muscle maladaptations in D-HF patients?

To provide detailed insight into the potential mechanisms limiting skeletal muscle O_2 extraction and/or O_2 utilization in CHF and D-HF patients, direct *pectoralis major* muscle sampling was performed during routine pacemaker and ICD/CRT device implantations (Results II). We anticipated upper limb sampling would better mitigate against the potential confounding influences of detraining that often impact lower limb skeletal muscle in patients with CHF (Olsen *et al.*, 2005; Reynolds *et al.*, 2007; Rehn *et al.*, 2012). Using high-resolution respirometry, we demonstrated that skeletal muscle mitochondrial respiration was significantly lower in D-HF patients compared to control, CHF and DM patients. However, the majority of these differences were negated after normalizing to mitochondrial content with the exception of complex I-supported OXPHOS. Consequently, we concluded that D-HF induces both qualitative as well as quantitative impairments to mitochondrial function. The respiratory control ratio (RCR), a measure of mitochondrial coupling at complex I, was also impaired in D-HF compared to CHF patients. Complex I-supported O_2 flux closely correlated with $\dot{V}O_{2peak}$, which provided the first evidence of a novel mechanism that may mediate exercise intolerance in D-HF patients. We reinforced these finding in Results III, where we demonstrated that D-HF patients also exhibit similar mitochondrial impairments in the *vastus lateralis* muscle of the leg. As these mitochondrial impairments were of a similar degree and well correlated to the chest muscle and $\dot{V}O_{2peak}$, we concluded that a systemic mechanism is likely acting to limit whole-body exercise tolerance via reduced mitochondrial content and a complex I-specific myopathy.

7.3.1 Mitochondrial dysfunction

7.3.1.1 Reduced mitochondrial content

$\dot{V}O_{2peak}$ measured at the limit of tolerance is dictated by the capacity of several integrative whole-body organ systems to uptake, transport, extract, and utilise atmospheric O_2 for cellular ATP resynthesis that provides the necessary energy for repeated skeletal muscle contractions. In its most simplistic form, $\dot{V}O_{2peak}$ is determined by the two products of the Fick equation: cardiac output ($\dot{Q}$) and skeletal muscle O_2 extraction. In healthy individuals, $\dot{V}O_{2peak}$ is primarily limited by $\dot{Q}$ and convective O_2 delivery to the muscles (Mortensen *et al.*, 2005; Saltin & Calbet, 2006) and therefore the capacity for skeletal muscle oxidative phosphorylation exceeds the capacity for O_2 delivery (Boushel *et al.*, 2011). However, diffusive O_2 delivery (Roca *et al.*, 1989) and mitochondrial O_2 utilisation (McAllister & Terjung, 1990) have also been shown to significantly impact $\dot{V}O_{2peak}$ in health, with both thought to provide a major limitation to exercise tolerance in CHF patients (Poole *et al.*, 2018). Indeed this is supported by ^{31}P MRS studies demonstrating that CHF patients have an impaired *in vivo* mitochondrial oxidative capacity (Wilson *et al.*, 1985; Wiener *et al.*, 1986; Massie *et al.*, 1987a; Massie *et al.*, 1987b; Rajagopalan *et al.*, 1988; Mancini *et al.*, 1989; Mancini *et al.*, 1990; Stratton *et al.*, 1994; van der Ent *et al.*, 1998; Okita *et al.*, 2001; Nakae *et al.*, 2005). As similar findings have been reported in DM patients (Petersen *et al.*, 2004; Schrauwen-Hinderling *et al.*, 2007; Phielix *et al.*, 2008; Bajpeyi *et al.*, 2011; Tecilazich *et al.*, 2013), and given the current findings from this thesis, it may be speculated that a reduction in mitochondrial content represents a potential limitation to exercise tolerance in D-HF patients (i.e. by impairing O_2 extraction and limiting myocyte O_2 diffusion/and or consumption). This is supported by the strong correlation between skeletal muscle mitochondrial

content and $\dot{V}O_{2peak}$ (Holloszy & Coyle, 1984). Similarly, we also observed a significant positive correlation between a validated marker of mitochondrial content (i.e. complex IV activity (Larsen *et al.*, 2012a)) and $\dot{V}O_{2peak}$ (Figure 2.8). Given that mitochondrial OXPHOS is the primary pathway of aerobic ATP resynthesis, a reduction in mitochondrial content would be expected to reduce $\dot{V}O_{2peak}$.

To further elucidate the mechanisms contributing to reduced mitochondrial content, we probed muscle fibre type isoforms as well as the mRNA expression of PGC-1α (Results IV). As previous studies have shown a shift in phenotype from mitochondria-rich type I oxidative fibres to type II glycolytic fibres in both CHF and DM (Sullivan *et al.*, 1990; Schaufelberger *et al.*, 1995; Massie *et al.*, 1996; Schaufelberger *et al.*, 1997; Sullivan *et al.*, 1997; Oberbach *et al.*, 2006; Mogensen *et al.*, 2007), we hypothesized that this fibre type switch may be an underlying pathway mediating reduced mitochondrial content in D-HF patients. Furthermore we also hypothesized that D-HF patients may have a reduced expression of the transcriptional coactivator PGC-1α, as this regulates mitochondrial biogenesis and is reduced in both CHF (Garnier *et al.*, 2003; Zoll *et al.*, 2006; Seiler *et al.*, 2016) and DM (Sreekumar *et al.*, 2002; Yang *et al.*, 2002; Mootha *et al.*, 2003; Patti *et al.*, 2003). Surprisingly, however, while this study did not observe a consistent fibre type shift to type II fibres, there was a tendency for PGC-1α expression to be reduced across the patient cohorts by ~25%. Therefore further studies are required to elucidate the specific mechanisms contributing to reduced mitochondrial content in D-HF patients.

7.3.1.2 Complex I mitochondrial dysfunction

Complex I and complex II are the two initial electron-accepting enzymes which enable ATP resynthesis via oxidative phosphorylation. Both of these complexes facilitate redox reactions that result in electrons being transported to complex IV, where O_2 acts as the final electron acceptor. Of these two complexes, complex I has a higher phosphate-to-oxygen ratio, thus it resynthesizes more ATP per molecule O_2 consumed (Hinkle, 2005). As such, complex I has a higher capacity for ATP resynthesis and any limitation in electron flow from complex I to complex IV has the potential to reduce proton translocation across the inner mitochondrial membrane and decrease the proton-motive force required for ATP resynthesis at complex V. Therefore an impairment in complex I is likely to reduce ATP resynthesis and limit exercise tolerance. Aside from a quantitative decrease in mitochondrial content, this thesis also observed a decrease in qualitative complex I function in D-HF patients, thereby suggesting that electron flow is impaired through this complex. Thereafter, we measured the gene expression of two key subunits of complex I (NDUFS1 and NDUFS3) that are involved in transporting electrons following NADH oxidation to the electron carrier ubiquinone. Of these, we found a lower gene expression of NDUFS1, which is the largest subunit of complex I (Hirst, 2013). Hence, it may be postulated that D-HF impairs complex I function, possibly by impacting NDUFS1, which subsequently decreases electron flow and oxidative ATP resynthesis to limit exercise tolerance.

7.3.2 Atrophy

Several previous studies have shown that skeletal muscle mass is reduced in CHF and DM (Anker *et al.*, 1997; Park *et al.*, 2006; Park *et al.*, 2009; Kim *et al.*, 2010; Leenders *et al.*, 2013; Kim *et al.*, 2014) and this contributes to exercise intolerance, in terms of reduced muscle strength and endurance (Minotti *et al.*, 1993; Cetinus *et al.*, 2005; Park *et al.*, 2006; Park *et al.*, 2007; Fulster *et al.*, 2013; Almurdhi *et al.*, 2016; Almurdhi *et al.*, 2017). This reduction in muscle mass has been attributed to fibre atrophy i.e. a reduction in the fibre cross-sectional area in both CHF and DM (Almond & Enser, 1984; Lipkin *et al.*, 1988; Mancini *et al.*, 1989; Sullivan *et al.*, 1990; Massie *et al.*, 1996; Delp *et al.*, 1997; Xu *et al.*, 1998; Larsen *et al.*, 2002; Li *et al.*, 2007; Yu *et al.*, 2008; Kemp *et al.*, 2009). Importantly, skeletal muscle wasting and weakness are associated with reduced $\dot{V}O_{2peak}$ (Fulster *et al.*, 2013), insulin resistance (Park *et al.*, 2006; Doehner *et al.*, 2015) as well as an increased risk for hospitalisations and mortality (Anker *et al.*, 1997; Anker *et al.*, 2003; Rossignol *et al.*, 2015). In the current thesis, we identified that fibre atrophy was type II specific in D-HF patients (Results IV). This finding has been previously reported in both CHF (Sullivan *et al.*, 1990; Xu *et al.*, 1998; Li *et al.*, 2007; Yu *et al.*, 2008) and DM (Almond & Enser, 1984; Kemp *et al.*, 2009). As type II fibres are larger and primarily support fast rapid contractions (Schiaffino & Reggiani, 2011), a type II fibre-specific atrophy likely contributes to muscle wasting and reduced skeletal muscle power and force generating capacity observed in both CHF and DM (Buller *et al.*, 1991; Minotti *et al.*, 1991; Minotti *et al.*, 1992; Harrington *et al.*, 1997; Sayer *et al.*, 2005; Park *et al.*, 2006; Park *et al.*, 2007; Toth *et al.*, 2010; Volpato *et al.*, 2012; Leenders *et al.*, 2013; Almurdhi *et al.*, 2016). Indeed, during the ageing process, type II fibre atrophy has been implicated in the decline of functional capacity (Deschenes, 2004;

Brunner *et al.*, 2007; Picard *et al.*, 2011a; Nilwik *et al.*, 2013; Murgia *et al.*, 2017). Therefore type II fibre-specific atrophy likely contributes to the greater exercise intolerance observed in D-HF patients. Nevertheless, future studies are required to examine how D-HF impacts upon the numerous pathways involved in muscle protein synthesis and degradation.

7.3.3 Capillary rarefaction

In addition to type II fibre atrophy, we also observed a type II fibre-specific capillary rarefaction (Results IV). This reduction in capillarity has the potential to reduce both convective and diffusive O_2 delivery to the mitochondria, thereby potentially decreasing $\dot{V}O_{2peak}$, as previously shown (Roca *et al.*, 1989). Several previous studies have shown a reduced skeletal muscle capillarisation in both CHF and DM (Marin *et al.*, 1994; Schaufelberger *et al.*, 1995; Schaufelberger *et al.*, 1997; Duscha *et al.*, 1999; Nusz *et al.*, 2003; Williams *et al.*, 2004; Groen *et al.*, 2014). However, the majority of these studies have relied on global indices of capillarity that are not as sensitive as localised indices, which give an indication of fibre-type specific O_2 delivery (Al-Shammari *et al.*, 2014). This reduction in capillarity, possibly in combination with endothelial dysfunction and sympathetic vasoconstriction, which both occur in the microvasculature of DM and CHF patients (Bauer *et al.*, 2007; Muniyappa & Sowers, 2013; Piepoli & Crisafulli, 2014; Poole *et al.*, 2018), may impair O_2 delivery further. This localised tissue hypoxic is known to deactivate complex I (Galkin *et al.*, 2009) and may also contribute to the mitochondrial complex I impairments we observed in the D-HF patients. Nevertheless further research is required to ascertain if a fibre

type-specific capillary rarefaction does actually induce localised tissue hypoxia and deactivate complex I in D-HF.

7.4 What upstream pathways possibly contribute to mitochondrial dysfunction and fibre atrophy?

Several studies have implicated oxidative stress in both fibre atrophy and mitochondrial dysfunction in both CHF and DM (Bonnard *et al.*, 2008; Koves *et al.*, 2008; Anderson *et al.*, 2009; Ohta *et al.*, 2011). Therefore we determined mitochondrial ROS production and mitochondrial antioxidant enzyme gene expression in Results IV. This demonstrated that D-HF patients exhibit increased mitochondrial ROS generation and a reduced gene expression of manganese SOD2, a matrix-specific antioxidant. Several distinct pathways involved in CHF and DM are known to induce oxidative stress, atrophy and mitochondrial dysfunction. Given that these pathways represent potential therapeutic targets in D-HF patients, each of these warrant further discussion.

7.4.1 Does oxidative stress contribute to complex I mitochondrial dysfunction?

One hypothesis generated from this thesis is that increased oxidative stress induces a mitochondrial complex I specific myopathy in D-HF. Given that complex I function, the respiratory control ratio (RCR), and NDUFS1 gene expression were all lower in parallel to elevated mitochondrial ROS production in D-HF compared to CHF, it may be speculated that increased mitochondrial ROS production damages the large complex I subunit NDUFS1. NDUFS1 damage would be predicted to limit the capacity of the iron-sulphur chains of

complex I to transfer electrons from NADH oxidation to the ubiquinone binding site and electrons may react with O_2 instead to form superoxide anion ($O_2^{\cdot-}$). In theory, this could create a vicious cycle between complex I damage and increased mitochondrial ROS production. Indeed complex I inhibition has previously been shown to increase mitochondrial ROS production (Li *et al.*, 2003a). Furthermore, our finding that ROS production was greatest with complex I substrates supports such a process and this is consistent with the finding that increased ROS production is associated with a decreased protein abundance of complex I subunits in insulin-resistant individuals (Lefort *et al.*, 2010). Given that many previous studies in DM and CHF have demonstrated that increased circulating angiotensin II, inflammation, hyperglycaemia- and hyperlipidaemia-induced substrate overload all contribute to oxidative stress, mitochondrial dysfunction and fibre atrophy, it is worth discussing how each of these pathways may interact in D-HF patients.

7.4.2 Role of circulating pro-inflammatory cytokines in D-HF

Both CHF and DM are associated with a chronically increased concentration of circulating and muscle pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α) and the interleukins (IL), IL-1β and IL-6 (Gielen *et al.*, 2003; Donath & Shoelson, 2011; Fulster *et al.*, 2013; Esser *et al.*, 2014; Lackey & Olefsky, 2016; Seiler *et al.*, 2016; Lavine & Sierra, 2017; von Haehling *et al.*, 2017). These inflammatory cytokines induce oxidative stress by increasing ROS production in mitochondria as well as via NADPH oxidase and NOS (Supinski & Callahan, 2007). Additionally, inflammation and oxidative stress promote atrophy via the ubiquitin-proteasome system, which is activated by the nuclear factor-κB

(NF-κB)-inhibitor of κB kinase (IκK) signalling pathway (Li *et al.*, 1998; Reid *et al.*, 2002; Li *et al.*, 2003b; Cai *et al.*, 2004; Hunter & Kandarian, 2004; Tantiwong *et al.*, 2010; Jackman *et al.*, 2013) as well as the p38 mitogen activated protein kinase pathway (Li *et al.*, 2005). This ubiquitin-proteasome pathway is coordinated by some key E3 ubiquitin-ligases, which include muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx)/Atrogin1 (Bodine *et al.*, 2001; Gomes *et al.*, 2001). These E3 ligases, which are upregulated by the forkhead box O1 (FOXO1) and FOXO3 transcription factors (Kamei *et al.*, 2004; Sandri *et al.*, 2004), promote muscle catabolism by increasing ubiquitin conjugation to muscle proteins that are to be degraded by the proteasome pathway (Bodine & Baehr, 2014). In CHF and DM, there is evidence that inflammation and oxidative stress upregulate the ubiquitin-proteasome system and promote atrophy (Fulster *et al.*, 2013; Bechara *et al.*, 2014; Cleasby *et al.*, 2016; Seiler *et al.*, 2016). Furthermore, inducible NOS (iNOS) promotes atrophy via apoptosis and this is associated with exercise intolerance in CHF (Adams *et al.*, 1997; Adams *et al.*, 1999; Hambrecht *et al.*, 1999a; Vescovo *et al.*, 2000). Finally, CHF and DM have also been associated with a reduced expression of several catabolic protein synthesis pathways, including insulin-like growth factor-1 (Hambrecht *et al.*, 2002) and AKT phosphorylation (Wang *et al.*, 2006). Collectively, therefore, it may be postulated that increased inflammation and oxidative stress in D-HF patients contribute to mitochondrial dysfunction and fibre atrophy.

7.4.3 Role of hyperglycaemia and hyperlipidaemia in D-HF

It has been suggested that the chronic hyperlipidaemia and hyperglycaemia associated with DM and insulin-resistant states promotes mitochondrial

substrate overload, which results in electrons being diverted towards $O_2^{\cdot-}$ formation (Muoio, 2014). Such excessive $O_2^{\cdot-}$ formation, in combination with a decrease in antioxidant defences, as evidenced by a decreased SOD2 gene expression in this thesis, may explain the greater ROS production and mitochondrial dysfunction observed in the D-HF patients. Indeed, it has been demonstrated that diet-induced substrate overload promotes mitochondrial dysfunction and oxidative stress which interfere with insulin signalling pathways (Bonnard *et al.*, 2008; Koves *et al.*, 2008; Anderson *et al.*, 2009). The prevailing hypothesis is that mitochondrial dysfunction and oxidative stress precede insulin resistance by reducing the capacity to oxidize free fatty acids, which subsequently leads to the accumulation of ectopic intramyocellular lipids (IMCLs) that interfere with insulin signalling pathways (Schrauwen-Hinderling *et al.*, 2007; Muoio, 2010; Szendroedi *et al.*, 2011; Hoeks & Schrauwen, 2012). This inhibition of the insulin signalling pathway downregulates muscle glucose transport and phosphorylation thereby promoting insulin resistance (Roden *et al.*, 1996). Recent studies in D-HF patients have shown that this pathway may be involved, as evidenced from lower SOD2 enzyme activity levels accompanied with increased markers of oxidative stress, including protein carbonylation and nitrotyrosine residue formation (Ramirez-Sanchez *et al.*, 2013).

In addition to mitochondrial dysfunction, impaired insulin signalling has been implicated in skeletal muscle atrophy. For instance, suppression of the insulin signalling pathway in a *db/db* mouse model has been shown to phosphorylate FOXO transcriptions factors and increase the expression of MuRF1 and MAFbx (Wang *et al.*, 2006). Furthermore, in CHF patients, insulin resistance alone is associated with reduced muscle strength (Doehner *et al.*, 2015). Therefore,

given that mitochondrial ROS production is increased in type II fibres (Anderson & Neufer, 2006), and oxidative stress is implicated in impaired insulin signalling and atrophy, it may be hypothesised that the type II fibre-specific atrophy observed in D-HF patients in the current study was induced by ROS.

The chronic hyperglycaemia associated with D-HF also likely exerts several deleterious effects on many of the integrative pathways involved in skeletal muscle mitochondrial oxygen consumption. Aside from potentially interfering with intracellular metabolic pathways, hyperglycaemia is known to impair micro- and macrovascular function. In particular, hyperglycaemia is known to induce endothelial dysfunction, which subsequently impairs vasodilation to peripheral tissues and decreases glucose uptake. Furthermore endothelial dysfunction is associated with microvascular complications, such as retinopathy and neuropathy, as well as the macrovascular development of atherosclerosis. However, there is currently limited research into how hyperglycaemia-induced endothelial dysfunction impacts upon the microvasculature and skeletal muscle mitochondrial function in D-HF patients. Therefore further research is warranted to assess the role of endothelial dysfunction in the pathogenesis of D-HF.

7.4.4 Role of angiotensin II in D-HF

One potentially unifying mechanism by which both CHF and DM may contribute to oxidative stress, mitochondrial dysfunction and fibre atrophy is the overactivity of the renin-angiotensin aldosterone system (RAAS) seen in these conditions (Henriksen *et al.*, 2011; Tsutsui *et al.*, 2011). CHF increases both peripheral and central angiotensin II (Ang II) expression and plasma renin activity to modulate

sympathetic activity (Zucker *et al.*, 2004) whilst Ang II is also increased in DM by hyperglycaemia-induced oxidative stress (Dikalov & Nazarewicz, 2013). In each case, Ang II binds to receptors on the skeletal muscle membrane to activate NADPH oxidase, which produces $O_2^{\cdot-}$ by the one-electron reduction of O_2 and oxidation of NADPH (Wei *et al.*, 2006; Dikalov & Nazarewicz, 2013). This pathway decreases insulin signalling in myotubes, a process which was negated by the AT1R and NADPH oxidase inhibitors, losartan and apocynin, and with small interfering RNA (siRNA) targeted against p47^{phox}, a cytosolic subunit of NADPH oxidase (Wei *et al.*, 2006). Interestingly, this same pathway has been implicated in diaphragm muscle contractile dysfunction (Ahn *et al.*, 2015; Bost *et al.*, 2015) as well as mitochondrial dysfunction (Ohta *et al.*, 2011) in animal models of CHF. In two of these studies, RAAS overactivation enhanced skeletal muscle oxidative stress by increased NADPH oxidase activity, which subsequently decreased insulin-stimulated serine phosphorylation of Akt and glucose transporter-4 translocation to the cell membrane, resulting in insulin resistance (Wei *et al.*, 2006; Ohta *et al.*, 2011). Furthermore, Ang II inhibition with olmesartan has also been shown to improve exercise capacity in DM mice by attenuating mitochondrial dysfunction induced by oxidative stress (Takada *et al.*, 2013).

Additionally, Ang II is known to promote muscle atrophy via FOXO increased expression of MuRF1 and MAFbx (Sukhanov *et al.*, 2011; Yoshida *et al.*, 2013; Du Bois *et al.,* 2015; Yoshida & Delafontaine, 2015). Indeed, Ang II administration to mice has also been shown to induce mitochondrial dysfunction, including reduced complex I and citrate synthase activities, in addition to fibre atrophy (Kadoguchi *et al.*, 2015). Collectively, these studies suggest that

increased concentrations of circulating Ang II may play a role in mitochondrial dysfunction, increased ROS and fibre atrophy, all of which were observed in the current study in D-HF patients.

7.4.5 Is ROS-mediated complex I dysfunction caused by hypoxic deactivation of the enzyme?

ROS can directly inhibit complex I when it has undergone a structural conformational change from the active to deactive form of the enzyme (Clementi *et al.*, 1998; Gostimskaya *et al.*, 2006; Galkin & Moncada, 2007). Prolonged hypoxia has been shown to induce deactivation **(Galkin et al., 2009)** by exposing a cysteine residue, Cys39, located within a hydrophilic loop connecting two transmembrane helices on the matrix side of the mitochondrially encoded ND3 subunit of complex I (Galkin *et al.*, 2008). This exposure renders complex I susceptible to covalent modifications by ROS products (Galkin *et al.*, 2008). It has been hypothesised that these modifications produce a shift in the ND3 subunit loop which disrupts the quinone binding site, where electrons are accepted by ubiquinone, reducing it to ubiquinol (Galkin & Moncada, 2017). As this redox reaction is catalytically coupled with proton translocation (Ripple *et al.*, 2013), this deactivation of complex I reduces electron flow and the mitochondrial membrane potential, which consequently decrease O_2 flux and ATP resynthesis. It may be hypothesised that the decreased type II fibre-specific capillarity observed in D-HF patients in the current thesis may promote hypoxia, thus encouraging the active-deactive transition of complex I. Therefore it may be postulated that diffusive skeletal muscle O_2 delivery may be compromised in D-HF patients, reducing tissue O_2 levels and deactivating complex I. Figure 7.2 provides an overview of the potential pathways that may contribute to

mitochondrial dysfunction, oxidative stress, fibre atrophy, and, ultimately, exercise intolerance in D-HF patients.
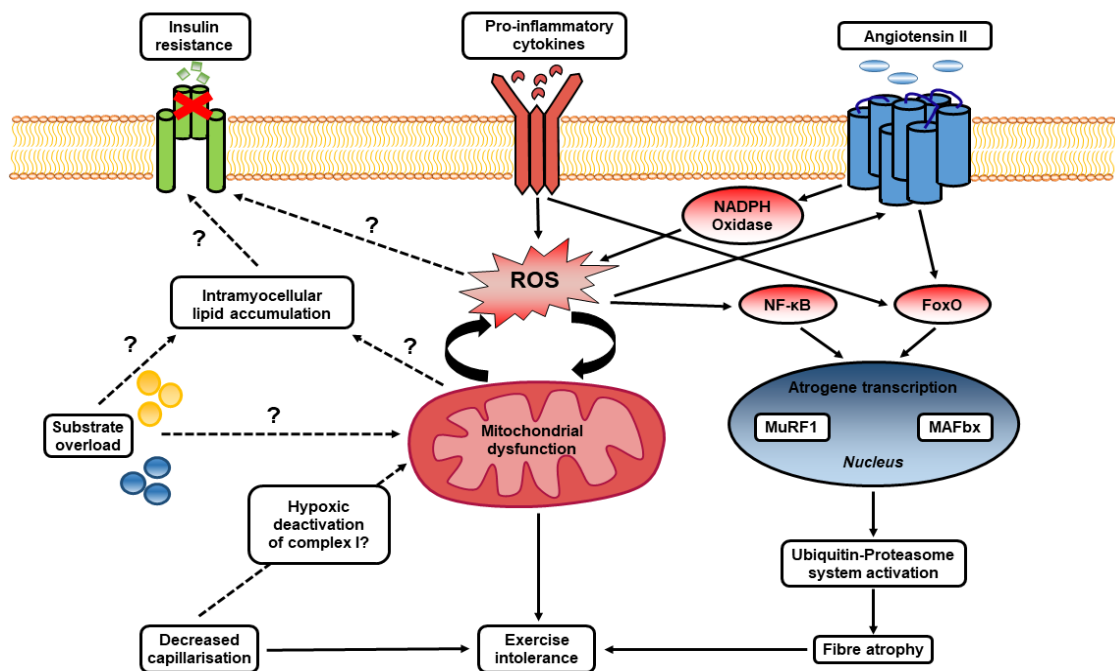
Figure 7.2. General overview of putative pathways that may mediate exercise intolerance in D-HF patients, as derived from the data collected in this thesis. See text for expanded details.

7.5 Experimental considerations and limitations

7.5.1 Methodology

Ex vivo measures of mitochondrial function using high-resolution respirometry are thought to reflect mitochondrial function and metabolism *in vivo*. Previous studies have validated this assumption by showing that *in vivo* measurements of mitochondrial function strongly correlate with *ex vivo* measurements obtained from high-resolution respirometry using both isolated mitochondria (Lanza *et al.*, 2011) and saponin-permeabilized muscle fibres (Ryan *et al.*, 2014). Nevertheless, it may be argued that the artificial hyperoxygenated environment and substrate saturation protocol employed in this current thesis is not reflective of *in vivo* mitochondrial function. Of particular relevance is the bolus saturating

concentration of ADP (2.5 mM), which is not accurately reflective of *in vivo* function where ADP concentrations are in constant flux as dictated by the demand for ATP resynthesis. Therefore future experiments, as recently demonstrated (Gonzalez-Freire *et al.*, 2018), may wish to incorporate several titrations of ADP that cover a spectrum of more physiologically-relevant ADP concentrations seen *in vivo*. Nevertheless, given that our methodology was consistent across all patient groups, the finding of reduced complex I OXPHOS is still of significance. Indeed, previous studies have shown that *in situ* mitochondrial respiration in the presence of saturating ADP significantly correlates with $\dot{V}O_{2peak}$ across a range of athletic abilities (Zoll *et al.*, 2002). To corroborate the current findings, future studies should assess *in vivo* skeletal muscle mitochondrial function using ^{31}P MRS in D-HF patients.

7.5.2 Patients

Clinical human research is often impacted by marked heterogeneity between patients. This limits studies, such as the current thesis, to observational evidence, which can demonstrate associations but not prove causality. As with all human studies, we aimed to match the four patient cohorts as best as possible, in terms of age, sex and weight, to ameliorate the confounding effects that each of these are known to have on skeletal muscle mitochondrial function (Trounce *et al.*, 1989; Duscha *et al.*, 2001; Lesnefsky & Hoppel, 2006; Karakelides *et al.*, 2010; Murgia *et al.*, 2017). Nevertheless, future studies may wish to employ animal models to acquire better mechanistic insights in to the effects of D-HF on skeletal muscle mitochondrial function, atrophy, capillarisation and oxidative stress.

7.5.3 Drug therapy

Medications administered to patients always have potential to confound research findings. Of particular note in the current thesis is the drug metformin, which has been shown to inhibit complex I (Owen *et al.*, 2000; Brunmair *et al.*, 2004a; Brunmair *et al.*, 2004b) and reduce *in vivo* and *ex vivo* skeletal muscle oxidative capacity in a rat model of DM (Wessels *et al.*, 2014). However, human studies with DM patients have shown that metformin does not inhibit complex I (Larsen *et al.*, 2012b). In patients that provided *pectoralis major* samples, metformin use did not affect complex I O_2 flux in either the DM or D-HF cohorts (both $P > 0.05$; Figure 7.3). As such, these provide strong evidence that metformin cannot explain the severe mitochondrial deficits we observed in D-HF patients.
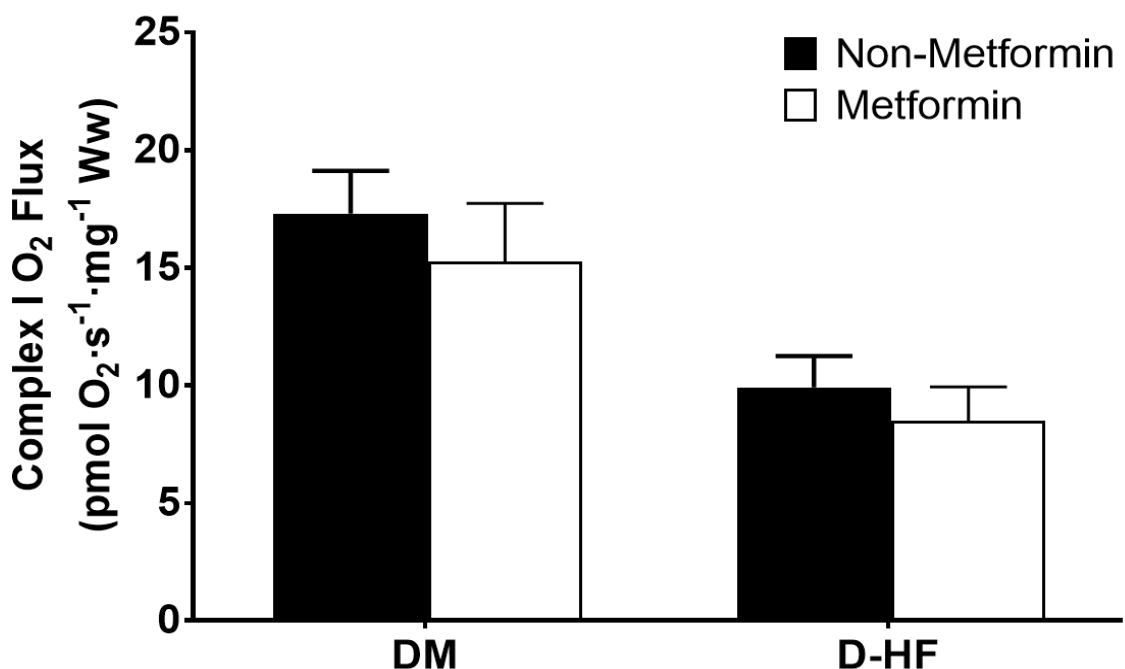
Figure 7.3. Metformin use did not affect complex I O_2 flux in either DM or D-HF patients. $P > 0.05$ using unpaired Student's *t*-tests.

7.6 Clinical implications and future directions

The first study of this thesis demonstrated the efficacy of a novel exercise protocol that arguably should be used in clinical settings in CHF patients. Firstly, the RISE-95 protocol is a more accurate method for confirming $\dot{V}O_{2max}$ in CHF patients. This is of significant clinical importance as $\dot{V}O_{2max}$ can accurately inform on the prognosis/symptomatic status of the patient and guide future therapeutic treatment(s) and/or clinical intervention(s). Secondly, this test may be used to delineate patients that have an "aerobic reserve".

Further research is required to identify the pathways that contribute to mitochondrial impairments and how these may be ameliorated. Exercise training remains a significant therapy for increasing mitochondrial content via contractile-induced upregulation of mitochondrial biogenesis pathways (Essig, 1996; Baar *et al.*, 2002). Several studies have shown the beneficial effects of regular aerobic exercise training on skeletal muscle mitochondrial function and content as well as metabolic flexibility and insulin sensitivity in DM patients (Toledo *et al.*, 2007; Meex *et al.*, 2010; Phielix *et al.*, 2010; Little *et al.*, 2011). Similarly, in CHF patients, exercise training can improve mitochondrial function (Hambrecht *et al.*, 1995; Hambrecht *et al.*, 1997; Santoro *et al.*, 2002; Williams *et al.*, 2007; Esposito *et al.*, 2011) and improve outcomes in terms of mortality and hospitalisations (O'Connor *et al.*, 2009). These improvements have been associated with exercise-induced anti-inflammatory (Gielen *et al.*, 2003; Gielen *et al.*, 2005), anti-oxidative (Linke *et al.*, 2005; Brinkmann *et al.*, 2012; de Oliveira *et al.*, 2012), and anti-atrophic (Lenk *et al.*, 2012) effects. Exercise training can also increase $\dot{V}O_{2peak}$ in D-HF patients, although this is comparably blunted compared to CHF patients (Banks *et al.*, 2016).

Given the close associations between Ang II and oxidative stress with mitochondrial dysfunction and fibre atrophy, previous studies in CHF and DM animal models have demonstrated that inhibiting these pathways can improve exercise tolerance (Ohta *et al.*, 2011; Takada *et al.*, 2013; Okutsu *et al.*, 2014). Therefore future studies may wish to investigate the effects of antioxidant treatments on skeletal muscle mitochondrial function and exercise intolerance in D-HF patients. However, antioxidant supplements should be used cautiously to avoid inhibiting the exercise training adaptations that low levels of ROS are known to induce (Gomez-Cabrera *et al.*, 2008; Mankowski *et al.*, 2015).

In addition to these interventional studies, future studies in animal models are required to further elucidate the mechanisms of mitochondrial dysfunction in D-HF, particularly in terms of identifying the pathways reducing complex I function, as this may be considered a potential therapeutic target. Additionally, it may be possible to determine the aetiology and pathophysiology behind the development of D-HF by inducing both DM and CHF in separate animal groups and chronicling the development and incidence of the other condition. This may potentially uncover what mechanisms drive the close association between these two conditions.

7.7 Thesis conclusions

The studies outlined in this thesis have revealed novel findings related to the aetiology of diabetic heart failure. In the first study, a novel exercise protocol (RISE-95) was employed to demonstrate that nearly half of CHF and D-HF

patients have an "aerobic reserve", but surprisingly these patients were characterised by a poorer prognosis. An improved understanding of the mechanisms underlying this "aerobic reserve" may potentially lead to the development of pharmacological treatments that can acutely increase $\dot{V}O_{2peak}$ in order to ameliorate symptoms of exercise intolerance in these patients.

Given that patients tested in the first study exhibited evidence of intramuscular impairments, the second and third studies of this thesis obtained skeletal muscle biopsies to identify that D-HF patients present with both quantitative and qualitative mitochondrial impairments, the latter of which resides at complex I. Comparable impairments were observed in both leg and chest muscle, suggesting that a systemic factor may be mediating these effects. Importantly, these mitochondrial impairments correlated with $\dot{V}O_{2peak}$, thereby suggesting that mitochondrial dysfunction may underlie the exacerbated exercise intolerance in D-HF patients.

The final study of this thesis assessed potential mechanisms underlying mitochondrial dysfunction in D-HF. This study identified that D-HF patients have type II fibre-specific atrophy and capillary rarefaction as well as an increase in mitochondrial ROS production. Furthermore this study also identified decrements in the expression of complex I and a mitochondrial antioxidant gene in D-HF patients. These findings suggest that certain pathways involved in both CHF and DM, such as chronic inflammation, elevated Ang II concentrations, and substrate overload may coalesce to increase oxidative stress, mitochondrial

dysfunction and fibre atrophy, all of which likely contribute to exercise intolerance (Figure 7.2).

Collectively, the evidence obtained from these studies demonstrate that D-HF patients exhibit various skeletal muscle impairments, many of which likely contribute to exercise intolerance. It is now of paramount importance to further elucidate the mechanisms and pathways contributing to these impairments such that effective therapeutic treatments can be developed to improve the symptoms, prognosis and outcomes in the growing population of D-HF patients

References

Abdul-Ghani MA & DeFronzo RA (2010). Pathogenesis of insulin resistance in skeletal muscle. *J Biomed Biotechnol*, **2010**, 1-19.

Abdul-Ghani MA, DeFronzo RA, Del Prato S, Chilton R, Singh R & Ryder REJ (2017). Cardiovascular disease and type 2 diabetes: Has the dawn of a new era arrived? *Diabetes Care*, **40**(7), 813-820.

Abraham WT, Fisher WG, Smith AL, Delurgio DB, Leon AR, Loh E,... & Messenger J (2002). Cardiac resynchronization in chronic heart failure. *N Engl J Med*, **346**(24), 1845-1853.

Abraham WT & Smith SA (2013). Devices in the management of advanced, chronic heart failure. *Nat Rev Cardiol*, **10**(2), 98-110.

Adams V, Yu J, Möbius-Winkler S, Linke A, Weigl C, Hilbrich L,... & Hambrecht R (1997). Increased inducible nitric oxide synthase in skeletal muscle biopsies from patients with chronic heart failure. *Biochem Mol Med*, **61**(2), 152-160.

Adams V, Jiang H, Yu J, Möbius-Winkler S, Fiehn E, Linke A,... & Hambrecht R (1999). Apoptosis in skeletal myocytes of patients with chronic heart failure is associated with exercise intolerance. *J Am Coll Cardiol*, **33**(4), 959-965.

Ahmed SK, Egginton S, Jakeman PM, Mannion AF & Ross HF (1997). Is human skeletal muscle capillary supply modelled according to fibre size or fibre type? *Exp Physiol*, **82**(1), 231-234.

Ahn B, Beharry AW, Frye GS, Judge AR & Ferreira LF (2015). NAD(P)H oxidase subunit p47^{phox} is elevated, and p47^{phox} knockout prevents diaphragm contractile dysfunction in heart failure. *Am J Physiol Lung Cell Mol Physiol* **309**(5), L497-L505.

Al-Shammari AA, Gaffney EA & Egginton S (2014). Modelling capillary oxygen supply capacity in mixed muscles: capillary domains revisited. *J Theor Biol*, **356**, 47-61.

Almond RE & Enser M (1984). A histochemical and morphological study of skeletal muscle from obese hyperglycaemic *ob/ob* mice. *Diabetologia*, **27**(3), 407-413.

Almurdhi MM, Reeves ND, Bowling FL, Boulton AJ, Jeziorska M & Malik RA (2016). Reduced lower-limb muscle strength and volume in patients with type 2 diabetes in relation to neuropathy, intramuscular fat, and vitamin D levels. *Diabetes Care*, **39**(3), 441-447.

Almurdhi MM, Reeves ND, Bowling FL, Boulton AJ, Jeziorska M & Malik RA (2017). Distal lower limb strength is reduced in subjects with impaired glucose tolerance and is related to elevated intramuscular fat level and vitamin D deficiency. *Diabet Med*, **34**(3), 356-363.

AlZadjali MA, Godfrey V, Khan F, Choy A, Doney AS, Wong AK,... & Lang CC (2009). Insulin resistance is highly prevalent and is associated with reduced exercise tolerance in nondiabetic patients with heart failure. *J Am Coll Cardiol*, **53**(9), 747-753.

Anderson EJ & Neufer PD (2006). Type II skeletal myofibers possess unique properties that potentiate mitochondrial H_2O_2 generation. *Am J Physiol Cell Physiol*, **290**(3), C844-C851.

Anderson EJ, Lustig ME, Boyle KE, Woodlief TL, Kane DA, Lin CT,... & Neufer PD (2009). Mitochondrial H_2O_2 emission and cellular redox state link

excess fat intake to insulin resistance in both rodents and humans. *J Clin Invest*, **119**(3), 573-581.

Andreassen CS, Jensen JM, Jakobsen J, Ulhøj BP & Andersen H (2014). Striated muscle fiber size, composition, and capillary density in diabetes in relation to neuropathy and muscle strength. *J Diabetes*, **6**(5), 462-471.

Anker SD, Ponikowski P, Varney S, Chua TP, Clark AL, Webb-Peploe KM,... & Coats AJS (1997). Wasting as independent risk factor for mortality in chronic heart failure. *Lancet*, **349**(9058), 1050-1053.

Anker SD, Negassa A, Coats AJS, Afzal R, Poole-Wilson PA, Cohn JN & Yusuf S (2003). Prognostic importance of weight loss in chronic heart failure and the effect of treatment with angiotensin-converting-enzyme inhibitors: an observational study. *Lancet*, **361**(9363), 1077-1083.

Arena R, Myers J, Aslam SS, Varughese EB & Peberdy MA (2004). Peak $\dot{V}O_2$ and $\dot{V}_E/\dot{V}CO_2$ slope in patients with heart failure: a prognostic comparison. *Am Heart J*, **147**(2), 354-360.

Astorino TA, Bediamol N, Cotoia S, Ines K, Koeu N, Menard N,... & Cruz GV (2018). Verification testing to confirm $\dot{V}O_{2max}$ attainment in persons with spinal cord injury. *J Spinal Cord Med*, 1-8.

Azevedo P, Bhammar D, Babb T, Bowen T, Witte K, Rossiter H,... & Turnes T (2018). Commentaries on Viewpoint: $\dot{V}O_{2peak}$ is an acceptable estimate of cardiorespiratory fitness but not $\dot{V}O_{2max}$. *J Appl Physiol*, **125**(1), 233.

Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M,... & Holloszy JO (2002). Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. *FASEB J*, **16**(14), 1879-1886.

Bajpeyi S, Pasarica M, Moro C, Conley K, Jubrias S, Sereda O,... & Smith SR (2011). Skeletal muscle mitochondrial capacity and insulin resistance in type 2 diabetes. *J Clin Endocrinol Metab*, **96**(4), 1160-1168.

Banks AZ, Mentz RJ, Stebbins A, Mikus CR, Schulte PJ, Fleg JL,... & O'Connor CM (2016). Response to exercise training and outcomes in patients with heart failure and diabetes mellitus: insights from the HF-ACTION trial. *J Card Fail*, **22**(7), 485-491.

Barbosa MR, Sampaio IH, Teodoro BG, Sousa TA, Zoppi CC, Queiroz AL,... & Silveira LR (2013). Hydrogen peroxide production regulates the mitochondrial function in insulin resistant muscle cells: effect of catalase overexpression. *Biochim Biophys Acta*, **1832**(10), 1591-1604.

Bauer TA, Reusch JE, Levi M & Regensteiner JG (2007). Skeletal muscle deoxygenation after the onset of moderate exercise suggests slowed microvascular blood flow kinetics in type 2 diabetes. *Diabetes Care*, **30**(11), 2880-2885.

Bayley JS, Pedersen TH & Nielsen OB (2016). Skeletal muscle dysfunction in the *db/db* mouse model of type 2 diabetes. *Muscle Nerve*, **54**(3), 460-468.

Beagley J, Guariguata L, Weil C & Motala AA (2014). Global estimates of undiagnosed diabetes in adults. *Diabetes Res Clin Pract*, **103**(2), 150-160.

Beaver WL, Wasserman K & Whipp BJ (1986). A new method for detecting anaerobic threshold by gas exchange. *J Appl Physiol*, **60**(6), 2020-2027.

Bechara LR, Moreira JB, Jannig PR, Voltarelli VA, Dourado PM, Vasconcelos AR,... & Brum PC (2014). NADPH oxidase hyperactivity induces plantaris atrophy in heart failure rats. *Int J Cardiol*, **175**(3), 499-507.

Bekelman DB, Havranek EP, Becker DM, Kutner JS, Peterson PN, Wittstein IS,... & Dy SM (2007). Symptoms, depression, and quality of life in patients with heart failure. *J Card Fail*, **13**(8), 643-648.

Belardinelli R, Georgiou D, Cianci G & Purcaro A (2012). 10-year exercise training in chronic heart failure: a randomized controlled trial. *J Am Coll Cardiol*, **60**(16), 1521-1528.

Benda NM, Seeger JP, Stevens GG, Hijmans-Kersten BT, van Dijk AP, Bellersen L,... & Thijssen DH (2015). Effects of high-intensity interval training versus continuous training on physical fitness, cardiovascular function and quality of life in heart failure patients. *PLoS One*, **10**(10), e0141256.

Bergström J (1962). Muscle electrolytes in man determined by neutron activation analysis on needle biopsy specimens. *Scand J Clin Lab Invest*, **14**(Suppl 68), 11-13.

Betts TR & de Bono JP (2010). Devices for heart failure. *Medicine*, **38**(9), 479-483.

Bianchi L & Volpato S (2016). Muscle dysfunction in type 2 diabetes: a major threat to patient's mobility and independence. *Acta Diabetol*, **53**(6), 879-889.

Bloom MW, Greenberg B, Jaarsma T, Januzzi JL, Lam CSP, Maggioni AP,... & Butler J (2017). Heart failure with reduced ejection fraction. *Nat Rev Dis Primers*, **3**, 1-19.

Boden MJ, Brandon AE, Tid-Ang JD, Preston E, Wilks D, Stuart E,... & Kraegen EW (2012). Overexpression of manganese superoxide dismutase ameliorates high-fat diet-induced insulin resistance in rat skeletal muscle. *Am J Physiol Endocrinol Metab*, **303**(6), E798-E805.

Bodine SC, Latres E, Baumhueter S, Lai VK-M, Nunez L, Clarke BA,... & Dharmarajan K (2001). Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science*, **294**(5547), 1704-1708.

Bodine SC & Baehr LM (2014). Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and MAFbx/atrogin-1. *Am J Physiol Endocrinol Metab*, **307**(6), E469-E484.

Bonnard C, Durand A, Peyrol S, Chanseaume E, Chauvin MA, Morio B,... & Rieusset J (2008). Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J Clin Invest*, **118**(2), 789-800.

Bonner JS, Lantier L, Hasenour CM, James FD, Bracy DP & Wasserman DH (2013). Muscle-specific vascular endothelial growth factor deletion induces muscle capillary rarefaction creating muscle insulin resistance. *Diabetes*, **62**(2), 572-580.

Bost ER, Frye GS, Ahn B & Ferreira LF (2015). Diaphragm dysfunction caused by sphingomyelinase requires the $p47^{phox}$ subunit of NADPH oxidase. *Resp Physiol & Neurobi*, **205**, 47-52.

Boushel R, Gnaiger E, Schjerling P, Skovbro M, Kraunsoe R & Dela F (2007). Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle. *Diabetologia*, **50**(4), 790-796.

Boushel R, Gnaiger E, Calbet JA, Gonzalez-Alonso J, Wright-Paradis C, Sondergaard H,... & Saltin B (2011). Muscle mitochondrial capacity exceeds maximal oxygen delivery in humans. *Mitochondrion*, **11**(2), 303-307.

Boveris A & Chance B (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J*, **134**(3), 707-716.

Bowen TS, Cannon DT, Begg G, Baliga V, Witte KK & Rossiter HB (2012a). A novel cardiopulmonary exercise test protocol and criterion to determine

maximal oxygen uptake in chronic heart failure. *J Appl Physiol*, **113**(3), 451-458.

Bowen TS, Cannon DT, Murgatroyd SR, Birch KM, Witte KK & Rossiter HB (2012b). The intramuscular contribution to the slow oxygen uptake kinetics during exercise in chronic heart failure is related to the severity of the condition. *J Appl Physiol*, **112**(3), 378-387.

Bowen TS, Mangner N, Werner S, Glaser S, Kullnick Y, Schrepper A,... & Adams V (2015a). Diaphragm muscle weakness in mice is early-onset post-myocardial infarction and associated with elevated protein oxidation. *J Appl Physiol*, **118**(1), 11-19.

Bowen TS, Schuler G & Adams V (2015b). Skeletal muscle wasting in cachexia and sarcopenia: molecular pathophysiology and impact of exercise training. *J Cachexia Sarcopenia Muscle*, **6**(3), 197-207.

Bowler MW, Montgomery MG, Leslie AG & Walker JE (2006). How azide inhibits ATP hydrolysis by the F-ATPases. *Proc Natl Acad Sci USA*, **103**(23), 8646-8649.

Brassard P, Maltais F, Noël M, Doyon J-F, LeBlanc P, Allaire J,... & Jobin J (2006). Skeletal muscle endurance and muscle metabolism in patients with chronic heart failure. *Can J Cardiol*, **22**(5), 387-392.

Bravard A, Lefai E, Meugnier E, Pesenti S, Disse E, Vouillarmet J,... & Rieusset J (2011). FTO is increased in muscle during type 2 diabetes, and its overexpression in myotubes alters insulin signaling, enhances lipogenesis and ROS production, and induces mitochondrial dysfunction. *Diabetes*, **60**(1), 258-268.

Brinkmann C, Chung N, Schmidt U, Kreutz T, Lenzen E, Schiffer T,... & Brixius K (2012). Training alters the skeletal muscle antioxidative capacity in non-insulin-dependent type 2 diabetic men. *Scand J Med Sci Sports*, **22**(4), 462-470.

Bristow MR (2012). Pharmacogenetic targeting of drugs for heart failure. *Pharmacol Ther*, **134**(1), 107-115.

Brunmair B, Lest A, Staniek K, Gras F, Scharf N, Roden M,... & Furnsinn C (2004a). Fenofibrate impairs rat mitochondrial function by inhibition of respiratory complex I. *J Pharmacol Exp Ther*, **311**(1), 109-114.

Brunmair B, Staniek K, Gras F, Scharf N, Althaym A, Clara R,... & Waldhäusl W (2004b). Thiazolidinediones, like metformin, inhibit respiratory complex I: a common mechanism contributing to their antidiabetic actions? *Diabetes*, **53**(4), 1052-1059.

Brunner F, Schmid A, Sheikhzadeh A, Nordin M, Yoon J & Frankel V (2007). Effects of aging on Type II muscle fibers: a systematic review of the literature. *J Aging Phys Activ*, **15**(3), 336-348.

Buchfuhrer MJ, Hansen JE, Robinson TE, Sue DY, Wasserman K & Whipp BJ (1983). Optimizing the exercise protocol for cardiopulmonary assessment. *J Appl Physiol Respir Environ Exerc Physiol*, **55**(5), 1558-1564.

Bui AL, Horwich TB & Fonarow GC (2011). Epidemiology and risk profile of heart failure. *Nat Rev Cardiol*, **8**(1), 30-41.

Buller NP, Jones D & Poole-Wilson PA (1991). Direct measurement of skeletal muscle fatigue in patients with chronic heart failure. *Br Heart J*, **65**(1), 20-24.

Cai D, Frantz JD, Tawa NE, Jr., Melendez PA, Oh BC, Lidov HG,... & Shoelson SE (2004). IKKβ/NF-κB activation causes severe muscle wasting in mice. *Cell*, **119**(2), 285-298.

Cardinale DA, Gejl KD, Ortenblad N, Ekblom B, Blomstrand E & Larsen FJ (2018). Reliability of maximal mitochondrial oxidative phosphorylation in permeabilized fibers from the *vastus lateralis* employing high-resolution respirometry. *Physiol Rep*, **6**(4), 1-8.

Cetinus E, Buyukbese MA, Uzel M, Ekerbicer H & Karaoguz A (2005). Hand grip strength in patients with type 2 diabetes mellitus. *Diabetes Res Clin Pract*, **70**(3), 278-286.

Chatterjee S, Khunti K & Davies MJ (2017). Type 2 diabetes. *Lancet*, **389**(10085), 2239-2251.

Chen L, Magliano DJ & Zimmet PZ (2012). The worldwide epidemiology of type 2 diabetes mellitus - present and future perspectives. *Nat Rev Endocrinol*, **8**(4), 228-236.

Chomentowski P, Coen PM, Radikova Z, Goodpaster BH & Toledo FG (2011). Skeletal muscle mitochondria in insulin resistance: differences in intermyofibrillar versus subsarcolemmal subpopulations and relationship to metabolic flexibility. *J Clin Endocrinol Metab*, **96**(2), 494-503.

Church TS, LaMonte MJ, Barlow CE & Blair SN (2005). Cardiorespiratory fitness and body mass index as predictors of cardiovascular disease mortality among men with diabetes. *Arch Intern Med*, **165**(18), 2114-2120.

Clark AL, Davies LC, Francis DP & Coats AJ (2000). Ventilatory capacity and exercise tolerance in patients with chronic stable heart failure. *Eur J Heart Fail*, **2**(1), 47-51.

Clark AL & Cleland JG (2013). Causes and treatment of oedema in patients with heart failure. *Nat Rev Cardiol*, **10**(3), 156-170.

Cleasby ME, Jamieson PM & Atherton PJ (2016). Insulin resistance and sarcopenia: mechanistic links between common co-morbidities. *J Endocrinol*, **229**(2), R67-R81.

Cleland JG, Daubert J-C, Erdmann E, Freemantle N, Gras D, Kappenberger L & Tavazzi L (2005). The effect of cardiac resynchronization on morbidity and mortality in heart failure. *N Engl J Med*, **352**(15), 1539-1549.

Clementi E, Brown GC, Feelisch M & Moncada S (1998). Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc Natl Acad Sci USA*, **95**(13), 7631-7636.

Coirault C, Guellich A, Barbry T, Samuel JL, Riou B & Lecarpentier Y (2007). Oxidative stress of myosin contributes to skeletal muscle dysfunction in rats with chronic heart failure. *Am J Physiol Heart Circ Physiol*, **292**(2), H1009-H1017.

Conrad N, Judge A, Tran J, Mohseni H, Hedgecott D, Crespillo AP,... & Rahimi K (2018). Temporal trends and patterns in heart failure incidence: a population-based study of 4 million individuals. *Lancet*, **391**(10120), 572-580.

Corra U, Piepoli MF, Adamopoulos S, Agostoni P, Coats AJ, Conraads V,... & Ponikowski PP (2014). Cardiopulmonary exercise testing in systolic heart failure in 2014: the evolving prognostic role. A position paper from the committee on exercise physiology and training of the Heart Failure Association of the ESC. *Eur J Heart Fail*, **16**(9), 929-941.

Cowie MR (2017). The heart failure epidemic: a UK perspective. *Echo Res Pract*, **4**(1), R15-R20.

Cubbon RM, Adams B, Rajwani A, Mercer BN, Patel PA, Gherardi G,... & Kearney MT (2013). Diabetes mellitus is associated with adverse

prognosis in chronic heart failure of ischaemic and non-ischaemic aetiology. *Diab Vasc Dis Res*, **10**(4), 330-336.

Cunha TF, Bacurau AV, Moreira JB, Paixao NA, Campos JC, Ferreira JC,... & Brum PC (2012). Exercise training prevents oxidative stress and ubiquitin-proteasome system overactivity and reverse skeletal muscle atrophy in heart failure. *PLoS One*, **7**(8), e41701.

Dauriz M, Targher G, Laroche Ce, Temporelli PL, Ferrari R, Anker S,... & Tavazzi L (2017). Association between diabetes and 1-year adverse clinical outcomes in a multinational cohort of ambulatory patients with chronic heart failure: results from the ESC-HFA heart failure long-term registry. *Diabetes Care*, **40**, 671-678.

Day JR, Rossiter HB, Coats EM, Skasick A & Whipp BJ (2003). The maximally attainable $\dot{V}O_2$ during exercise in humans: the peak vs. maximum issue. *J Appl Physiol*, **95**(5), 1901-1907.

de Groote P, Lamblin N, Mouquet F, Plichon D, McFadden E, Van Belle E & Bauters C (2004). Impact of diabetes mellitus on long-term survival in patients with congestive heart failure. *Eur Heart J*, **25**(8), 656-662.

de Oliveira VN, Bessa A, Jorge ML, Oliveira RJ, de Mello MT, De Agostini GG,... & Espindola FS (2012). The effect of different training programs on antioxidant status, oxidative stress, and metabolic control in type 2 diabetes. *Appl Physiol Nutr Metab*, **37**(2), 334-344.

De Sousa E, Veksler V, Bigard X, Mateo P & Ventura-Clapier R (2000). Heart failure affects mitochondrial but not myofibrillar intrinsic properties of skeletal muscle. *Circulation*, **102**(15), 1847-1853.

Dei Cas A, Khan SS, Butler J, Mentz RJ, Bonow RO, Avogaro A,... & Fonarow GC (2015). Impact of diabetes on epidemiology, treatment, and outcomes of patients with heart failure. *JACC Heart Fail*, **3**(2), 136-145.

Delp MD, Duan C, Mattson JP & Musch TI (1997). Changes in skeletal muscle biochemistry and histology relative to fiber type in rats with heart failure. *J Appl Physiol*, **83**(4), 1291-1299.

Deschenes MR (2004). Effects of aging on muscle fibre type and size. *Sports Med*, **34**(12), 809-824.

DiFranco M, Quinonez M, Shieh P, Fonarow GC, Cruz D, Deng MC,... & Middlekauff HR (2014). Action potential-evoked calcium release is impaired in single skeletal muscle fibers from heart failure patients. *PLoS One*, **9**(10), e109309.

Dikalov SI & Nazarewicz RR (2013). Angiotensin II-induced production of mitochondrial reactive oxygen species: potential mechanisms and relevance for cardiovascular disease. *Antioxid Redox Signal*, **19**(10), 1085-1094.

DiMarco JP (2003). Implantable Cardioverter-Defibrillators. *N Engl J Med*, **349**(19), 1836-1847.

Distefano G, Standley RA, Dube JJ, Carnero EA, Ritov VB, Stefanovic-Racic M,... & Coen PM (2017). Chronological age does not influence *ex-vivo* mitochondrial respiration and quality control in skeletal muscle. *J Gerontol A Biol Sci Med Sci*, **72**(4), 535-542.

Doehner W, Rauchhaus M, Ponikowski P, Godsland IF, von Haehling S, Okonko DO,... & Anker SD (2005). Impaired insulin sensitivity as an independent risk factor for mortality in patients with stable chronic heart failure. *J Am Coll Cardiol*, **46**(6), 1019-1026.

Doehner W, Turhan G, Leyva F, Rauchhaus M, Sandek A, Jankowska EA,... & Anker SD (2015). Skeletal muscle weakness is related to insulin resistance in patients with chronic heart failure. *ESC Heart Fail*, **2**(2), 85-89.

Donath MY & Shoelson SE (2011). Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol*, **11**(2), 98-107.

Drexler H, Riede U, Munzel T, Konig H, Funke E & Just H (1992). Alterations of skeletal muscle in chronic heart failure. *Circulation*, **85**(5), 1751-1759.

Du Bois P, Pablo Tortola C, Lodka D, Kny M, Schmidt F, Song K,... & Fielitz J (2015). Angiotensin II induces skeletal muscle atrophy by activating TFEB-mediated MuRF1 expression. *Circ Res*, **117**(5), 424-436.

Duscha BD, Kraus WE, Keteyian SJ, Sullivan MJ, Green HJ, Schachat FH,... & Annex BH (1999). Capillary density of skeletal muscle: a contributing mechanism for exercise intolerance in class II-III chronic heart failure independent of other peripheral alterations. *J Am Coll Cardiol*, **33**(7), 1956-1963.

Duscha BD, Annex BH, Keteyian SJ, Green HJ, Sullivan MJ, Samsa GP,... & Kraus WE (2001). Differences in skeletal muscle between men and women with chronic heart failure. *J Appl Physiol*, **90**(1), 280-286.

Egginton S (1990a). Numerical and areal density estimates of fibre type composition in a skeletal muscle (rat extensor digitorum longus). *J Anat*, **168**, 73.

Egginton S (1990b). Morphometric analysis of tissue capillary supply. *In:* RG Boutilier, ed. *Vertebrate gas exchange: from environment to cell.* Berlin, Germany: Springer Berlin Heidelberg, 73-141.

Eigentler A, Fontana-Ayoub M & Gnaiger E (2013). O2k-Fluorometry: HRR and H_2O_2 production in mouse cardiac tissue homogenate. *Mitochondr Physiol Network*, **18.05**, 1-6.

Eigentler A, Draxl A, Wiethüchter A, Kuznetsov A, Lassing B & Gnaiger E (2015). Laboratory Protocol: Citrate synthase a mitochondrial marker enzyme. *Mitochondr Physiol Network*, **17.04**(1-11),

Elliott JE, Omar TS, Mantilla CB & Sieck GC (2016). Diaphragm muscle sarcopenia in Fischer 344 and Brown Norway rats. *Exp Physiol*, **101**(7), 883-894.

Ennezat PV, Malendowicz SL, Testa M, Colombo PC, Cohen-Solal A, Evans T & LeJemtel TH (2001). Physical training in patients with chronic heart failure enhances the expression of genes encoding antioxidative enzymes. *J Am Coll Cardiol*, **38**(1), 194-198.

Eriksson K-F, Saltin B & Lindgärde F (1994). Increased skeletal muscle capillary density precedes diabetes development in men with impaired glucose tolerance: a 15-year follow-up. *Diabetes*, **43**(6), 805-808.

Eshima H, Tamura Y, Kakehi S, Kurebayashi N, Murayama T, Nakamura K,... & Watada H (2017). Long-term, but not short-term high-fat diet induces fiber composition changes and impaired contractile force in mouse fast-twitch skeletal muscle. *Physiol Rep*, **5**(7), 1-12.

Esposito F, Reese V, Shabetai R, Wagner PD & Richardson RS (2011). Isolated quadriceps training increases maximal exercise capacity in chronic heart failure: the role of skeletal muscle convective and diffusive oxygen transport. *J Am Coll Cardiol*, **58**(13), 1353-1362.

Esser N, Legrand-Poels S, Piette J, Scheen AJ & Paquot N (2014). Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. *Diabetes Res Clin Pract*, **105**(2), 141-150.

Essig DA (1996). Contractile activity-induced mitochondrial biogenesis in skeletal muscle. *Exerc Sport Sci Rev*, **24**(1), 289-320.

Estacio RO, Regensteiner JG, Wolfel EE, Jeffers B, Dickenson M & Schrier RW (1998). The association between diabetic complications and exercise capacity in NIDDM patients. *Diabetes Care*, **21**(2), 291-295.

Evans WJ, Phinney SD & Young VR (1982). Suction applied to a muscle biopsy maximizes sample size. *Med Sci Sports Exerc*, **14**(1), 101-102.

Fang JC (2009). Rise of the machines - left ventricular assist devices as permanent therapy for advanced heart failure. *N Engl J Med*, **361**(23), 2282-2285.

Fang ZY, Sharman J, Prins JB & Marwick TH (2005). Determinants of exercise capacity in patients with type 2 diabetes. *Diabetes Care*, **28**(7), 1643-1648.

Fasching M, Sumbalova Z & Gnaiger E (2013). O2k-fluorometry: HRR and H_2O_2 production in mouse brain mitochondria. *Mitochondr Physiol Network*, **17.17**, 1-4.

Fasching M, Fontana-Ayoub M & Gnaiger E (2014a). Mitochondrial Respiration Medium - MiR06. *Mitochondr Physiol Network*, **14.13**, 1-4.

Fasching M & Gnaiger E (2014). Polarographic oxygen sensors: calibration, accuracy and quality control SOP. *Mitochondr Physiol Network*, **06.03**, 1-8.

Fasching M, Gradl P & Gnaiger E (2014b). The O2k-fluorescence LED2-module. *Mitochondr Physiol Network*, **17.05**, 1-6.

Fontana-Ayoub M, Fasching M & Gnaiger E (2014). Selected media and chemicals for respirometry with mitochondrial preparations. *Mitochondr Physiol Network*, **03.02**, 1-9.

Forman DE, Daniels KM, Cahalin LP, Zavin A, Allsup K, Cao P,... & Lecker SH (2014). Analysis of skeletal muscle gene expression patterns and the impact of functional capacity in patients with systolic heart failure. *J Card Fail*, **20**(6), 422-430.

Franciosa JA, Park M & Levine TB (1981). Lack of correlation between exercise capacity and indexes of resting left ventricular performance in heart failure. *Am J Cardiol*, **47**(1), 33-39.

From AM, Leibson CL, Bursi F, Redfield MM, Weston SA, Jacobsen SJ,... & Roger VL (2006). Diabetes in heart failure: prevalence and impact on outcome in the population. *Am J Med*, **119**(7), 591-599.

Fuchsberger C, Flannick J, Teslovich TM, Mahajan A, Agarwala V, Gaulton KJ,... & McCarthy MI (2016). The genetic architecture of type 2 diabetes. *Nature*, **536**(7614), 41-47.

Fulle S, Protasi F, Di Tano G, Pietrangelo T, Beltramin A, Boncompagni S,... & Fanò G (2004). The contribution of reactive oxygen species to sarcopenia and muscle ageing. *Exp Gerontol*, **39**(1), 17-24.

Fulster S, Tacke M, Sandek A, Ebner N, Tschope C, Doehner W,... & von Haehling S (2013). Muscle wasting in patients with chronic heart failure: results from the studies investigating co-morbidities aggravating heart failure (SICA-HF). *Eur Heart J*, **34**(7), 512-519.

Galkin A & Moncada S (2007). S-nitrosation of mitochondrial complex I depends on its structural conformation. *J Biol Chem*, **282**(52), 37448-37453.

Galkin A, Meyer B, Wittig I, Karas M, Schagger H, Vinogradov A & Brandt U (2008). Identification of the mitochondrial ND3 subunit as a structural component involved in the active/deactive enzyme transition of respiratory complex I. *J Biol Chem*, **283**(30), 20907-20913.

Galkin A, Abramov AY, Frakich N, Duchen MR & Moncada S (2009). Lack of oxygen deactivates mitochondrial complex I: implications for ischemic injury? *J Biol Chem*, **284**(52), 36055-36061.

Galkin A & Moncada S (2017). Modulation of the conformational state of mitochondrial complex I as a target for therapeutic intervention. *Interface Focus*, **7**(2), 20160104.

Garnier A, Fortin D, Delomenie C, Momken I, Veksler V & Ventura-Clapier R (2003). Depressed mitochondrial transcription factors and oxidative capacity in rat failing cardiac and skeletal muscles. *J Physiol*, **551**(2), 491-501.

Garnier A, Fortin D, Zoll J, N'Guessan B, Mettauer B, Lampert E,... & Ventura-Clapier R (2005). Coordinated changes in mitochondrial function and biogenesis in healthy and diseased human skeletal muscle. *FASEB J*, **19**(1), 43-52.

Gaster M, Staehr P, Beck-Nielsen H, Schroder HD & Handberg A (2001). GLUT4 is reduced in slow muscle fibers of type 2 diabetic patients: is insulin resistance in type 2 diabetes a slow, type 1 fiber disease? *Diabetes*, **50**(6), 1324-1329.

Geng T, Li P, Yin X & Yan Z (2011). PGC-1α promotes nitric oxide antioxidant defenses and inhibits FOXO signaling against cardiac cachexia in mice. *Am J Pathol*, **178**(4), 1738-1748.

Gibbons RJ, Balady GJ, Beasley JW, Bricker JT, Duvernoy WF, Froelicher VF,... & Thompson PD (1997). ACC/AHA guidelines for exercise testing: a report of the American College of Cardiology/American Heart Association task force on practice guidelines (committee on exercise testing). *J Am Coll Cardiol*, **30**(1), 260-311.

Gielen S, Adams V, Möbius-Winkler S, Linke A, Erbs S, Yu J,... & Hambrecht R (2003). Anti-inflammatory effects of exercise training in the skeletal muscle of patients with chronic heart failure. *J Am Coll Cardiol*, **42**(5), 861-868.

Gielen S, Adams V, Linke A, Erbs S, Möbius-Winkler S, Schubert A,... & Hambrecht R (2005). Exercise training in chronic heart failure: correlation between reduced local inflammation and improved oxidative capacity in the skeletal muscle. *Eur J Cardiov Prev R*, **12**(4), 393-400.

Gillis TE, Klaiman JM, Foster A, Platt MJ, Huber JS, Corso MY & Simpson JA (2016). Dissecting the role of the myofilament in diaphragm dysfunction during the development of heart failure in mice. *Am J Physiol Heart Circ Physiol*, **310**(5), H572-H586.

Glancy B, Hartnell LM, Malide D, Yu ZX, Combs CA, Connelly PS,... & Balaban RS (2015). Mitochondrial reticulum for cellular energy distribution in muscle. *Nature*, **523**(7562), 617-620.

Gnaiger E, Lassnig B, Kuznetsov AV & Margreiter R (1998). Mitochondrial respiration in the low oxygen environment of the cell. Effect of ADP on oxygen kinetics. *Biochim Biophys Acta*, **1365**(1-2), 249-254.

Gnaiger E (2014). *Mitochondrial pathways and respiratory control: an introduction to OXPHOS analysis.* 4th ed. Innsbruck, Austria: Oroboros MiPNet Publications.

Gomes MD, Lecker SH, Jagoe RT, Navon A & Goldberg AL (2001). Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc Natl Acad Sci USA*, **98**(25), 14440-14445.

Gomes MJ, Martinez PF, Campos DH, Pagan LU, Bonomo C, Lima AR,... & Okoshi MP (2016). Beneficial effects of physical exercise on functional

capacity and skeletal muscle oxidative stress in rats with aortic stenosis-induced heart failure. *Oxid Med Cell Longev*, **2016**, 1-12.

Gomez-Cabrera M-C, Domenech E, Romagnoli M, Arduini A, Borras C, Pallardo FV,... & Vina J (2008). Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. *Am J Clin Nutr*, **87**(1), 142-149.

Gonzalez-Freire M, Scalzo P, D'Agostino J, Moore ZA, Diaz-Ruiz A, Fabbri E,... & Ferrucci L (2018). Skeletal muscle *ex vivo* mitochondrial respiration parallels decline *in vivo* oxidative capacity, cardiorespiratory fitness, and muscle strength: The Baltimore Longitudinal Study of Aging. *Aging Cell*, **17**(2), 1-11.

Gostimskaya IS, Cecchini G & Vinogradov AD (2006). Topography and chemical reactivity of the active-inactive transition-sensitive SH-group in the mitochondrial NADH:ubiquinone oxidoreductase (Complex I). *Biochim Biophys Acta*, **1757**(9-10), 1155-1161.

Grassi B, Majerczak J, Bardi E, Buso A, Comelli M, Chlopicki S,... & Pellegrino MA (2017). Exercise training in Tgα_q*44 mice during the progression of chronic heart failure: cardiac vs. peripheral (soleus muscle) impairments to oxidative metabolism. *J Appl Physiol*, **123**(2), 326-336.

Gray MW, Burger G & Lang BF (1999). Mitochondrial evolution. *Science*, **283**(5407), 1476-1481.

Groen BB, Hamer HM, Snijders T, van Kranenburg J, Frijns D, Vink H & van Loon LJ (2014). Skeletal muscle capillary density and microvascular function are compromised with aging and type 2 diabetes. *J Appl Physiol*, **116**(8), 998-1005.

Guazzi M, Brambilla R, Pontone G, Agostoni P & Guazzi MD (2002). Effect of non-insulin-dependent diabetes mellitus on pulmonary function and exercise tolerance in chronic congestive heart failure. *Am J Cardiol*, **89**(2), 191-197.

Guazzi M, Tumminello G, Matturri M & Guazzi MD (2003). Insulin ameliorates exercise ventilatory efficiency and oxygen uptake in patients with heart failure-type 2 diabetes comorbidity. *J Am Coll Cardiol*, **42**(6), 1044-1050.

Gustafsson I, Brendorp B, Seibaek M, Burchardt H, Hildebrandt P, Kober L,... & Mortality on Dofetilde Study G (2004). Influence of diabetes and diabetes-gender interaction on the risk of death in patients hospitalized with congestive heart failure. *J Am Coll Cardiol*, **43**(5), 771-777.

Guzman Mentesana G, Baez AL, Lo Presti MS, Dominguez R, Cordoba R, Bazan C,... & Paglini-Oliva P (2014). Functional and structural alterations of cardiac and skeletal muscle mitochondria in heart failure patients. *Arch Med Res*, **45**(3), 237-246.

Hambrecht R, Niebauer J, Fiehn E, Kalberer B, Offner B, Hauer K,... & Schuler G (1995). Physical training in patients with stable chronic heart failure: effects on cardiorespiratory fitness and ultrastructural abnormalities of leg muscles. *J Am Coll Cardiol*, **25**(6), 1239-1249.

Hambrecht R, Fiehn E, Yu J, Niebauer J, Weigl C, Hilbrich L,... & Schuler G (1997). Effects of endurance training on mitochondrial ultrastructure and fiber type distribution in skeletal muscle of patients with stable chronic heart failure. *J Am Coll Cardiol*, **29**(5), 1067-1073.

Hambrecht R, Adams V, Gielen S, Linke A, Möbius-Winkler S, Yu J,... & Schuler G (1999a). Exercise intolerance in patients with chronic heart failure and increased expression of inducible nitric oxide synthase in the skeletal muscle. *J Am Coll Cardiol*, **33**(1), 174-179.

Hambrecht R, Fiehn E, Weigl C, Gielen S, Hamann C, Kaiser R,... & Schuler G (1999b). Regular physical exercise corrects endothelial dysfunction and improves exercise capacity in patients with chronic heart failure. *Cardiopulm Phys Ther J*, **10**(1), 23.

Hambrecht R, Schulze PC, Gielen S, Linke A, Möbius-Winkler S, Yu J,... & Schuler G (2002). Reduction of insulin-like growth factor-I expression in the skeletal muscle of noncachectic patients with chronic heart failure. *J Am Coll Cardiol*, **39**(7), 1175-1181.

Harper ME, Bevilacqua L, Hagopian K, Weindruch R & Ramsey JJ (2004). Ageing, oxidative stress, and mitochondrial uncoupling. *Acta Physiol Scand*, **182**(4), 321-331.

Harrington D, Anker SD, Chua TP, Webb-Peploe KM, Ponikowski PP, Poole-Wilson PA & Coats AJ (1997). Skeletal muscle function and its relation to exercise tolerance in chronic heart failure. *J Am Coll Cardiol*, **30**(7), 1758-1764.

Hartupee J & Mann DL (2017). Neurohormonal activation in heart failure with reduced ejection fraction. *Nat Rev Cardiol*, **14**(1), 30-38.

Hawkins MN, Raven PB, Snell PG, Stray-Gundersen J & Levine BD (2007). Maximal oxygen uptake as a parametric measure of cardiorespiratory capacity. *Med Sci Sports Exerc*, **39**(1), 103-107.

He J, Watkins S & Kelley DE (2001). Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. *Diabetes*, **50**(4), 817-823.

Heidenreich PA, Albert NM, Allen LA, Bluemke DA, Butler J, Fonarow GC,... & Stroke C (2013). Forecasting the impact of heart failure in the United States: a policy statement from the American Heart Association. *Circ Heart Fail*, **6**(3), 606-619.

Hennessey JV, Chromiak JA, Della Ventura S, Guertin J & MacLean DB (1997). Increase in percutaneous muscle biopsy yield with a suction-enhancement technique. *J Appl Physiol*, **82**(6), 1739-1742.

Henriksen EJ, Diamond-Stanic MK & Marchionne EM (2011). Oxidative stress and the etiology of insulin resistance and type 2 diabetes. *Free Radic Biol Med*, **51**(5), 993-999.

Hey-Mogensen M, Hojlund K, Vind BF, Wang L, Dela F, Beck-Nielsen H,... & Sahlin K (2010). Effect of physical training on mitochondrial respiration and reactive oxygen species release in skeletal muscle in patients with obesity and type 2 diabetes. *Diabetologia*, **53**(9), 1976-1985.

Hill A & Lupton H (1923). Muscular exercise, lactic acid, and the supply and utilization of oxygen. *QJM*, **16**(62), 135-171.

Hinkle PC (2005). P/O ratios of mitochondrial oxidative phosphorylation. *Biochim Biophys Acta*, **1706**(1-2), 1-11.

Hirst J (2013). Mitochondrial complex I. *Annu Rev Biochem*, **82**, 551-575.

Ho KKL, Pinsky JL, Kannel WB & Levy D (1993). The epidemiology of heart failure: The Framingham Study. *J Am Coll Cardiol*, **22**(4), A6-A13.

Hoeks J & Schrauwen P (2012). Muscle mitochondria and insulin resistance: a human perspective. *Trends Endocrinol Metab*, **23**(9), 444-450.

Holloszy J & Coyle EF (1984). Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. *J Appl Physiol*, **56**(4), 831-838.

Holloszy JO & Booth FW (1976). Biochemical adaptations to endurance exercise in muscle. *Annu Rev Physiol*, **38**(1), 273-291.

Holmstrom MH, Iglesias-Gutierrez E, Zierath JR & Garcia-Roves PM (2012). Tissue-specific control of mitochondrial respiration in obesity-related insulin resistance and diabetes. *Am J Physiol Endocrinol Metab*, **302**(6), E731-E739.

Holzmeister J & Leclercq C (2011). Implantable cardioverter defibrillators and cardiac resynchronisation therapy. *Lancet*, **378**(9792), 722-730.

Howley ET, Bassett DR & Welch HG (1995). Criteria for maximal oxygen uptake: review and commentary. *Med Sci Sports Exerc*, **27**(9), 1292-1301.

Hunter RB & Kandarian SC (2004). Disruption of either the *Nfkb1* or the *Bcl3* gene inhibits skeletal muscle atrophy. *J Clin Invest*, **114**(10), 1504-1511.

Ingelsson E, Sundström J, Ärnlöv J, Zethelius B & Lind L (2005). Insulin resistance and risk of congestive heart failure. *JAMA*, **294**(3), 334-341.

Ingle L, Reddy P, Clark AL & Cleland JG (2006). Diabetes lowers six-minute walk test performance in heart failure. *J Am Coll Cardiol*, **47**(9), 1909-1910.

Iribarren C, Karter AJ, Go AS, Ferrara A, Liu JY, Sidney S & Selby JV (2001). Glycemic control and heart failure among adult patients with diabetes. *Circulation*, **103**(22), 2668-2673.

Izawa KP, Watanabe S, Hiraki K, Osada N & Omiya K (2013). Muscle strength in heart failure male patients complicated by diabetes mellitus. *Int J Cardiol*, **168**(1), 551-552.

Jackman RW, Cornwell EW, Wu CL & Kandarian SC (2013). Nuclear factor-κB signalling and transcriptional regulation in skeletal muscle atrophy. *Exp Physiol*, **98**(1), 19-24.

Jarcho JA (2005). Resynchronizing ventricular contraction in heart failure. *N Engl J Med*, **352**(15), 1594-1597.

Johannsen DL & Ravussin E (2009). The role of mitochondria in health and disease. *Curr Opin Pharmacol*, **9**(6), 780-786.

Johnson ML, Robinson MM & Nair KS (2013). Skeletal muscle aging and the mitochondrion. *Trends Endocrinol Metab*, **24**(5), 247-256.

Kadoguchi T, Kinugawa S, Takada S, Fukushima A, Furihata T, Homma T,... & Tsutsui H (2015). Angiotensin II can directly induce mitochondrial dysfunction, decrease oxidative fibre number and induce atrophy in mouse hindlimb skeletal muscle. *Exp Physiol*, **100**(3), 312-322.

Kahn SE, Cooper ME & Del Prato S (2014). Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future. *Lancet*, **383**(9922), 1068-1083.

Kamei Y, Miura S, Suzuki M, Kai Y, Mizukami J, Taniguchi T,... & Ezaki O (2004). Skeletal muscle FOXO1 (FKHR) transgenic mice have less skeletal muscle mass, down-regulated type I (slow twitch/red muscle) fiber genes, and impaired glycemic control. *J Biol Chem*, **279**(39), 41114-41123.

Kannel WB & McGee DL (1979). Diabetes and cardiovascular disease: the Framingham Study. *JAMA*, **241**(19), 2035-2038.

Karakelides H, Irving BA, Short KR, O'Brien P & Nair KS (2010). Age, obesity, and sex effects on insulin sensitivity and skeletal muscle mitochondrial function. *Diabetes*, **59**(1), 89-97.

Kearney MT (2015). Chronic heart failure and type 2 diabetes mellitus: The last battle? *Diab Vasc Dis Res*, **12**(4), 226-227.

Kelley DE & Mandarino LJ (2000). Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes*, **49**(5), 677-683.

Kelley DE, He J, Menshikova EV & Ritov VB (2002). Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes*, **51**(10), 2944-2950.

Kemp CD & Conte JV (2012). The pathophysiology of heart failure. *Cardiovasc Pathol*, **21**(5), 365-371.

Kemp GJ, Thompson CH, Stratton JR, Brunotte F, Conway M, Adamopoulos S,... & Rajagopalan B (1996). Abnormalities in exercising skeletal muscle in congestive heart failure can be explained in terms of decreased mitochondrial ATP synthesis, reduced metabolic efficiency, and increased glycogenolysis. *Heart*, **76**(1), 35-41.

Kemp GJ, Meyerspeer M & Moser E (2007). Absolute quantification of phosphorus metabolite concentrations in human muscle *in vivo* by ^{31}P MRS: a quantitative review. *NMR Biomed*, **20**(6), 555-565.

Kemp GJ, Ahmad RE, Nicolay K & Prompers JJ (2015). Quantification of skeletal muscle mitochondrial function by ^{31}P magnetic resonance spectroscopy techniques: a quantitative review. *Acta Physiol*, **213**(1), 107-144.

Kemp JG, Blazev R, Stephenson DG & Stephenson GM (2009). Morphological and biochemical alterations of skeletal muscles from the genetically obese (*ob/ob*) mouse. *Int J Obesity*, **33**(8), 831-841.

Keteyian SJ, Patel M, Kraus WE, Brawner CA, McConnell TR, Piña IL,... & Fonarow GC (2016). Variables measured during cardiopulmonary exercise testing as predictors of mortality in chronic systolic heart failure. *J Am Coll Cardiol*, **67**(7), 780-789.

Khan H, Kunutsor SK, Kauhanen J, Kurl S, Gorodeski EZ, Adler AI,... & Laukkanen JA (2014). Fasting plasma glucose and incident heart failure risk: a population-based cohort study and new meta-analysis. *J Card Fail*, **20**(8), 584-592.

Kim JK (2009). Hyperinsulinemic-euglycemic clamp to assess insulin sensitivity *in vivo. In:* C Stocker, ed. *Type 2 diabetes methods and protocols.* Berlin, Germany: Springer, 221-238.

Kim KS, Park KS, Kim MJ, Kim SK, Cho YW & Park SW (2014). Type 2 diabetes is associated with low muscle mass in older adults. *Geriatr Gerontol Int*, **14**(Suppl. 1), 115-121.

Kim TN, Park MS, Yang SJ, Yoo HJ, Kang HJ, Song W,... & Choi KM (2010). Prevalence and determinant factors of sarcopenia in patients with type 2 diabetes: the Korean Sarcopenic Obesity Study (KSOS). *Diabetes Care*, **33**(7), 1497-1499.

Kirkeby S, Mandel U & Vedtofte P (1993). Identification of capillaries in sections from skeletal muscle by use of lectins and monoclonal antibodies reacting with histo-blood group ABH antigens. *Glycoconjugate J*, **10**(2), 181-188.

Kissane RWP, Egginton S & Askew GN (2018). Regional variation in the mechanical properties and fibre-type composition of the rat extensor digitorum longus muscle. *Exp Physiol*, **103**(1), 111-124.

Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O,... & Muoio DM (2008). Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab*, **7**(1), 45-56.

Kowaltowski AJ, de Souza-Pinto NC, Castilho RF & Vercesi AE (2009). Mitochondria and reactive oxygen species. *Free Radic Biol Med*, **47**(4), 333-343.

Krum H & Abraham WT (2009). Heart failure. *Lancet*, **373**(9667), 941-955.

Krum H & Teerlink JR (2011). Medical therapy for chronic heart failure. *Lancet*, **378**(9792), 713-721.

Krumschnabel G, Makrecka-Kuka M, Kumphune S, Fontana-Ayoub M, Fasching M & Gnaiger E (2014). O2k-fluorometry: HRR and simultaneous

determination of H_2O_2 production with Amplex Red. *Mitochondr Physiol Network*, **19.20**, 1-6.

Krumschnabel G, Fontana-Ayoub M, Fasching M & Gnaiger E (2015a). O2k-fluorometry: HRR and simultaneous determination of H_2O_2 production with Amplex Red. *Mitochondr Physiol Network*, **20.14**(1-6),

Krumschnabel G, Fontana-Ayoub M, Sumbalova Z, Heidler J, Gauper K, Fasching M & Gnaiger E (2015b). Simultaneous high-resolution measurement of mitochondrial respiration and hydrogen peroxide production. *In:* V Weissig and M Edeas, eds. *Mitochondrial medicine. Volume I, probing mitochondrial function.* New York, NY: Humana Press, 245-261.

Kuznetsov AV, Schneeberger S, Seiler R, Brandacher G, Mark W, Steurer W,... & Gnaiger E (2004). Mitochondrial defects and heterogeneous cytochrome *c* release after cardiac cold ischemia and reperfusion. *Am J Physiol Heart Circ Physiol*, **286**(5), H1633-1641.

Kuznetsov AV, Veksler V, Gellerich FN, Saks V, Margreiter R & Kunz WS (2008). Analysis of mitochondrial function *in situ* in permeabilized muscle fibers, tissues and cells. *Nat Protoc*, **3**(6), 965-976.

Kuznetsov AV & Gnaiger E (2010). Oxygraph assay of cytochrome *c* oxidase activity: chemical background correction. *Mitochondr Physiol Network*, **06.06**, 1-4.

Lackey DE & Olefsky JM (2016). Regulation of metabolism by the innate immune system. *Nat Rev Endocrinol*, **12**(1), 15-28.

Laitano O, Ahn B, Patel N, Coblentz PD, Smuder AJ, Yoo JK,... & Ferreira LF (2016). Pharmacological targeting of mitochondrial reactive oxygen species counteracts diaphragm weakness in chronic heart failure. *J Appl Physiol*, **120**(7), 733-742.

Lamarra N, Whipp BJ, Ward SA & Wasserman K (1987). Effect of interbreath fluctuations on characterizing exercise gas exchange kinetics. *J Appl Physiol*, **62**(5), 2003-2012.

Lang RM, Badano LP, Mor-Avi V, Afilalo J, Armstrong A, Ernande L,... & Voigt JU (2015). Recommendations for cardiac chamber quantification by echocardiography in adults: an update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. *Eur Heart J Cardiovasc Imaging*, **16**(3), 233-270.

Lanza IR, Bhagra S, Nair KS & Port JD (2011). Measurement of human skeletal muscle oxidative capacity by ^{31}P-MRS: a cross-validation with *in vitro* measurements. *J Magn Reson Imaging*, **34**(5), 1143-1150.

Larsen AI, Lindal S, Aukrust P, Toft I, Aarsland T & Dickstein K (2002). Effect of exercise training on skeletal muscle fibre characteristics in men with chronic heart failure. Correlation between skeletal muscle alterations, cytokines and exercise capacity. *Int J Cardiol*, **83**(1), 25-32.

Larsen S, Ara I, Rabol R, Andersen JL, Boushel R, Dela F & Helge JW (2009). Are substrate use during exercise and mitochondrial respiratory capacity decreased in arm and leg muscle in type 2 diabetes? *Diabetologia*, **52**(7), 1400-1408.

Larsen S, Stride N, Hey-Mogensen M, Hansen CN, Andersen JL, Madsbad S,... & Dela F (2011). Increased mitochondrial substrate sensitivity in skeletal muscle of patients with type 2 diabetes. *Diabetologia*, **54**(6), 1427-1436.

Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N,... & Hey-Mogensen M (2012a). Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiol*, **590**(14), 3349-3360.

Larsen S, Rabol R, Hansen CN, Madsbad S, Helge JW & Dela F (2012b). Metformin-treated patients with type 2 diabetes have normal mitochondrial complex I respiration. *Diabetologia*, **55**(2), 443-449.

Larsen S, Kraunsoe R, Gram M, Gnaiger E, Helge JW & Dela F (2014). The best approach: homogenization or manual permeabilization of human skeletal muscle fibers for respirometry? *Anal Biochem*, **446**, 64-68.

Lavine KJ & Sierra OL (2017). Skeletal muscle inflammation and atrophy in heart failure. *Heart Fail Rev*, **22**(2), 179-189.

Leenders M, Verdijk LB, van der Hoeven L, Adam JJ, van Kranenburg J, Nilwik R & van Loon LJ (2013). Patients with type 2 diabetes show a greater decline in muscle mass, muscle strength, and functional capacity with aging. *J Am Med Dir Assoc*, **14**(8), 585-592.

Lefort N, Glancy B, Bowen B, Willis WT, Bailowitz Z, De Filippis EA,... & Mandarino LJ (2010). Increased reactive oxygen species production and lower abundance of complex I subunits and carnitine palmitoyltransferase 1B protein despite normal mitochondrial respiration in insulin-resistant human skeletal muscle. *Diabetes*, **59**(10), 2444-2452.

Lehrke M & Marx N (2017). Diabetes Mellitus and Heart Failure. *Am J Med*, **130**(6S), S40-S50.

Lemieux H, Votion M-D & Gnaiger E (2014). Mitochondrial respiration in permeabilized fibres: needle biopsies from horse skeletal muscle. *Mitochondr Physiol Network*, **12.23**, 1-4.

Lenk K, Erbs S, Hollriegel R, Beck E, Linke A, Gielen S,... & Adams V (2012). Exercise training leads to a reduction of elevated myostatin levels in patients with chronic heart failure. *Eur J Prev Cardiol*, **19**(3), 404-411.

Lesnefsky EJ & Hoppel CL (2006). Oxidative phosphorylation and aging. *Ageing Res Rev*, **5**(4), 402-433.

Letellier T, Malgat M, Coquet M, Moretto B, Parrot-Roulaud F & Mazat JP (1992). Mitochondrial myopathy studies on permeabilized muscle fibers. *Pediatr Res*, **32**(1), 17-22.

Levene H (1961). Robust tests for equality of variances. *In:* I Olkin, ed. *Contributions to probability and statistics.* Palo Alto, CA: Stanford University Press, 279-292.

Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA & Robinson JP (2003a). Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J Biol Chem*, **278**(10), 8516-8525.

Li P, Waters RE, Redfern SI, Zhang M, Mao L, Annex BH & Yan Z (2007). Oxidative phenotype protects myofibers from pathological insults induced by chronic heart failure in mice. *Am J Pathol*, **170**(2), 599-608.

Li Y-P, Schwartz RJ, Waddell ID, Holloway BR & Reid MB (1998). Skeletal muscle myocytes undergo protein loss and reactive oxygen-mediated NF-κB activation in response to tumor necrosis factor α. *FASEB J*, **12**(10), 871-880.

Li Y-P, Chen Y, Li AS & Reid MB (2003b). Hydrogen peroxide stimulates ubiquitin-conjugating activity and expression of genes for specific E2 and E3 proteins in skeletal muscle myotubes. *Am J Physiol Cell Physiol*, **285**(4), C806-C812.

Li Y-P, Chen Y, John J, Moylan J, Jin B, Mann DL & Reid MB (2005). TNF-α acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. *FASEB J*, **19**(3), 362-370.

Lillioja S, Young AA, Culter CL, Ivy JL, Abbott WG, Zawadzki JK,... & Bogardus C (1987). Skeletal muscle capillary density and fiber type are possible determinants of *in vivo* insulin resistance in man. *J Clin Invest*, **80**(2), 415-424.

Linke A, Adams V, Schulze PC, Erbs S, Gielen S, Fiehn E,... & Hambrecht R (2005). Antioxidative effects of exercise training in patients with chronic heart failure: increase in radical scavenger enzyme activity in skeletal muscle. *Circulation*, **111**(14), 1763-1770.

Lipkin DP, Jones DA, Round JM & Poole-Wilson PA (1988). Abnormalities of skeletal muscle in patients with chronic heart failure. *Int J Cardiol*, **18**(2), 187-195.

Little JP, Gillen JB, Percival ME, Safdar A, Tarnopolsky MA, Punthakee Z,... & Gibala MJ (2011). Low-volume high-intensity interval training reduces hyperglycemia and increases muscle mitochondrial capacity in patients with type 2 diabetes. *J Appl Physiol*, **111**(6), 1554-1560.

Lunde PK, Dahlstedt AJ, Bruton JD, Lannergren J, Thoren P, Sejersted OM & Westerblad H (2001). Contraction and intracellular Ca^{2+} handling in isolated skeletal muscle of rats with congestive heart failure. *Circ Res*, **88**(12), 1299-1305.

Lunde PK, Sejersted OM, Thorud HM, Tonnessen T, Henriksen UL, Christensen G,... & Bruton J (2006). Effects of congestive heart failure on Ca^{2+} handling in skeletal muscle during fatigue. *Circ Res*, **98**(12), 1514-1519.

MacDonald MR, Petrie MC, Hawkins NM, Petrie JR, Fisher M, McKelvie R,... & McMurray JJ (2008a). Diabetes, left ventricular systolic dysfunction, and chronic heart failure. *Eur Heart J*, **29**(10), 1224-1240.

MacDonald MR, Petrie MC, Varyani F, Ostergren J, Michelson EL, Young JB,... & Investigators C (2008b). Impact of diabetes on outcomes in patients with low and preserved ejection fraction heart failure: an analysis of the Candesartan in Heart failure: Assessment of Reduction in Mortality and morbidity (CHARM) programme. *Eur Heart J*, **29**(11), 1377-1385.

Machado MV, Martins RL, Borges J, Antunes BR, Estato V, Vieira AB & Tibirica E (2016). Exercise training reverses structural microvascular rarefaction and improves endothelium-dependent microvascular reactivity in rats with diabetes. *Metab Syndr Relat Disord*, **14**(6), 298-304.

Makrecka-Kuka M, Krumschnabel G & Gnaiger E (2015). High-resolution respirometry for simultaneous measurement of oxygen and hydrogen peroxide fluxes in permeabilized cells, tissue homogenate and isolated mitochondria. *Biomolecules*, **5**(3), 1319-1338.

Malhotra R, Bakken K, D'Elia E & Lewis GD (2016). Cardiopulmonary exercise testing in heart failure. *JACC Heart Fail*, **4**(8), 607-616.

Mancini DM, Coyle E, Coggan A, Beltz J, Ferraro N, Montain S & Wilson JR (1989). Contribution of intrinsic skeletal muscle changes to ^{31}P NMR skeletal muscle metabolic abnormalities in patients with chronic heart failure. *Circulation*, **80**(5), 1338-1346.

Mancini DM, Schwartz M, Ferraro N, Seestedt R, Chance B & Wilson JR (1990). Effect of dobutamine on skeletal muscle metabolism in patients with congestive heart failure. *Am J Cardiol*, **65**(16), 1121-1126.

Mancini DM, Eisen H, Kussmaul W, Mull R, Edmunds L & Wilson J (1991). Value of peak exercise oxygen consumption for optimal timing of cardiac transplantation in ambulatory patients with heart failure. *Circulation*, **83**(3), 778-786.

Mancini DM, Walter G, Reichek N, Lenkinski R, McCully KK, Mullen JL & Wilson JR (1992). Contribution of skeletal muscle atrophy to exercise intolerance and altered muscle metabolism in heart failure. *Circulation*, **85**(4), 1364-1373.

Mangner N, Weikert B, Bowen TS, Sandri M, Hollriegel R, Erbs S,... & Adams V (2015). Skeletal muscle alterations in chronic heart failure: differential effects on quadriceps and diaphragm. *J Cachexia Sarcopenia Muscle*, **6**(4), 381-390.

Mangner N, Bowen TS, Werner S, Fischer T, Kullnick Y, Oberbach A,... & Adams V (2016). Exercise training prevents diaphragm contractile dysfunction in heart failure. *Med Sci Sports Exerc*, **48**(11), 2118-2124.

Mankowski RT, Anton SD, Buford TW & Leeuwenburgh C (2015). Dietary antioxidants as modifiers of physiologic adaptations to exercise. *Med Sci Sports Exerc*, **47**(9), 1857-1868.

Marin P, Andersson B, Krotkiewski M & Bjorntorp P (1994). Muscle fiber composition and capillary density in women and men with NIDDM. *Diabetes Care*, **17**(5), 382-386.

Martinez PF, Bonomo C, Guizoni DM, Junior SA, Damatto RL, Cezar MD,... & Okoshi MP (2015). Influence of *N*-acetylcysteine on oxidative stress in slow-twitch soleus muscle of heart failure rats. *Cell Physiol Biochem*, **35**(1), 148-159.

Maskin CS, Forman R, Sonnenblick EH, Frishman WH & LeJemtel TH (1983). Failure of dobutamine to increase exercise capacity despite hemodynamic improvement in severe chronic heart failure. *Am J Cardiol*, **51**(1), 177-182.

Mason SA, Della Gatta PA, Snow RJ, Russell AP & Wadley GD (2016). Ascorbic acid supplementation improves skeletal muscle oxidative stress and insulin sensitivity in people with type 2 diabetes: findings of a randomized controlled study. *Free Radic Biol Med*, **93**, 227-238.

Massie B, Conway M, Yonge R, Frostick S, Ledingham J, Sleight P,... & Rajagopalan B (1987a). Skeletal muscle metabolism in patients with congestive heart failure: relation to clinical severity and blood flow. *Circulation*, **76**(5), 1009-1019.

Massie BM, Conway M, Yonge R, Frostick S, Sleight P, Ledingham J,... & Rajagopalan B (1987b). ^{31}P nuclear magnetic resonance evidence of abnormal skeletal muscle metabolism in patients with congestive heart failure. *Am J Cardiol*, **60**(4), 309-315.

Massie BM, Simonini A, Sahgal P, Wells L & Dudley GA (1996). Relation of systemic and local muscle exercise capacity to skeletal muscle characteristics in men with congestive heart failure. *J Am Coll Cardiol*, **27**(1), 140-145.

Matsushita K, Blecker S, Pazin-Filho A, Bertoni A, Chang PP, Coresh J & Selvin E (2010). The association of hemoglobin A1c with incident heart failure among people without diabetes: the atherosclerosis risk in communities study. *Diabetes*, **59**(8), 2020-2026.

McAllister RM & Terjung RL (1990). Acute inhibition of respiratory capacity of muscle reduces peak oxygen consumption. *Am J Physiol Cell Physiol*, **259**(6), C889-C896.

McAuley PA, Myers JN, Abella JP, Tan SY & Froelicher VF (2007). Exercise capacity and body mass as predictors of mortality among male veterans with type 2 diabetes. *Diabetes Care*, **30**(6), 1539-1543.

McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Bohm M, Dickstein K,... & Zeiher A (2012). ESC guidelines for the diagnosis and treatment of acute

and chronic heart failure 2012. The task force for the diagnosis and treatment of acute and chronic heart failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. *Eur Heart J*, **33**(14), 1787-1847.

Meex RC, Schrauwen-Hinderling VB, Moonen-Kornips E, Schaart G, Mensink M, Phielix E,... & Hesselink MK (2010). Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity. *Diabetes*, **59**(3), 572-579.

Mehra MR, Canter CE, Hannan MM, Semigran MJ, Uber PA, Baran DA,... & Zuckermann A (2016). The 2016 International Society for Heart Lung Transplantation listing criteria for heart transplantation: a 10-year update. *J Heart Lung Transplant*, **35**(1), 1-23.

Metra M & Teerlink JR (2017). Heart failure. *Lancet*, **390**(10106), 1981-1995.

Mettauer B, Zoll J, Sanchez H, Lampert E, Ribera F, Veksler V,... & Ventura-Clapier R (2001). Oxidative capacity of skeletal muscle in heart failure patients versus sedentary or active control subjects. *J Am Coll Cardiol*, **38**(4), 947-954.

Mettauer B, Zoll J, Garnier A & Ventura-Clapier R (2006). Heart failure: a model of cardiac and skeletal muscle energetic failure. *Pflugers Arch*, **452**(6), 653-666.

Mezzani A, Agostoni P, Cohen-Solal A, Corra U, Jegier A, Kouidi E,... & Vanhees L (2009). Standards for the use of cardiopulmonary exercise testing for the functional evaluation of cardiac patients: a report from the Exercise Physiology Section of the European Association for Cardiovascular Prevention and Rehabilitation. *Eur J Cardiovasc Prev Rehabil*, **16**(3), 249-267.

Middlekauff HR (2010). Making the case for skeletal myopathy as the major limitation of exercise capacity in heart failure. *Circ Heart Fail*, **3**(4), 537-546.

Middlekauff HR, Vigna C, Verity MA, Fonarow GC, Horwich TB, Hamilton MA,... & Tupling AR (2012). Abnormalities of calcium handling proteins in skeletal muscle mirror those of the heart in humans with heart failure: a shared mechanism? *J Card Fail*, **18**(9), 724-733.

Middlekauff HR, Verity MA, Horwich TB, Fonarow GC, Hamilton MA & Shieh P (2013). Intact skeletal muscle mitochondrial enzyme activity but diminished exercise capacity in advanced heart failure patients on optimal medical and device therapy. *Clin Res Cardiol*, **102**(8), 547-554.

Miller MS, VanBuren P, LeWinter MM, Braddock JM, Ades PA, Maughan DW,... & Toth MJ (2010). Chronic heart failure decreases cross-bridge kinetics in single skeletal muscle fibres from humans. *J Physiol*, **588**, 4039-4053.

Minotti JR, Christoph I, Oka R, Weiner MW, Wells L & Massie BM (1991). Impaired skeletal muscle function in patients with congestive heart failure. Relationship to systemic exercise performance. *J Clin Invest*, **88**(6), 2077-2082.

Minotti JR, Pillay P, Chang L, Wells L & Massie BM (1992). Neurophysiological assessment of skeletal muscle fatigue in patients with congestive heart failure. *Circulation*, **86**(3), 903-908.

Minotti JR, Pillay P, Oka R, Wells L, Christoph I & Massie BM (1993). Skeletal muscle size: relationship to muscle function in heart failure. *J Appl Physiol*, **75**(1), 373-381.

Mogensen M, Sahlin K, Fernstrom M, Glintborg D, Vind BF, Beck-Nielsen H & Hojlund K (2007). Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. *Diabetes*, **56**(6), 1592-1599.

Mootha VK, Lindgren CM, Eriksson K-F, Subramanian A, Sihag S, Lehar J,... & Laurila E (2003). PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet*, **34**(3), 267-273.

Morino K, Petersen KF, Dufour S, Befroy D, Frattini J, Shatzkes N,... & Shulman GI (2005). Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. *J Clin Invest*, **115**(12), 3587-3593.

Mortensen SP, Dawson EA, Yoshiga CC, Dalsgaard MK, Damsgaard R, Secher NH & Gonzalez-Alonso J (2005). Limitations to systemic and locomotor limb muscle oxygen delivery and uptake during maximal exercise in humans. *J Physiol*, **566**, 273-285.

Moss AJ, Hall WJ, Cannom DS, Klein H, Brown MW, Daubert JP,... & Higgins SL (2009). Cardiac-resynchronization therapy for the prevention of heart-failure events. *N Engl J Med*, **361**(14), 1329-1338.

Moss AJ (2010). Preventing heart failure and improving survival. *N Engl J Med*, **363**(25), 2456-2457.

Motulsky HJ & Brown RE (2006). Detecting outliers when fitting data with nonlinear regression - a new method based on robust nonlinear regression and the false discovery rate. *BMC Bioinformatics*, **7**, 123.

Mudd JO & Kass DA (2008). Tackling heart failure in the twenty-first century. *Nature*, **451**(7181), 919-928.

Muniyappa R & Sowers JR (2013). Role of insulin resistance in endothelial dysfunction. *Rev Endocr Metab Disord*, **14**(1), 5-12.

Muoio DM (2010). Intramuscular triacylglycerol and insulin resistance: guilty as charged or wrongly accused? *Biochim Biophys Acta*, **1801**(3), 281-288.

Muoio DM (2014). Metabolic inflexibility: when mitochondrial indecision leads to metabolic gridlock. *Cell*, **159**(6), 1253-1262.

Murgia M, Toniolo L, Nagaraj N, Ciciliot S, Vindigni V, Schiaffino S,... & Mann M (2017). Single muscle fiber proteomics reveals fiber-type-specific features of human muscle aging. *Cell Rep*, **19**(11), 2396-2409.

Murias JM, Pogliaghi S & Paterson DH (2018). Measurement of a true $\dot{V}O_{2max}$ during a ramp incremental test is not confirmed by a verification phase. *Front Physiol*, **9**(143), 1-8.

Murphy MP (2009). How mitochondria produce reactive oxygen species. *Biochem J*, **417**(1), 1-13.

Myers J, Prakash M, Froelicher V, Do D, Partington S & Atwood JE (2002). Exercise capacity and mortality among men referred for exercise testing. *N Engl J Med*, **346**(11), 793-801.

Nagai T, Okita K, Yonezawa K, Yamada Y, Hanada A, Ohtsubo M,... & Kitabatake A (2004). Comparisons of the skeletal muscle metabolic abnormalities in the arm and leg muscles of patients with chronic heart failure. *Circ J*, **68**(6), 573-579.

Nakae I, Mitsunami K, Matsuo S, Inubushi T, Morikawa S, Koh T & Horie M (2005). Detection of calf muscle alterations in patients with chronic heart failure by P magnetic resonance spectroscopy: Impaired adaptation to continuous exercise. *Exp Clin Cardiol*, **10**(1), 4-8.

Nilwik R, Snijders T, Leenders M, Groen BB, van Kranenburg J, Verdijk LB & van Loon LJ (2013). The decline in skeletal muscle mass with aging is mainly

attributed to a reduction in type II muscle fiber size. *Exp Gerontol*, **48**(5), 492-498.

Noakes TD (2008). Maximal oxygen uptake as a parametric measure of cardiorespiratory capacity: comment. *Med Sci Sports Exerc*, **40**(3), 585.

Nojiri H, Shimizu T, Funakoshi M, Yamaguchi O, Zhou H, Kawakami S,... & Shirasawa T (2006). Oxidative stress causes heart failure with impaired mitochondrial respiration. *J Biol Chem*, **281**(44), 33789-33801.

Nordsborg NB, Connolly L, Weihe P, Iuliano E, Krustrup P, Saltin B & Mohr M (2015). Oxidative capacity and glycogen content increase more in arm than leg muscle in sedentary women after intense training. *J Appl Physiol*, **119**(2), 116-123.

Nusz DJ, White DC, Dai Q, Pippen AM, Thompson MA, Walton GB,... & Annex BH (2003). Vascular rarefaction in peripheral skeletal muscle after experimental heart failure. *Am J Physiol Heart Circ Physiol*, **285**(4), H1554-H1562.

Nyholm B, Qu Z, Kaal A, Pedersen SB, Gravholt CH, Andersen JL,... & Schmitz O (1997). Evidence of an increased number of type IIb muscle fibers in insulin-resistant first-degree relatives of patients with NIDDM. *Diabetes*, **46**(11), 1822-1828.

Nylen ES, Kokkinos P, Myers J & Faselis C (2010). Prognostic effect of exercise capacity on mortality in older adults with diabetes mellitus. *J Am Geriatr Soc*, **58**(10), 1850-1854.

O'Connor CM, Whellan DJ, Lee KL, Keteyian SJ, Cooper LS, Ellis SJ,... & Blumenthal JA (2009). Efficacy and safety of exercise training in patients with chronic heart failure: HF-ACTION randomized controlled trial. *JAMA*, **301**(14), 1439-1450.

Oberbach A, Bossenz Y, Lehmann S, Niebauer J, Adams V, Paschke R,... & Punkt K (2006). Altered fiber distribution and fiber-specific glycolytic and oxidative enzyme activity in skeletal muscle of patients with type 2 diabetes. *Diabetes Care*, **29**(4), 895-900.

Ogata T & Yamasaki Y (1997). Ultra-high-resolution scanning electron microscopy of mitochondria and sarcoplasmic reticulum arrangement in human red, white, and intermediate muscle fibers. *Anat Rec*, **248**(2), 214-223.

Ohta Y, Kinugawa S, Matsushima S, Ono T, Sobirin MA, Inoue N,... & Tsutsui H (2011). Oxidative stress impairs insulin signal in skeletal muscle and causes insulin resistance in postinfarct heart failure. *Am J Physiol Heart Circ Physiol*, **300**(5), H1637-H1644.

Okita K, Yonezawa K, Nishijima H, Hanada A, Ohtsubo M, Kohya T,... & Kitabatake A (1998). Skeletal muscle metabolism limits exercise capacity in patients with chronic heart failure. *Circulation*, **98**(18), 1886-1891.

Okita K, Yonezawa K, Nishijima H, Hanada A, Nagai T, Murakami T & Kitabatake A (2001). Muscle high-energy metabolites and metabolic capacity in patients with heart failure. *Med Sci Sports Exerc*, **33**(3), 442-448.

Okita K, Kinugawa S & Tsutsui H (2013). Exercise intolerance in chronic heart failure - skeletal muscle dysfunction and potential therapies. *Circ J*, **77**(2), 293-300.

Okutsu M, Call JA, Lira VA, Zhang M, Donet JA, French BA,... & Yan Z (2014). Extracellular superoxide dismutase ameliorates skeletal muscle abnormalities, cachexia, and exercise intolerance in mice with congestive heart failure. *Circ Heart Fail*, **7**(3), 519-530.

Olsen DB, Sacchetti M, Dela F, Ploug T & Saltin B (2005). Glucose clearance is higher in arm than leg muscle in type 2 diabetes. *J Physiol*, **565**(2), 555-562.

Ostler JE, Maurya SK, Dials J, Roof SR, Devor ST, Ziolo MT & Periasamy M (2014). Effects of insulin resistance on skeletal muscle growth and exercise capacity in type 2 diabetic mouse models. *Am J Physiol Endocrinol Metab*, **306**(6), E592-E605.

Owen MR, Doran E & Halestrap AP (2000). Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochemical Journal*, **348**(3), 607-614.

Park SW, Goodpaster BH, Strotmeyer ES, de Rekeneire N, Harris TB, Schwartz AV,... & Newman AB (2006). Decreased muscle strength and quality in older adults with type 2 diabetes: the health, aging, and body composition study. *Diabetes*, **55**(6), 1813-1818.

Park SW, Goodpaster BH, Strotmeyer ES, Kuller LH, Broudeau R, Kammerer C,... & Newman AB (2007). Accelerated loss of skeletal muscle strength in older adults with type 2 diabetes: the health, aging, and body composition study. *Diabetes Care*, **30**(6), 1507-1512.

Park SW, Goodpaster BH, Lee JS, Kuller LH, Boudreau R, de Rekeneire N,... & Newman AB (2009). Excessive loss of skeletal muscle mass in older adults with type 2 diabetes. *Diabetes Care*, **32**(11), 1993-1997.

Parsons D, McIntyre K, Schulz W & Stray-Gundersen J (1993). Capillarity of elite cross-country skiers: a lectin (*Ulex europaeus* I) marker. *Scand J Med Sci Sports*, **3**(2), 89-98.

Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S,... & Saccone R (2003). Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1. *Proc Natl Acad Sci USA*, **100**(14), 8466-8471.

Perry BD, Caldow MK, Brennan-Speranza TC, Sbaraglia M, Jerums G, Garnham A,... & Levinger I (2016). Muscle atrophy in patients with Type 2 Diabetes Mellitus: roles of inflammatory pathways, physical activity and exercise. *Exerc Immunol Rev*, **22**, 94-109.

Perry CG, Kane DA, Lin CT, Kozy R, Cathey BL, Lark DS,... & Neufer PD (2011). Inhibiting myosin-ATPase reveals a dynamic range of mitochondrial respiratory control in skeletal muscle. *Biochem J*, **437**(2), 215-222.

Pesta D & Gnaiger E (2011). Preparation of permeabilized muscle fibres for diagnosis of mitochondrial respiratory function. *Mitochondr Physiol Network*, **14.14**, 1-5.

Pesta D & Gnaiger E (2012). High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. *Methods Mol Biol*, **810**, 25-58.

Petersen KF, Dufour S, Befroy D, Garcia R & Shulman GI (2004). Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med*, **350**(7), 664-671.

Phielix E, Schrauwen-Hinderling VB, Mensink M, Lenaers E, Meex R, Hoeks J,... & Schrauwen P (2008). Lower intrinsic ADP-stimulated mitochondrial respiration underlies *in vivo* mitochondrial dysfunction in muscle of male type 2 diabetic patients. *Diabetes*, **57**(11), 2943-2949.

Phielix E, Meex R, Moonen-Kornips E, Hesselink MK & Schrauwen P (2010). Exercise training increases mitochondrial content and *ex vivo* mitochondrial function similarly in patients with type 2 diabetes and in control individuals. *Diabetologia*, **53**(8), 1714-1721.

Picard M, Ritchie D, Thomas MM, Wright KJ & Hepple RT (2011a). Alterations in intrinsic mitochondrial function with aging are fiber type-specific and do not explain differential atrophy between muscles. *Aging Cell*, **10**(6), 1047-1055.

Picard M, Taivassalo T, Gouspillou G & Hepple RT (2011b). Mitochondria: isolation, structure and function. *J Physiol*, **589**(18), 4413-4421.

Picard M, Taivassalo T, Ritchie D, Wright KJ, Thomas MM, Romestaing C & Hepple RT (2011c). Mitochondrial structure and function are disrupted by standard isolation methods. *PLoS One*, **6**(3), e18317.

Piepoli MF, Corrà U, Agostoni PG, Belardinelli R, Cohen-Solal A, Hambrecht R & Vanhees L (2006). Statement on cardiopulmonary exercise testing in chronic heart failure due to left ventricular dysfunction: recommendations for performance and interpretation. Part I: definition of cardiopulmonary exercise testing parameters for appropriate use in chronic heart failure. *Eur J Cardiov Prev R*, **13**(2), 150-164.

Piepoli MF & Crisafulli A (2014). Pathophysiology of human heart failure: importance of skeletal muscle myopathy and reflexes. *Exp Physiol*, **99**(4), 609-615.

Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JGF, Coats AJS,... & van der Meer P (2016). 2016 ESC guidelines for the diagnosis and treatment of acute and chronic heart failure: The task force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur Heart J*, **37**(27), 2129-2200.

Poole DC & Jones AM (2017). Measurement of the maximum oxygen uptake $\dot{V}O_{2max}$: $\dot{V}O_{2peak}$ is no longer acceptable. *J Appl Physiol*, **122**(4), 997-1002.

Poole DC, Richardson RS, Haykowsky MJ, Hirai DM & Musch TI (2018). Exercise limitations in heart failure with reduced and preserved ejection fraction. *J Appl Physiol*, **124**(1), 208-224.

Rabøl R, Hojberg PM, Almdal T, Boushel R, Haugaard SB, Madsbad S & Dela F (2009). Effect of hyperglycemia on mitochondrial respiration in type 2 diabetes. *J Clin Endocrinol Metab*, **94**(4), 1372-1378.

Rabøl R, Larsen S, Hojberg PM, Almdal T, Boushel R, Haugaard SB,... & Dela F (2010). Regional anatomic differences in skeletal muscle mitochondrial respiration in type 2 diabetes and obesity. *J Clin Endocrinol Metab*, **95**(2), 857-863.

Rajagopalan B, Conway MA, Massie B & Radda GK (1988). Alterations of skeletal muscle metabolism in humans studied by phosphorus 31 magnetic resonance spectroscopy in congestive heart failure. *Am J Cardiol*, **62**(8), 53e-57e.

Ramirez-Sanchez I, Taub PR, Ciaraldi TP, Nogueira L, Coe T, Perkins G,... & Villarreal F (2013). (-)-Epicatechin rich cocoa mediated modulation of oxidative stress regulators in skeletal muscle of heart failure and type 2 diabetes patients. *Int J Cardiol*, **168**(4), 3982-3990.

Rasmussen HN & Rasmussen UF (2003). Oxygen solubilities of media used in electrochemical respiration measurements. *Anal Biochem*, **319**(1), 105-113.

Regensteiner JG, Sippel J, McFarling ET, Wolfel EE & Hiatt WR (1995). Effects of non-insulin-dependent diabetes on oxygen consumption during treadmill exercise. *Med Sci Sports Exerc*, **27**(5), 661-667.

Rehn TA, Munkvik M, Lunde PK, Sjaastad I & Sejersted OM (2012). Intrinsic skeletal muscle alterations in chronic heart failure patients: a disease-

specific myopathy or a result of deconditioning? *Heart Fail Rev*, **17**(3), 421-436.

Reid MB, Lannergren J & Westerblad H (2002). Respiratory and limb muscle weakness induced by tumor necrosis factor-α: involvement of muscle myofilaments. *Am J Respir Crit Care Med*, **166**(4), 479-484.

Reynolds THt, Supiano MA & Dengel DR (2007). Regional differences in glucose clearance: effects of insulin and resistance training on arm and leg glucose clearance in older hypertensive individuals. *J Appl Physiol*, **102**(3), 985-991.

Ripple MO, Kim N & Springett R (2013). Mammalian complex I pumps 4 protons per 2 electrons at high and physiological proton motive force in living cells. *J Biol Chem*, **288**(8), 5374-5380.

Ritov VB, Menshikova EV, He J, Ferrell RE, Goodpaster BH & Kelley DE (2005). Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. *Diabetes*, **54**(1), 8-14.

Ritov VB, Menshikova EV, Azuma K, Wood R, Toledo FG, Goodpaster BH,... & Kelley DE (2010). Deficiency of electron transport chain in human skeletal muscle mitochondria in type 2 diabetes mellitus and obesity. *Am J Physiol Endocrinol Metab*, **298**(1), E49-E58.

Roca J, Hogan MC, Story D, Bebout DE, Haab P, Gonzalez R,... & Wagner PD (1989). Evidence for tissue diffusion limitation of $\dot{V}O_{2max}$ in normal humans. *J Appl Physiol*, **67**(1), 291-299.

Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL, Cline GW & Shulman GI (1996). Mechanism of free fatty acid-induced insulin resistance in humans. *J Clin Invest*, **97**(12), 2859-2865.

Rodriguez-Reyes N, Rodriguez-Zayas AE, Javadov S & Frontera WR (2016). Single muscle fiber contractile properties in diabetic rat muscle. *Muscle Nerve*, **53**(6), 958-964.

Rossignol P, Masson S, Barlera S, Girerd N, Castelnovo A, Zannad F,... & Val-He FTI (2015). Loss in body weight is an independent prognostic factor for mortality in chronic heart failure: insights from the GISSI-HF and Val-HeFT trials. *Eur J Heart Fail*, **17**(4), 424-433.

Rossiter HB, Kowalchuk JM & Whipp BJ (2006). A test to establish maximum O_2 uptake despite no plateau in the O_2 uptake response to ramp incremental exercise. *J Appl Physiol*, **100**(3), 764-770.

Rullman E, Andersson DC, Melin M, Reiken S, Mancini DM, Marks AR,... & Gustafsson T (2013). Modifications of skeletal muscle ryanodine receptor type 1 and exercise intolerance in heart failure. *J Heart Lung Transplant*, **32**(9), 925-929.

Rush JW, Green HJ, Maclean DA & Code LM (2005). Oxidative stress and nitric oxide synthase in skeletal muscles of rats with post-infarction, compensated chronic heart failure. *Acta Physiol Scand*, **185**(3), 211-218.

Ryan TE, Brophy P, Lin CT, Hickner RC & Neufer PD (2014). Assessment of *in vivo* skeletal muscle mitochondrial respiratory capacity in humans by near-infrared spectroscopy: a comparison with *in situ* measurements. *J Physiol*, **592**(15), 3231-3241.

Saks VA, Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T,... & Kunz WS (1998). Permeabilized cell and skinned fiber techniques in studies of mitochondrial function *in vivo. In:* S V.A., V-C R., L X., R A. and R M., eds. *Bioenergetics of the cell: quantitative aspects. Developments in molecular and cellular biochemistry, vol 25.* 1998/09/24 ed. Boston, MA: Springer, 81-100.

Saltin B & Calbet JA (2006). Point: in health and in a normoxic environment, $\dot{V}O_{2max}$ is limited primarily by cardiac output and locomotor muscle blood flow. *J Appl Physiol*, **100**(2), 744-745.

Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A,... & Goldberg AL (2004). Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell*, **117**(3), 399-412.

Santoro C, Cosmas A, Forman D, Morghan A, Bairos L, Levesque S,... & Manfredi T (2002). Exercise training alters skeletal muscle mitochondrial morphometry in heart failure patients. *J Cardiovasc Risk*, **9**(6), 377-381.

Sayer AA, Dennison EM, Syddall HE, Gilbody HJ, Phillips DI & Cooper C (2005). Type 2 diabetes, muscle strength, and impaired physical function: the tip of the iceberg? *Diabetes Care*, **28**(10), 2541-2542.

Saynor ZL, Barker AR, Oades PJ & Williams CA (2013). A protocol to determine valid $\dot{V}O_{2max}$ in young cystic fibrosis patients. *J Sci Med Sport*, **16**(6), 539-544.

Schaufelberger M, Eriksson BO, Grimby G, Held P & Swedberg K (1995). Skeletal muscle fiber composition and capillarization in patients with chronic heart failure: relation to exercise capacity and central hemodynamics. *J Card Fail*, **1**(4), 267-272.

Schaufelberger M, Eriksson BO, Held P & Swedberg K (1996). Skeletal muscle metabolism during exercise in patients with chronic heart failure. *Heart*, **76**(1), 29-34.

Schaufelberger M, Eriksson BO, Grimby G, Held P & Swedberg K (1997). Skeletal muscle alterations in patients with chronic heart failure. *Eur Heart J*, **18**(6), 971-980.

Schiaffino S & Reggiani C (2011). Fiber types in mammalian skeletal muscles. *Physiol Rev*, **91**(4), 1447-1531.

Schmittgen TD & Livak KJ (2008). Analyzing real-time PCR data by the comparative CT method. *Nat Protoc*, **3**(6), 1101-1108.

Schrauwen-Hinderling VB, Kooi ME, Hesselink MK, Jeneson JA, Backes WH, van Echteld CJ,... & Schrauwen P (2007). Impaired *in vivo* mitochondrial function but similar intramyocellular lipid content in patients with type 2 diabetes mellitus and BMI-matched control subjects. *Diabetologia*, **50**(1), 113-120.

Scott AC, Davies LC, Coats AJ & Piepoli M (2002). Relationship of skeletal muscle metaboreceptors in the upper and lower limbs with the respiratory control in patients with heart failure. *Clin Sci*, **102**(1), 23-30.

Scrutinio D, Lagioia R, Ricci A, Clemente M, Boni L & Rizzon P (1994). Prediction of mortality in mild to moderately symptomatic patients with left ventricular dysfunction. The role of the New York Heart Association classification, cardiopulmonary exercise testing, two-dimensional echocardiography and Holter monitoring. *Eur Heart J*, **15**(8), 1089-1095.

Seferovic PM, Petrie MC, Filippatos GS, Anker SD, Rosano G, Bauersachs J,... & McMurray JJV (2018). Type 2 diabetes mellitus and heart failure: a position statement from the Heart Failure Association of the European Society of Cardiology. *Eur J Heart Fail*, **20**(5), 853-872.

Seibæk M, Vestergaard H, Burchardt H, Sloth C, Torp-Pedersen C, Nielsen SL,... & Pedersen O (2003). Insulin resistance and maximal oxygen uptake. *Clin Cardiol*, **26**(11), 515-520.

Seiler M, Bowen TS, Rolim N, Dieterlen MT, Werner S, Hoshi T,... & Adams V (2016). Skeletal muscle alterations are exacerbated in heart failure with

reduced compared with preserved ejection fraction: mediated by circulating cytokines? *Circ Heart Fail*, **9**(9), e003027.

Seyoum B, Estacio RO, Berhanu P & Schrier RW (2006). Exercise capacity is a predictor of cardiovascular events in patients with type 2 diabetes mellitus. *Diab Vasc Dis Res*, **3**(3), 197-201.

Shah AD, Langenberg C, Rapsomaniki E, Denaxas S, Pujades-Rodriguez M, Gale CP,... & Hemingway H (2015). Type 2 diabetes and incidence of cardiovascular diseases: a cohort study in 1·9 million people. *Lancet Diabetes Endo*, **3**(2), 105-113.

Shah AM & Mann DL (2011). In search of new therapeutic targets and strategies for heart failure: recent advances in basic science. *Lancet*, **378**(9792), 704-712.

Shah RV, Altman RK, Park MY, Zilinski J, Leyton-Mange J, Orencole M,... & Das S (2012). Usefulness of hemoglobin A_{1c} to predict outcome after cardiac resynchronization therapy in patients with diabetes mellitus and heart failure. *Am J Cardiol*, **110**(5), 683-688.

Shanely RA, Zwetsloot KA, Triplett NT, Meaney MP, Farris GE & Nieman DC (2014). Human skeletal muscle biopsy procedures using the modified Bergström technique. *JoVE*, (91), 51812.

Shindler DM, Kostis JB, Yusuf S, Quinones MA, Pitt B, Stewart D,... & Wilson AC (1996). Diabetes mellitus, a predictor of morbidity and mortality in the Studies Of Left Ventricular Dysfunction (SOLVD) trials and registry. *Am J Cardiol*, **77**, 1017-1020.

Shulman GI, Rothman DL, Jue T, Stein P, DeFronzo RA & Shulman RG (1990). Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by ^{13}C nuclear magnetic resonance spectroscopy. *N Engl J Med*, **322**(4), 223-228.

Simonini A, Long CS, Dudley GA, Yue P, McElhinny J & Massie BM (1996). Heart failure in rats causes changes in skeletal muscle morphology and gene expression that are not explained by reduced activity. *Circ Res*, **79**(1), 128-136.

Simonini A, Chang K, Yue P, Long CS & Massie BM (1999). Expression of skeletal muscle sarcoplasmic reticulum calcium-ATPase is reduced in rats with postinfarction heart failure. *Heart*, **81**(3), 303-307.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD,... & Klenk DC (1985). Measurement of protein using bicinchoninic acid. *Anal Biochem*, **150**(1), 76-85.

Solomon SD, Sutton MS, Lamas GA, Plappert T, Rouleau JL, Skali H,... & Pfeffer MA (2002). Ventricular remodeling does not accompany the development of heart failure in diabetic patients after myocardial infarction. *Circulation*, **106**(10), 1251-1255.

Sreekumar R, Halvatsiotis P, Schimke JC & Nair KS (2002). Gene expression profile in skeletal muscle of type 2 diabetes and the effect of insulin treatment. *Diabetes*, **51**(6), 1913-1920.

Srere P (1969). Citrate synthase. *Methods Enzymol*, **13**, 3-11.

Starkov AA (2010). Measurement of mitochondrial ROS production. *Methods Mol Biol*, **648**, 245-255.

Stratton IM, Adler AI, Neil HAW, Matthews DR, Manley SE, Cull CA,... & Holman RR (2000). Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. *BMJ*, **321**(7258), 405-412.

Stratton JR, Kemp GJ, Daly RC, Yacoub M & Rajagopalan B (1994). Effects of cardiac transplantation on bioenergetic abnormalities of skeletal muscle in congestive heart failure. *Circulation*, **89**(4), 1624-1631.

Sukhanov S, Semprun-Prieto L, Yoshida T, Michael Tabony A, Higashi Y, Galvez S & Delafontaine P (2011). Angiotensin II, oxidative stress and skeletal muscle wasting. *Am J Med Sci*, **342**(2), 143-147.

Sullivan MJ, Green HJ & Cobb FR (1990). Skeletal muscle biochemistry and histology in ambulatory patients with long-term heart failure. *Circulation*, **81**(2), 518-527.

Sullivan MJ, Duscha BD, Klitgaard H, Kraus WE, Cobb FR & Saltin B (1997). Altered expression of myosin heavy chain in human skeletal muscle in chronic heart failure. *Med Sci Sports Exerc*, **29**(7), 860-866.

Supinski GS & Callahan LA (2005). Diaphragmatic free radical generation increases in an animal model of heart failure. *J Appl Physiol*, **99**(3), 1078-1084.

Supinski GS & Callahan LA (2007). Free radical-mediated skeletal muscle dysfunction in inflammatory conditions. *J Appl Physiol*, **102**(5), 2056-2063.

Suskin N, McKelvie RS, Burns RJ, Latini R, Pericak D, Probstfield J,... & Yusuf S (2000). Glucose and insulin abnormalities relate to functional capacity in patients with congestive heart failure. *Eur Heart J*, **21**(16), 1368-1375.

Swan JW, Anker SD, Walton C, Godsland IF, Clark AL, Leyva F,... & Coats AJ (1997). Insulin resistance in chronic heart failure: relation to severity and etiology of heart failure. *J Am Coll Cardiol*, **30**(2), 527-532.

Swank AM, Horton J, Fleg JL, Fonarow GC, Keteyian S, Goldberg L,... & Kraus WE (2012). Modest increase in peak $\dot{V}O_2$ is related to better clinical outcomes in chronic heart failure patients: results from heart failure and a controlled trial to investigate outcomes of exercise training. *Circ Heart Fail*, **5**(5), 579-585.

Szendroedi J, Phielix E & Roden M (2011). The role of mitochondria in insulin resistance and type 2 diabetes mellitus. *Nat Rev Endocrinol*, **8**(2), 92-103.

Szentesi P, Bekedam MA, van Beek-Harmsen BJ, van der Laarse WJ, Zaremba R, Boonstra A,... & Stienen GJM (2005). Depression of force production and ATPase activity in different types of human skeletal muscle fibers from patients with chronic heart failure. *J Appl Physiol*, **99**(6), 2189-2195.

Takada S, Kinugawa S, Hirabayashi K, Suga T, Yokota T, Takahashi M,... & Sobirin MA (2013). Angiotensin II receptor blocker improves the lowered exercise capacity and impaired mitochondrial function of the skeletal muscle in type 2 diabetic mice. *J Appl Physiol*, **114**(7), 844-857.

Takada S, Kinugawa S, Matsushima S, Takemoto D, Furihata T, Mizushima W,... & Tsutsui H (2015). Sesamin prevents decline in exercise capacity and impairment of skeletal muscle mitochondrial function in mice with high-fat diet-induced diabetes. *Exp Physiol*, **100**(11), 1319-1330.

Tantiwong P, Shanmugasundaram K, Monroy A, Ghosh S, Li M, DeFronzo RA,... & Musi N (2010). NF-κB activity in muscle from obese and type 2 diabetic subjects under basal and exercise-stimulated conditions. *Am J Physiol Endocrinol Metab*, **299**(5), E794-E801.

Tarnopolsky MA, Pearce E, Smith K & Lach B (2011). Suction-modified Bergström muscle biopsy technique: experience with 13,500 procedures. *Muscle Nerve*, **43**(5), 717-725.

Taub PR, Ramirez-Sanchez I, Ciaraldi TP, Perkins G, Murphy AN, Naviaux R,... & Villarreal F (2012). Alterations in skeletal muscle indicators of

mitochondrial structure and biogenesis in patients with type 2 diabetes and heart failure: effects of epicatechin rich cocoa. *Clin Transl Sci*, **5**(1), 43-47.

Tecilazich F, Dinh T, Lyons TE, Guest J, Villafuerte RA, Sampanis C,... & Veves A (2013). Postexercise phosphocreatine recovery, an index of mitochondrial oxidative phosphorylation, is reduced in diabetic patients with lower extremity complications. *J Vasc Surg*, **57**(4), 997-1005.

Tibb AS, Ennezat PV, Chen JA, Haider A, Gundewar S, Cotarlan V,... & Le Jemtel TH (2005). Diabetes lowers aerobic capacity in heart failure. *J Am Coll Cardiol*, **46**(5), 930-931.

Toledo FG, Menshikova EV, Ritov VB, Azuma K, Radikova Z, DeLany J & Kelley DE (2007). Effects of physical activity and weight loss on skeletal muscle mitochondria and relationship with glucose control in type 2 diabetes. *Diabetes*, **56**(8), 2142-2147.

Toth MJ, Shaw AO, Miller MS, VanBuren P, LeWinter MM, Maughan DW & Ades PA (2010). Reduced knee extensor function in heart failure is not explained by inactivity. *Int J Cardiol*, **143**(3), 276-282.

Toth MJ, Miller MS, Ward KA & Ades PA (2012). Skeletal muscle mitochondrial density, gene expression, and enzyme activities in human heart failure: minimal effects of the disease and resistance training. *J Appl Physiol*, **112**(11), 1864-1874.

Townsend N, Williams J, Bhatnagar P, Wickramasinghe K & Rayner M (2014). *Cardiovascular Disease Statistics 2015.* London, UK: British Heart Foundation.

Trounce I, Byrne E & Marzuki S (1989). Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in ageing. *Lancet*, **333**(8639), 637-639.

Tsutsui H, Ide T, Hayashidani S, Suematsu N, Shiomi T, Wen J,... & Takeshita A (2001). Enhanced generation of reactive oxygen species in the limb skeletal muscles from a murine infarct model of heart failure. *Circulation*, **104**(2), 134-136.

Tsutsui H, Kinugawa S & Matsushima S (2011). Oxidative stress and heart failure. *Am J Physiol Heart Circ Physiol*, **301**(6), H2181-H2190.

Turner DL, Hoppeler H, Claassen H, Vock P, Kayser B, Schena F & Ferretti G (1997). Effects of endurance training on oxidative capacity and structural composition of human arm and leg muscles. *Acta Physiol Scand*, **161**(4), 459-464.

Turner N & Heilbronn LK (2008). Is mitochondrial dysfunction a cause of insulin resistance? *Trends Endocrinol Metab*, **19**(9), 324-330.

van Breda E, Schoffelen PFM & Plasqui G (2017). Clinical $\dot{V}O_{2peak}$ is "part of the deal". *J Appl Physiol*, **122**(5), 1370.

van der Ent M, Jeneson JA, Remme WJ, Berger R, Ciampricotti R & Visser F (1998). A non-invasive selective assessment of type I fibre mitochondrial function using ^{31}P NMR spectroscopy. Evidence for impaired oxidative phosphorylation rate in skeletal muscle in patients with chronic heart failure. *Eur Heart J*, **19**(1), 124-131.

van Deursen VM, Urso R, Laroche C, Damman K, Dahlstrom U, Tavazzi L,... & Voors AA (2014). Co-morbidities in patients with heart failure: an analysis of the European Heart Failure Pilot Survey. *Eur J Heart Fail*, **16**(1), 103-111.

Veksler VI, Kuznetsov AV, Sharov VG, Kapelko VI & Saks VA (1987). Mitochondrial respiratory parameters in cardiac tissue: a novel method of

assessment by using saponin-skinned fibers. *Biochim Biophys Acta*, **892**(2), 191-196.

Vermes E, Ducharme A, Bourassa MG, Lessard M, White M & Tardif J-C (2003). Enalapril reduces the incidence of diabetes in patients with chronic heart failure: insight from the Studies Of Left Ventricular Dysfunction (SOLVD). *Circulation*, **107**(9), 1291-1296.

Vescovo G, Volterrani M, Zennaro R, Sandri M, Ceconi C, Lorusso R,... & Dalla Libera L (2000). Apoptosis in the skeletal muscle of patients with heart failure: investigation of clinical and biochemical changes. *Heart*, **84**(4), 431-437.

Volpato S, Bianchi L, Lauretani F, Lauretani F, Bandinelli S, Guralnik JM,... & Ferrucci L (2012). Role of muscle mass and muscle quality in the association between diabetes and gait speed. *Diabetes Care*, **35**(8), 1672-1679.

von Haehling S & Anker SD (2014). Prevalence, incidence and clinical impact of cachexia: facts and numbers - update 2014. *J Cachexia Sarcopenia Muscle*, **5**(4), 261-263.

von Haehling S, Ebner N, Dos Santos MR, Springer J & Anker SD (2017). Muscle wasting and cachexia in heart failure: mechanisms and therapies. *Nat Rev Cardiol*, **14**(6), 323-341.

Vondra K, Rath R, Bass A, Slabochova Z, Teisinger J & Vitek V (1977). Enzyme activities in quadriceps femoris muscle of obese diabetic male patients. *Diabetologia*, **13**(5), 527-529.

Wang X, Hu Z, Hu J, Du J & Mitch WE (2006). Insulin resistance accelerates muscle protein degradation: activation of the ubiquitin-proteasome pathway by defects in muscle cell signaling. *Endocrinology*, **147**(9), 4160-4168.

Wang Y, Negishi T, Negishi K & Marwick TH (2015). Prediction of heart failure in patients with type 2 diabetes mellitus - a systematic review and meta-analysis. *Diabetes Res Clin Pract*, **108**(1), 55-66.

Wei M, Gibbons LW, Mitchell TL, Kampert JB, Lee CD & Blair SN (1999). The association between cardiorespiratory fitness and impaired fasting glucose and type 2 diabetes mellitus in men. *Ann Intern Med*, **130**(2), 89-96.

Wei Y, Sowers JR, Nistala R, Gong H, Uptergrove GM, Clark SE,... & Stump CS (2006). Angiotensin II-induced NADPH oxidase activation impairs insulin signaling in skeletal muscle cells. *J Biol Chem*, **281**(46), 35137-35146.

Wessels B, Ciapaite J, van den Broek NM, Nicolay K & Prompers JJ (2014). Metformin impairs mitochondrial function in skeletal muscle of both lean and diabetic rats in a dose-dependent manner. *PLoS One*, **9**(6), e100525.

Wiener DH, Fink LI, Maris J, Jones RA, Chance B & Wilson JR (1986). Abnormal skeletal muscle bioenergetics during exercise in patients with heart failure: role of reduced muscle blood flow. *Circulation*, **73**(6), 1127-1136.

Williams AD, Selig S, Hare DL, Hayes A, Krum H, Patterson J,... & Carey MF (2004). Reduced exercise tolerance in CHF may be related to factors other than impaired skeletal muscle oxidative capacity. *J Card Fail*, **10**(2), 141-148.

Williams AD, Carey MF, Selig S, Hayes A, Krum H, Patterson J,... & Hare DL (2007). Circuit resistance training in chronic heart failure improves skeletal muscle mitochondrial ATP production rate - a randomized controlled trial. *J Card Fail*, **13**(2), 79-85.

Williams CA, Saynor ZL, Barker AR, Oades PJ & Tomlinson OW (2017). Measurement of $\dot{V}O_{2max}$ in clinical groups is feasible and necessary. *J Appl Physiol*, **123**(4), 1017.

Wilson JR, Martin JL & Ferraro N (1984a). Impaired skeletal muscle nutritive flow during exercise in patients with congestive heart failure: role of cardiac pump dysfunction as determined by the effect of dobutamine. *Am J Cardiol*, **53**(9), 1308-1315.

Wilson JR, Martin JL, Schwartz D & Ferraro N (1984b). Exercise intolerance in patients with chronic heart failure: role of impaired nutritive flow to skeletal muscle. *Circulation*, **69**(6), 1079-1087.

Wilson JR, Fink L, Maris J, Ferraro N, Power-Vanwart J, Eleff S & Chance B (1985). Evaluation of energy metabolism in skeletal muscle of patients with heart failure with gated phosphorus-31 nuclear magnetic resonance. *Circulation*, **71**(1), 57-62.

Witte K, Thackray S, Nikitin N, Cleland J & Clark A (2003). Pattern of ventilation during exercise in chronic heart failure. *Heart*, **89**(6), 610-614.

Wu C, Xu G, Tsai SA, Freed WJ & Lee CT (2017). Transcriptional profiles of type 2 diabetes in human skeletal muscle reveal insulin resistance, metabolic defects, apoptosis, and molecular signatures of immune activation in response to infections. *Biochem Biophys Res Commun*, **482**(2), 282-288.

Wüst RC, Myers DS, Stones R, Benoist D, Robinson PA, Boyle JP,... & Rossiter HB (2012). Regional skeletal muscle remodeling and mitochondrial dysfunction in right ventricular heart failure. *Am J Physiol Heart Circ Physiol*, **302**(2), H402-H411.

Xu L, Poole DC & Musch TI (1998). Effect of heart failure on muscle capillary geometry: implications for O_2 exchange. *Med Sci Sports Exerc*, **30**(8), 1230-1237.

Yang X, Pratley RE, Tokraks S, Bogardus C & Permana PA (2002). Microarray profiling of skeletal muscle tissues from equally obese, non-diabetic insulin-sensitive and insulin-resistant Pima Indians. *Diabetologia*, **45**(11), 1584-1593.

Yokota T, Kinugawa S, Hirabayashi K, Matsushima S, Inoue N, Ohta Y,... & Tsutsui H (2009). Oxidative stress in skeletal muscle impairs mitochondrial respiration and limits exercise capacity in type 2 diabetic mice. *Am J Physiol Heart Circ Physiol*, **297**(3), H1069-H1077.

Yoshida T, Tabony AM, Galvez S, Mitch WE, Higashi Y, Sukhanov S & Delafontaine P (2013). Molecular mechanisms and signaling pathways of angiotensin II-induced muscle wasting: potential therapeutic targets for cardiac cachexia. *Int J Biochem Cell Biol*, **45**(10), 2322-2332.

Yoshida T & Delafontaine P (2015). Mechanisms of cachexia in chronic disease states. *Am J Med Sci*, **350**(4), 250-256.

Young JB, Abraham WT, Smith AL, Leon AR, Lieberman R, Wilkoff B,... & Hall S (2003). Combined cardiac resynchronization and implantable cardioversion defibrillation in advanced chronic heart failure: the MIRACLE ICD trial. *JAMA*, **289**(20), 2685-2694.

Yu Z, Li P, Zhang M, Hannink M, Stamler JS & Yan Z (2008). Fiber type-specific nitric oxide protects oxidative myofibers against cachectic stimuli. *PLoS One*, **3**(5), e2086.

Zheng Y, Ley SH & Hu FB (2018). Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nat Rev Endocrinol*, **14**(2), 88-98.

Zhou M, Diwu Z, Panchuk-Voloshina N & Haugland RP (1997). A stable nonfluorescent derivative of resorufin for the fluorometric determination of

trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal Biochem*, **253**(2), 162-168.

Zhu J, Vinothkumar KR & Hirst J (2016). Structure of mammalian respiratory complex I. *Nature*, **536**(7616), 354-358.

Ziaeian B & Fonarow GC (2016). Epidemiology and aetiology of heart failure. *Nat Rev Cardiol*, **13**(6), 368-378.

Zisa D, Shabbir A, Mastri M, Suzuki G & Lee T (2009). Intramuscular VEGF repairs the failing heart: role of host-derived growth factors and mobilization of progenitor cells. *Am J Physiol Regul Integr Comp Physiol*, **297**(5), R1503-R1515.

Zoll J, Sanchez H, N'Guessan B, Ribera F, Lampert E, Bigard X,... & Mettauer B (2002). Physical activity changes the regulation of mitochondrial respiration in human skeletal muscle. *J Physiol*, **543**(1), 191-200.

Zoll J, Monassier L, Garnier A, N'Guessan B, Mettauer B, Veksler V,... & Geny B (2006). ACE inhibition prevents myocardial infarction-induced skeletal muscle mitochondrial dysfunction. *J Appl Physiol*, **101**(2), 385-391.

Zucker IH, Schultz HD, Li YF, Wang Y, Wang W & Patel KP (2004). The origin of sympathetic outflow in heart failure: the roles of angiotensin II and nitric oxide. *Prog Biophys Mol Biol*, **84**(2-3), 217-232.

Appendix A - Research Ethics Committee (11/YH/0291) approval

NHS

National Research Ethics Service

NRES Committee Yorkshire & The Humber - Leeds West

First Floor
Millside
Mill Pond Lane
Leeds
LS6 4RA

Telephone: 0113 3050122
Facsimile:

06 September 2011

Dr Klaus Witte
Senior Lecturer in Cardiology
Academic Unit of Cardiovascular Medicine
Leeds Institute for Genetics, Health and Therapeutics
The LIGHT Laboratories
University of Leeds
Clarendon Road, Leeds
LS3 9JT

Dear Dr Witte

Study title: Pectoralis muscle biopsy during pacemaker
implantation: a simple way to explore skeletal muscle
abnormalities in chronic heart failure?

REC reference: 11/YH/0291

Thank you for your letter of 29 August 2011, responding to the Committee's request for
further information on the above research.

The further information has been considered on behalf of the Committee by the Chair and
Professor Bird.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the
above research on the basis described in the application form, protocol and supporting
documentation, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to
management permission being obtained from the NHS/HSC R&D office prior to the start of
the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of
the study.

Management permission or approval must be obtained from each host organisation prior to
the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Evidence of insurance or indemnity		10 September 2010
GP/Consultant Information Sheets	1	17 June 2011
Investigator CV		11 July 2011
Other: Map to Physiology Lab		
Participant Consent Form	1	28 June 2011
Participant Information Sheet	1	28 June 2011
Participant Information Sheet: Controls	1	28 June 2011
Protocol	1	17 June 2011
REC application		02 July 2011
Response to Request for Further Information		29 August 2011

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback
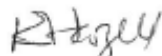
You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

| 11/YH/0291 | Please quote this number on all correspondence |

With the Committee's best wishes for the success of this project

Yours sincerely

Dr Rhona Bratt
Dr Rhona Bratt
Chair

Email: Elaine.hazell@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: Mrs Rachel   De Souza
                          Derek Norfolk, Leeds Teaching Hospitals NHS Trust

Appendix B - Declaration of Helsinki

Declaration of Helsinki

World Medical Association Declaration of Helsinki

Ethical Principles for Medical Research Involving Human Subjects

Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964; amended by the 29th WMA General Assembly, Tokyo, Japan, October 1975; 35th WMA General Assembly, Venice, Italy, October 1983; 41st WMA General Assembly, Hong Kong, September 1989; 48th WMA General Assembly, Somerset West, Republic of South Africa, October 1996, and the 52nd WMA General Assembly, Edinburgh, Scotland, October 2000

A. Introduction

1.   The World Medical Association has developed the Declaration of Helsinki as a statement of ethical principles to provide guidance to physicians and other participants in medical research involving human subjects. Medical research involving human subjects includes research on identifiable human material or identifiable data.

2.   It is the duty of the physician to promote and safeguard the health of the people. The physician's knowledge and conscience are dedicated to the fulfillment of this duty.

3.   The Declaration of Geneva of the World Medical Association binds the physician with the words, "The health of my patient will be my first consideration," and the International Code of Medical Ethics declares that, "A physician shall act only in the patient's interest when providing medical care which might have the effect of weakening the physical and mental condition of the patient."

4.   Medical progress is based on research which ultimately must rest in part on experimentation involving human subjects.

5.   In medical research on human subjects, considerations related to the well-being of the human subject should take precedence over the interests of science and society.

6.   The primary purpose of medical research involving human subjects is to improve prophylactic, diagnostic and therapeutic procedures and the understanding of the aetiology and pathogenesis of disease. Even the best proven prophylactic, diagnostic, and therapeutic methods must continuously be challenged through research for their effectiveness, efficiency, accessibility and quality.

7.   In current medical practice and in medical research, most prophylactic, diagnostic and therapeutic procedures involve risks and burdens.

8.   Medical research is subject to ethical standards that promote respect for all human beings and protect their health and rights. Some research populations are vulnerable and need special protection. The particular needs of the economically and medically disadvantaged must be recognized. Special attention is also required for those who cannot give or refuse consent for themselves, for those who may be subject to giving consent under duress, for those who will not benefit personally from the research and for those for whom the research is combined with care.

9.   Research Investigators should be aware of the ethical, legal and regulatory requirements for research on human subjects in their own countries as well as applicable international requirements. No national ethical, legal or regulatory requirement should be allowed to reduce or eliminate any of the protections for human subjects set forth in this Declaration.

B. Basic principles for all medical research

10. It is the duty of the physician in medical research to protect the life, health, privacy, and dignity of the human subject.

11. Medical research involving human subjects must conform to generally accepted scientific principles, be based on a thorough knowledge of the scientific literature, other relevant sources of information, and on adequate laboratory and, where appropriate, animal experimentation.

12. Appropriate caution must be exercised in the conduct of research which may affect the environment, and the welfare of animals used for research must be respected.

13. The design and performance of each experimental procedure involving human subjects should be clearly formulated in an experimental protocol. This protocol should be submitted for consideration, comment, guidance, and where appropriate, approval to a specially appointed ethical review committee, which must be independent of the investigator, the sponsor or any other kind of undue influence. This independent committee should be in conformity with the laws and regulations of the country in which the research experiment is performed. The committee has the right to monitor ongoing trials. The researcher has the obligation to provide monitoring information to the committee, especially any serious adverse events. The researcher should also submit to the committee, for review, information regarding funding, sponsors, institutional affiliations, other potential conflicts of interest and incentives for subjects.

14. The research protocol should always contain a statement of the ethical considerations involved and should indicate that there is compliance with the principles enunciated in this Declaration.

15. Medical research involving human subjects should be conducted only by scientifically qualified persons and under the supervision of a clinically competent medical person. The responsibility for the human subject must always rest with a medically qualified person and never rest on the

subject of the research, even though the subject has given consent.

16. Every medical research project involving human subjects should be preceded by careful assessment of predictable risks and burdens in comparison with foreseeable benefits to the subject or to others. This does not preclude the participation of healthy volunteers in medical research. The design of all studies should be publicly available.

17. Physicians should abstain from engaging in research projects involving human subjects unless they are confident that the risks involved have been adequately assessed and can be satisfactorily managed. Physicians should cease any investigation if the risks are found to outweigh the potential benefits or if there is conclusive proof of positive and beneficial results.

18. Medical research involving human subjects should only be conducted if the importance of the objective outweighs the inherent risks and burdens to the subject. This is especially important when the human subjects are healthy volunteers.

19. Medical research is only justified if there is a reasonable likelihood that the populations in which the research is carried out stand to benefit from the results of the research.

20. The subjects must be volunteers and informed participants in the research project.

21. The right of research subjects to safeguard their integrity must always be respected. Every precaution should be taken to respect the privacy of the subject, the confidentiality of the patient's information and to minimize the impact of the study on the subject's physical and mental integrity and on the personality of the subject.

22. In any research on human beings, each potential subject must be adequately informed of the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail. The subject should be informed of the right to abstain from participation in the study or to withdraw consent to participate at any time without reprisal. After ensuring that the subject has understood the information, the physician should then obtain the subject's freely-given informed consent, preferably in writing. If the consent cannot be obtained in writing, the non-written consent must be formally documented and witnessed.

23. When obtaining informed consent for the research project the physician should be particularly cautious if the subject is in a dependent relationship with the physician or may consent under duress. In that case the informed consent should be obtained by a well-informed physician who is not engaged in the investigation and who is completely independent of this relationship.

24. For a research subject who is legally incompetent, physically or mentally incapable of giving consent or is a legally incompetent minor, the investigator must obtain informed consent from the legally authorized representative in accordance with applicable law. These groups should not be included in research unless the research is necessary to promote the health of the population represented and this research cannot instead be performed on legally competent persons.

25. When a subject deemed legally incompetent, such as a minor child, is able to give assent to decisions about participation in research, the investigator must obtain that assent in addition to the consent of the legally authorized representative.

26. Research on individuals from whom it is not possible to obtain consent, including proxy or advance consent, should be done only if the physical/mental condition that prevents obtaining informed consent is a necessary characteristic of the research population. The specific reasons for involving research subjects with a condition that renders them unable to give informed consent should be stated in the experimental protocol for consideration and approval of the review committee. The protocol should state that consent to remain in the research should be obtained as soon as possible from the individual or a legally authorized surrogate.

27. Both authors and publishers have ethical obligations. In publication of the results of research, the investigators are obliged to preserve the accuracy of the results. Negative as well as positive results should be published or otherwise publicly available. Sources of funding, institutional affiliations and any possible conflicts of interest should be declared in the publication. Reports of experimentation not in accordance with the principles laid down in this Declaration should not be accepted for publication.

C. Additional principles for medical research combined with medical care

28. The physician may combine medical research with medical care, only to the extent that the research is justified by its potential prophylactic, diagnostic or therapeutic value. When medical research is combined with medical care, additional standards apply to protect the patients who are research subjects.

29. The benefits, risks, burdens and effectiveness of a new method should be tested against those of the best current prophylactic, diagnostic, and therapeutic methods. This does not exclude the use of placebo, or no treatment, in studies where no proven prophylactic, diagnostic or therapeutic method exists.

30. At the conclusion of the study, every patient entered into the study should be assured of access to the best proven prophylactic, diagnostic and therapeutic methods identified by the study.

31. The physician should fully inform the patient which aspects of the care are related to the research. The refusal of a patient to participate in a study must never interfere with the patient–physician relationship.

32. In the treatment of a patient, where proven prophylactic, diagnostic and therapeutic methods do not exist or have been ineffective, the physician, with informed consent from the patient, must be free to use unproven or new prophylactic, diagnostic and therapeutic measures, if in the physician's judgement it offers hope of saving life, re-establishing health or alleviating suffering. Where possible, these measures should be made the object of research, designed to evaluate their safety and efficacy. In all cases, new information should be recorded and, where appropriate, published. The other relevant guidelines of this Declaration should be followed. ∎

Appendix C - Participant information sheet

Dr Klaus Witte
Academic Unit of Cardiovascular Medicine
Leeds Institute for Genetics, Health and Therapeutics
The LIGHT Laboratories
University of Leeds
Leeds LS2 9JT

Information sheet

Chief Investigator: Dr. Klaus K Witte (Tel: 0113 3926642)

Title: Pectoralis muscle biopsy during pacemaker implantation: a simple way to explore skeletal muscle abnormalities in chronic heart failure? – Thigh muscle biopsy substudy

You are being asked to take part in a research study. Before agreeing to participate in this study, it is important that you read and understand the following explanation of the proposed study procedures. The following information describes the purpose, procedures, benefits, discomforts, risks and precautions associated with this study. It also describes your right to refuse to participate or withdraw from the study at any time. In order to decide whether you wish to participate in this research study, you should understand enough about its risks and benefits to be able to make an informed decision. This is known as the informed consent process. Please ask the study doctor or study staff to explain any words you don't understand before signing this consent form. Make sure all your questions have been answered to your satisfaction before signing the consent form.

Purpose
Chronic heart failure is a condition most people associate with a weakened heart. However, there are lots of other features. One feature that is often overlooked is weakness of the muscles of the body to do with movement and exercise such as the thigh muscles which are known as skeletal muscles. From samples (biopsies) taken from the thigh muscles of patients with heart failure we know that there are changes associated with heart failure that make these muscles function much less effectively with reduced power and endurance. Even in patients whose hearts get better or receive a transplant these muscle abnormalities persist and contribute to ongoing symptoms. We don't really know why the skeletal muscles degenerate like this but there are lots of tests we could do to examine this and hopefully find treatments. One reason that progress has been so slow is that getting samples of muscle tissue usually requires a small operation under local anaesthetic on the thigh which can be painful. Many people don't really want to undergo an operation, even a small one, when it is done purely for research purposes. We have come up with a unique solution which we hope will take this field much further very rapidly.

Pacemaker implantation, which your doctor will already have discussed with you, requires an operation below the collar bone under local anaesthetic. The pacemaker generator is placed under the skin but onto the pectoralis muscle which is the major chest muscle.

Dr Klaus Witte
Academic Unit of Cardiovascular Medicine
Leeds Institute for Genetics, Health and Therapeutics
The LIGHT Laboratories
University of Leeds
Leeds LS2 9JT

This provides us with an opportunity to take a small sample of this muscle from lots of patients without the need for an extra procedure.

Why have I been invited to take part?
We have invited you take part in this study because you are receiving a pacemaker as part of your treatment. This provides an opportunity to sample your muscle. We will then use that small sample to carefully look at all of the aspects of muscle function to find out which are abnormal in heart failure so that eventually we can work on treatments.

Do I have to take part?
It is up to you to decide. Dr Witte (the principle investigator) will telephone you in a week to allow you the opportunity to ask questions, but there are telephone numbers at the end of this information sheet if you wish to call yourself. We will describe the study and go through this information sheet, which we will then give to you. We will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

What will happen if I take part?
If you agree to participate in this study, we will invite you to come to the Cardiology Department at Leeds General Infirmary or the Exercise Physiology Laboratory at the University of Leeds on three separate occasions. The Exercise Physiology Laboratory is on the 5th **floor (there is a lift) of the Garstang Building** (just along the same road as the Jubilee Building where you go for your outpatient visits) (please see the enclosed map).

Each test will be performed on a stationary bicycle, and during the test we will sample the air you breathe in and out through a mouthpiece to measure and the amount of air you bearth in and out and the concentrations of oxygen and carbon dioxide. You will also have an "electrocardiogram" or ECG (a tracing of your heart activity) measured throughout and we will attach a small plastic clip to your earlobe (called a pulse oximeter) which shines a red light and measures the amount of oxygen in your blood. You may have previously experienced one of these monitors on your finger tip on the wards or in clinic. We will also strap a monitor to your thigh (this is called a near-infrared oximeter), which will allow us to measure the amount of oxygen in your leg muscles. This machine works in the same way (using light absorption) as the ear probe clip and is completely painless. We will ask you to bring shorts to wear during each test.

During this visit we will ask you to perform what is called a "progressive exercise test" on the exercise bike. During this test, the work rate will start very low and increase gradually every minute. The idea is that you keep going until you can do no more. The test will be stopped when you decide you can go no longer. During this test you will be asked score your level of breathlessness at each stage during the exercise. This is an

Dr Klaus Witte
Academic Unit of Cardiovascular Medicine
Leeds Institute for Genetics, Health and Therapeutics
The LIGHT Laboratories
University of Leeds
Leeds LS2 9JT

accurate way to measure your exercise capacity.

At the second and third visits the set-up will remain the same but the intensity of the work will be less. Instead of the work rate gradually rising constantly throughout the test, the work rate will change instantaneously between one level and another - we call this "constant work rate exercise". At each visit you will be asked to perform two constant work rate exercise bouts; each lasting 6 minutes separated by 6 minutes of rest. The work rates for the constant work rate exercise bouts will be around 50% of your maximum intensity (i.e. they are submaximal tests).

At these visits we will also measure your leg muscle strength. We will ask you to sit on a large seat and we will strap your dominant leg into an arm extending below the seat. We will then ask you to straighten your leg as hard as you can. This brief test will take only a few seconds but we will repeat it three times and record the best result. At one of these visits we will do some blood tests for kidney function, liver function and tests to look at your stores of iron, vitamins, and markers of immune function. Many of these tests are done prior to the pacemaker procedure anyway.

At one of these visits, or possibly just before the pacemaker procedure, we will measure your handgrip strength. This is done using a small machine that you hold firmly in your palm. We will ask you to grip as tightly as you can for a few seconds and then release. We will ask you to repeat this test three times in total and we will record the strongest reading we get.

During the pacemaker procedure, Dr Witte will take a small sample (about 0.5cm x 1.0cm) from your pectoralis muscle and a small sample of the fat when he inserts the pacemaker. The sampling is unlikely to be painful. The pacemaker procedure will otherwise be entirely normal and the sampling will take less than 5 minutes.

The muscle and fat samples will immediately be sent to laboratories in the University of Leeds where scientists will look at each phase of the function of the muscle cells and the arteries supplying the fat cells. Unused samples will be stored in an approved facility or destroyed immediately. We would also like to take a small sample of your blood (about 10mls – as much as two teaspoonfuls) at the same time as the routine tests you'll have done on admission to look at measures of inflammation, sugar balance and other markers of heart failure and diabetes severity. This will also be analysed immediately or stored in an approved facility at the University of Leeds.

The following day Dr Witte will come and check the pacemaker wound and arrange the discharge and follow-up.

Optional Substudy
The following section describes an additional procedure which is optional. If you decide

Dr Klaus Witte
Academic Unit of Cardiovascular Medicine
Leeds Institute for Genetics, Health and Therapeutics
The LIGHT Laboratories
University of Leeds
Leeds LS2 9JT

that you do not want to have this done you can still participate in the rest of the study.

In order to compare the information from the pectoralis muscle with a muscle used for walking we would very much like to take a small sample from your thigh muscle. To sample this requires an additional procedure which involves a small cut in the skin of around 5mm just above the knee. The procedure is done under local anaesthetic and takes about ten minutes. This part of the study will be performed immediately prior to the pacemaker procedure. The thigh muscle biopsy leaves a small scar just above the knee that might ache for a day or two but will not interfere with your walking. You should consider not doing any strenuous exercise for 4-5 days after the thigh muscle biopsy.

Following the procedure, the biopsy wound is dressed in several layers, including adhesive skin closure tape, a soft adhesive sterile dressing, a waterproof vapour permeable dressing and a pressure dressing comprising gauze pads and an elastic bandage. Below is some further advice on how to care for your dressing in the days following the biopsy:

How do I care for my dressing?
We advise that you follow the following regime strictly, ensuring that you keep the dressing on over the indicated time and keep the dressing dry:

a. Remove the pressure dressing the day after your biopsy. If need be it can be removed earlier (even after 3-4 hours) but most participants are more comfortable if it is retained overnight
b. Leave the waterproof dressing for a minimum of 24 and preferably 48 hours. This gives time for the wound to form a bacterial-proof seal.
c. Leave the wound dressing and skin closure tapes for 4-5 days. Although these are not waterproof the dressings will normally remain in place even if they get wet during showering. They are not bath proof! After removing the dressings you can cover the wound with a simple Airstrip or Elastoplast dressing for additional protection.

What about bathing?
We would advise against getting the primary dressings wet as the elastic bandage is not waterproof and the gauze pads absorb water.

If you must bathe (and we would encourage showering) the following seems to work:

- Push your leg through a plastic shopping bag so that the hole fits tightly round the thigh just below your dressing.
- Tie the top of the bag snugly round your thigh above the dressing.
- Try not to saturate the dressing in the shower.
  Cling film round the dressing does not work!

Dr Klaus Witte
Academic Unit of Cardiovascular Medicine
Leeds Institute for Genetics, Health and Therapeutics
The LIGHT Laboratories
University of Leeds
Leeds LS2 9JT

- If you want to have a bath, protect your leg as above and keep your leg out of the water

After you have removed the pressure dressing you can shower as normal. We would advise against soaking in the bath until you are ready to remove the wound closure dressings (4-5 days) and you will find that they are easier to remove when wet. Should you have any questions, please telephone the ward on 0113 3927114 or the Cardiovascular Clinical Research Facility on 0113 39 28240.

Risks

The risk of any adverse event with this project is very small. It is possible, although unlikely that the bruising from the pacemaker procedure might be slightly more as a result of the muscle biopsy, but this will settle. The removal of this small biopsy sample will not affect the function of the pectoralis muscle.

The thigh muscle biopsy will initially leave a small bruise and then a small scar on the lower portion of your thigh just above the knee. This might ache for a day or two but we do not expect any long term effects from this procedure.

Exercise testing can leave you feeling a little tired following the test, but you should rest for the remainder of the day. Exercise tests are safe in patients with heart problems.

Benefits

We want to learn about why patients with heart failure feel tired and breathless during exercise. It may be that by examining the effects of skeletal muscle function, we can develop treatments that target the muscle weakness specifically. You are unlikely to benefit personally benefit from participation in this study. However, information learned may benefit future patients with heart problems.

Participation

Your participation in this study is voluntary. You can choose not to participate or you may withdraw at any time without affecting your medical care. There will be compensation for reasonable for travel expenses.

Compensation

If you become ill or are physically injured as a result of participation in this study, medical treatment will be provided. In no way does signing this consent form waive your legal rights nor does it relieve the investigators, sponsors or involved institutions from their legal and professional responsibilities.

Part 2

Complaints

Dr Klaus Witte
Academic Unit of Cardiovascular Medicine
Leeds Institute for Genetics, Health and Therapeutics
The LIGHT Laboratories
University of Leeds
Leeds LS2 9JT

If you have a concern about any aspect of this study, you should ask to speak to Dr Klaus Witte who will do his best to answer your questions (0113 3926642). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against Leeds University or Leeds Teaching Hospitals NHS Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Confidentiality
All information obtained during the study will be held in strict confidence. You will be identified with a study number only. No names or identifying information will be used in any publication or presentations. No information identifying you will be transferred outside the investigators in this study. During the regular monitoring of your study or in the event of an audit, your medical record may be reviewed by the Hospital Research Ethics Board. We will however make your General Practitioner aware that you are participating in this study.

What will happen to the results of the study?
We will inform all participants of the results of the study and the results will be published in international peer-reviewed journals.

Who has reviewed the study?
All research in the NHS and the University of Leeds is looked at by independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by Leeds West Research Ethics Committee.

Questions
If you have any further questions about the study, or would like to be included in this research, please call Dr Klaus K Witte on 0113 3926642 or write to Dr Witte at the Cardiology Department, G-floor, Jubilee Wing, Leeds General Infirmary, Great George Street, Leeds, LS1 3EX.

Appendix D - Consent form

Dr Klaus Witte
Division of Cardiovascular and Diabetes Research
Leeds Institute for Cardiovascular and Metabolic Medicine
The LIGHT Laboratories
University of Leeds
Leeds LS2 9JT

Centre Number: 1

Study Number: 1

Patient Identification Number for this trial:

CONSENT FORM

Title of Project: Pectoralis muscle biopsy during pacemaker implantation: a simple way to explore skeletal muscle abnormalities in chronic heart failure?

Name of Investigator: Dr Klaus Witte

Please initial box

1. I confirm that I have read and understand the information sheet dated **24th April 2016 (Version 3.1)** 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 am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I confirm that I have agreed to undergo a thigh muscle biopsy as described at the time of the pacemaker implant procedure.

4. I understand that relevant sections of my medical notes and data collected during the study may be looked at by regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

5. I agree to my GP being informed of my participation in the study

6. I agree to take part in the above study.

_____   _____   _____
Name of Subject   Date   Signature

_____   _____   _____
Name of Person   Date   Signature
taking consent

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Appendix E - Good Clinical Practice certificate

NHS
National Institute for
Health Research

Clinical Research Network

Certificate of Completion

Jack Garnham

has completed

Introduction to Good Clinical Practice (GCP)
e-learning course

A practical guide to ethical
and scientific quality standards in clinical research

on

23 June2015

Modules completed:

Introduction to Research in the NHS
Good Clinical Practice and Standards in Research
Study Set Up and Responsibilities
The Process of Informed Consent
Data Collection and Documentation
Safety Reporting

National Institute for
Health Research
Clinical Research Network

Appendix F - NIHR informed consent workshop certificate

NHS
National Institute for
Health Research
Clinical Research Network
Yorkshire and Humber

Certificate of Attendance

This is to certify that

Jack Garnham

attended the:

Yorkshire and Humber
Informed Consent Workshop

Monday 29 June 2015

Sessions included:
1. History and Background
2. UK Regulations and Guidelines
3. Genetics
4. Responsibilities

5. PPI
6. The Consent Process
7. Vulnerable Groups

"Wonderful ambassador for this important message"
Henrietta Lacks Foundation 2012

Signed _____
Dr Christine Davey, Research Advisor
CRN: Yorkshire and Humber