

BEET-HF: The Effects of Dietary Inorganic Nitrate Supplementation on
Aerobic Exercise Performance, Vascular Function, Cardiac Performance and
Mitochondrial Respiration in Patients with Heart Failure with Reduced Ejection
Fraction

Submitted by:

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Abstract

Chronic heart failure (CHF) is characterised by an inability of the heart to pump enough blood to meet the body's metabolic needs, resulting in exercise intolerance. A reduction in nitric oxide (NO) bioavailability has been implicated as an initiator and/or contributor to many of the peripheral skeletal tissue dysfunctions that contribute to the exercise intolerance in patients with CHF. Inorganic nitrate supplementation has been identified as an important mediator of exercise tolerance via increasing NO bioavailability, but the potential efficacy of this on patients with heart failure with reduced ejection fraction (HFrEF) as well as the effect on vascular function is not well understood and was the focus of *Study 1*. Additionally, to our knowledge, no previous study has examined the potential impact of nitrate supplementation on cardiac performance during submaximal exercise and mitochondrial respiration in individuals with HFrEF. These were the foci of *Studies 2* and *3* respectively.

Study 1: The effect of dietary inorganic nitrate supplementation on exercise tolerance and vascular function in patients with HFrEF

The primary aim of this study was to determine the effect of chronic inorganic nitrate supplementation on exercise tolerance, as measured by peak aerobic capacity ($\text{VO}_{2\text{peak}}$) and time to exhaustion (TTE), during treadmill exercise in patients with HFrEF. A secondary aim was to determine the effect of chronic supplementation on vascular function (endothelial function) in these patients. **Methods:** Sixteen patients with HFrEF (15 men and 1 woman, 63 ± 4 y, BMI: 31.8 ± 2.1 $\text{kg}\cdot\text{m}^{-2}$) completed the primary outcome of this study (exercise tolerance), and 12 completed the vascular function component. Participants were randomly allocated, in a double-blind, crossover design, to consume either a nitrate rich beetroot juice (16mmol nitrate/day), or a nitrate-

depleted placebo for five days prior to the first testing visit. Participants then continued daily dosing until they completed a cardiopulmonary exercise test (CPX) and a battery of vascular function assessments (peripheral and central blood pressure (BP) as well as aortic stiffness and brachial artery flow mediated dilation (BAFMD)). **Results:** There were significant increases in both plasma nitrate ($p < 0.001$) and nitrite ($p < 0.05$) following nitrate supplementation. No significant differences were observed in either $\text{VO}_{2\text{peak}}$ (nitrate $18.5 \pm 5.7 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, placebo: $19.3 \pm 1.4 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $p = 0.13$) or TTE (nitrate: $1165 \pm 92 \text{ sec}$, placebo: $1207 \pm 96 \text{ sec}$, $p = 0.16$) between the two interventions. Similarly, there were no significant ($p > 0.05$) changes in peripheral tissue oxygenation during exercise, as measured non-invasively with near-infrared spectroscopy (NIRS). There were no differences in the brachial blood pressure measurements including systolic blood pressure (SBP) (nitrate: $130 \pm 4 \text{ mmHg}$, placebo: $132 \pm 5 \text{ mmHg}$, $p = 0.58$), diastolic blood pressure (DBP) (nitrate: $80 \pm 3 \text{ mmHg}$, placebo: $81 \pm 3 \text{ mmHg}$, $p = .74$) and mean arterial pressure (MAP) (nitrate: $96 \pm 3 \text{ mmHg}$, placebo: $98 \pm 4 \text{ mmHg}$, $p = 0.67$). There were also no significant differences in aortic pressure or stiffness. BAFMD reactive hyperaemic percent change tended to improve (nitrate: $5.7\% \pm 1.1$, placebo: $4.1\% \pm 0.7$, ($p = 0.06$), and this change had a moderate effect size (ES) (Cohen's d 0.607). **Conclusions:** Results from this study indicate the nitrate appears ineffective at improving exercise tolerance and vascular function in HFrEF. Future studies should explore alternative interventions to improve peripheral muscle tissue function in HFrEF.

Study 2: The effect of dietary nitrate supplementation on cardiac output and stroke volume during submaximal exercise in men with HFrEF: a pilot study

The primary aim of this exploratory study was to determine the effect of chronic inorganic nitrate supplementation on cardiac performance during three submaximal exercise bouts. **Methods:** Five male patients with HFrEF (61 ± 3 y) completed this pilot study. Participants consumed either the nitrate-rich beetroot juice (16 mmol nitrate) or the placebo an average of 13 ± 2 days prior to the testing visit. They completed a three-stage (15-25 watts, 25-40 watts and 35-60 watts) discontinuous exercise protocol on an echo-compatible recumbent cycle ergometer with simultaneous Doppler echocardiography. Cardiac output (\dot{Q}) and stroke volume (SV) were derived using the Doppler velocity time integral via the Huntsman method. **Results:** There were significant increases in both plasma nitrate ($p=0.004$, $ES=3.54$) and nitrite ($p=0.01$, $ES=0.82$) following nitrate supplementation. Although not statistically significant (all $p>0.27$), the differences in \dot{Q} during stage two and stage three had medium to large ES (stage two: nitrate: $6.4 \pm .4$ L \cdot min $^{-1}$, placebo: $5.3 \pm .2$ L \cdot min $^{-1}$, $ES=1.51$; stage three: nitrate: 7.5 ± 0.6 L \cdot min $^{-1}$, placebo: 6.4 ± 0.7 L \cdot min $^{-1}$, $ES=0.50$) exercise. Changes in \dot{Q} were accompanied by medium to large ES changes in SV (stage two: $ES=0.97$ and stage three: $ES=0.57$) and medium to large increases in heart rate (HR) at rest and all exercise stages. These differences were likely mediated by a reduction in total peripheral resistance (TPR) at stage two ($ES=-1.62$) and stage three ($ES=-0.81$). **Conclusions:** We report potentially clinically important improvements in measures of cardiac performance during submaximal exercise following nitrate supplementation in patients with HFrEF. The initial findings from this pilot study warrant further investigation in larger and more diverse samples in order to determine the efficacy of this intervention.

Study 3: The effect of dietary nitrate supplementation on mitochondrial respiration in men with HFrEF

The primary aim of this exploratory study was to determine the effect of chronic inorganic nitrate supplementation on parameters of mitochondrial respiration in patients with HFrEF. **Methods:** Seven male participants (62 ± 2 y) completed this invasive study. Participants consumed the nitrate rich beetroot juice (16mmol nitrate/day) or a placebo for an average of 15 ± 2 days prior to their muscle biopsy. Muscle samples were taken from the vastus lateralis. Mitochondrial respiration was assessed using high resolution respirometry. Western blot analysis was used to assess the protein content of mechanistic target of rapamycin complex 1 (mTORC1), p38 mitogen activated protein kinase (p38MAPK), protein kinase B (Akt), and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α). **Results:** Plasma nitrate increased (831%, $p < 0.001$) following supplementation. Plasma nitrite also increased (100%) but this was not statistically significant ($p = 0.22$). There were no differences in skeletal muscle maximal oxidative phosphorylation capacity as assessed as either mass-specific ($p = 0.93$) or mitochondrial-specific ($p = 0.68$) respiratory function of (CI+CII)_p, nor were there any significant differences in other parameters of mitochondrial respiration (all $p > 0.05$). Similarly, there were no differences in mitochondrial content, as assessed by citrate synthase activity ($p = 0.73$) and no differences were noted in total and phosphorylated forms of mTORC1, p38MAPK, Akt, or PGC-1 α (all $p > 0.10$).

Conclusions: Short-term nitrate supplementation, as a standalone treatment, may not be an effective way to improve mitochondrial function in patients with HFrEF and, as such, it may be clinically important to combine nitrate supplementation with other interventions known to affect mitochondrial function, such as exercise training.

General Conclusions

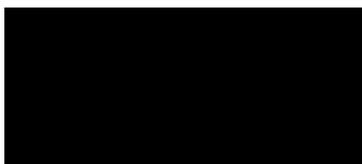
Short-term inorganic nitrate supplementation had no effect on exercise tolerance (*Study 1-Chapter 4*), peripheral tissue oxygenation (*Study 1- Chapter 4*), or mitochondrial respiration (*Study 3- Chapter 6*) in patients with HFrEF. However, it may have a meaningful clinical effect on \dot{Q} and SV during submaximal exercise (*Study 2- Chapter 5*). It may also improve vascular function (**Chapter 4**), reduce TPR (**Chapter 5**) and reduce DBP and MAP during submaximal exercise (**Chapter 5**) in these patients.

Overall the data suggest that nitrate supplementation may be used in conjunction with other pharmacological and non-pharmacological (exercise training) interventions to improve clinical outcomes in this population. This hypothesis should be explored in the future by conducting a large-scale clinical trial.

Declaration

I, Mary N. Woessner, declare that the PhD thesis entitled “BEET-HF: The Effects of Dietary Inorganic Nitrate Supplementation on Aerobic Exercise Performance, Vascular Function, Cardiac Performance and Mitochondrial Respiration in Patients with Heart Failure with Reduced Ejection” is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature



Date 02/09/2019

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2. Woessner, M. N., McIlvenna, L. C., Ortiz de Zevallos, J., Neil, C. J., & Allen, J. D. (2018). Dietary nitrate supplementation in cardiovascular health: an ergogenic aid or exercise therapeutic? *Am J Physiol Heart Circ Physiol*, 314(2), H195-H212. doi:10.1152/ajpheart.00414.2017

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List of abbreviations

ACE	Angiotensin-Converting Enzyme
ACE-Inhibitor	Angiotensin-Converting Enzyme Inhibitor
ADL	Activities of Daily Living
ADP	Adenosine Diphosphate
AIX	Augmentation Index
Akt	Protein kinase B
AMPK	Adenosine Monophosphate-Activated Protein Kinase
ANOVA	Analysis of Variance
AP	Augmentation Pressure
ARB	Angiotensin II Receptor Blockers
ATP	Adenosine Triphosphate
a-VO ₂ diff	Arterial-Venous Oxygen Difference
BAFMD	Brachial Artery Flow Mediated Dilation
β-blockers	Beta-Adrenergic Blockers
BP	Blood Pressure
Ca ⁺⁺	Calcium
CAD	Coronary Artery Disease
cGMP	Cyclic Guanosine Monophosphate
CHF	Chronic Heart Failure
CI	Cardiac Index
CPX	Cardiopulmonary Exercise Test
CVD	Cardiovascular Disease
CL	Chemiluminescence
CS	Citrate Synthase

DASH	Dietary Approaches to Stop Hypertension
DBP	Diastolic Blood Pressure
eNOS	Endothelial Nitric Oxide Synthase
ECG	Electrocardiogram
EF	Ejection Fraction
ES	Effect Size
ETS	Electron Transport System
FHS	Framingham Heart Study
FMD	Flow Mediated Dilation
GET	Gas Exchange Threshold
GTN	Glyceryl Trinitrate
HCl	Hydrochloric Acid
HF	Heart Failure
HFmrEF	Heart Failure Mid-Range Ejection Fraction
HFpEF	Heart Failure Preserved Ejection Fraction
HFrEF	Heart Failure Reduced Ejection Fraction
HbO ₂	Oxygenated Haemoglobin
Hb _{tot}	Total Haemoglobin
HHb	Deoxygenated Haemoglobin
HR	Heart Rate
HRR	Heart Rate Recovery
HTN	Hypertension
iNOS	Inducible Nitric Oxide Synthase
L-NMMA	NG-Monomethyl-L-Arginine
LV	Left Ventricle

LVEF	Left Ventricular Ejection Fraction
L VH	Left Ventricular Hypertrophy
LVOT	Left Ventricular Outflow Tract Diameter
MAP	Mean Arterial Pressure
MI	Myocardial Infarction
mTORC1	Mechanistic Target of Rapamycin Complex 1
NIDCM	Non-Ischaemic Dilated Cardiomyopathy
NIRS	Near-Infra-Red Spectroscopy
nNOS	Neuronal Nitric Oxide Synthase
NHNAES	National Health and Nutrition Examination Survey
NO ₃ ⁻	Nitrate
NO	Nitric Oxide
NO ₂ ⁻	Nitrite
NOA	Nitric Oxide Analyser
NO _x	NO Metabolites
NOS	Nitric Oxide Synthase
NYHA	New York Heart Association
PAD	Peripheral Arterial Disease
PGC-1 α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha
PP	Pulse Pressure
PWV	Pulse Wave Velocity
p38MAPK	
\dot{Q}	Cardiac Output
QoL	Quality of Life
RAAS	Renin-Angiotensin-Aldosterone System

RER	Respiratory Exchange Ration
RF	Rectus Femoris
ROS	Reactive Oxygen Species
RPE	Rating of Perceived Exertion
RV	Right Ventricle
SBP	Systolic Blood Pressure
SEM	Standard Error of the Mean
SNS	Sympathetic Nervous System
SUIT	Substrate-Uncoupler-Inhibitor Titration
SV	Stroke Volume
TBST	Tris-Buffered Saline with Tween-20
TPR	Total Peripheral Resistance
TTE	Time to Exhaustion
T2DM	Type 2 Diabetes Mellitus
VCl ₃	Vanadium Chloride
VCO ₂	Carbon Dioxide Output
VE	Ventilation
VL	Vastus Lateralis
VM	Vastus Medius
$\dot{V}O_2$	Oxygen Uptake
$\dot{V}O_{2max}$	Maximal Oxygen Consumption
$\dot{V}O_{2peak}$	Peak Oxygen Uptake

1 Introduction

Chronic heart failure (CHF) is a syndrome characterised by the inability of the heart to pump a sufficient amount of blood to meet the metabolic demands of the body. It affects over 26 million people worldwide and the prevalence within Australia is 1 to 2% of the population (congruent with the prevalence in Europe and North America) (Sahle et al., 2016; Savarese et al., 2017). CHF is a multifarious syndrome that presents with different physiological impairments depending on age, medical history, pathology, and left ventricular ejection fraction (LVEF) status (Bui et al., 2011). While the aetiology may vary, those with CHF are plagued by the hallmark symptoms of exercise intolerance (low aerobic capacity), dyspnoea and fatigue (Pina et al., 2003). This reduced aerobic capacity independently predicts morbidity and mortality and directly contributes to a reduced quality of life (QoL) (Cicoira et al., 2004; Francis et al., 2000; Jeng et al., 2004). Impaired cardiac performance contributes to the exercise intolerance; however, the emergence of the skeletal muscle hypothesis in the 1990's illustrated how maladaptations within the peripheral tissues (in response to the initial cardiac insult) are critical limiters in the exercise capacity of patients with CHF (Coats, 1996; Coats et al., 1994). More specifically, a reduction in the endothelial production of nitric oxide (NO), a key regulatory molecule for vasodilation and blood flow modulation, has been identified as a mediator/contributor to many of the peripheral skeletal muscle dysfunctions. Thus, interventions targeting improvements in NO bioavailability could have significant implications for improving patients' functionality, well-being and mortality.

The diagnosis of CHF and subsequent classification into one of the three types relies heavily on the assessment of the function of the left ventricle (LV) and specifically the

LVEF. HFrEF, often described as “classic heart failure,” is defined as those individuals with an ejection fraction (EF) <40% (Moayedi et al., 2015). Those with the hallmark signs and symptoms of CHF, but with a preserved ejection fraction (EF>50%) are classified as heart failure preserved ejection fraction (HFpEF) (Nadar et al., 2018). The recent emergence of the heart failure midrange ejection fraction (HFmrEF) classification (EF 40-49%) has complicated the interpretation of many of the previously published studies in HFrEF because individuals in what was once deemed to be a “grey area” of EF percentage between HFrEF and HFpEF were included in the HFrEF studies at the researchers’ discretion. The literature on the HFmrEF classification is limited, but initial evidence suggests that their clinical presentation seems to be intermediary between the two classifications (Lopatin, 2018). Thus, studies that include both HFrEF and HFmrEF must be interpreted with caution.

In comparison with healthy controls, individuals with CHF have significantly lower $\dot{V}O_{2peak}$ (Dhakal et al., 2015; Haykowsky et al., 2011; Sullivan et al., 1989). Within the CHF cohort, those with HFrEF have even lower aerobic capacities than other classifications (Batalli et al., 2017). It was historically assumed that the reduced $\dot{V}O_{2peak}$ was primarily due to the concomitant reduction in \dot{Q} . Indeed, the Fick equation clearly identifies both \dot{Q} and muscle tissue oxygen delivery and extraction as contributors to aerobic exercise capacity (Albouaini et al., 2007; Fick, 1870). However, it is now accepted that cardiac dysfunctions cause downstream maladaptations to the peripheral tissues, including vascular dysfunction, rarefaction of type I fibres and decreased mitochondrial function) and it is these dysfunctions within the muscle tissues that have a greater impact on the reduction in exercise capacity seen in patients with CHF (Nilsson et al., 2008; Pina et al., 2003).

In CHF, the reduced \dot{Q} creates a competition for the limited available blood supply: the muscle tissues require oxygenated blood, and BP must be maintained to perfuse vital organs. Perfusion of the vital organs takes precedence and this is accomplished via an increased activation of the sympathetic nervous system (SNS). Upregulation of the SNS results in an increase in the chronotropic and inotropic response in cardiac tissue and vasoconstriction of the arterial tree. This results in increases in \dot{Q} and TPR (Nilsson et al., 2008) While the increase in \dot{Q} is compensatory, chronically the increased TPR requires the heart to pump against a higher afterload, further exacerbating the progression of CHF. Additionally, continued over-activation results in chronic under perfusion of the skeletal muscle tissues, which contributes to endothelial dysfunction, mitochondrial dysfunction and reduced peripheral tissue perfusion, shifting these patients to a more glycolytic phenotype (Duscha et al., 1999; Rosca et al., 2010; Sullivan et al., 1990, 1991; Williams et al., 2004).

NO is a key regulator of blood flow within the vascular system that is released by the endothelium (Nosarev et al., 2015). In healthy individuals, it is released primarily in response to shear stress, such as that which occurs from the increased blood flow during exercise (Moncada, 1997; Nosarev et al., 2015). This response is critical as the release of NO induces vasodilation, augmenting blood flow to the tissues to meet the increased metabolic demand. Patients with HFrEF have marked endothelial dysfunction and a concomitant decrease in NO bioavailability both at rest and during exercise, which has been implicated as a contributing factor to the exercise intolerance (Katz et al., 1997; Pina et al., 2003). The endothelial dysfunction and the reduction in NO bioavailability contribute to exercise intolerance primarily due to the NO role in regulation blood flow

(via vasodilation of the endothelium) and mitochondrial function (Drexler, 1998; Gevaert et al., 2017). The potential implications of reduced NO bioavailability for exercise intolerance in HFrEF has brought forward an alternative research focus such that several studies are now targeting improvements within the peripheral tissue function (specifically increases in NO bioavailability) to restore exercise tolerance and vascular function (Marti et al., 2012).

An emerging approach to increasing NO bioavailability is via the consumption of dietary inorganic nitrate, found in kale, green leafy vegetables or beetroot juice (Lundberg et al., 2008; Stanaway et al., 2017). This occurs via a two-step process whereby nitrate is first swallowed and absorbed via the gut before it is released into the circulation. Approximately ~25% of the nitrate becomes highly concentrated in the salivary glands and is then secreted and subsequently reduced via oral commensal bacteria to nitrite, swallowed and absorbed (Spiegelhalder et al., 1976; Woessner et al., 2016). Circulating nitrite in the plasma may act as a relatively protected NO species that can be reduced to NO in hypoxic environments, such as in tissues with low partial pressure of oxygen and/or during exercise (Shiva, 2013). The skeletal muscle tissues of patients with HFrEF are an ideal environment for the reduction of nitrite to NO, given that they are chronically less perfused than healthy participants at rest and become further hypoxic during exercise (Wilson et al., 1989).

Studies in healthy populations have demonstrated a myriad of benefits in exercise performance and BP following inorganic nitrate supplementation, including increases in time to exhaustion (TTE), oxygen consumption efficiency (during submaximal exercise), total power output as well as systemic reductions in BP (Bailey et al., 2009;

Cermak et al., 2012; Kapil et al., 2010; Lansley et al., 2011a; Lansley et al., 2011b; Larsen et al., 2011; Larsen et al., 2010; Pinna et al., 2014; Webb et al., 2008). It is suggested that inorganic nitrate supplementation has the potential to be more efficacious in clinical populations first because they cannot adequately produce it endogenously and second because it may be an effective way of assisting in the targeted redistribution of blood flow in the underperfused peripheral tissues (Lundberg et al., 2008). Indeed several clinical trials have demonstrated the beneficial effects of nitrate supplementation on aerobic capacity in patients with chronic obstructive pulmonary disease (COPD) and peripheral arterial disease (Kenjale et al., 2011; Kerley et al., 2015; Woessner et al., 2018a). Moreover, some studies have indicated that increases in NO bioavailability can lead to improvements in mitochondrial function and biogenesis. As mitochondrial dysfunction is a characteristic of many clinical pathologies, including CHF, supplementation could be particularly beneficial in this population (Larsen et al., 2011; Nisoli et al., 2004).

Overall, the study of inorganic nitrate supplementation in patients with CHF is new. At the onset of this thesis, there was a single study published which had demonstrated significant improvements in exercise capacity following nitrate supplementation in patients with HFpEF (Zamani et al., 2015). Since 2015, six additional interventional studies have emerged. Of these seven total studies, three examined the effect of inorganic nitrate on patients with HFpEF, and four studies included patients with HFrEF (<40%) (Coggan et al., 2018; Coggan et al., 2015; Eggebeen et al., 2016; Hirai et al., 2017; Kerley et al., 2016; Zamani et al., 2015; Zamani et al., 2017).

While all of the studies in patients with HFpEF have shown improvements in measures of exercise capacity (i.e., $\dot{V}O_{2peak}$ or TTE), the results in the HFrEF studies are much more inconsistent. Only one study of exclusively patients with HFrEF (n=10) has assessed aerobic capacity following inorganic nitrate supplementation. They showed no significant changes in TTE or $\dot{V}O_{2peak}$ (Hirai et al., 2017). The remaining three studies which included patients with HFrEF have similarly small samples sizes (n=8, 9 and 11), single aetiology recruitment and the dilution of the samples with patients with HFmrEF which may influence the applicability of the findings (Coggan et al., 2018; Coggan et al., 2015; Kerley et al., 2016). Of note, each study utilised different modalities and methodologies for the assessment of aerobic capacity (6-minute walk, incremental shuttle walk test and exercise capacity test on a cycle ergometer), which complicates the interpretation of the findings. These limitations leave many questions unanswered with regards to the efficacy of nitrate supplementation in patients with HFrEF (Coggan et al., 2018; Kerley et al., 2016). Moreover, no study has assessed the potential effects of nitrate supplementation on aerobic capacity during upright treadmill exercise. As walking exercise more closely mirrors the physical activity these individuals would perform during normal activities of daily living, assessing aerobic capacity using a similar modality provides more translatable outcomes.

Cardiac defects are the established precursor to many of the peripheral dysfunctions associated with CHF. However, the presence of abnormalities within both the cardiac and skeletal systems create a dynamic positive feedback loop whereby dysfunctions in each further exacerbate the degradation of the other (Coats, 1996; Coats et al., 1994). The maladaptations in the peripheral tissues must be addressed to see improvements in exercise performance, however, if interventions targeting these peripheral

maladaptations could be coupled with those that target improvements in cardiac performance (e.g., \dot{Q} and SV), there could be a synergistic effect providing benefits beyond that which either intervention alone could achieve. Inorganic nitrate supplementation has the potential to target both the cardiac and skeletal muscle tissues, but its effect on cardiac performance during exercise has not been well explored.

To date, no study has examined the effect of nitrate supplementation on vascular endothelial function in patients HFrEF. Given the established benefits of nitrate supplementation on BP in both healthy and other clinical cohorts, and the potential effect on endothelial function, it is imperative to have a study design incorporate assessment and testing techniques appropriate for comprehensively examining the potential effect on vascular function in HFrEF (Eggebeen et al., 2016; Gilchrist et al., 2013; Kapil et al., 2015; Kenjale et al., 2011; Kerley et al., 2016; Velmurugan et al., 2016; Zamani et al., 2017).

Despite the known regulatory role of the peripheral tissues on exercise capacity in CHF, few studies have investigated the potential effects of nitrate supplementation on skeletal muscle respiration. In a healthy cohort, Larsen et al., reported improvements in mitochondrial respiration following three days of supplementation (Larsen et al., 2011). However, a subsequent work failed to reproduce these results (Whitfield et al., 2016). The potential effect of nitrate supplementation in participants with established impairments in the structure and function of the mitochondria remains unknown.

Increasing NO bioavailability via inorganic nitrate supplementation has the potential to positively effect changes in the vascular system, skeletal muscle tissues and cardiac

performance, all of which could lead to improvements in aerobic capacity in patients with CHF. Given that exercise intolerance is an independent predictor of mortality in this population, novel ways to improve exercise capacity could contribute to a refinement in the treatment and management of CHF in a clinical setting.

Accordingly, the primary of this thesis was to explore the effects of inorganic nitrate supplementation on exercise capacity and vascular function (*Study 1- Chapter 4*), cardiac performance during submaximal exercise (*Study 2- Chapter 5*) and mitochondrial respiration (*Study 3- Chapter 6*) in patients with HFrEF.

1.1 Significance of the PhD

Exercise intolerance is the hallmark symptom associated with CHF and is independently linked to morbidity and mortality. Interventions that can improve exercise tolerance could lead to better patient outcomes and an improved QoL. Dysfunctions within the vasculature and the peripheral skeletal muscle tissues have been implicated as contributing to this exercise intolerance. More specifically, a reduction in NO bioavailability is associated with many of the noted maladaptations in the peripheral tissues and thus has been implicated as a contributor to the decreased exercise capacity. In other clinical populations with skeletal muscle abnormalities like those seen in CHF, nitrate supplementation has been beneficial. This thesis explored how the novel use of inorganic nitrate supplementation could improve NO bioavailability and potentially positively impact exercise performance via improvements in cardiac performance, vascular function and mitochondrial respiration in HFrEF. Findings from this thesis have the potential to enhance the clinical management as well as improve the functional capacity of patients with HFrEF. This is the largest trial to assess the effects of

inorganic nitrate supplementation on aerobic capacity, the first to measure it during upright exercise, and the only one to explore the potential effects on endothelial function, cardiac function, and mitochondrial respiration in patients with HFrEF.

2 Literature review

2.1 Chronic heart failure: background

CHF is a complex, multifarious syndrome characterised by an inability of the left ventricle to pump sufficient volume of blood to meet the body's metabolic needs (Kemp et al., 2012). This syndrome is widespread, affecting over 26 million people worldwide and over 500,000 in Australia (Sahle et al., 2016). CHF carries a significant personal and economic burden, costing the Australian health system more than two and a half billion dollars per year. This, in part, is due to its high hospitalisation rate of over one million days per year (Chan et al., 2016). While there have been marked improvements in the treatment of CHF, the prognosis remains poor, with 10 to 30% of patients dying within 30 days after the initial diagnosis. Of those who survive, 20 to 37% will die within the first year, and the five-year mortality rate still lies between 40 and 65% (Bleumink et al., 2004; Bytyçi et al., 2015). The incidence of CHF increases with age, and the mean age of diagnosis is between 70 to 75 years (Ho et al., 1993; Sahle et al., 2016). There is no consensus in the literature concerning CHF prevalence based on sex, with some studies indicating a higher incident rate in men and others reporting a 20% lifetime risk independent of sex (Bleumink et al., 2004; Lloyd-Jones et al., 2002; Mahmood et al., 2013).

Heart failure (HF) can be classified as left-ventricular HF, right-ventricular HF or both. In right-sided HF, increased pressure in the right ventricle can lead to excess fluid in circulation and cause oedema in the ankles and lower limbs. However, most HF begins in the LV, which results in an accumulation of fluid in the lungs leading to shortness of breath. The function of the LV has critical implications for the classification of the CHF.

2.1.1 Chronic heart failure: classifications

Originally, CHF was defined as a single syndrome with one “type.” Known formally as systolic heart failure, now termed “HFrEF”, this diagnosis was given to an individual with left ventricular EF <40%. (Yancy et al., 2013). The classification of CHF diversified with the introduction of HFpEF (formally known as diastolic dysfunction). The HFpEF classification was developed in response to the growing population of individuals presenting with a preserved EF, but signs and symptoms of CHF. While patients with an EF between 40 and 49% used to fall into a “grey area” of CHF diagnosis, recently this range has been given its own classification entity: HFmrEF (Nadar & Tariq, 2018). This group is not yet well understood. While the prevalence of this classification amongst all patients with CHF is estimated to be comparatively lower than the other two classifications, somewhere between 13 and 24% of all CHF cases, studies have indicated that these patients are demographically and clinically intermediaries between HFpEF and HFrEF. In contrast to the relatively low prevalence of HFmrEF, HFrEF and HFpEF are equally prevalent across patients with CHF (Ponikowski et al., 2016). However, while the diagnosis of HFrEF is clear, EF% <40, there are challenges associated with the identification and diagnosis of HFpEF due to differing clinical definitions (Oktay et al., 2015).

While most diagnostic criteria for HFpEF include evidence of diastolic dysfunction, the presence of HF symptomology and a normal EF, the exact cut-offs for each criterion in research studies is inconsistent. For example, some studies have classified patients with an EF as low as 40% as HFpEF, while other studies utilised a cut-off value of 50% (Borlaug et al., 2015; Eggebeen et al., 2016). Considering the recent emergence of the HFmrEF classification, studies utilising EF percentage cut-off criteria in the 40-49%

range for HFpEF or HFrEF must be interpreted with caution. Another factor adversely impacting HFpEF diagnosis is the absence of routine assessment of diastolic function in clinics and hospitals as well as ongoing challenges with obtaining sufficiently high-quality echocardiographic images to make an objective diagnosis. As a consequence, HFpEF continues to be under diagnosed (Bhuiyan et al., 2011).

The focus of this thesis will be on patients with HFrEF. While the research questions pertain specifically to HFrEF, given the extensive overlap in the aetiologies of CHF, the following sections will include aetiological development relevant for the two primary classifications of HF, HFpEF and HFrEF.

2.1.2 Chronic heart failure: aetiologies

The initiation and progression of the CHF syndrome is complex and multifactorial (Figure 2.1), with an estimated 17 primary aetiologies (Ziaieian et al., 2016). HF can occur as the result of either an acute incident, such as a myocardial infarction (MI), or due to the continued progression of a congenital heart defect or a chronic vascular disease such as hypertension (HTN) or coronary artery disease (CAD) (Minicucci et al., 2011; Velagaleti et al., 2007). While the presence of an acute event or a chronic comorbidity does not guarantee the development of CHF, they do put an individual at a higher risk of developing it in the future. Figure 2.1 illustrates some of the complexities associated with the progression of the syndrome. The figure synthesises information from both the American College of Cardiology (ACC)/American Heart Association (AHA) classification of HF development, which is based on the structural changes to the heart, and the New York Heart Association (NYHA) classification system which focuses on symptomology (Ahmed, 2003; Hunt et al., 2001). As illustrated below, the

presence of cardiac dysfunctions alone does not dictate a CHF diagnosis. If the individual has these dysfunctions, but is asymptomatic, they are classified as having preclinical HF (Stage B in Figure 2.1). However, when symptoms are present, the individuals are classified as having CHF (Stage C).

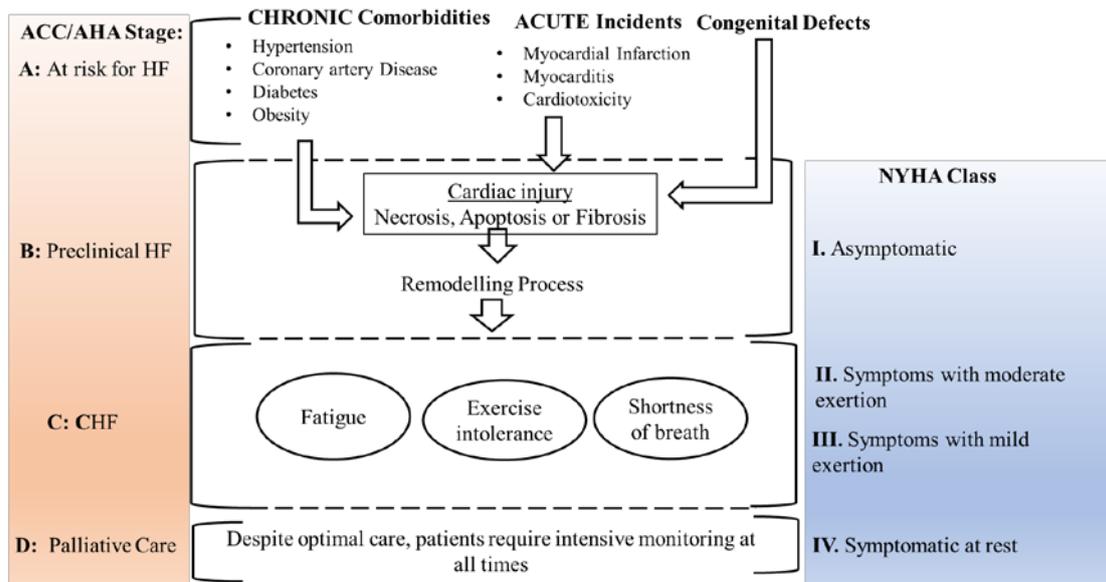


Figure 2.1 Progression pathway of HF

This combines figures from the ACC/AHA stages of Heart Failure and the NYHA classification of severity of HF based on symptomology Ahmed (2003); Hunt et al. (2001). Abbreviations: ACC- American College of Cardiology, American Heart Association- AHA, CHF- chronic heart failure, HF- heart failure, NYHA- New York Heart Association

Whether the injury results from an acute or chronic condition, the damage to the cardiac tissue manifests as necrosis, apoptosis or fibrosis. All of these result in the heart having a diminished capacity to effectively contract and/or fill the LV, resulting in a reduced SV and Q at rest. During exercise, these complications are further exacerbated. The presence of these cardiac abnormalities creates a competition in the body between maintaining adequate BP (to perfuse vital organs) and maintaining an appropriate supply of blood to meet the demands of the skeletal muscle tissues.

In response to the reduced \dot{Q} , multiple compensatory mechanisms are triggered within the cardiac and skeletal muscle tissues to preserve BP and maintain vital organ perfusion (Triposkiadis et al., 2009). The SNS increases its activity to preserve \dot{Q} by increasing HR, (known as resting tachycardia) (Kemp et al., 2012). The over-activation of the SNS also induces vasoconstriction of blood flow to inactive tissues, thereby assisting in the shunting of blood to the vital organs and increasing TPR (Triposkiadis et al., 2009). The increase in TPR augments the afterload, inducing extensive cardiac remodelling (Sabbah, 2000). The specifics of how the heart is remodelled are partly dependent on the initial aetiology of the CHF (**Sections 2.1.2.1 and 2.1.2.2**).

As described previously, the aetiology of CHF has implications for the development and progression of the syndrome. The two most common aetiologies are HTN and CAD (Ambrosy et al., 2014; Gheorghiade et al., 1998; Velagaleti et al., 2007). HTN is the most prevalent antecedent, with the Framingham Heart Study (FHS) reporting over 90% of patients with CHF as having HTN (Levy et al., 1996). For CAD, data accumulated across several studies indicate that it precedes a CHF diagnosis in 23 to 73% patients. While it is possible to have either HTN or CAD, many patients suffer from both (Gheorghiade et al., 1998). The following sections will provide a brief review of both the HTN and CAD aetiology.

2.1.2.1 Hypertension

Both the FHS and data from the more recent National Health and Nutrition Examination Survey (NHNAES) identify HTN as a leading cause of CHF, with prevalence of HTN in the NHNAES study exceeding 90% (Ho et al., 1993; Komanduri et al., 2017).

Chronic HTN can lead to increases in the afterload via over activation of the renin-

angiotensin-aldosterone system (RAAS), and either initiate left ventricular hypertrophy (LVH), or, if already present, exacerbate the damage.

Recent guidelines from the ACC and AHA Task Force on Clinical Practice Guidelines define two stages of hypertension: stage 1 hypertension is when the SBP is between 130-139 mmHg and/or the diastolic blood pressure (DBP) is between 80-89 mmHg, and stage 2 HTN is when the SBP is ≥ 140 mmHg and/or the DBP is ≥ 90 mmHg (Whelton et al., 2018). Individuals with HTN are often characterised as having increased TPR, due to arterial vasoconstriction (Velagaleti et al., 2007). Over 90% of cases are classed as essential HTN, which may involve several underlying but unclear physiological disorders. These often include hyperactivity of the SNS, endothelial dysfunction, dysfunctions within the RAAS or genetic factors (Carretero Oscar et al., 2000).

The RAAS is a critical regulator of BP and fluid balance. A fall in BP within the kidneys, or an increase in SNS stimulation causes the kidneys to release the enzyme renin (**Figure 2.2**) (Powers et al., 2015). When the renin reaches the blood, it is converted to angiotensin I, which is further converted to angiotensin II by the angiotensin-converting enzyme (ACE) in the lungs. Angiotensin II is a powerful vasoconstrictor, which acts to increase BP. In tandem with this, decreases in the plasma volume or increases in sodium (Na^+) concentration activate the subsequent release of aldosterone which regulates BP by initiating the reabsorption of Na^+ and the release of potassium (Powers et al., 2015).

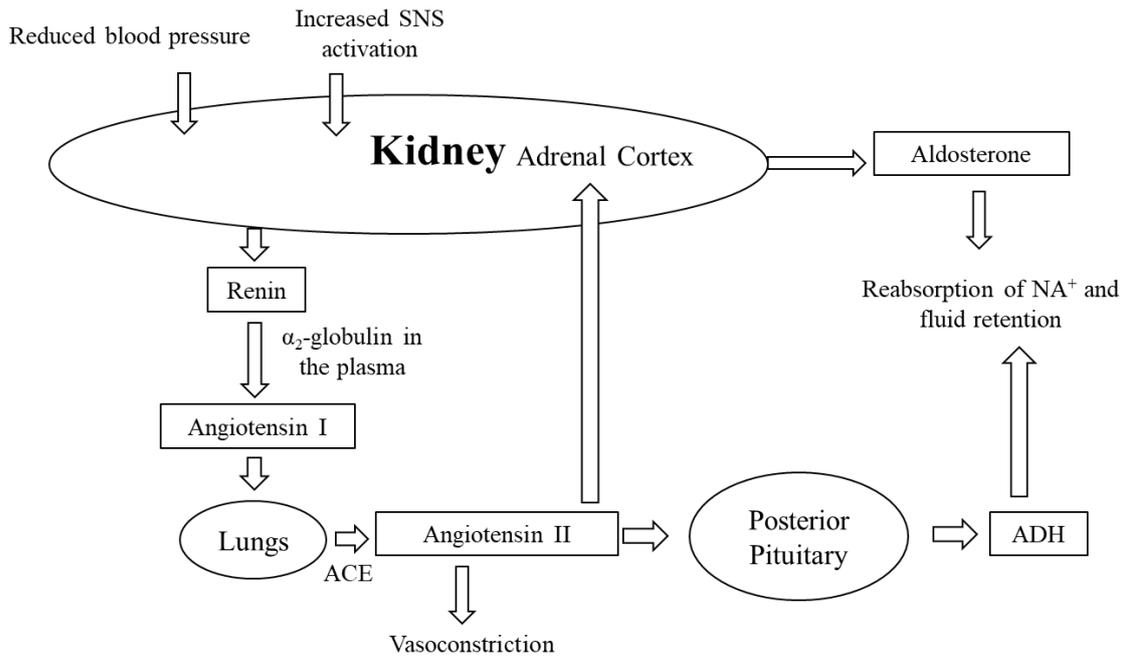


Figure 2.2 The renin-angiotensin-aldosterone system

Adapted from Levinger (2004). Abbreviations: ACE-angiotensin converting enzyme, NA+-sodium, SNS-sympathetic nervous system, ADH-antidiuretic hormone.

In healthy humans, the RAAS helps to maintain normal plasma volume and BP (Patel et al., 2017). In patients with CHF, the RAAS can be over activated, causing increases in venous and arterial tone (increasing both preload and afterload). Ultimately, this leads to water retention and peripheral oedema (Jackson et al., 2000). The increased preload and afterload can either initiate or progress LVH and lead to a worsening of the CHF syndrome.

In response to a chronic pressure overload, concentric hypertrophy (a precursor to CHF) can occur. The LV walls become thickened due to the addition of sarcomeres to the myocytes in parallel, which helps preserve contractile force (Glezeva et al., 2014; Volpe et al., 2010). While thickening of the LV provides short-term benefits to overcome an elevated afterload, chronically fibrotic stiffening occurs, which prevents full relaxation

in diastole, thereby reducing end diastolic volume as well as \dot{Q} (Inamdar et al., 2016). These specific diastolic abnormalities are characteristic of the HFpEF classification. However, as indicated previously, HTN is associated with 90% of all patients with CHF, and thus the over activation of the SNS and RAAS systems are important considerations for all patients.

2.1.2.2 *Coronary artery disease*

CAD is a major risk factor for the development of CHF and is the leading cause of death in the developing world. It accounts for nearly 20% of the total deaths each year (Cassar et al., 2009; Gheorghide et al., 2006). CAD occurs when the arteries supplying blood to the heart have an accumulation of plaque that restricts normal blood flow (Torpy et al., 2009). If the plaque becomes inflamed or ruptures, it can lead to a coronary artery blockage, a subsequent MI and/or the development of CHF (Cassar et al., 2009; Smith et al., 2015).

In patients with CHF, the presence of CAD is associated with higher mortality (Hwang et al., 2014b). In some cases, it can present as clinically silent, where patients are asymptomatic. In fact, as many as a third of the individuals with HF are found to have undiagnosed CAD at the time of their death (Uretsky et al., 2000). However, in about half of the patients, CAD first manifests as pain/tightness in the chest, known as angina. This pain can radiate into the jaw, shoulders and arms (Cassar et al., 2009; Kannel et al., 1972). Angina typically presents following exertion or stress and subsides within a few minutes of activity cessation. It is caused by the mismatch between the increased cardiac oxygen demand during exercise and the limited supply of oxygenated blood due

to blood flow restrictions. This detrimental combination of high demand and low supply often precedes an acute MI and the ultimate development of CHF (Cassar et al., 2009).

An MI can lead to tissue necrosis and fibrosis, which impede the heart's capacity to contract (Glezeva et al., 2014). These trigger a critical remodelling process within the cardiac tissue which can lead to impairments in either systolic function, diastolic function or both (Pazos-López et al., 2011). An MI most often results in eccentric hypertrophy due to a volume overload. In this instance, new sarcomeres are added in series within the cardiac tissues, leading to a dilated LV with thin walls (Mihl et al., 2008). While this does not improve contractile force, it increases the size of the LV, allowing for an increased diastolic filling volume, thereby assisting in the acute preservation of \dot{Q} . The majority of the remodelling that occurs following an MI is irreversible. Thus, interventions aiming to improve functionality in these patients by necessity must also target dysfunctions outside of the cardiac system.

Individuals with CAD that progresses to CHF often have MI as a precursor (Cowie et al., 1999). However, patients with CAD can also develop CHF in the absence of an MI. When the CAD is severe enough to cause chronic hypo perfusion without a total occlusion and tissue death, the heart can become stiff, inflamed and fibrous, thereby negatively affecting the heart's capacity to relax and fill (Velagaleti et al., 2007). CHF rarely initiates from a singular event or dysfunction. Rather, the development and progression of the syndrome is more akin to a web of interrelated comorbidities and precursors that cumulatively result in the cardiac impairments and symptomology present in patients with CHF. Indeed, despite the myriad of differential aetiological

pathways, patients with CHF are all characterised by the hallmark symptoms of fatigue and exercise intolerance.

2.1.3 The clinical and functional importance of exercise capacity in patients with CHF

2.1.3.1 Peak aerobic capacity

Individuals with CHF have a reduced peak aerobic exercise capacity compared to age-matched healthy controls (Dhakal et al., 2015; Haykowsky et al., 2011; Sullivan et al., 1989). Peak aerobic exercise capacity is best objectively quantified by measuring the individual's maximal oxygen consumption ($\dot{V}O_{2max}$) during an incremental exercise test. However, in some cases patients with chronic conditions, including CHF, are unable to achieve a true $\dot{V}O_{2max}$, due to exercise intolerance caused by dysfunctions in the skeletal muscle tissue. For these individuals, the maximal achieved value of oxygen consumption is termed a $\dot{V}O_{2peak}$.

Patients with CHF can have reductions in $\dot{V}O_{2peak}$ of 50% or more compared to healthy individuals (Dhakal et al., 2015; Haykowsky et al., 2011; Sullivan et al., 1989). It has been reported that patients with HF_rEF have significantly lower aerobic exercise capacity (~9%) compared to those with HF_pEF (Dhakal et al., 2015). There is no clear aerobic capacity data for patients with HF_mrEF due to the classification's relatively recent emergence. However, all patients with CHF are at risk of having their $\dot{V}O_{2peak}$ below $14 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ which is considered by many to be a minimal functioning threshold value for oxygen uptake ($\dot{V}O_2$) and is a qualifying criterion for a heart transplant (de Jonge et al., 2008; Mancini et al., 1991). In CHF there is an association between a low $\dot{V}O_{2peak}$ and a poor prognosis (**Figure 2.3**). Improving $\dot{V}O_{2peak}$ is the

primary target for interventions in patients with CHF as every 6% increase ($\sim 1 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) equates to a 5% lower risk of hospitalisation and mortality (Francis et al., 2000; Swank et al., 2012).

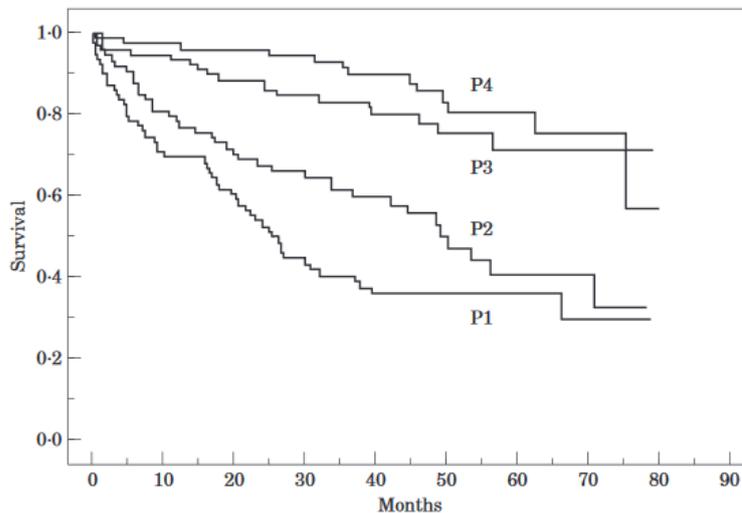


Figure 2.3 Kaplan-Meier survival curve of patients with HFrEF

Those with HFrEF and a higher $\dot{V}O_{2\text{peak}}$ (P3 and P4) have a lower risk of mortality over an 80 month period. The survival curves for patients with CHF grouped into quartiles by their $\dot{V}O_{2\text{peak}}$: P1= $<13.0 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; P2= $13-16.5 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; P3= $16.6-21.6 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; P4= $>21.6 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$. The median follow-up for survivors was 47 months. Adapted from Francis et al. (2000).

2.1.3.2 Submaximal exercise

While peak aerobic capacity is a strong predictor of mortality, submaximal measures of aerobic capacity offer differential or additive insights into patient functionality (Gitt et al., 2002; Nyasavajjala et al., 2009). While it is known that \dot{Q} is modulated by both SV and HR, the relative contributions of each are intensity-driven. During submaximal exercise, \dot{Q} is primarily modulated via changes in SV, whereas subsequent increases in exercise intensity rely predominantly on increases in HR (Powers et al., 2015). Thus, combining a peak exercise capacity assessment with submaximal measures could provide complementary prognostic information (Rickli et al., 2003). Additionally, some evidence suggests that while $\dot{V}O_{2\text{peak}}$ could be underestimated in clinical populations

due to lack of patient motivation or premature exercise test termination, submaximal measures of aerobic function could be derived without these limitations (Gitt et al., 2002).

One such submaximal measure of oxygen utilisation is the gas exchange threshold (GET). Physiologically, it represents a surrogate measure of anaerobic threshold. The identification of the GET can be accomplished via various methodologies, but the most widely used is the V-slope methodology (Beaver et al., 1986). This method requires a visual examination of the carbon dioxide output ($\dot{V}CO_2$) versus $\dot{V}O_2$ plot and the subsequent identification of the first point of departure from the linearity between the two (**Figure 2.4**). This point represents the GET.

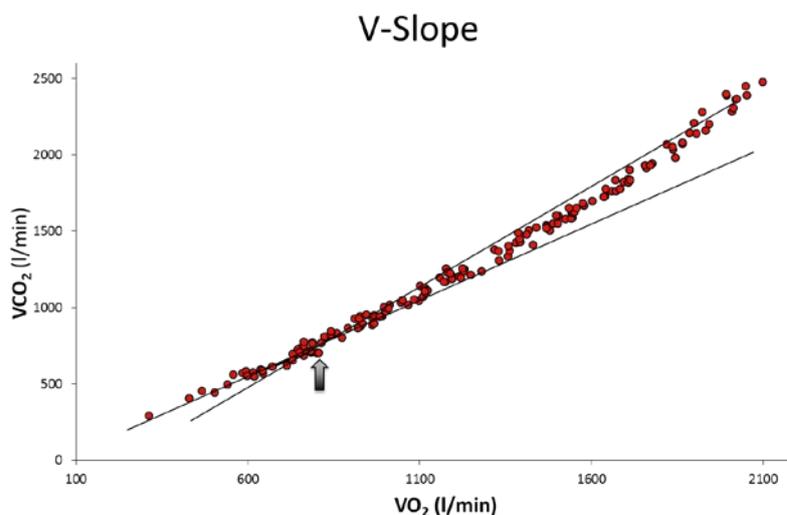


Figure 2.4 Example of V-slope method for determination of GET

Representative slopes for $\dot{V}CO_2$ and $\dot{V}O_2$ are illustrated above with the arrow indicating the GET. Abbreviations: $\dot{V}CO_2$ - carbon dioxide output, $\dot{V}O_2$ - oxygen consumption

In CHF, the GET occurs at approximately 50 to 85% of $\dot{V}O_{2peak}$. The GET in CHF occurs earlier than healthy individuals suggesting those with CHF become anaerobic at

a relatively low percentage of their $\dot{V}O_{2\text{peak}}$. This is a likely contributor to early fatigue and the low exercise tolerance which characterises these individuals (Nyasavajjala et al., 2009; Tomono et al., 2016). The low GET also has negative implications for mortality with one study demonstrating that patients with HFrEF who have a relative GET lower than $11 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ have a 5.3-fold increased risk of death at six months (Gitt et al., 2002).

One of the benefits of GET quantification is that it occurs at an intensity level that more closely mirrors what patients with CHF might achieve in performing their activities of daily living (ADL). This suggests that changes to the GET may also provide benefits translatable to completing ADL. Individuals with CHF have a higher relative metabolic demand during ADL as compared to age-matched controls (Spruit et al., 2011). This, combined with increased levels of fatigue, suggests that these patients are working harder to accomplish the same activities, likely due to inefficiencies in oxygen delivery and utilisation during low-intensity exercise. Additionally, symptoms such as fatigue and dyspnoea often occur in relatively low intensity stages of exercises, resulting in early termination of aerobic exercise tests. Quantifying the mismatch in oxygen supply and demand in CHF by measuring submaximal exercise parameters such as $\dot{V}O_2$ at steady state exercise could further illustrate functional impairments that are present at intensities similar to ADL.

Exercise intolerance can and should be quantified by assessments beyond those of just acute exercise bouts. The ability to perform exercise is limited both by acute onset of fatigue as well as factors that negatively impact the recovery from exercise, thus inhibiting subsequent bouts of exercise. For instance, a longer heart rate recovery time

following exercise is associated with lower $\dot{V}O_{2\text{peak}}$ and is a significant predictor of all-cause cardiovascular mortality (Bilsel et al., 2006; Nishime et al., 2000). An abnormal HR recovery is defined as a HR that declines ≤ 18 beats per minute after one minute of exercise cessation (Bilsel et al., 2006; Watanabe et al., 2001). Similarly, a delayed recovery of $\dot{V}O_2$ following exercise cessation is also indicative of impairments in the ability of the circulatory system to rapidly deliver oxygenated blood to the peripheral tissues. Patients with CHF have a demonstrably longer time to recovery for HR (twice as long as healthy individuals) and a slower recovery of $\dot{V}O_2$ following exercise cessation (Kriatselis et al., 2012). While few studies report this response within their manuscripts, the value is simple to calculate and can be used as another prognostic marker in HFrEF. Though it is not prevalent in all patients with HFrEF, the frequency of an attenuated HR recovery following exercise is higher than in healthy controls (Imai et al., 1994).

2.1.4 Pharmacological and non-pharmacological interventions for CHF and their effect on exercise capacity

Historically, the aim of both pharmacological and non-pharmacological (exercise-based) interventions in CHF was to reduce morbidity and hospitalisations and reduce premature mortality. As these rates have improved in recent decades, the focus has shifted to reducing symptomology, improving the QoL and enhancing functional and aerobic capacity (Berliner et al., 2017). Pharmacological and exercise interventions have been key components for the treatment of patients with CHF.

2.1.4.1 Pharmacological interventions

The primary purpose of pharmacological interventions in patients with CHF are to reduce the strain and pressure placed on the heart and to prevent/minimise the

detrimental effects of the over activated SNS (Berliner et al., 2017; McKenzie et al., 2003). While the pharmacological interventions primarily target the cardiac abnormalities associated with CHF, many have both negative and positive effects on vascular function and exercise capacity. The following section will differentiate three of the most common medications prescribed to patients with HFrEF and describe the potential effects on exercise and vascular function.

2.1.4.1.1 Beta-blockers

Over activation of the SNS is linked to the deterioration of cardiac performance as well as increased mortality and increased hospitalisations in patients with CHF (Metra et al., 1994). Beta-adrenergic blockers (β -blockers) bind to the beta-adrenergic receptors, inhibiting the effect of catecholamines (e.g., norepinephrine and epinephrine) and suppressing the over expression of the SNS. They act as cardio protectives, preventing the over activation of the heart resulting in decreases in HR, BP and myocardial oxygen demand (Zoghbi et al., 2008) Long-term use of β -blockers reduces the risk of death and improves the QoL in patients with CHF (Ram, 2010). β -blockers also have beneficial effects on vascular function largely through the reduction of TPR. A meta-analysis on vascular function and use of β -blockers indicates that they significantly improve endothelial function and lead to reductions in BP (Peller et al., 2015).

The effects of β -blockers on exercise performance in patients with CHF is unclear due to several reasons including the type of β -blocker (selective vs non selective) and/or the modes/types of the exercise interventions (de Milliano et al., 2001; Fisher et al., 1994; Olsen et al., 1995; Waagstein et al., 1993). A recent meta-analysis identified that β -blocker therapy is associated with a significant improvement in time to exhaustion, but

provided no meaningful changes in 6-minute walk distance or in $\dot{V}O_{2\text{peak}}$, suggesting that they have an effect on exercise tolerance independent of oxygen uptake (Abdulla et al., 2006).

2.1.4.1.2 *Angiotensin converting enzyme inhibitors*

Angiotensin-converting enzyme inhibitors (ACE-inhibitors) combined with β -blockers are the foundation of pharmacologic interventions for patients with HFrEF (Willenheimer et al., 2005). As previously discussed, over activation of the SNS and RAAS can lead to increased levels of angiotensin II, which has been linked to progressive cardiac hypertrophy, as well as thicker and stiffer blood vessels (Sweitzer, 2003). ACE-inhibitors are antihypertensive agents that work by inhibiting ACE, which decreases the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor (Davies et al., 2000). The reduction in vasoconstriction aids in lowering BP. In turn, the lower BP helps decrease the workload of the heart and aids in the prevention of further deterioration in function.

While the beneficial effects of ACE-inhibitors on vascular function and mortality are well established, their effect on exercise capacity remains inconclusive (Coppola et al., 2008). Some studies have indicated that individualised dosing regimens can result in a small improvement (7%) in aerobic capacity (Cooke et al., 2002), while others showed no changes in exercise capacity following long-term treatment with an ACE-inhibitor (Dayi et al., 2005; Gundersen et al., 1994). There are a few potential side effects associated with the use of ACE-inhibitors, such as hyperkalaemia, a dry cough or a skin rash; however, use of appropriate medications and dosing regimens have reduced the risk of these adverse responses (Izzo et al., 2011).

2.1.4.1.3 *Diuretics*

Diuretics are primarily used to achieve euvolemia and manage symptoms associated with fluid overload. Sodium and water retention are hallmark characteristics of patients with CHF due to the over stimulation of the RAAS (McKenzie et al., 2003). The fluid retention can either be in the lungs (pulmonary oedema) or in the lower limbs (peripheral oedema) (Clark et al., 2013). When the fluid accumulates within the lungs, patients can develop a severe cough, shortness of breath, and, if left untreated, can result in hospitalisation or, in extreme cases, death.

Treatment with diuretics reduces both hospital admissions and mortality rates in patients with CHF (Faris et al., 2006). The effects on exercise capacity are less understood. This is primarily due to the limited number of randomised controlled trials examining the effect of a diuretic versus an active control on exercise capacity in patients with CHF (McKenzie et al., 2003; Richardson et al., 1987). Moreover, there are concerns with prescribing diuretics to patients who do not have clinical signs and symptoms of congestion as diuretics can cause an increase in LV filling pressures and a decrease in the SV index (i.e., the volume of blood pumped divided by the body surface area) (Francis et al., 1985). Thus, while diuretics are an essential tool for the treatment of patients with CHF who have fluid overload, they are not indicated for general use to improve aerobic or vascular function.

2.1.4.2 *Exercise training interventions*

Initially, it was believed that patients recovering from cardiac events such as an MI required bed rest and limited activity (Burch G et al., 1965). This attitude began to shift in the late 1960's with the publication of the Dallas Bed Rest and Exercise Study, which

identified the beneficial effects of exercise in contrast to the detrimental effects of bed rest (Saltin et al., 1968). However, the development of medications led to a focus on pharmacological interventions to produce short-term benefits rather than cardiac rehabilitation efforts (Mampuya, 2012). Due to the assumption that there was a high risk associated with exercise training patients with CHF, it has only been in recent decades that patients have been considered for cardiac rehabilitation. Today, exercise training is an established and crucial component of the rehabilitation process for patients with CHF (Selig et al., 2010; Taylor et al., 2014).

Seminal work by Sullivan et al. indicated the safety and efficacy of long-term (four to six months) exercise training in patients with CHF and regular aerobic exercise is now a Class 1A recommendation (Sullivan et al., 1988; Yancy et al., 2013). Since that initial trial, a myriad of small clinical trials have also supported the benefits of aerobic exercise training at improving patients' aerobic capacity, vascular health (endothelial function) and QoL (Beckers et al., 2008; Coats et al., 1992; Giannuzzi et al., 2003; Gielen et al., 2003). A recent meta-analysis indicates that on average, $\dot{V}O_{2\text{peak}}$ increases approximately 16% following aerobic training (Smart et al., 2004). However, even modest improvements in $\dot{V}O_{2\text{peak}}$ ($1 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ or ~6%) are associated with significant reductions (8%) in the combined risk of mortality and hospitalisations in patients with CHF (Swank et al., 2012).

The beneficial changes in aerobic capacity and function are achieved primarily via the reversing of peripheral abnormalities associated with CHF (including endothelial dysfunction and skeletal muscle atrophy) (Papathanasiou et al., 2008). Current evidence is conflicting regarding the potential for a dose-dependent response (both in intensity

and frequency) to exercise training for patients with CHF (Keteyian et al., 2012). Indeed, some studies have indicated that the improvements in exercise capacity are intensity dependent, with higher intensity interval training leading to significantly greater improvements as compared to continuous moderate intensity exercise (Warburton et al., 2005; Wisloff et al., 2007). Other studies have demonstrated no superior effect of high intensity training as compared to moderate continuous training (Ellingsen et al., 2017). However, in all training modalities, intensity of exercise, patient attendance and long-term adherence are primary limitations. While three to five days of aerobic exercise per week is recommended for patients with CHF, studies are reporting that patients attend 1.7-1.8 sessions per week on average (McKelvie et al., 2002; O'Connor et al., 2009). Even when patients attend the sessions, many do not achieve or maintain the desired exercise intensity. The largest trial to date in HF and exercise training showed that only 40% of all participants exercised at or above the prescribed exercise duration in the first year (O'Connor et al., 2009).

The factors leading to low adherence rates in cardiac rehabilitation are the subject of ongoing debate and investigation, but this limitation in the effectiveness of exercise training provides a strong rationale for exploring alternative interventions that can improve exercise capacity in those who cannot or do not want to exercise.

2.2 Central and peripheral pathophysiology and exercise intolerance in chronic heart failure

Exercise intolerance is a hallmark symptom of patients with CHF. Identifying the mechanisms responsible for this intolerance has been an area of intense research focus. From the Fick principle, it is understood that aerobic capacity is influenced by both Q

(cardiac output) and the oxygen extraction/utilisation capacity of the skeletal muscle tissues (Albouaini et al., 2007; Fick, 1870). Patients with HFrEF have significant dysfunctions within both the cardiac system and peripheral skeletal muscle tissues that negatively impact exercise performance.

2.2.1 Cardiac abnormalities

\dot{Q} is a significant contributor to exercise capacity and patients with HFrEF have up to 50% reduction in \dot{Q} during exercise compared to healthy age-matched controls (Dhakal et al., 2015). This impaired \dot{Q} response is mediated by reductions in SV as well as chronotropic incompetence. Previous studies have indicated that the SV achieved at peak exercise is lower in patients with CHF as compared to healthy controls and that patients with CHF often reach plateau levels of SV during lower intensity exercise compared to healthy controls (Fukuda et al., 2012; Sullivan et al., 1989). These reductions deprive the muscles and tissues of oxygen. Because a lack of oxygen can result in early fatigue, this mismatch in supply and demand is especially critical in conditions with a high oxygen requirement, such as during exercise.

Chronotropic incompetence is defined as the inability of the heart to increase the rate of its contractions (i.e., HR) in proportion to the increase in activity (Brubaker et al., 2006). It is common in patients with HFrEF and is largely mediated by imbalances in the parasympathetic system and SNS activity (Brubaker et al., 2006; Brubaker et al., 2011). Chronic increases in SNS activity in CHF results primarily in the inability of the heart to pump enough blood to adequately perfuse the skeletal muscle tissues while also maintaining BP. BP is prioritised in order to maintain vital organ perfusion, which requires a higher TPR (Ledoux et al., 2003). The increased TPR is achieved via

vasoconstriction induced by the SNS. During exercise, the competition between muscle tissue oxygen demand and vital organ perfusion intensifies, and neither system receives an adequate supply of oxygenated blood (Jackson et al., 2000).

2.2.2 The muscle hypothesis

While cardiac dysfunctions were initially considered to be major limiting factors in exercise performance in CHF, it is now known that pathologies in the periphery play a crucial role in limiting exercise capacity, perhaps more than the central abnormalities (Jondeau et al., 1992). There are several factors that may contribute to this theory, first EF is not correlated with $\dot{V}O_{2\text{peak}}$ (Bocchi et al., 1995). Second, pharmaceutical interventions (e.g., dobutamine) that restore \dot{Q} have not translated to a significant increases in aerobic capacity or reduced symptoms of exercise intolerance (Maskin et al., 1983). Similarly, interventions that have improved aerobic capacity in patients with CHF have not always resulted in improvements in systolic or diastolic functions of the heart (Haykowsky et al., 2012; Kitzman et al., 2013).

The role of the periphery, and in particular skeletal muscle, in limiting exercise capacity in these patients was first described by Professor Andrew Coats in the 1990's. Termed "the muscle hypothesis," Coats described it as a deleterious pattern of dysfunctions associated with CHF in which the initial dysfunction in the LV induces several physiological impairments including decreased blood flow (i.e. reduced \dot{Q}), endothelial dysfunction, physical inactivity and excessive catabolic activity within the skeletal muscle (**Figure 2.5**). (Coats, 1996; Coats et al., 1994). These changes cumulatively create a more glycolytic profile which induces early fatigue in patients with CHF, further contributing to physical inactivity and muscle atrophy (Coats, 1996; Coats et al.,

1994). Thus, the CHF syndrome can be viewed as being a vicious cyclical where the dysfunctions of each system result in the further deterioration of others.

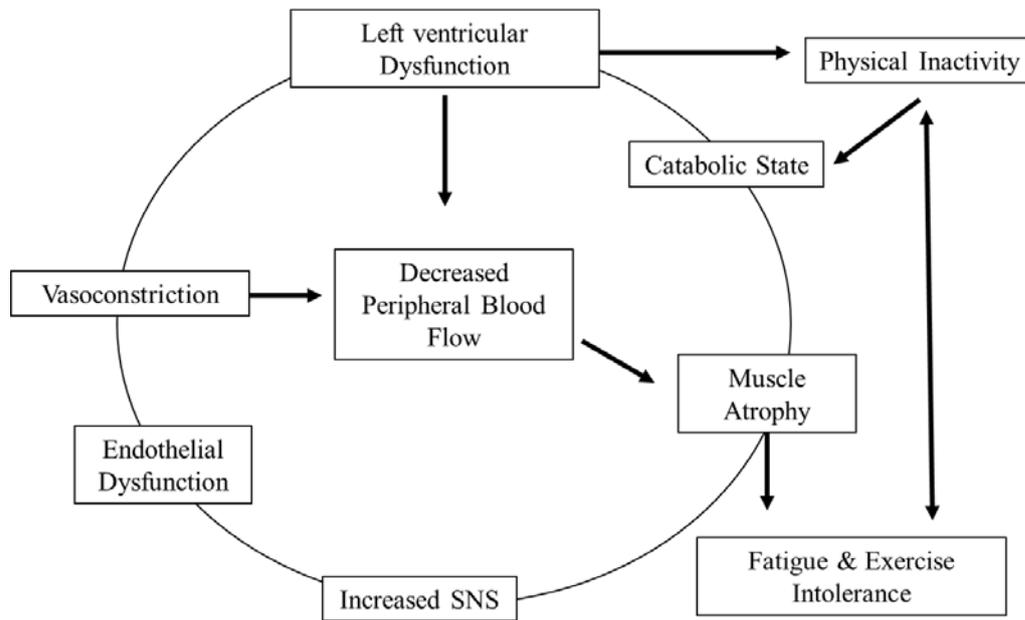


Figure 2.5 Skeletal muscle hypothesis

The CHF syndrome is cyclical whereby cardiac dysfunctions lead to maladaptations in the skeletal muscle tissues which result in exercise intolerance. The exercise intolerance and excessive fatigue feeds back into decreasing physical activity, further exacerbating the muscle atrophy. Increased SNS stimulation occurs in response to LV dysfunction but can also chronically worsen LV function. Adapted from Coats et al. (1994).

Some of the dysfunctions within the skeletal muscle result from both disuse and chronic underperfusion. Reductions in capillary density, for example, correspond to a smaller surface area for oxygen exchange and can negatively affect exercise capacity and contributes to exercise intolerance seen in these patients with CHF (Chilibeck et al., 1997). Indeed, decreased capillary density around the myofibrils significantly correlates with lower $\dot{V}O_{2peak}$ in these patients suggesting that improvements within the capillaries could also aid in restoring exercise capacity (Kitzman et al., 2014).

In addition to the significant impact on the capillaries, chronic deconditioning and underperfusion also results in the oxygen deprived skeletal muscle tissues preferentially utilising anaerobic metabolic pathways for energy production. While these patients experience a reduction in all muscle fibre types compared to age-matched controls, the reduction in Type I fibres is demonstrably higher (Schaufelberger et al., 1995; Sullivan et al., 1990). In concordance with this, there is also a relative shift to a higher composition of Type II fibres making them less resistant to fatigue and further contributing to their exercise intolerance (Schaufelberger et al., 1995).

While the reduction in capillary density and rarefaction of Type I fibres are both critical dysfunctions in the skeletal muscles of patients with CHF, they are not ideal targets for short term interventions (<2 weeks) as adaptations to these systems require a longer, chronic stimulus. They have been briefly reviewed here due to their noted contribution to the glycolytic phenotype of patients with CHF, but will not be the primary focus of this work. Rather, this thesis focuses on targeting blood flow and perfusion (endothelial function and tissue perfusion) as well as energy production and efficiency (mitochondrial function). If these measures can be improved in patients with CHF, future studies could further determine whether these improvements in tissue perfusion and aerobic energy production (mitochondrial function) can have a subsequent positive effect on the maladaptations (reduced capillary density and rarefaction of type I skeletal muscle fibres).

2.3 Nitric oxide bioavailability

NO is a diatomic, lipid-soluble gas that has been implicated in numerous physiological functions including neurotransmission, immune defence, and blood flow regulation,

among others (Ghimire et al., 2017). NO exists in many of the organs and systems within the human body, and thus has a modulatory role on the function (and dysfunction) of those organs and systems. Patients with CHF have reduced NO bioavailability, which has been identified as a mediating mechanism as well as associated factor in the initiation and progression of many of the impairments within both the cardiac system and peripheral tissues (Drexler, 1998; Gevaert et al., 2017).

Figure 2.6 illustrates the potential pathway by which a reduction in NO bioavailability influences \dot{Q} , mitochondrial function and peripheral tissue perfusion. Cumulatively these dysfunctions lead to exercise intolerance. A detailed description of these peripheral dysfunctions and the link between them and reduced NO bioavailability in CHF will be described below.

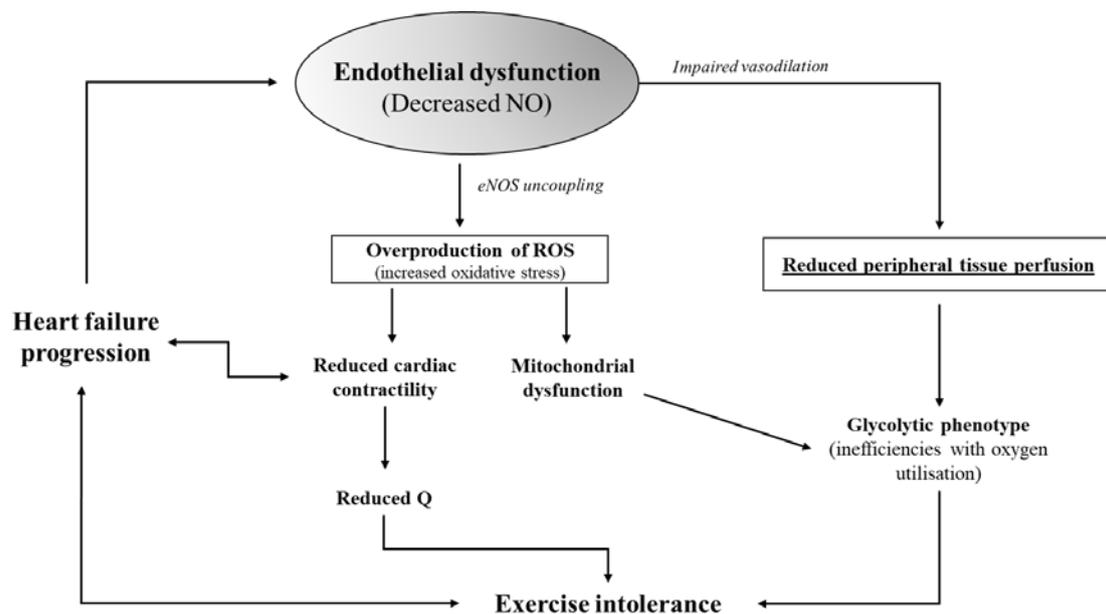


Figure 2.6 Role of NO in peripheral and central dysfunctions in HFrEF

Endothelial dysfunction (reduced NO) is both a precursor and progressor of many of the peripheral tissue abnormalities associated with CHF. It is also a progressor of the CHF syndrome itself primarily through the negative impacts of increased oxidative stress on cardiac contractility. Abbreviations: NO- nitric oxide, \dot{Q} - cardiac output, ROS- reactive oxygen species.

The following sections are substantially based on a first-author review paper I published exploring the role of dietary nitrate supplementation in cardiovascular health (Woessner et al., 2018c). The manuscript focused on comparing the beneficial effects of inorganic nitrate supplementation in healthy individuals and those with CVD. While much of the manuscript is reproduced herein, the original content of the paper has been adapted and expanded to align directly with the HFrEF syndrome, which is the focus of this thesis.

NO can be produced by three isoforms of NO synthase (NOS), all of which use L-arginine and oxygen as substrates. Inducible nitric oxide synthase (iNOS) is expressed in many different types of cells. It is calcium (Ca^{++}) independent and generates NO that has cytostatic effects on parasitic cells. Thus, iNOS plays an important role in immune defence system. The other two forms, neuronal NOS (nNOS) and endothelial NOS (eNOS), are both constitutive isoforms that are Ca^{++} -dependent. nNOS is expressed in both central and peripheral neurons in the nervous tissue and skeletal muscle, and it functions in the central regulation of BP, smooth muscle relaxation and vasodilation via the peripheral nitrenergic nerves (Förstermann et al., 2012). Within the endothelium, eNOS has a critical role in vascular health as it involves in blood flow regulation (via blood vessel dilation), modulates BP, and has other anti-atherosclerotic actions. Given the active role of the endothelium in blood vessel relaxation and vasodilatation in response to exercise via the production and release of vasodilators, eNOS production of NO will be the focus of this thesis. However, iNOS and nNOS will be referenced (Deanfield et al., 2007).

2.3.1 eNOS production of NO

The release of NO from the endothelium via eNOS can be activated by humoral, mechanical or pharmacological stimuli (Omar et al., 2014). In terms of messenger

activation, eNOS activity can be upregulated by the Ca^{++} -binding messenger protein, calmodulin. When Ca^{++} rises, inducing the binding of calmodulin to eNOS, NO production increases (Förstermann et al., 2012). eNOS can also be activated by mechanical stimuli, such as shear stress induced by increased blood flow during exercise (Moncada, 1997; Nosarev et al., 2015).

The shear stress on the endothelial cells initiates a cascade of reactions which involve mechanosensors as well as enzymes that ultimately target and activate eNOS and increase NO bioavailability (**Figure 2.7**). When L-arginine and other cofactors (such as tetrahydrobiopterin) are present, eNOS produces NO. However, when these are limited, eNOS can become uncoupled from the production of NO and can instead produce reactive oxygen species (ROS) such as superoxide (Montezano et al., 2012). Superoxide and NO spontaneously combine to produce peroxynitrite, an unstable, highly reactive oxidant which causes damage to cells and proteins and has been linked to myocardial contractile failure (Ferdinandy et al., 2000).

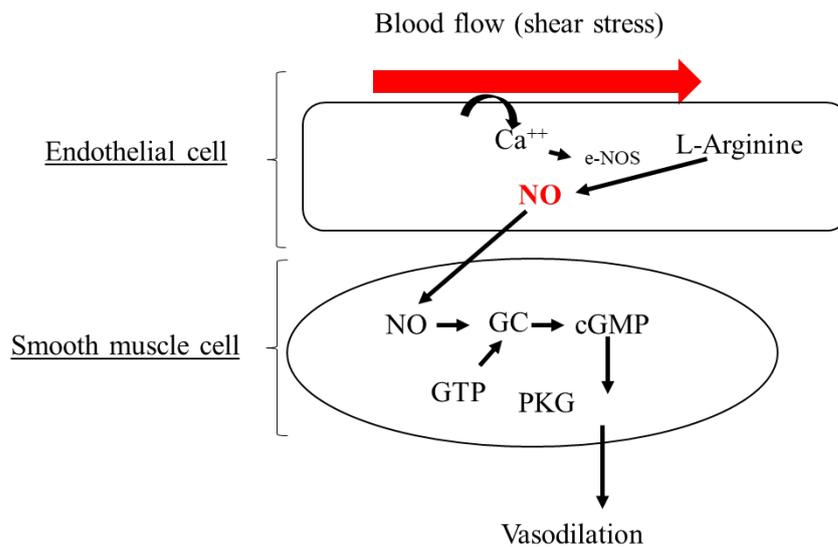


Figure 2.7 NO production via shear stress

Abbreviations: Ca^{++} - calcium, GC- guanylate cyclase, cGMP- cyclic guanosine monophosphate, GTP- guanosine 5'-triphosphate, NO- nitric oxide, PKG- protein kinase G. This figure is adapted from Sandoo et al. (2010).

2.3.2 Methods to increase NO bioavailability

Increasing NO bioavailability as a means for restoring central and peripheral defects to improve aerobic capacity in CHF is of emerging clinical interest. This section will detail the measurement of NO bioavailability as well as potential interventions to improve NO bioavailability.

2.3.2.1 Nitrite as a biomarker for NO

Once NO was identified as a potential target to improve exercise capacity, determining an accurate measure for the levels of circulating NO became imperative. In the presence of oxygen, NO oxidises to nitrite within seconds. This short half-life (two to six seconds) poses obvious challenges to measuring NO directly in human models, necessitating the identification of a stable marker of NO (Bryan et al., 2007).

Despite decades-long knowledge that nitrite acts as a vasodilator at supra-physiological (micromolar) concentrations, it was regarded within biological systems as an inactive “NO-sink,” which was ultimately excreted by the kidneys (Furchgott et al., 1953). While it was understood that NO could be oxidised to nitrite and subsequently to nitrate, it was originally assumed to be a unidirectional pathway (left side of **Figure 2.8**). Recently, however, nitrite (along with S-nitrosothiols, N-nitroso proteins and iron-nitrosyl complexes) has been shown to be reduced back to NO under hypoxic conditions (right side of **Figure 2.8**) (Govoni et al., 2008; Rassaf et al., 2002; Stamler et al., 1992). Thus, nitrite and the more stable nitrate are frequently used as markers of NO bioavailability.

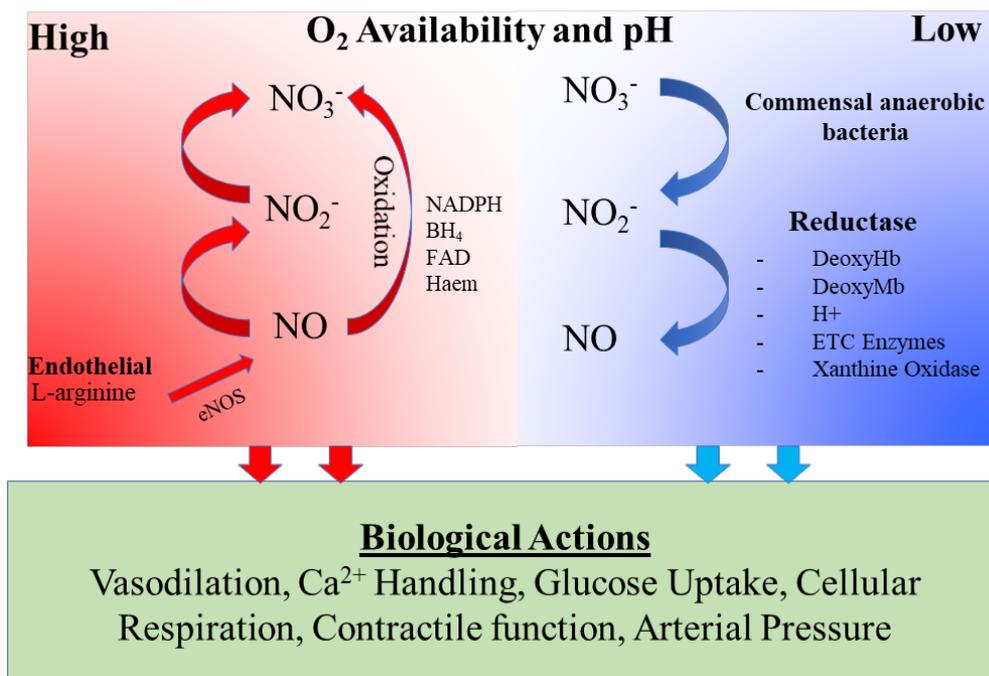


Figure 2.8 Nitrate-nitrite-NO pathway in oxygen rich and hypoxic environments

The left side in red is indicative of environments with high oxygen, and the right side in blue represents hypoxic environments. The oxidation of NO to NO₂⁻ to NO₃⁻ occurs in conditions of oxygen, whereas the reduction occurs preferentially in hypoxia in a two-step process via commensal oral bacteria and then a reductase. Abbreviations: BH₄-tetrahydrobiopterin, deoxyHb- deoxygenated haemoglobin, DeoxyMb- deoxygenated myoglobin, ETC- electron transport chain, FAD-flavin adenine dinucleotide, H⁺- hydrogen ion, NADPH- nicotinamide adenine dinucleotide phosphate, NO₃⁻-nitrate, NO- nitric oxide, NO₂⁻-nitrite. This figure is reproduced here from its original publication Woessner et al. (2018c).

Plasma nitrite is now commonly utilised as a surrogate marker of NO bioavailability due to its low background concentration (50-150nM) and its ability to reflect changes in regional eNOS activity (Lauer et al., 2001). Conversely, nitrate concentrations do not show such sensitivity to acute changes in eNOS activity, and concentrations can often be 100-fold higher than in plasma nitrite (Lauer et al., 2001). This is likely due to nitrate's relatively long half-life compared to nitrite and the fact that nitrate levels are known to be influenced by a myriad of factors unrelated to eNOS (e.g., diet, renal function and syntheses within the bowel) (Kelm, 1999). The literature remains controversial regarding the circulating levels of NO and NO metabolites in patients with CHF due to the lack of consistency in how and what is measured in quantifying these levels.

There is a need to delineate and differentiate the various measurement terminologies for circulating NO concentrations. For instance, NO metabolites (NO_x) is a measure that is inclusive of nitrate, nitrite, NO adducts, and NO-metal and haem complexes and is therefore representative of the overall balance between NO production, clearance and dietary intake. In HFREF, total levels of circulating NO metabolites (NO_x) can be similar to non-HF controls (Chirinos et al., 2016). However, as NO_x is not specific to NO derived from eNOS, high levels of NO_x do not necessarily equate to high vascular NO bioavailability. Indeed, it is thought that high NO_x could be reflective of increased activation of iNOS (Chirinos et al., 2016; Winlaw et al., 1994). This theory is further supported by the fact that plasma concentrations of NO_x are not linked to worsening HF. Thus while NO_x is sometimes utilised in studies, likely due to the added complexity associated with quantifying nitrate, nitrite and NO separately, it may not represent the best biomarker of circulating bioavailable NO. In contrast, plasma nitrite is a commonly

used surrogate marker of NO in studies in both healthy and CHF populations seeking to increase NO bioavailability to enhance performance outcomes (Hirai et al., 2017; Thompson et al., 2017; Wylie et al., 2016; Zamani et al., 2017).

Initial studies in patients with PAD (another CVD population with similar peripheral tissue dysfunctions to those seen in CHF) have demonstrated that changes in plasma nitrite are related to changes in exercise performance (Allen et al., 2009; Allen et al., 2014). This suggests that interventions targeting increases in plasma nitrite could be an attractive means of improving exercise tolerance in patients with HFrEF (Thompson et al., 2016; Wilkerson et al., 2012; Wylie et al., 2013).

2.3.2.2 Exogenous interventions to improve NO bioavailability

Supplementation with nitrite or nitrate is an established means of increasing NO bioavailability (McDonagh et al., 2019; Stanaway et al., 2017). Supplementation with nitrite/nitrate can be achieved through medications (e.g., GTN), oral supplementation (e.g., sodium nitrite/nitrate) or within the standard diet (e.g., beetroot juice and kale). These interventions can be either organic or inorganic, and while both increase NO bioavailability, the chemical structures, pharmacokinetics, and mechanism of actions make the therapeutic utility of organic and inorganic interventions distinct (Omar et al., 2012). For example, organic nitrite/nitrate have the benefit of being fast acting, but the vasodilatory effects are extensive, non-specific and acute resulting in widespread vasodilation. Patients may also develop tolerance to doses in such interventions. In contrast, inorganic compounds, which require a two-step metabolising process, have a longer lasting effect and the ultimate release of NO is more targeted (i.e., NO is released

from nitrite in hypoxic environments). The following sections will describe the different pathways associated with organic versus inorganic nitrite/nitrate consumption.

2.3.2.3 *Organic nitrite/nitrate*

Organic nitrate/nitrite are medicinally synthesised products formed by the reaction of nitric acid and an alcohol group, resulting in a hydrocarbon chain attached to a nitroxyl-radical. This synthesised compound produces a vasodilatory response within the blood vessel via the NO-mediated mechanisms described previously. Initially, organic nitrite compounds were used to induce widespread vasodilation for the treatment of angina (Nossaman et al., 2010). Shortly after its introduction, the use of organic nitrite compounds (specifically, glyceryl trinitrate (GTN)) emerged as a superior treatment option due to its more rapid effect, with patients indicating symptoms abating shortly after consumption. However, because of their chemical structure, when organic nitrates are consumed orally, they undergo first-pass metabolism within the liver that results in as much as 90% of the drug being quickly metabolised (Omar et al., 2012). Thus, organic nitrates must be administered sublingually or trans-dermally to increase the absorption percentage. Due to the use of these types of administration, and the organic nitrates being highly lipophilic, the effects of dosing are both immediate and widespread. Peripheral venodilation and blood flow redistribution result within one to three minutes, which lead to decreases in both left ventricular end-diastolic pressure and pulmonary artery pressure, thereby reducing cardiac preload (Klemenska et al., 2009). Reductions in the preload decrease the myocardial oxygen demand but can also cause rapid hypotension. In some cases, this can lead to syncope (Thadani et al., 2006)

While the rapid effect of the organic nitrate makes it an ideal immediate intervention for CVD patients suffering from angina, long-term treatment has been shown to have a negative impact on endothelial function due to further attenuation of eNOS (Munzel et al., 2000). Additionally, long-term use of these drugs is limited by the propensity of patients to develop tolerance to the vasodilatory effects of the medications (Omar et al., 2012). While the exact mechanisms of the developed tolerance are still debated, one potential cause is the depletion of aldehyde dehydrogenase (ALDH2) reductase. ALDH2 catalyses the conversion of GTN to 1,2-GDN and nitrite, and this reaction oxidises ALDH2, which then needs to be reduced in order to continue to catalyse the GTN reaction (Bantle et al., 2008). It is thought that continued dosing with GTN depletes the system of the ALDH2 reductase, thus slowing the production of nitrite and NO, and attenuating the downstream vasodilatory benefits (Mayer et al., 2008).

2.3.2.3.1 Inorganic nitrite/nitrate supplementation

Inorganic nitrite/nitrate supplementation has been shown to be a simple, non-invasive means of exogenously increasing plasma nitrite concentration, and consequently NO bioavailability, under suitable conditions (Lundberg et al., 2008). Inorganic nitrite/nitrate can be obtained through the daily diet. Average daily intake of nitrite is reported to be between 0-20mg per day (primarily from cured meats), while the average daily intake for nitrate ranges from 53-300mg per day (primarily from green vegetables) (Pennington, 1998). While dietary inorganic nitrate consumption has been shown to be safe even at relatively high concentrations, high levels of nitrite supplementation are not recommended due to the risk of acute nitrite toxicity and the formation of nitrosamines also from interaction with acid in stomach (Lundberg et al., 2011). Thus, most studies

today utilise inorganic nitrate supplementation as a means for increasing NO bioavailability.

Green leafy vegetables are the predominant source (85%) of daily dietary inorganic nitrate intake, with vegetables like kale and beetroot having the highest relative concentrations. Accordingly, while the average daily diet contains about 1.2 mmol of inorganic nitrate, a vegetarian diet yields closer to 4.3mmol of nitrate per day (Scanlan et al., 1996). Other dietary interventions, like the Dietary Approaches to Stop Hypertension (DASH), which utilises a high inorganic nitrate dietary intervention, have demonstrated both inorganic nitrate's preventative role in the development of vascular disease and its short-term effect on reducing BP (Gilchrist et al., 2010). The potential benefits of dietary nitrate supplementation are becoming more widely recognised and the DASH diet is recommended by the American Heart Association, the American Diabetes Association and the Dietary Guidelines for Americans (Appel et al., 2006; Bantle et al., 2008; U.S. Department of Health and Human Services, 2005).

Augmenting NO bioavailability using dietary inorganic nitrate works via the nitrate-nitrite-nitric oxide reduction pathway, which is a discrete yet complementary system to oxygen-dependent eNOS production. The following subsections will describe this pathway in detail and provide a summary of complications and considerations associated with nitrate supplementation.

2.3.2.3.1.1 Nitrate-nitrite-NO pathway

Oral inorganic nitrate supplementation works in a two-step process. In the first step, after consumption, nitrate is swallowed and absorbed into the circulation, with ~75%

being subsequently excreted by the kidneys (**Figure 2.9**) and the remaining ~25% being concentrated in the salivary glands (up to 10 times the plasma concentration) (Spiegelhalter et al., 1976). In the second step, nitrate secreted in saliva is reduced to nitrite via commensal oral bacteria on the dorsal surface of the tongue. The oral nitrite is then swallowed and absorbed into circulation. Plasma nitrite concentrations take approximately two to three hours to peak due to this two-step process; however, there is a steady concentration for around six hours, which avoids the short-lived bolus effects of direct oral nitrite administration (Webb et al., 2008; Wilkerson et al., 2012). Plasma nitrite can then be reduced to NO in low-oxygen environments, exerting beneficial biological effects.

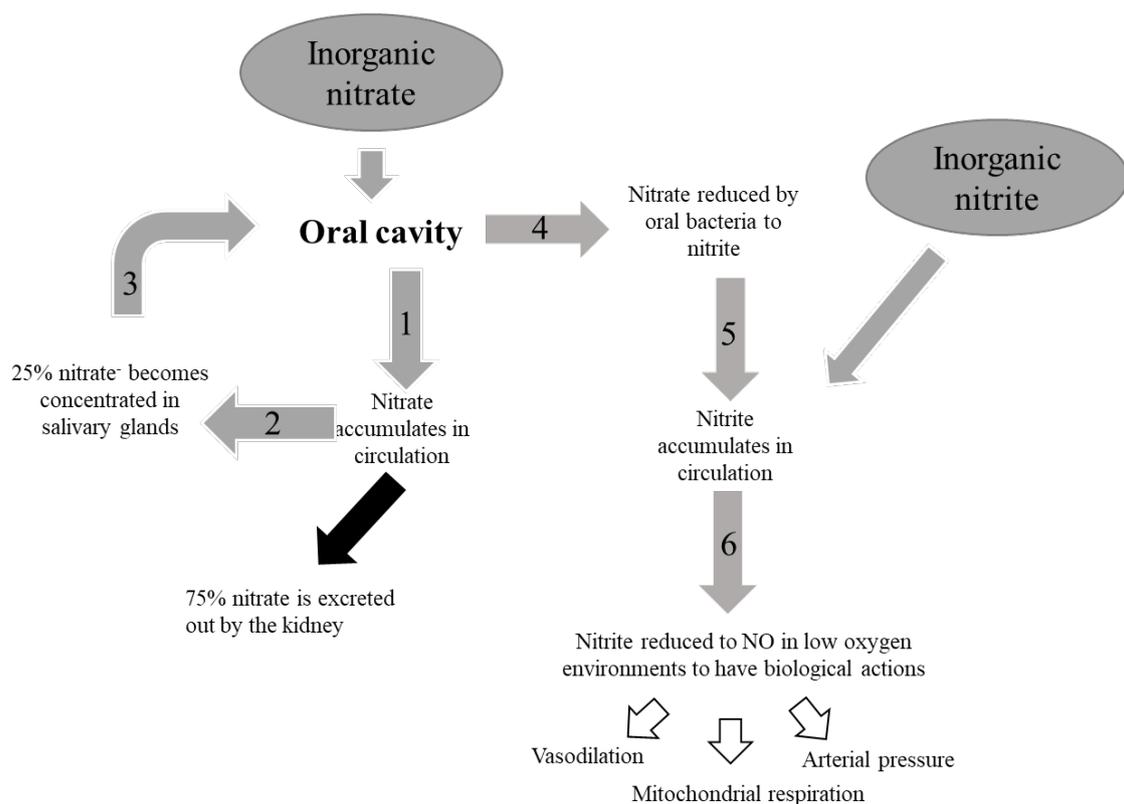


Figure 2.9 Two-step reduction pathway of nitrate to increase NO bioavailability-

Adapted from Kapil et al. (2010).

While this reduction pathway is an attractive option to target improvements in NO bioavailability, the reduction of nitrate to nitrite within the oral cavity is highly dependent on the profile of the oral bacteria. Commensal oral bacteria utilise nitrate as a terminal respiratory electron acceptor for ATP synthesis, reducing nitrate to nitrite. Oral nitrate reduction appears to occur mainly on the dorsal surface of the tongue and is predominantly mediated via two broad categories of bacteria: the strict anaerobes *Veillonella spp*, and the facultative anaerobes *Actinomyces spp* (Doel et al., 2005). The current literature limits our ability to draw far-reaching conclusions about the importance of the specific species and abundance of nitrate-reducing bacteria in the oral cavity on the conversion of inorganic nitrate to plasma nitrite. However, studies which have eradicated or inhibited these bacteria, via the use of antiseptic and antibacterial mouthwash treatments, have been shown to attenuate salivary and plasma nitrite concentrations, thereby negating the beneficial effects of nitrite on systemic BP (**Figure 2.10**) (Govoni et al., 2008; Kapil et al., 2013; Woessner et al., 2016).

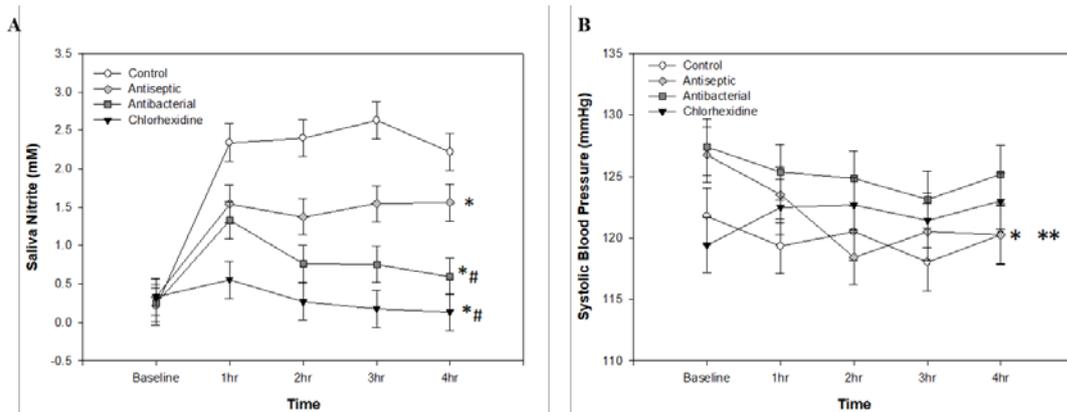


Figure 2.10 Effects of 8.4 mmol inorganic nitrate ingestion and mouthwash treatment on plasma nitrite levels and SBP response

All data are expressed relative to the control condition (ingestion of nitrate with just a water rinse). In **Figure 2.10A**, plasma nitrite values showed a stepwise reduction in total concentration four hours post ingestion in the chlorhexidine and antibacterial mouthwash conditions as compared to the no mouthwash control and the antiseptic mouthwash. SBP (**Figure 2.10B**) was consequently significantly reduced only in the control and anti-septic conditions. Adapted from Woessner et al. (2016). In **Figure 2.10A**: *indicates $p \leq 0.05$ lower than control treatment, **indicates $p \leq 0.01$ than control treatment, # indicates $p \leq 0.05$ lower than antiseptic treatment. In **Figure 2.10B**: * indicates $p \leq 0.05$ lower than antibacterial and chlorhexidine treatment, ** indicates $p \leq 0.01$ lower than antibacterial and chlorhexidine control treatment.

While it is not possible to control for all the factors that could impact the stability of an individual's oral microbiome, it is now considered best practice to instruct participants to avoid mouthwash for the duration of their participation in the trial.

2.3.2.3.1.2 Dosing considerations

Dosing amount and duration of supplementation are important factors to consider. A recent crossover study in 10 healthy males showed that an acute dosage of 4.2, 8.4 and 16.8 mmol inorganic nitrate, via beetroot juice, increased plasma nitrite in a dose-dependent manner with peak concentrations occurring at approximately two to three hours post consumption (Wylie et al., 2013). This same study also demonstrated that nitrate supplementation appears to have a dose-dependent response with vascular (**Figure 2.11**) and aerobic capacity (**Figure 2.12**) outcomes. The doses at 8.4 mmol of

nitrate and above appeared to be more efficacious at reducing BP and improving aerobic capacity.

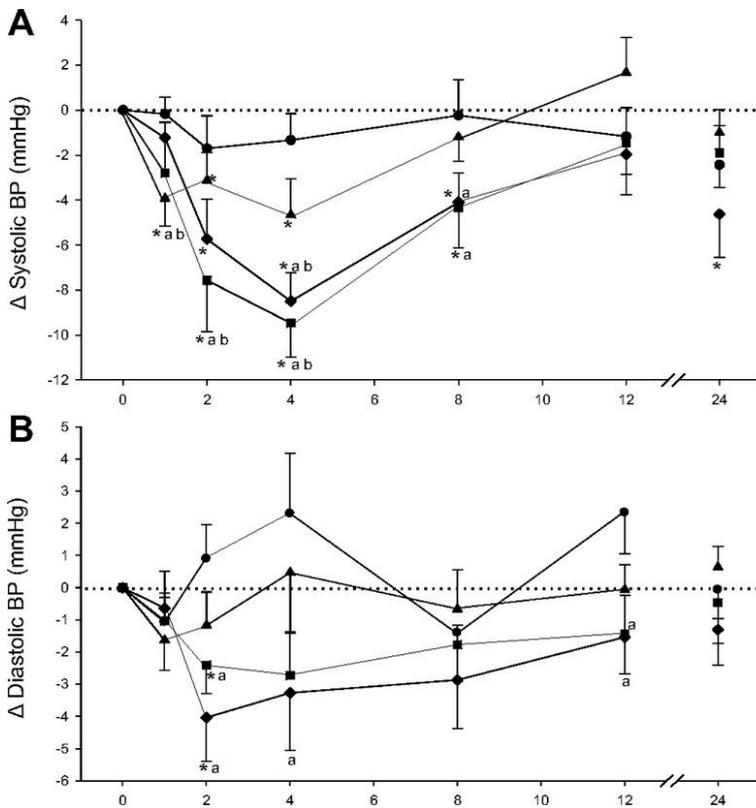


Figure 2.11 Dose-dependent response of blood pressure to nitrate supplementation

Change (Δ) relative to pre-supplementation baseline in systolic blood pressure (BP; A) and diastolic BP (B) following consumption of water (control; ●) and 4.2 (▲), 8.4 (■), and 16.8 (◆) mmol nitrate (group mean \pm SEM). There were significant reductions in SBP following all doses of nitrate supplementation at two and four hours post supplementation as compared to pre-supplementation baseline. Only the 8.4 mmol and 16.8 mmol doses elicited significant reductions in SBP as compared to the control condition, with the reductions remaining significant eight hours post dosing. For DBP, again both the 8.4 mmol and 16.8 mmol doses induced significant reductions compared to both pre-supplementation and the control condition at two hours post supplementation. At four hours, only the highest dose was significantly reduced compared to the control. * indicates a significant difference from pre-supplementation baseline ($p < 0.05$); ^a indicates a significant difference from control ($p < 0.05$); ^b indicates a significant difference from 4.2 mmol inorganic nitrate ($p < 0.05$). Adapted from Wylie et al. (2013)

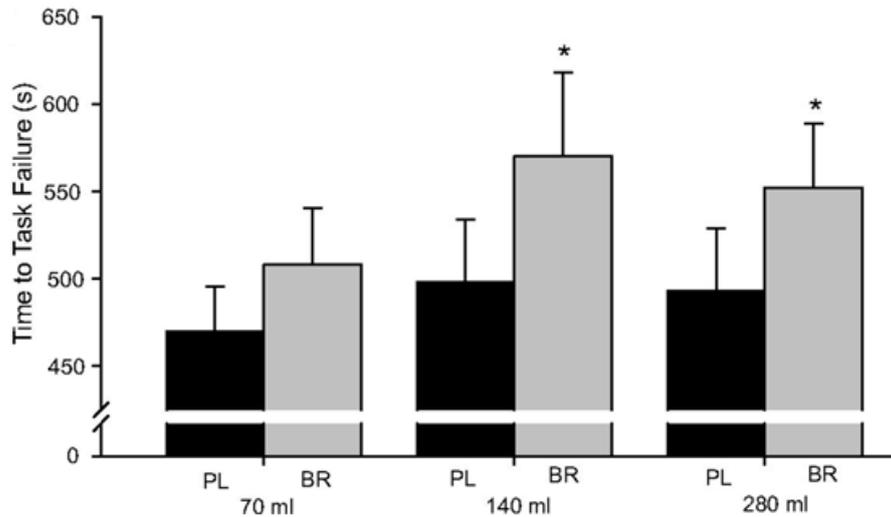


Figure 2.12 Dose-dependent response of time to failure and nitrate supplementation

Mean \pm SEM for time-to-task failure during severe-intensity exercise following consumption of 70, 140, and 280 ml nitrate-rich beetroot juice (gray bars) or nitrate-depleted beetroot juice (placebo=black bars). There were significant increases in time to task failure following 140 ml of beetroot juice (8.4 mmol nitrate), but no further increases at 280 ml (16.8 mmol nitrate). *indicates a significant difference from placebo ($p < 0.05$).

Comparisons between acute and chronic dosing of inorganic nitrate suggest that chronic dosing (15 days) maintains exercise economy benefits and can potentially have a greater effect on peak power output and time trial performance as compared to acute doses (Boorsma et al., 2014; Vanhatalo et al., 2010). A recent systematic review and meta-analysis on endurance exercise performance showed a positive trend toward improvements in TTE when utilising chronic nitrate supplementation, defined as repeated doses >6 hours apart (McMahon et al., 2017). It has also been reported that longer-term nitrate supplementation (5-7 days) can result in changes in mitochondrial and contractile proteins that would be expected to enhance skeletal muscle metabolic and mechanical efficiency (Hernandez et al., 2012; Larsen et al., 2011). It would seem unlikely that these changes could be fully effected within a few hours of nitrate ingestion and therefore the duration of nitrate supplementation is likely to introduce

variability into the potential efficacy of nitrate on the physiological responses to exercise.

While dose-dependent responses have not been evaluated in clinical populations, a previous clinical trial utilising patients with PAD has demonstrated that a dose of 18.1 mmol/day of inorganic nitrate was feasible and safe for patients, with doses as high as 12.9 mmol nitrate/day having been utilised in the CHF population (Hirai et al., 2017; Kenjale et al., 2011; Shaltout et al., 2017).

As no serious adverse events or side effects have been linked to higher doses of inorganic nitrate, and there appears to be the potential for greater efficacy when utilising a higher dose over five days, current studies are predominantly utilising a high acute or chronic dose of >11.9 mmol nitrate (Hirai et al., 2017; Kerley et al., 2016; Zamani et al., 2017). As such, a high-concentration dose of 16 mmol and a chronic supplementation period of at least five days were utilised in this thesis.

2.4 Effect of increasing NO bioavailability in chronic heart failure

In healthy individuals, supplementation can be used as an ergogenic aid to augment normal levels of bioavailable NO in exercising tissues to enhance physical performance, stamina and/or recovery (Jones, 2014). Supplementation within the clinical cohort, however, takes a therapeutic approach with the primary aim of restoring deficient NO bioavailability, correcting physiological dysfunctions, and recovering exercise capacity/performance and health (McDonagh et al., 2019). Moreover, given that nitrite is reduced to NO in low-oxygen and acidic conditions, the underperfused muscle tissues of patients with HFrEF could be an ideal environment for the potentiation of nitrate supplementation.

2.4.1 Nitrate supplementation and aerobic capacity

The effects of inorganic nitrate supplementation on aerobic capacity in CHF is not clear.

This section will provide an overview of the literature on inorganic nitrate supplementation and aerobic capacity in both healthy individuals and those with CHF.

To date, there have been a limited few studies that have examined the effects of inorganic nitrate supplementation on aerobic exercise in patients with CHF (**Table 2.1**).

While the studies in HFpEF all indicate positive improvements in aerobic capacity following supplementation, the results are split with regards to inorganic nitrate's efficacy for improving exercise tolerance in patients with HFrEF; half of the studies indicate improvements in measures of aerobic capacity and the others showed no effect of supplementation. There are some complicating factors, however. Of the four studies inclusive of HFrEF, only three assessed peak aerobic capacity, two of which indicated beneficial effects (Coggan et al., 2018; Kerley et al., 2016) and one of which had no effect (Hirai et al., 2017). The two more favourable studies, should be interpreted with caution due to the use of alternative modalities: an incremental shuttle walk test and a peak exercise test on a recumbent cycle ergometer.

Table 2.1 Published studies with inorganic nitrate and CHF

CHF type	Author	N	EF %	Duration	Design	Dose/Administration	Exercise Outcome	Description
HFpEF	Zamani et al. (2015)	17	≥ 50	Acute	Double-blind, randomized, crossover	12.9 mmol nitrate	↑	Increase in $\dot{V}O_{2peak}$ (p=0.005) Increase in TTE (p=0.02) No change in maximal exercise efficiency
	Eggebeen et al. (2016)	18	≥ 50	Acute	A:Crossover design	6.1 mmol nitrate	↔	No change in submaximal TTE
			≥ 50	Chronic	B:All treated	7days- 6.1 mmol nitrate	↑	Increase in submaximal TTE (p=0.02)
	Zamani et al. (2017)	12	≥ 50	Chronic	Single Blind	7days 12 mmol potassium nitrate	↔	No change in $\dot{V}O_{2peak}$
≥ 50			Single Blind		Above followed by 7days 18 mmol potassium nitrate	↑	Increase in TTE: (p=0.002)	
HF+rEF	Hirai et al. (2017)	10	≤ 40	Chronic	Double-blind, randomized crossover	9 days-12.9 mmol nitrate	↔	No change in exercise performance measures
HF+rEF/HFmrEF	Coggan et al. (2015)	9	≤ 45	Acute	Double-blind, crossover	11.2 mmol nitrate	↔	No change in 6-minute walk test
	Kerley et al. (2016)	11	15-50	Acute	Double-blind, crossover	12.9 mmol nitrate	↑	Increase in incremental shuttle walk test (p<0.05)
	Coggan et al. (2018)	8	≤ 45	Acute	Double-blind, crossover	11.2 mmol nitrate	↑	Increase in exercise duration, peak power and $\dot{V}O_{2peak}$ during incremental exercise test (p<0.05) Supine cycle ergometer

An inclusive table of all published studies examining the effects of inorganic nitrate supplementation on aerobic capacity in patients with CHF. Abbreviations: $\dot{V}O_{2peak}$ - maximal oxygen consumption, TTE- time to exhaustion.

The trial by Kerley et al. indicated improvements in aerobic capacity via an 18% increase in incremental shuttle walk test distance following an acute dose of inorganic nitrate (12.9 mmol) in patients with non-ischaemic dilated cardiomyopathy (NIDCM) (Kerley et al., 2016). There are, however, several considerations to account for in this study. First, while the incremental shuttle walk test has been shown to correlate with $\dot{V}O_{2\text{peak}}$, it is not currently widely used (due in part to the inability to extract detailed physiological data), and it has several limitations. This test increases intensity by requiring individuals to gradually increase their walking speed. Thus, if a participant is unable to maintain an adequate speed, the test is terminated (Holland et al., 2015). Incremental tests on treadmills or cycle ergometers can individualise the increases in intensity by making changes in speed or workload in order to meet the patient's needs and ensure test termination is due to exertion (Arnardóttir et al., 2006). Additionally, the use of only patients with NIDCM limits the applicability to HFrEF as a whole. Significantly, the inclusion criteria allowed for patients with an EF of up to 50% to participate, suggesting there was a mix of HFrEF and HFmrEF participants. There are no studies to date assessing the effects of inorganic nitrate on HFmrEF only, but initial evidence suggests these patients have clinical profiles and responses which are intermediary between HFpEF and HFrEF (Nadar et al., 2018). As supplementation has been more efficacious in HFpEF, this is a possible explanation for the positive results seen.

The other clinical trial with favourable findings in HFrEF is the most recent (2018), where the authors noted a significant increase in $\dot{V}O_{2\text{peak}}$ and TTE (8% and 7%, respectively) during a recumbent incremental cycle test following an acute dose (11.2 mmol) of inorganic nitrate (Coggan et al., 2018). Supine positioning augments venous return, which can lessen the impact of the central impairments in CHF, potentially enhancing the efficacy of supplementation. This study is also complicated in a similar way to the previous study in that

they also only recruited patients with a non-ischaemic aetiology and had an EF (<45%) cut-off which, again, did not exclude recruitment of patients with HFmrEF.

The single published study examining the effect of inorganic nitrate on aerobic capacity in patients with HFrEF (EF < 40%) was a chronic (nine days) daily dose of 12.1 mmol nitrate or placebo (Hirai et al., 2017). This study reported no improvements in TTE or $\dot{V}O_{2\text{peak}}$ following nitrate supplementation. The authors suggest the negative findings could be due to these patients having relatively normal values of arterial-venous oxygen difference ($a-vO_2\text{dif}$), thus there could be less capacity for improving on this parameter as oxygen extraction was already optimal.

While most studies have focused on the potential effects of inorganic nitrate supplementation on peak exercise performance outcomes, in clinical populations, outcomes associated with submaximal intensities (or those that reflect the energy requirement of ADLs) could be equally pertinent. Despite the absolute $\dot{V}O_2$ required to complete the five ADLs being similar between patients and healthy controls, the relative percentage of $\dot{V}O_{2\text{peak}}$ required for ADLs was 7-14% higher for patients with CHF (Spruit et al., 2011). This higher metabolic demand is accompanied by significantly higher fatigue-related symptoms, suggesting that interventions which can lower the relative metabolic demand of submaximal activities could result in improvements in daily functioning and QoL in patients with CHF.

Of the two studies that have measured submaximal exercise outcomes following nitrate supplementation, neither found any beneficial effect of the intervention (Coggan et al., 2015; Hirai et al., 2017). The earliest study by Coggan et al. in 2015 found no significant improvements in a 6-minute walk test following an acute dose, and the chronic dose study by

Hirai et al. saw no improvements in the oxygen cost of exercise at a low or high intensity (Coggan et al., 2015; Hirai et al., 2017).

The recruitment of a single aetiology of HFrEF is a complicating factor in this body of literature. While the Hirai et al. study recruited predominantly patients with ischaemic heart disease, the other three trials utilised patients with non-ischaemic histories. It has been suggested that the discrepancy in the findings of the trials could be in part explained by these aetiological differences. However, no study to date has been diverse enough to examine this potential. Additionally, the use of a variety of different maximal capacity protocols (e.g., incremental shuttle walk test, supine cycle ergometry and upright cycle ergometry) also presents challenges with regards to the clear interpretation of findings. Moreover, no study to date has explored the potential effect of inorganic nitrate supplementation with extensive physiological outcomes in the most real-world translatable mode of exercise, upright walking. There is a need for studies in HFrEF which include a larger more diverse sample of patients and which utilises treadmill exercise to assess submaximal and maximal aerobic capacity. *Study 1* was designed in an effort to address these previous limitations.

2.4.2 The effect of NO on cardiac function in HFrEF

2.4.2.1 Cardiac function in health and HFrEF

In healthy individuals, both HR and SV are increased in response to exercise in order to increase \dot{Q} . As previously discussed in **Section 2.2.1**, patients with HFrEF display significant reductions in \dot{Q} both at rest and during exercise compared to healthy individuals and those with HFpEF (Dhakal et al., 2015). These reductions in cardiac performance are due to a myriad of impairments in both the structure and function of the cardiac system as a result of the cardiac remodeling (as reviewed in **Section 2.1.2**).

2.4.2.2 *NO and cardiac function in HFrEF*

A mounting body of evidence is emerging which suggests that the myocardial defects associated with CHF are, at least in part, due to the imbalances in NO bioavailability and oxidative stress (ROS production) causing endothelial dysfunction (Marti et al., 2012).

Indeed, the degree of cardiac dysfunction has been shown to be proportional to the reduction of NO-dependent reserves in the coronary arteries. This reduction in NO impairs coronary blood flow and the reduced blood flow is inversely correlated to the EF (Mohri et al., 1997). This suggests that reduced NO bioavailability could negatively impact measures of cardiac function/performance and that interventional measures for increasing NO bioavailability could potentially ameliorate some of these central deficits.

It has been previously shown that eNOS is downregulated in CHF (Bauersachs et al., 2008; Comini et al., 1996; Smith Carolyn et al., 1996). In support of this, eNOS deficient mice are characterised by increased LV hypertrophy compared to mice with over expressed eNOS (Jones et al., 2003). Furthermore, over expression of eNOS in mice also improves survival (Jones et al., 2003). While it is unclear whether it is the eNOS from coronary arteries or from the cardiac myocytes that exerts these positive effects on LV remodeling, NO from eNOS does appear to potentially offset the cardiac hypertrophy and fibrosis that is prominent in CHF (Ruetten et al., 2005). This suggests that interventions targeting either upregulation of eNOS or increased NO bioavailability (without superoxide generation) could have clinically beneficial effects in the cardiac tissue and result in corresponding improvements in cardiac performance of patients with HFrEF.

Inorganic nitrate supplementation in healthy animal models has been shown to improve Ca^{++} signaling in the cardiac tissues and elicit beneficial effects on LV contractile function (Pironti et al., 2016). A subsequent six-month supplementation study in humans at risk for or with

type 2 diabetes mellitus (T2DM) showed a 5% reduction in LV volume (Faconti et al., 2019). These promising benefits to cardiac structure, however, have not consistently translated to improvements in cardiac performance. A study in healthy individuals indicated a reduced TPR at rest and during exercise following 15 days of supplementation, but there were no corresponding increases in \dot{Q} or SV (Lee et al., 2015). Similarly, in older adults, one week of inorganic nitrate dosing (12 mmol/day) did not modify \dot{Q} at rest or during exercise (Oggioni et al., 2018).

To date, only two studies have examined the potential effects of inorganic nitrate supplementation on cardiac performance during exercise in CHF (Hirai et al., 2017; Zamani et al., 2015). In HFpEF, utilising a non-invasive echocardiograph imaging, acute supplementation significantly increased \dot{Q} at peak exercise. This was likely mediated by a concomitant decrease in TPR (Zamani et al., 2015). The single study in HFrEF utilised an indirect impedance cardiography technique (Physioflow, Manatec Biomedical, Petit Ebersviller, France) and found no significant changes in \dot{Q} , SV or HR following inorganic nitrate supplementation (Hirai et al., 2017). However, these results must be interpreted with caution as the device used is relatively new, and, to the best of our knowledge, this device can overestimate \dot{Q} in heart failure (Kemps et al., 2008). Given the positive results seen in HFpEF, and the complications with the methodology used in the single HFrEF study, there remains an ongoing need to further explore the potential effects of inorganic nitrate supplementation on cardiac performance in patients with HFrEF. This is explored in *Study 2*.

2.4.3 Peripheral effect of NO in HFrEF

As introduced previously in the skeletal muscle hypothesis (**Section 2.2.2**) there are a myriad of dysfunctions within the skeletal muscle of patients with HFrEF which can contribute to the

exercise intolerance and clinical disability evidenced in this population. This section will review the peripheral pathophysiology in CHF and highlight the role of NO and the potential efficacy of nitrate supplementation.

2.4.3.1 *Vascular function in health and HFrEF*

The production of NO via eNOS is dependent on a healthy endothelial layer. In healthy individuals, the release of NO is stimulated during exercise via shear stress on the arterial wall (Moncada, 1997; Nosarev et al., 2015). As large- and small-vessel vasodilation is a crucial contributor to exercise capacity via the modulation of blood flow and tissue perfusion, dysfunctions in the endothelium can lead to increases in BP, upregulation of TPR and ultimately reduce exercise performance (Bauersachs et al., 2008; Patel et al., 2001; Patel et al., 2005; Pina et al., 2003). Individuals with CHF are characterised by an inability of the endothelium to upregulate NO during exercise, leading to early fatigue and exercise intolerance (Bauersachs et al., 2008; Pina et al., 2003). Endothelial dysfunction is also correlated with a reduction in NYHA classification and is an independent predictor of cardiac hospitalisations and deaths in patients with CHF (Nakamura et al., 1994). As such, endothelial dysfunction and reductions in NO bioavailability are considered to be critical modulations of vascular function and exercise capacity (Fischer et al., 2005; Katz et al., 1997).

Animal studies lend further insight into the role of NO bioavailability on vascular function in HFrEF. Mice deficient in eNOS were shown to be at a higher risk for the development of CHF, while those with an enhanced production have increased protection against CVD development and progression (Bhushan et al., 2014). The link between decreased NO production and blood flow is best demonstrated in studies utilising an inhibitor of NO

synthesis, such as NG-nitro-L-arginine methyl ester (L-NAME) or NG-monomethyl-L-arginine (L-NMMA). A rat model showed significant decreases in skeletal muscle blood flow in healthy rats following the use of L-NAME, but no significant reduction was seen in the rats with CHF (Hirai et al., 1995). A similar phenomena was reported in the coronary arteries in humans with HF, whereby the vasoconstrictor response following L-NMMA was reduced in patients with CHF compared to healthy controls (Mohri et al., 1997). This suggests that in CHF, there is already substantial attenuation in NO production in both the central and peripheral vasculature which results in a reduction in overall blood flow via decreased vasodilation and increased TPR (Hirai et al., 1995). As such, studies that will explore exogenous methods to increase NO bioavailability in patients with HFrEF may result in improvements in endothelial function, BP and exercise capacity.

2.4.3.1.1 Effect of nitrate supplementation on vascular (dys)function

A reduction in BP is one of the most established benefits of inorganic nitrate supplementation in healthy and clinical cohorts (Jajja et al., 2014; Kenjale et al., 2011; Larsen et al., 2006; Siervo et al., 2013). Three days of sodium nitrate supplementation (0.1 mmol/kg/day) was shown to significantly reduce DBP by 3.7 mmHg in healthy volunteers (Larsen et al., 2006). An acute dose of inorganic nitrate (22.5 mmol) via beetroot juice also significantly reduced SBP and DBP by ~10 and 8 mmHg respectively in another cohort of healthy individuals (Webb et al., 2008). Furthermore, the reduction in BP was correlated to plasma nitrite concentrations, and both changes could be abolished by interruption of the enterosalivary conversion of nitrate to nitrite (via mouthwash) (Webb et al., 2008).

Similar benefits have been observed in studies of patients with HTN (Ghosh et al., 2013). A double-blind placebo-controlled study where 68 patients were given a 6 mmol dose of inorganic nitrate, via 250 ml beetroot juice for four weeks, demonstrated significant

reductions in clinic measured systolic and diastolic BP (~8/2.5 mmHg), 24-hour ambulatory-measured BP(~8/5 mmHg), and home measured (~8/4 mmHg) BP (Kapil et al., 2015). These reductions are clinically significant when one considers that every 1 mmHg reduction in SBP can lead to a 5% decrease in the risk of stroke (Grossman, 2011). Similarly, a reduction in DBP of just 5-6 mmHg was associated with a 38% reduction in the five-year risk of stroke and 23% reduction in five-year risk of coronary heart disease (Collins et al., 1990). Thus, even modest reductions in brachial SBP could offer significant clinical benefits, particularly to those individuals with HTN.

As discussed previously, the data available specifically in CHF is limited. Three studies in HFpEF reported a significant reductions in SBP following either acute or chronic doses (Eggebeen et al., 2016; Zamani et al., 2015; Zamani et al., 2017). In contrast, none of the trials which included HFrfEF, including those that also had HFmrEF, reported a reduction in SBP (Coggan et al., 2018; Coggan et al., 2015; Hirai et al., 2017; Kerley et al., 2016). It is important to note that while brachial BP was collected in the trials, it was not a primary outcome. This may affect the findings because participants were not in a fasted state and medications were taken as prescribed. Given the known effects of both food and medications on BP, the arterial pressure findings from these trials must be interpreted with caution. The differences between studies could be explained by the HFpEF cohort typically being characterized as older and having HTN as a frequent precursor. Patients with a high BP at baseline may be more responsive to therapies targeting reductions in BP (Bondonno et al., 2015; Kapil et al., 2015). There is an urgent need to optimise a study protocol to assess brachial and aortic BP to determine the potential efficacy of inorganic nitrate supplementation in HFrfEF. This is one of the foci of *Study 1*.

The potential efficacy of nitrate supplementation for improving endothelial function in healthy and clinical populations was investigated in a small number of publications. Healthy individuals have had seen mixed effects of nitrate supplementation on endothelial function, with one showing small increases in FMD following 200mg of spinach, while another showed no effect after a single dose (8mmol) of potassium nitrate (Bahra et al., 2012; Bondonno et al., 2012). In the elderly, acute ingestion of dietary nitrate via a single dose of beetroot juice increased BAFMD by 77% (de Oliveira et al., 2016). At altitude, nitrate supplementation abolished the negative impact of altitude-induced hypoxia on endothelial function (Bakker et al., 2015). While exposure to hypobaric hypoxia undeniably initiates several other physiological adaptations not present in conditions of peripheral hypoxia, these results and results from the elderly population do suggest that there is the potential for a greater efficacy of supplementation in older individuals with more hypoxic tissues. Indeed, longer dosing studies of four to six weeks of daily nitrate dosing found significant increases in endothelial function (20% and 26%) in patients with HTN and hypercholesterolemia, respectively (Kapil et al., 2015; Velmurugan et al., 2016). The only clinical population that has shown no consistent benefit to endothelial function with nitrate is T2DM, and this is thought to be due to the myriad of biochemical disturbances including hyperglycaemia and increased oxidative stress which are known to be present in some patients with HFrEF (Gilchrist et al., 2013). There are no published studies examining the effect of nitrate supplementation on endothelial function in CHF, but given the positive results in HTN and the improvements in hypoxic environments, it is hypothesised that supplementation could improve this parameter in HFrEF. This hypothesis is tested in *Study 1*.

The pressure and/or stiffness of the aortic arteries are strong predictors of future cardiovascular events (Pini et al., 2008; Zuo et al., 2018). Patients with CHF are characterised by chronic elevations in aortic pulse pressure (PP), pulse wave velocity (PWV) and/or

augmentation index (AIX) which are reflective of increased arterial stiffness (Chirinos et al., 2005; Marti et al., 2012). Recent technological advancements allow for the estimation of central pressures from peripheral pressures in order to improve prognosis in clinical patients (Laugesen et al., 2014). Reductions in aortic pressure and stiffness are of significant clinical interest in all individuals. High central PP can predict CHF development in the healthy aged population and both AIX and PWV are independently associated with systolic and diastolic dysfunction (Chae et al., 1999; Weber et al., 2008). In patients with CAD, increases of 10 mmHg in the PP can enhance the risk of premature mortality by 18% over ~4 years (Chirinos et al., 2005). NO is an established regulator of arterial stiffness and increases in NO bioavailability are related to decreases arterial stiffness (Anderson, 2006). Thus, interventions that target NO bioavailability potentially could improve arterial stiffness thereby providing clinical benefits to patients with HFREF. This hypothesis is tested in *Study 1*.

2.4.3.2 *Mitochondrial function*

2.4.3.2.1 *Mitochondrial function: overview*

The mitochondria are the site for cellular respiration in the body and the main source of energy production in the form of adenosine triphosphate (ATP) (Germann et al., 2005). The mitochondria are separated from the cytoplasm in the cell by an outer and inner membrane. The outer membrane is porous and allows for the free flow of substrates such as adenosine diphosphate (ADP) and ATP as well as ions such as hydrogen (Farrell et al., 2012). The inner membrane acts as a strong barrier that can only be crossed utilising specific transport proteins. Due to this selectivity and tight control, there is a membrane potential, which allows for the use of an electrochemical gradient via membrane protein complexes (Farrell et al., 2012). Thus, it is the inner membrane, through the use of the electrochemical gradient, that acts as the site of oxidative phosphorylation within the mitochondria.

Along this inner membrane, there are four membrane protein complexes involved in the respiratory chain of the mitochondria: complexes I, II, III and IV. These complexes, cumulatively named the electron transport system (ETS), couple the sequential transfer of electrons with the transfer of protons (hydrogen ions) across the membrane to establish an electrochemical gradient. This gradient then provides the energy to phosphorylate ADP to ATP (Kühlbrandt, 2015). Normal mitochondria respiration and function is critical for health outcomes as well as exercise capacity.

In addition to the necessity for intact ETS for effective respiration, there are also numerous regulatory proteins that are associated with mitochondrial function: PGC-1 α , mTORC1, Akt and p38MAPK. PGC-1 α is a signaling molecular involved in mitochondrial biogenesis, a shift to slow twitch fibre typing and angiogenesis (Arany, 2008; Lin et al., 2002). p38MAPK is a stress activated kinase that positively regulates PGC-1 α transcription (Akimoto et al., 2005). mTORC1 is responsible for protein synthesis and Akt is a critical mediator of mTOR (Yoon, 2017). Not only are these regulatory proteins associated with the healthy function of the mitochondria, but disruptions in the expression of these proteins and impaired mitochondrial respiration have also been implicated in the initiation and/or progression of CHF (Riehle et al., 2012; Sciarretta et al., 2014; Tobar et al., 2018).

2.4.3.2.2 *Mitochondrial (dys)function in HFrEF*

Mitochondrial function is associated with exercise capacity ($\dot{V}O_{2\text{peak}}$) in both healthy and HF cohorts (Drexler et al., 1992; Hoppeler et al., 1985; Hoppeler et al., 1973). In patients with CHF, there are marked dysfunctions in the structure and function of the mitochondria, downregulation of the mitochondrial proteins and reductions in overall ATP production (Knowlton et al., 2014; Rosca et al., 2009; Rosca et al., 2010, 2013). Impairments in

mitochondrial function are not limited to a single tissue type. Reductions in oxygen consumption and ATP production have been indicated in both oxidative tissues (brain) and glycolytic tissues (gastrocnemius) of patients with CHF (Nisoli et al., 2004). While mitochondrial dysfunction in other tissues of patients with CHF have severe consequences, impairments within the skeletal muscle mitochondria are particularly detrimental as these are linked to the hallmark symptom of exercise intolerance (**Figure 2.13**).

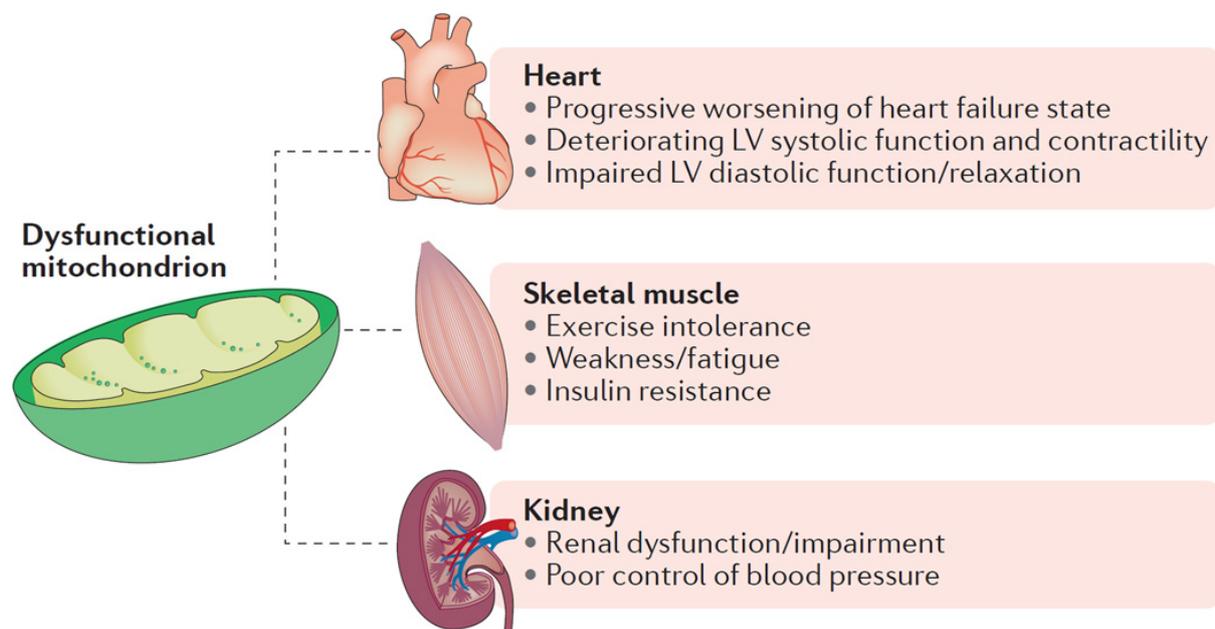


Figure 2.13 Mitochondrial dysfunction in tissues of patients with CHF
Reproduced from Brown et al. (2017)

NO is an established regulator of mitochondrial function and a stimulator of mitochondrial biogenesis via the activation of various cellular signaling pathways (Caballano-Infantes et al., 2017; Nisoli et al., 2004) (**Figure 2.14**). Reductions in NO bioavailability and impaired mitochondrial function are associated with the development and progression of CHF (Fischer et al., 2005; Nisoli et al., 2004).

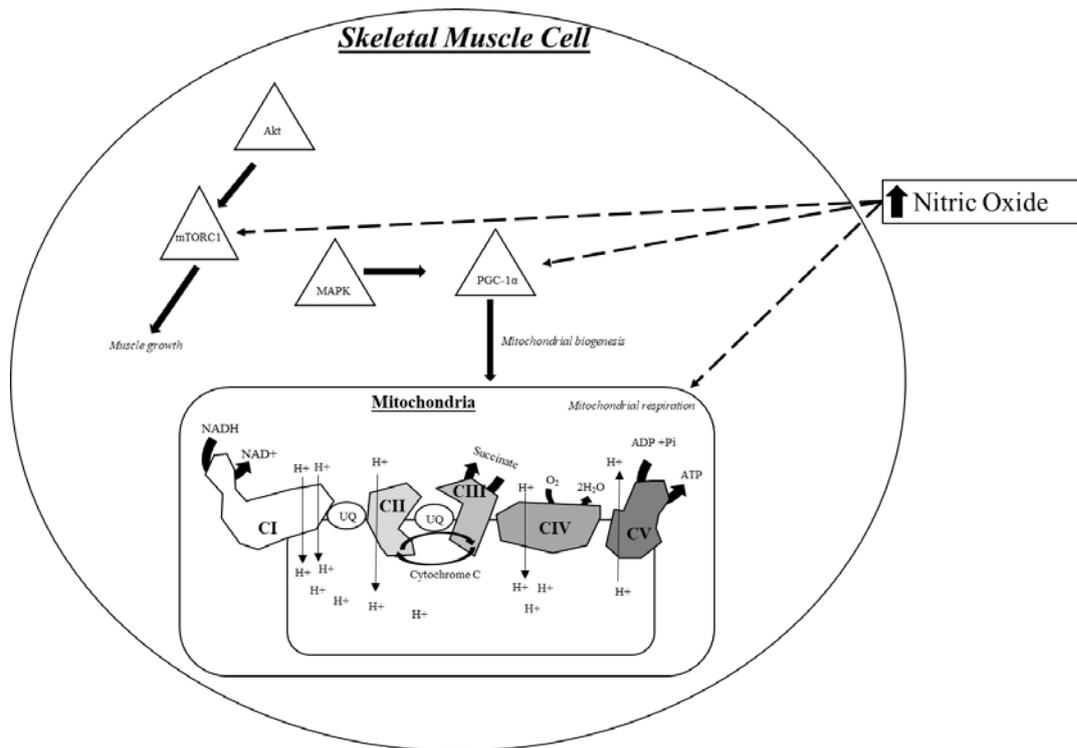


Figure 2.14 Mitochondrial respiratory chain

The potential effect of increasing NO bioavailability on protein signalling and mitochondrial respiration. A depiction of the structure and function of the mitochondrial complexes and the associated links with regulatory proteins. Abbreviations: ATP-adenosine triphosphate, ADP-adenosine diphosphate, Pi-inorganic phosphate, H⁺-hydrogen, NAD-nicotinamide adenine dinucleotide, UQ-ubiquinol, MAPK- p38 mitogen activated protein kinase, PGC-1α- peroxisome proliferator-activated receptor-γ coactivator 1α, Akt- protein kinase B, mTOR- mechanistic target of rapamycin. This figure was adapted from Kühlbrandt (2015) and Davies et al. (2013).

Independent studies in both human and animal CHF models have reported decreases in function in the individual mitochondrial complexes (Ide et al., 1999; Jarreta et al., 2000; Rosca et al., 2009). In patients with CHF, as well as those with dilated cardiomyopathy, there are dysfunctions in complexes III and IV., and significant reductions in ATP synthase activity (Guzmán Mentésana et al., 2014). NO is known to play a regulatory role in the function of some of the individual mitochondrial complexes (complex IV). This association is best illustrated by a study which found that the deletion of eNOS alone from mice led to significant decreases in mitochondrial number as well as reduced energy expenditure compare to control mice (Nisoli et al., 2003). This suggests that the reduced NO in CHF could be both a cause and consequence of the mitochondrial dysfunction and therefore

represents an intriguing target for interventions aiming to improve both mitochondrial function and aerobic capacity in these individuals.

Reductions in mitochondrial protein expression in patients with CHF also have significant impacts on the function of the mitochondria. However, the levels of protein expression in CHF relative to healthy controls remains poorly understood, with studies publishing conflicting results (Riehle et al., 2012). While low levels of PGC-1 α have been found in animal models of CHF and have been linked to the muscle atrophy seen in patients with CHF, other recent studies in humans have indicated no reductions in PGC-1 α (Hu et al., 2011; Souza et al., 2014). One potential influencer in the differential results in animal versus human studies could be the pharmacological interventions that the human participants are undergoing. For example, it is established that phosphodiesterase inhibitors could increase PGC-1 α expression, which could acutely and artificially augment PGC-1 α levels in these patients. As exogenously increasing NO bioavailability works in a similar manner, these types of medications need to be controlled for in studies seeking to determine the potential efficacy of increasing NO bioavailability. Despite some conflictual findings with regards to the relative levels of PGC-1 α in CHF, the link between reduced PGC-1 α and muscle wasting is clear. In a CHF mouse model, induced over expression of PGC-1 α offset the CHF-induced skeletal muscle atrophy via an upregulation of eNOS and iNOS production of NO and enhanced expression of Akt (Geng et al., 2011).

In addition, other regulators of PGC-1 α could also be upstream targets for interventions aiming to reduce muscle wasting. p38MAPK is a stress activated kinase that positively regulates/stimulates PGC-1 α transcription and could also be negatively regulated by NO (Akimoto et al., 2005; Kinugawa et al., 2005; Riehle et al., 2012). Other studies have also

found that reductions in the Akt/mTORC1 protein synthesis pathways are linked to the muscle wasting in CHF (Bacurau et al., 2016). In the skeletal muscle, Akt is a critical mediator of mTORC1, a protein kinase responsible for protein synthesis. Indeed both Akt and mTORC1 signaling are essential to promoting muscle hypertrophy (Yoon, 2017). mTORC1 is responsible for protein synthesis, and its function underpins hypertrophic growth in cardiac tissues. Indeed, an animal model has shown that reduced mTOR signaling can induce the development of HF due to its activation of hypertrophy during stress (Zhang et al., 2010). Interventional training studies have indicated that aerobic exercise training can restore Akt/mTORC1 pathway in patients with CHF and attenuate the muscle wasting (Bacurau et al., 2016). There is also evidence that mTORC1 may be regulated by NO-dependent mechanisms (Aguar et al., 2017; Ito et al., 2013). Akt is an upstream activator of mTORC1, whose activity is inhibited by NO-mediated S-nitrosylation (Yasukawa et al., 2005). Given a reduction in mTOR can both precede the CHF syndrome and/or worsen the associated pathology, interventions that can restore Akt/mTORC1 activity could be an intriguing option in this patient population.

2.4.3.2.3 Effect of inorganic nitrate supplementation on mitochondrial (dys)function

Several mechanistic animal studies in healthy models have shown that improved nitrite and NO signalling can affect mitochondrial function at key steps in order to potentially match respiration to oxygen availability (Basu et al., 2008; Shiva et al., 2007; Shiva et al., 2011). For example, during low-oxygen conditions, nitrite has been shown to inhibit complex I (NADH Coenzyme Q oxidoreductase) by S-nitrosylation leading to decreased mitochondrial ROS generation (Shiva et al., 2007). Similarly, the reduction of nitrite to NO, potentially via deoxymyoglobin or xanthine oxidase, has been shown to inhibit cytochrome oxidase (complex IV) (Brown et al., 2001). In addition, peroxynitrite may inhibit multiple respiratory

complexes under specific conditions (Brown et al., 2001). When oxygen availability is restored, these inhibitory mechanisms are reversed, NO is oxidised to nitrite (to resume ATP production), while inhibition of complex I is prolonged to limit ROS production (Shiva et al., 2007). These mechanisms have also been implicated in nitrite mediated cytoprotection following ischemia/reperfusion injury (Hendgen-Cotta et al., 2012; Shiva et al., 2007; Webb et al., 2004).

In humans, three days of dietary sodium nitrate supplementation reduced the oxygen cost of submaximal cycling in healthy individuals (Larsen et al., 2007). This improvement was likely mediated by increased mitochondrial efficiency via a decrease in leak respiration (Larsen et al., 2011). These results appear promising, but have yet to be replicated, and studies that have employed an NO-blockade approach to measure its effects on changes in skeletal muscle mitochondrial function and oxygen uptake in humans have been mainly negative (Schrage et al., 2010). The discord between the animal models and the human studies may be due to mechanisms employed in an intact model physiology or potentially multiple nitration and nitrosylation signalling pathways initiated by exogenous administration of NO species (as described above) (Tschakovsky et al., 2008). However, no studies to date have examined the effects of inorganic nitrate supplementation on mitochondrial respiration in patients with CHF, and this is the focus of *Study 3*.

There have been a limited number of studies examining the effect of nitrate supplementation on mitochondrial regulatory protein expression, and none were identified in CHF. The aforementioned study by Larsen et al. which demonstrated improvements in mitochondrial respiration following three days of nitrate dosing reported no change in PGC-1 α expression.

Similarly, experiments using healthy animal models have indicated no effect of eight to ten weeks of nitrate supplementation on Akt expression (Carlström et al., 2015). No studies were identified as having tested the effects of nitrate supplementation on MAPK or mTOR signalling; however, studies utilising dietary arginine supplementation, a precursor to NO, showed significant increases in mTOR signalling in the skeletal muscle tissue of both neonatal pigs and mice (Wang et al., 2018; Yao et al., 2008). The available literature on the efficacy of nitrate supplementation for improving mitochondrial protein signalling in CHF is non-existent. Studies in healthy human and animal models have demonstrated inconsistent findings with regards to specific proteins. However, the demonstrated links between reduced NO bioavailability, downregulation of mitochondrial proteins and the CHF syndrome suggest that nitrate supplementation could potentially improve mitochondrial protein signalling in HFrEF. This is examined in *Study 3*.

2.4.3.3 *Skeletal muscle perfusion*

In healthy individuals at rest, peripheral skeletal muscle tissues are usually adequately perfused; however, during exercise stress, the increased metabolic demands of skeletal muscles can outstrip the ability to supply blood flow and oxygen causing a decline in pH and intermyocyte and microvascular oxygen tensions (Ferguson et al., 2015; Richardson et al., 1995; Tanaka et al., 2016). This outstripping becomes even more pertinent in clinical populations such as patients with CHF who already suffer from dysfunctions in the oxygen uptake pathways (Dumitru et al., 2013). The exercise-induced hypoxia could be an ideal environment to reduce nitrite to NO and contribute to optimal matching of perfusion to metabolic demands (Dumitru et al., 2013).

2.4.3.3.1 *Skeletal muscle perfusion in health and HFrEF*

Control of blood flow to skeletal muscle is altered during the aging process, as identified by the attenuated vasodilator responses in forearm and leg exercises of older adults (Kirby et al., 2012; Lawrenson et al., 2003). As previously described, individuals with HFrEF have a significant decrease in \dot{Q} during exercise, increased SNS activation (vasoconstriction), and endothelial dysfunction, all of which will also negatively impact blood flow delivery and cause/progress the extensive maladaptations within the skeletal muscle tissues (Drexler et al., 1992).

Indeed, in CHF, the chronic physical deconditioning, coupled with a low delivery of oxygenated blood, results in oxygen-deprived skeletal muscle tissues preferentially utilising anaerobic metabolic pathways for energy production. While these patients experience a reduction in all muscle fibre types, the reduction in type I fibres (oxidative fibres) is demonstrably higher (Schaufelberger et al., 1995; Sullivan et al., 1990). In concordance with this, there is also a relative shift to a higher composition of type II fibres, making the muscles less resistant to fatigue and further contributing to exercise intolerance in individuals with CHF (Schaufelberger et al., 1995). The shift to predominantly type II skeletal muscle fibres in HFrEF may assist in the potentiation of nitrate supplementation. Additionally, skeletal muscle perfusion is demonstrably impaired in patients with HFrEF as compared to healthy individuals. This combination of fast fatigable fibre typing and low oxygenation creates an idea environment for the reduction of nitrite to NO in HFrEF, particularly during exercise.

At the microvascular level, there is significant impairment in the matching of oxygen delivery with oxygen uptake in CHF (Esposito et al., 2010; Hirai et al., 2015). While reductions in oxygen delivery to the peripheral tissues due to impaired \dot{Q} and peripheral vasoconstriction

have been previously discussed, there are also impairments in the diffusion of oxygen. In CHF, there is a reduction in capillary density and a concomitant reduction in the oxygen partial pressure within the microcirculation (Hirai et al., 2015; Poole et al., 2012). As the diffusion of oxygen relies on a pressure gradient between the red blood cells and the muscle fibre, the lower partial pressure of oxygen in circulation combined with the lower intracellular oxygen partial pressure in the muscle during contraction disrupts the free flow of oxygen, leaving the tissues underperfused (Hirai et al., 2015).

2.4.3.3.2 *Effects of inorganic nitrate supplementation on muscle tissue perfusion*

Animal models in CHF have suggested there is the potential for increased NO bioavailability to improve blood flow to the skeletal muscles during exercise (Ferguson et al., 2016; Glean et al., 2015). A rat model found increases in blood flow following a nitrite infusion primarily in those muscles comprised of predominantly type II fibres (Glean et al., 2015). Dietary nitrate supplementation ($1 \text{ mmol}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) also significantly increased skeletal muscle blood flow, but only during exercise (Ferguson et al., 2016). Taken together, these findings support the hypothesis that increasing NO bioavailability has beneficial effects on skeletal muscle perfusion in animal models of CHF during exercise.

The efficacy of increasing NO bioavailability to improve blood flow in healthy people is not clear and data available in CHF is limited. While an early study in healthy humans indicated nitrite reduction to NO via deoxygemoglobin has a beneficial vasodilator effect a more recent study showed NOS-inhibition and positron emission tomography scanning failed to show differences in blood flow between the various muscles that make up the quadriceps femoris; vastus intermedius, rectus femoris (RF), vastus medialis (VM), and vastus lateralis (VL) (Cosby et al., 2003; Heinonen et al., 2011). Similarly, Breese et al. saw no differences in the

spatial variance of absolute deoxyhemoglobin plus myoglobin kinetics, using near-infra-red spectroscopy (NIRS), across the RF, VL and VM muscles in healthy humans following the onset of heavy-step cycling (Breese et al., 2017). Studies in hypoxia seem to show more efficacy in healthy individuals, with sodium nitrite infusion leading to acute vasodilation in hypoxic but not normoxic tissues (Maher et al., 2008). While these results and others present a somewhat conflictual story, this evidence does suggest that augmenting NO bioavailability has the potential to increase blood flow to the muscle tissues, and is likely further potentiated in hypoxic tissues.

In CVD populations it is postulated that supplementation could be more efficacious, in part due endothelial dysfunction limiting the vasodilator response to stress. Indeed, significant increases in peripheral tissue oxygenation, as assessed by NIRS, were found in patients with PAD following an acute dose of 18 mmol of dietary nitrate (Kenjale et al., 2011). However, the only identified study utilising patients with HFrEF reported no significant changes in skeletal muscle oxygenation following nine days of dietary nitrate supplementation (Hirai et al., 2017). This finding may be due to the relative small sample size.

Due to the very small number of studies that examined the effect of nitrate supplementation on patients with CVD and in particular those with HFrEF, further studies are needed to explore the potential efficacy of nitrate supplementation. The effect of nitrate supplementation on tissue oxygenation in HFrEF is explored in *Study 1*.

2.5 Aims and hypotheses

CHF is a debilitating syndrome affecting exercise tolerance, functional capacity and QoL. The reduced exercise capacity seen in patients with CHF is mediated by both central and peripheral factors. As such, interventions must target both central and peripheral abnormalities for optimal improvements in exercise capacity. NO is a modulator of blood flow (via its role as a vasodilator) and a regulator of vascular and mitochondrial function. . Increasing NO bioavailability has emerged as a potential intervention to assist in the restoration exercise capacity by improving blood flow, vascular function, cardiac performance and mitochondrial respiration. Inorganic nitrate supplementation is an established means of increasing NO bioavailability in both healthy and clinical populations.

While there is an increasing interest in exploring the effects of inorganic nitrate on aerobic capacity in patients with CHF, there are a very limited number of studies on patients with HFrEF and the sample size of these studies is relatively small. Moreover, no study to date have explored the potential impact of inorganic nitrate supplementation on vascular function, cardiac performance or mitochondrial respiration in HFrEF. There is an urgent need to comprehensively examine the potential efficacy of this intervention in the HFrEF classification.

The effects of inorganic nitrate supplementation on exercise tolerance and the related central and peripheral contributors are investigated in this thesis. The thesis will test the following hypotheses:

In HFrEF, chronic supplementation with inorganic nitrate will:

Study 1:

1. Improve maximal exercise performance ($\dot{V}O_{2peak}$, TTE)
2. Improve vascular function via decreases in peripheral and central resting BP and increases in peak BAFMD response to reactive hyperaemia.
3. Improve peripheral tissue oxygenation

Study 2:

1. Increase cardiac output during exercise via concomitant decreases in total peripheral resistance (TPR)

Study 3:

1. Increase mitochondrial respiration
2. Improve mitochondrial protein signalling of PGC-1 α , mTORC1, Akt and p38MAPK

3 General methodology

The three studies of this thesis were individual components of a randomised, double-blind, placebo-controlled, crossover project that aimed to examine the effect of inorganic nitrate supplementation on exercise tolerance, vascular function, cardiac performance and mitochondrial respiration of individuals with HFrEF. This chapter provides the full overview of the study design as well as the methodology for the recruitment, screening, randomisation, supplementation and nitrate/nitrite analysis. The subsequent experimental chapters (Chapter 4, 5 and 6) are written in manuscript format (in preparation) with abbreviated methodologies specific to each study component.

3.1 Study design

The full study design is illustrated in **Figure 3.1**.

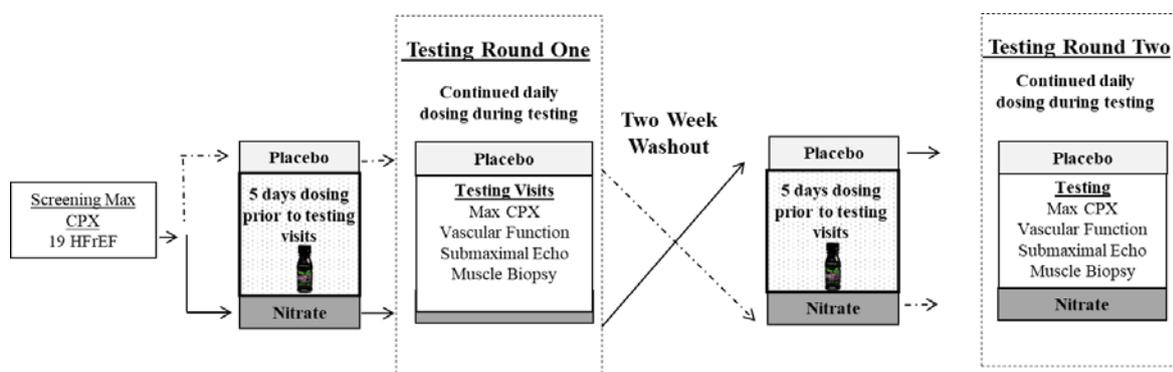


Figure 3.1 BEET-HF study design

Abbreviations: CPX-peak cardiopulmonary exercise test, Vascular Function-BAFMD, peripheral and central pressures, Submaximal Echo-submaximal exercise with echocardiograph, Muscle Biopsy- vastus lateralis muscle biopsy(Woessner et al., 2018b)

Following a screening visit, participants were randomised to consume either nitrate-rich beetroot juice or a nitrate-depleted placebo for five days (James White Drinks, Ipswich, UK). Following this five-day loading, the participants continued daily dosing until the completion of the four testing visits (described below). Due to the need for adequate rest days between

exercise visits, it took up to two weeks in total to complete all testing visits within each round. However, both the dosing days and testing order were matched for each participant between the two rounds of supplementation. Participants all underwent a two-week washout period between interventions.

3.1.1 Recruitment and eligibility

Participants were identified through medical chart reviews at the following clinics in and around Melbourne, Australia: Sunshine Hospital Heart Failure Clinic, One Heart Cardiology Clinic, Melbourne Heart Institute and Specialist Consulting Rooms. The primary recruitment site was Sunshine Hospital Heart Failure clinic and recruitment at this site was predominantly conducted in-person during the weekly HF clinic. Individuals who met inclusion criteria and were interested in the project were identified by the clinicians and either spoken to in-person or were mailed a recruitment letter from the clinic (Appendix A). Interested individuals were provided a detailed description of the nature of the study and those who chose to participate signed an informed consent document (Appendix B). Of the 882 medical charts of patients with HF that were reviewed over the two and a half years of active recruitment, fifteen males and one female aged 62.6 ± 3.6 years with diagnosed HF_{rEF} (EF 30.4 ± 1.8) completed this study (**Figure 3.2**). Of these individuals, 16 completed the peak exercise test, 12 completed the vascular function assessments, 5 completed the submaximal exercise with echocardiography and seven consent to the muscle biopsy visit.

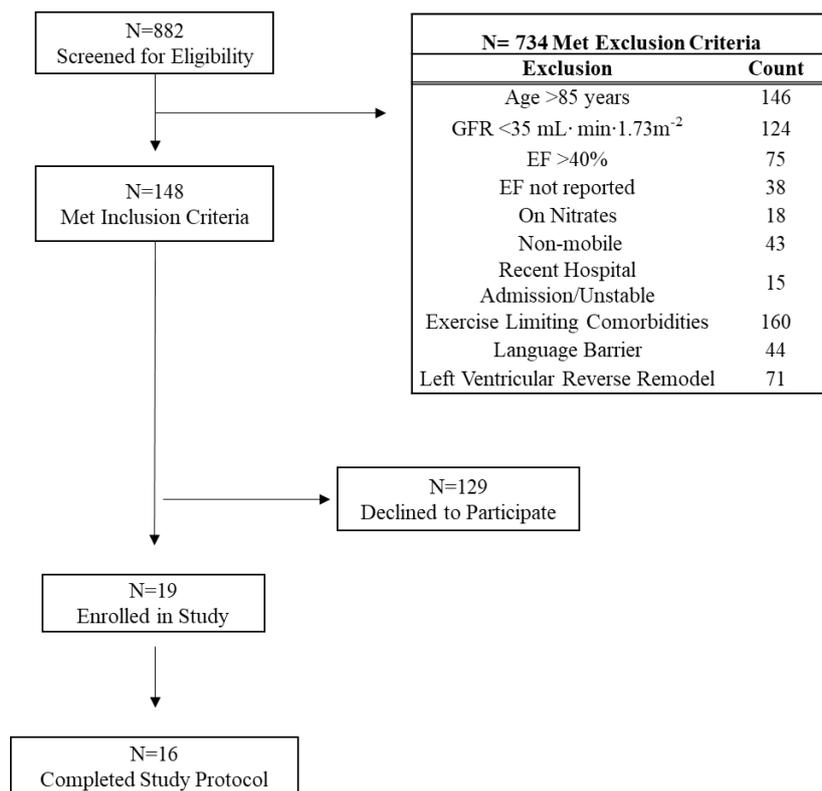


Figure 3.2 Participant recruitment flow

Table 3.1 Inclusion and exclusion criteria for BEET-HF Trial

Inclusion Criteria

- I. Aged between 18 and 85 years
- II. Diagnosed stable HFrEF (EF ≤ 40%)
- III. New York Heart Association classes I-III
- IV. On stable medications for at least three months
- V. $\dot{V}O_{2peak}$ less than <85% age predicted max

Exclusion Criteria

- I. A major cardiovascular event within the previous six weeks or a planned hospitalization within the next two months
- II. Patients with an EF between 41 and 49%
- III. Un-controlled diabetes (>9% HbA1C [glycated haemoglobin])—Can delay start of testing by three months until levels are controlled and stable
- IV. Foot ulcers/advanced neuropathy or other musculoskeletal condition that could limit exercise performance
- V. Abnormal response to cardiopulmonary exercise testing
- VI. Allergy to beets or hypersensitivity to proton pump inhibitors
- VII. Refusal or inability to abstain from the use of proton pump inhibitors for 24 hours before testing

3.1.2 Supplementation

Bottles of beetroot juice were chosen as the supplementation regimen for the nitrate intervention due to the ease of administration and the ability to effectively control the dose of nitrate. Beetroot juice was obtained from the James White Company (James White Drinks, Ipswich, UK) who produces a variety of beetroot juice products and specifically manufactures active and placebo bottles for use in research.

Previous research has indicated that high, chronic doses of inorganic nitrate are potentially more efficacious. Thus, a dose of 16 mmol/nitrate per day, delivered via three 70 ml bottles of beetroot juice was utilised in this study (Hirai et al., 2017; Kenjale et al., 2011; Shaltout et al., 2017). Participants were asked to consume the bottle containing ~6.4 mmol/nitrate with their morning meal, and to subsequently consume one lower dose of ~4.8 mmol/nitrate at both lunch and dinner. Participants consumed the juice for five days before they commenced testing. They then continued to consume the daily doses until completing all four testing visits. There was a two-week washout period between the two rounds. The order of testing visits was kept consistent for each participant between the nitrate and placebo testing rounds. On testing days, patients consumed the first bottle of either nitrate or placebo two and a half hours prior to the appointment time to coincide with peak levels of plasma nitrite (Kapil et al., 2010; Webb et al., 2008). To assess supplementation compliance, participants maintained a dosing log and returned all bottle caps to the research team.

Participants were given instructions regarding their outside activities and dietary patterns both at the commencement of the trial as well as prior to each testing visit. For the duration of the trial, all participants were asked to refrain from the use of any type of mouthwash due to the potential effect of mouthwash on oral bacteria involved in the conversion of nitrate to

nitrite (Woessner et al., 2016). While participants' diets were not controlled during the study, they were provided a list of foods high in dietary nitrate to avoid. They were also asked to maintain their normal dietary and exercise patterns for the duration of the study.

3.1.3 Peak exercise capacity

Peak aerobic exercise capacity was assessed during a CPX on a treadmill (**Figure 3.3**).

Participants performed a total of three CPX tests throughout the study: a screening CPX, utilised both as a screening for adverse responses to exercise and as a familiarisation to the protocol and two subsequent CPX tests during the intervention arms of the trial. All CPX tests were supervised by medical personnel. Prior to the CPX visit, participants were asked to refrain from smoking or caffeine on the morning of testing. They were also instructed to avoid rigorous exercise for the 24 hours prior to testing.

The CPX utilised a two-step treadmill protocol whereby all participants first completed six minutes of low-intensity walking at 1.4 km/hour at a 4% grade (Rickli et al., 2003).

Following this, the speed and/or incline was increased in an individualised manner until participants requested to stop, or before this if any clinical signs or symptoms of adverse responses to exercise occurred. Clinical criteria for stopping a CPX include the onset of moderate-to-severe angina; drop in SBP below standing resting pressure or drop in SBP with increasing workload accompanied by signs or symptoms; hypertensive response to exercise (>250 mmHg SBP or >120 mmHg DBP); signs of poor perfusion, including pallor, cyanosis, or cold and clammy skin; severe or unusual shortness of breath;



Figure 3.3 Participant with medgraphics mask

central nervous system symptoms (e.g., ataxia, vertigo, visual or gait problems, confusion); serious arrhythmias (e.g., second / third degree atrioventricular block, atrial fibrillation with fast ventricular response, increasing premature ventricular contractions or sustained ventricular tachycardia); technical inability to monitor the electrocardiogram (ECG); or at participant's request (to stop) (American College of Sports Medicine, 2012).

Expired respiratory gases were collected breath-by-breath through a mask attached to a gas analyser (Medgraphics, cardio2 and CPX/D System – Utilising Breezeex Software, 142090-001, Revia, Minnesota, USA) (**Figure 3.3**). The Medgraphics system was calibrated prior to each test using gases that have been calibrated at alpha standard. The mean values for every 10-second interval were calculated for both relative and absolute $\dot{V}O_2$ and $\dot{V}CO_2$ as well as ventilation ($\dot{V}E$). HR was monitored continuously via a 12-lead ECG (Mortara, X-Scribe II, Milwaukee, WI, USA), while BP and rating of perceived exertion (RPE), using the BORG 6-20 scale, were obtained at each stage of exercise. Mean values of the resting period, the last minute of the submaximal exercise bout, the last 10 seconds of each incremental workload and the five minutes of recovery data were used to determine $\dot{V}O_2$, $\dot{V}CO_2$ and $\dot{V}E$. The GET was calculated via the V-slope methodology (Beaver et al., 1986). VO_{2peak} will be calculated as the average $\dot{V}O_2$ over the final 30 seconds of the CPX test and TTE will be calculated as the total time (in seconds) of the CPX. Tissue oxygenation was captured noninvasively using a near-infrared spectrometry (NIRS, PortaMon, Artinis Medical Systems B.V., Netherlands) device positioned on the gastrocnemius muscle of the participant.

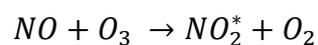
3.1.4 Plasma nitrate and nitrite concentrations

The measurement of plasma nitrate and nitrite was used to confirm adherence to supplementation and appropriate conversion of nitrate to nitrite. Venous blood draws were taken at each of the interventional CPX testing visits to ensure an appropriate plasma nitrate

and nitrite response following supplementation. Following five minutes of seated rest, a venous blood sample was drawn from the forearm. Blood was immediately transferred into five 1 ml micro tubes each containing 5 uL heparin (1 to 1000U/ml) which were then spun in refrigerated centrifuge (3 degrees C for 3 minutes a 5,000rpm). Plasma was then immediately snap frozen in liquid nitrogen and all samples were then transferred to a -80 degrees freezer for storage for analysis.

Analysis of plasma nitrite and nitrate concentrations was performed utilising chemiluminescence via a Sievers NOA model 280i (GE Analytical Instruments).

Chemiluminescence (CL) in combination with a reducing agent is considered to be the most sensitive methodology for the measurement of nitrate and nitrite as it allows for quantification of both nitrite and nitrate at the nanomolar level. CL detects the free-gas NO that is released when NO is oxidized in the presence of ozone to produce an excited state of nitrogen dioxide (see equations below). When the nitrogen dioxide returns to a ground state, it emits an intensity of light proportional to the concentration of NO, which can then be quantified in a nitric oxide analyser (NOA) (Bryan et al., 2007).



This reaction relies on the detection of free NO gas and therefore requires that nitrate and nitrite first be converted to NO gas. This conversion can be achieved with a variety of reduction agents, but the energy (e.g., heat and protons) required in the system is dependent on the NO metabolite being reduced (nitrate requiring greater energy than nitrite). The sample to be quantified can be injected into a reaction vessel containing the reducing agent,

whereby the NO gas is then purged by a carrier gas (such as argon) into the reaction chamber of the NO analyser where ozone is combined with the NO to initiate the reactions previously described.

The chemical reduction agents utilised for nitrite and nitrate differ, but the setup of the system is similar apart from the gas bubbler (which is only required for nitrite analysis) (Figure 3.4). For all nitrate and nitrite analyses, nitrite-free consumables were chosen to minimise cross-contamination. Additionally, syringes were rinsed between each injection and new pipette tips were used for every sample transfer. All NO metabolite concentrations were measured within 30 minutes of defrosting.

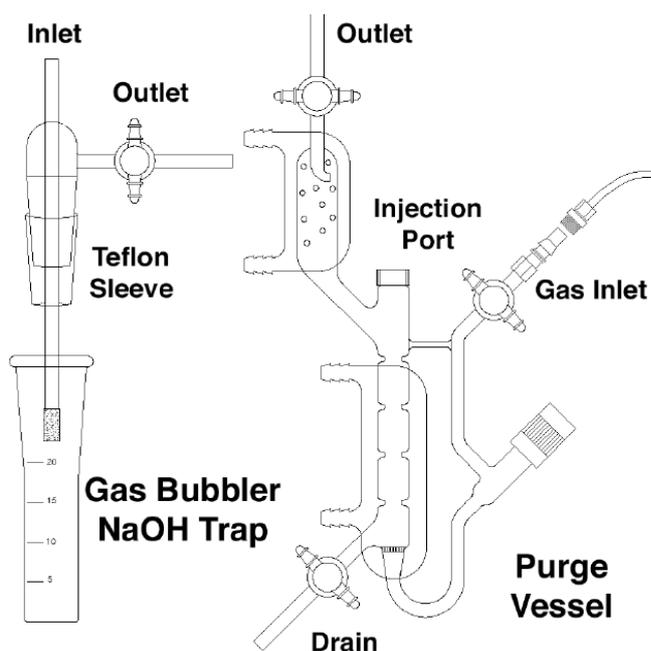


Figure 3.4 NOA purge vessel and gas bubbler setup

This figure is reproduced from the Sievers's user manual for the NOA.

3.1.4.1.1 Nitrate analysis

Upon consumption of inorganic nitrate, plasma nitrate levels will increase. Thus, the measurement of plasma nitrate confirms that participants have adhered to the supplementation protocol. The reduction of nitrate to NO requires a strong acid, vanadium chloride (VCl_3), diluted in hydrochloric acid (HCl). The reaction process is performed at 90 degrees C via the use of a circulating water bath to heat the purge vessel. Argon gas was the inert gas utilised for this reaction. All sample injections were made in duplicate. After 15-20 samples, the purge vessel was emptied and fresh VCl_3/HCl solution was added. Peak responses were determined via area under the curve analysis on Origin Lab software (Origin, Version 2019b. OriginLab Corporation, Northampton, MA, USA), and these values were assessed against a standard curve (**Figure 3.5**)

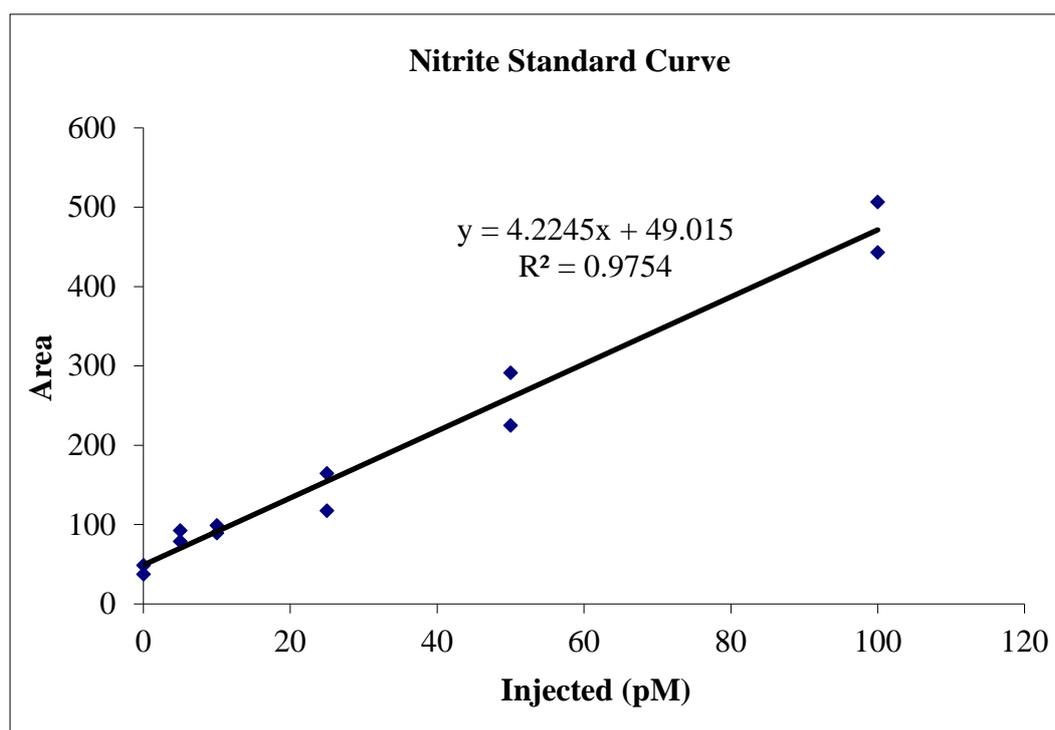


Figure 3.5 Nitrite standard curve

Abbreviations: pM-picomolar

3.1.4.1.2 *Nitrite analysis*

In conditions of low oxygen, such as during exercise, plasma nitrite can be directly reduced to NO to have biological effects. Thus, the measurement of the total plasma nitrite concentration determines the relative increase in NO bioavailability following supplementation with inorganic nitrate. The reduction of nitrite to NO does not require a strong acid or the addition of a heat source. For this reaction, potassium iodide dissolved in acetic acid was utilised. This reductant is insufficient to reduce any higher oxides of nitrogen such as nitrate and, is therefore relatively specific for nitrite concentrations. The injection of the samples, area under the curve analysis and subsequent measurement relative to a nitrite standard curve were completed in the same manner as previously described for nitrate analysis.

3.1.5 Ethics and governance approval

Ethical approval was obtained from both the Melbourne Health Ethics Committee [HREC/15/MH/166] as well as mirror approval by Victoria University Ethics Committee. In accordance with regulations, the trial was also registered in the Australian New Zealand Clinical Trials Registry [ACTRN12615000906550]. The study was primarily conducted at Sunshine Hospital out of the Western Health Centre for Research and Education. Therefore, site-specific governance approval was obtained and a collaboration agreement between Western Health and Victoria University was approved.

4 The effect of dietary inorganic nitrate supplementation on exercise tolerance and vascular function in patients with heart failure with reduced ejection fraction

4.1 Introduction

HFrEF is a syndrome characterised by inability of the heart to pump enough blood to meet the metabolic demand of the body (Moayedi et al., 2015). This mismatch in supply and demand is further exacerbated in high metabolic demand conditions such as during exercise, which results in exercise intolerance, shortness of breath and early fatigue. Improving exercise capacity, measured by $\dot{V}O_{2\text{peak}}$, is an important target for interventions aiming to positively impact mortality and morbidity and overall QoL in these patients (Cicoira et al., 2004; Francis et al., 2000; Jeng et al., 2004). An increase in $\dot{V}O_{2\text{peak}}$ as small as 6% ($\sim 1 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) can have important clinical outcomes, including a 5% reduction in the risk of hospitalisation and mortality (Swank et al., 2012).

It is well accepted that impairments in the peripheral tissues, resulting from the impairments in cardiac function, have a significant contribution to the reduced exercise capacity in patients with HFrEF (Coats, 1996; Coats et al., 1994). Indeed, individuals with CHF are characterised by endothelial dysfunction, decreased tissue perfusion, mitochondria dysfunction and other peripheral tissue maladaptations all of which are associated with exercise intolerance (Nakamura et al., 1994). A reduction in NO bioavailability has been implicated as a key factor in the initiation and progression of many of those peripheral tissue disturbances. As such, in recent years there is a vast interest in the role that exogenous NO supplementation may play in improving exercise capacity and vascular function in CHF.

Inorganic nitrate supplementation is an established means of increasing NO bioavailability via the nitrate-nitrite-NO reduction pathway (Webb et al., 2008; Wilkerson et al., 2012). Nitrate supplementation has shown promise for improving exercise capacity and BP in patients with HFpEF, as shown in several studies with relatively large sample sizes (Eggebeen et al., 2016; Zamani et al., 2015; Zamani et al., 2017). In contrast, limited studies with small sample sizes (n=8-11) have produced inconsistent results regarding the efficacy of supplementation in patients with HFrEF (Coggan et al., 2018; Coggan et al., 2015; Hirai et al., 2017; Kerley et al., 2016). Furthermore, those few published studies are complicated by broad EF percentage inclusion criteria, single aetiology recruitment and the use of varied testing modalities.

Therefore, the primary aim of this study was to test the hypothesis that chronic inorganic nitrate supplementation will improve peak aerobic capacity during treadmill exercise in patients with HFrEF. Secondary aims were to determine whether chronic supplementation with inorganic nitrate improves endothelial function or tissue perfusion in this population.

4.2 Methods

4.2.1 Study design

See General Methodology **Section 3.1**

4.2.2 Participant recruitment

See General Methodology **Section 3.1.1.**

4.2.3 Randomisation and supplementation

See General Methodology **Section 3.1.2.**

4.2.4 Maximal cardiopulmonary exercise

See General Methodology **Section 3.1.3.**

4.2.5 Vascular function

Participants arrived at the vascular laboratory in the morning following an overnight fast but having still consumed the beetroot juice (70 mls with 6.4 mmols nitrate) two and a half hours prior to testing initiation. They were instructed to abstain from exercise for the 24 hours prior testing and to avoid caffeine and smoking for at least three hours before testing. Participants were also asked to hold their morning medications until post-testing (Mahmud et al., 2001; Shechter et al., 2011). All vascular testing was performed following a minimum of 10 minutes of supine rest.

4.2.5.1 Endothelial function

BAFMD is an established reliable and stable measure of endothelial function in both healthy and clinical populations (Kenjale et al., 2011; Welsch et al., 2002). BAFMD was assessed using a high-resolution ultrasound (Terason, LifeHealthcare, New South Wales, Australia) externally-linked to ECG gating software set to trigger image capture on the R-wave (**Figure 4.1**). All images/video clips were recorded and stored onto an externally-linked computer. Ten-second video clips were captured in duplicate at baseline and during occlusion. A continuous two-minute video was captured after the occlusion cuff release (reactive hyperaemia). Peak reactive hyperaemia was calculated as the percentage change in brachial artery diameter from baseline to peak hyperaemia. BAFMD has been shown to be a

reproducible measure when performed under controlled conditions (Welsch et al., 2002). The time of day was kept consistent for all participants and a single experienced tester conducted all imaging and analysis.

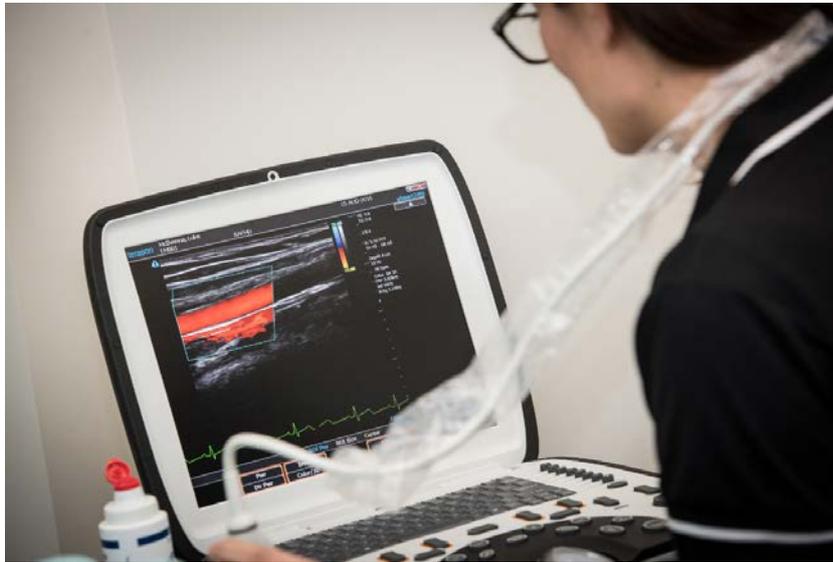


Figure 4.1 Imaging of the brachial artery for BAFMD assessment

4.2.5.2 Central and peripheral BP and aortic stiffness

For all BP measurements, the non-invasive a SphygomoCor® (AtCor Medical, Sydney, NSW, Australia) diagnostic system was utilised. It provides assessments of both the peripheral and central BP as well as measures of arterial stiffness. It has been shown to be valid and reproducible in healthy individuals as well as clinical populations (Hwang et al., 2014a; Laugesen et al., 2014). A SphygomoCor® brachial BP cuff was fitted on the upper arm. The system then automatically recorded pulsations at the brachial artery and produced (via a generalised transfer function) central aortic pressure waveforms. It then predicted central SBP, DBP, MAP, PP, AP, and AIX. Two measurements were captured, with the lower of the two readings recorded. If the two blood pressure readings were >6 mmHg apart, a third measure was recorded to ensure a true resting value.

4.2.6 Quality of life and health status questionnaires

Quality of life and health status assessments were utilised in order to assess the relative impacts of inorganic nitrate supplementation on the individual's activities of daily living and experience of exercise. These measures were assessed via the use of three questionnaires including the Minnesota Living with Heart Failure Questionnaire (MLHF), the Subjective Exercise Experience Scale (SEES), and a Physical Activity Questionnaire (PAQ) (Bilbao et al., 2016; McAuley et al., 1994; Naveiro-Rilo et al., 2010; Sallis et al., 1985). Participants completed the MLHF and the PAQ once before commencing each testing round (a total of two times) to ensure there was no change in how their CHF affects their daily life and how much they are exercising. Participants completed the SEES after every exercise test for the duration of the study to assess their psychological response to exercise.

4.2.7 Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (version 22 (SPSS Inc. Chicago, IL, USA)). The main outcomes data were analysed via paired-samples t-tests and statistical significance was set *a-priori* at $p \leq 0.05$. The magnitude of change was assessed for all measures of vascular function using Cohen's *d* ES which was defined as small, 0.2; moderate, 0.5 and large 0.8 or very large 1.2 (Cohen, 1988). ES has been established as being more representative of the clinical relevance of the results of smaller clinical trials than the p-value alone (Citrome, 2014). Graphs and figures were created utilising GraphPad Prism Version 7.00 for Windows (GraphPad Software, La Jolla, California USA). Unless otherwise indicated, all results are presented as mean \pm standard error of the mean (SEM). In the present study, a sample size of approximately 23 would be needed in order to obtain statistical power at the recommended .80 level (Faul et al., 2007). This power calculation was made utilizing data from Zamani et al. (2015). The target

recruitment for this study was 30. Similar studies in the HFpEF population have found significant differences with smaller sample sizes, however (Zamani et al., 2015; Zamani et al., 2017).

4.3 Results

Patients' anthropometrics and clinical characteristics are described in **Table 4.1**. All participants had an EF percentage below 40% and were on stable medications for a minimum of three months. The participants were predominantly NYHA functional class II, but some had other comorbidities including T2DM and chronic obstructive pulmonary disease. While 19 participants commenced to the trial, two participants dropped out following the screening visit (one had an aversion to the supplement and the other a reluctance to participate in the exercise testing). A third participant withdrew following completion of one round of testing due to gaining new employment and the required time commitment to complete the study. A total of 16 participants completed the two CPX testing visits, and 12 participants completed the vascular function assessments, with four individuals opting out of the vascular function component of the trial. There were no significant effects seen for the order of testing for any of the exercise or vascular function parameters.

Table 4.1 Participants' demographics

Variable	Value
Age, mean \pm SEM, y	62.6 \pm 3.6
Height, mean \pm SEM, cm	167.9 \pm 3.9
Mass, mean \pm SEM, kg	87.7 \pm 4.0
BMI	31.8 \pm 2.1
Male, n (%)	15 (93.75)
eGFR, mean \pm SEM, ml \cdot min ⁻¹ \cdot 1.73m ²	75.8 \pm 4.2
eGFR <60, n (%), ml \cdot min ⁻¹ \cdot 1.73m ²	5 (31.25)
EF	30.4 \pm 1.8
Aetiology	
Ischaemic	9
Non-Ischaemic Dilated Cardiomyopathy	6
Idiopathic Heart Disease	1
NYHA Class, n (%)	
Class I	3 (18.75)
Class II	10 (62.5)
Class III	3 (18.75)
Comorbidities, n (%)	
Diabetic	6 (37.5)
COPD	2 (12.5)
HTN	7 (43.75)
Current Smoker	3 (18.75)
Obese	9 (56.25)
Drug therapy, n (%)	
Metformin	4 (25)
β -Blockers	15 (93.75)
ACE Inhibitor	6 (37.5)
Statin	7 (43.75)
Aspirin	9 (56.25)
Diuretics	12 (75)

4.3.1 Supplementation

Adherence to the supplementation was ~98%, as confirmed by cross-checking of the supplementation logbooks and bottle cap returns. While there were no significant adverse effects of the supplementation, excluding discoloration of urine and stools, one participant had a strong aversion to the beetroot juice and thus dropped out after the screening visit.

4.3.2 Plasma nitrate/nitrite

Plasma nitrate increased (933%, $p < 0.001$) following nitrate supplementation in all but one participant (**Figure 4.2 A and B**). There was also a significant increase (94%, $p < 0.05$) in plasma nitrite (**Figure 4.2 C**), but the individualised data suggest that some participants may have had a diminished capacity to convert the nitrate to nitrite (**Figure 4.2 D**).

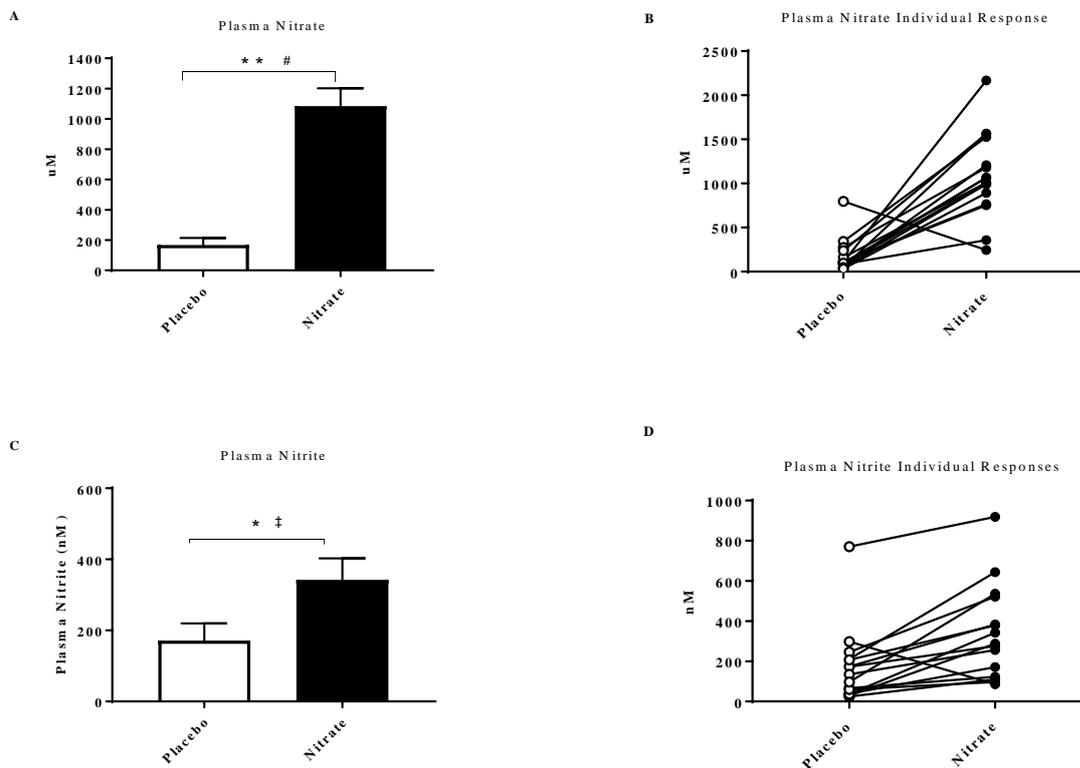


Figure 4.2 The effect of nitrate supplementation on plasma nitrate and nitrite

Mean plasma nitrate (**A**) and plasma nitrite (**C**) were significantly higher following supplementation. The individual responses for nitrate (**B**) and nitrite (**D**) are presented as well. One participant's nitrite data were excluded ($n=15$) due to abnormal levels (4SD above the mean). * indicates $p < 0.05$ level, ** indicates $p < 0.001$. Cohen's d ES of the change between interventions are indicated as follows: ‡ indicates a large ES ($> .8$); # indicates a very large ES (> 1.2).

4.3.3 Exercise outcomes:

Neither $\dot{V}O_{2peak}$ (**Figure 4.3 A**) nor TTE (**Figure 4.3 B**) were significantly different between the two interventions (nitrate: 18.5 ± 5.7 , placebo: 19.3 ± 1.4 $ml \cdot kg^{-1} \cdot min^{-1}$, nitrate: $1165 \pm$

92, placebo: 1207 ± 96 seconds, respectively). There were also no significant ($p > 0.05$) differences in recovery values for $\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}E$, and HR at minutes one, two or three.

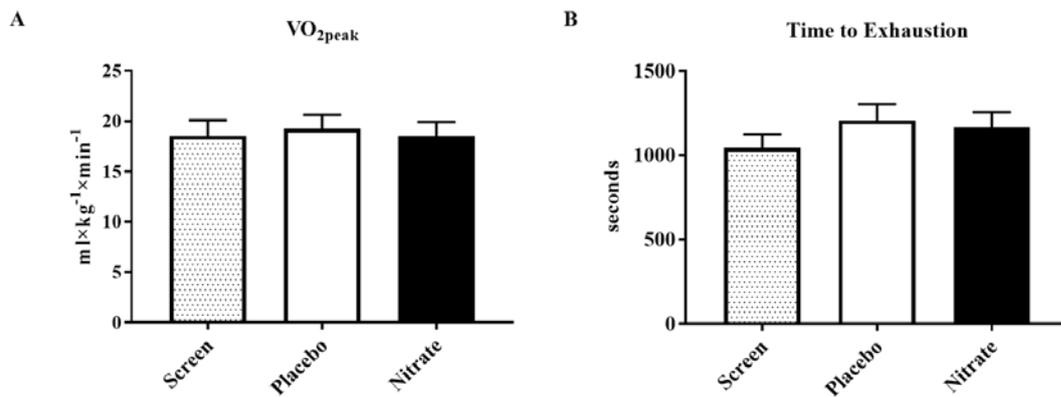


Figure 4.3 The effect of nitrate supplementation on $\dot{V}O_{2peak}$ and TTE

$\dot{V}O_{2peak}$ (A) and TTE (B) during the maximal exercise test were unchanged following supplementation. Data reported as mean \pm standard error of the mean (SEM).

There were no significant differences in either the absolute GET ($n=10$, nitrate: 1159.7 ± 65.7 ml·min⁻¹, placebo: 1132.4 ± 69.9 ml·min⁻¹, $p=0.53$), or the time to GET (nitrate: 611 ± 38 seconds, placebo: 611 ± 45 seconds, $p=1.00$).

Concentrations of deoxygenated haemoglobin (HHb), oxygenated haemoglobin (HbO₂) and total haemoglobin (Hb_{tot}) were also not significantly different during first six minutes of steady state exercise, nor during the first three stages of incremental exercise between the placebo and nitrate tests (Table 4.2 and Figure 4.4).

Table 4.2 Effect of nitrate supplementation on peripheral tissue oxygenation

	Placebo	Nitrate
[HHb]		
Submaximal stage (final 15 seconds)	8.88 ± 0.03	6.32 ± 0.09
Stage one (465 seconds)	11.82 ± 0.05	8.55 ± 0.04
Stage two (585 seconds)	13.41 ± 0.04	9.87 ± 0.04
Stage three (705 seconds)	14.52 ± 0.05	11.73 ± 0.04
[HbO₂]		
Submaximal stage (final 15 seconds)	-0.72 ± 0.07	-0.63 ± 0.12
Stage one (465 seconds)	-4.40 ± 0.06	-3.72 ± 0.08
Stage two (585 seconds)	-5.21 ± 0.08	-4.34 ± 0.08
Stage three (705 s)	-5.40 ± 0.10	-5.08 ± 0.03
[Hb_{tot}]		
Submaximal stage (final 15 seconds)	8.32 ± 0.06	8.97 ± 0.2
Stage one (465 seconds)	7.36 ± 0.07	9.14 ± 0.08
Stage two (585 seconds)	7.91 ± 0.09	9.74 ± 0.07
Stage three (705 seconds)	9.02 ± 0.09	11.0 ± 0.04

Absolute individual values for HHb, HbO₂ and Hb_{tot} were converted into change scores (Δ =absolute value-resting value). Values are mean \pm SEM. Data are displayed for n=12. Four participants were excluded from final NIRS analysis due to poor signal quality. Abbreviations: HHb, deoxygenated haemoglobin concentration; HbO₂, oxygenated haemoglobin concentration; Hb_{tot}, total haemoglobin concentration, Δ - change score.

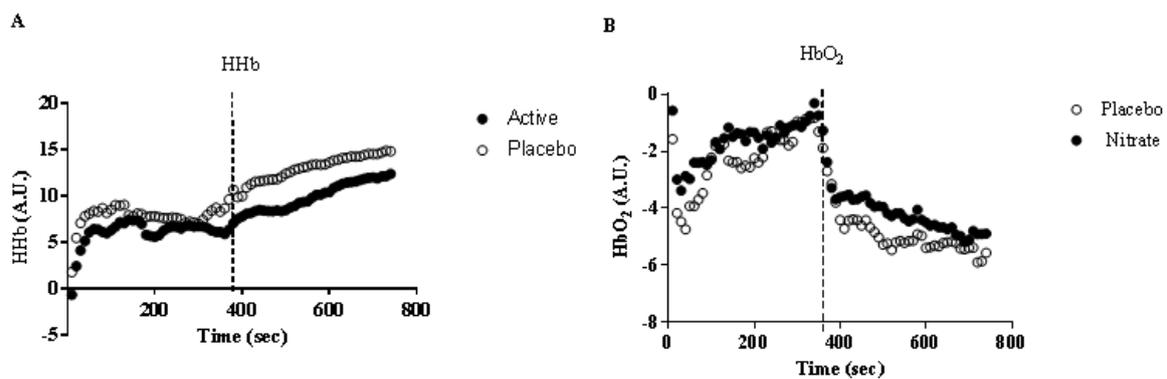


Figure 4.4 Effect of nitrate supplementation on HHb, HbO₂

These data represent the group data (10-second averages) from the NIRS for the six minutes of submaximal steady state exercise and the first three stages of incremental exercise. The vertical dotted line represents the transition between the steady state and incremental steps of the maximal CPX. The data are presented in arbitrary units (AU). To control for the alterations in arterial/venous capacitance during transition from rest to exercise, the “zero” point of each individual participant was selected as the point just following the initiation of exercise, after accounting GraphPad Prism plots. Paired t-tests were run for each individual point between the placebo and nitrate mean values, and there were no significant differences at any time points.

4.3.4 Vascular function

There were no significant differences in the resting brachial BPs (SBP, DBP and MAP) between the placebo and nitrate interventions ($\Delta = -2, -1, -2$ mmHg, all $p > 0.05$) (Figure 4.5).

All differences in BP had Cohen's d ES of < 0.17 .

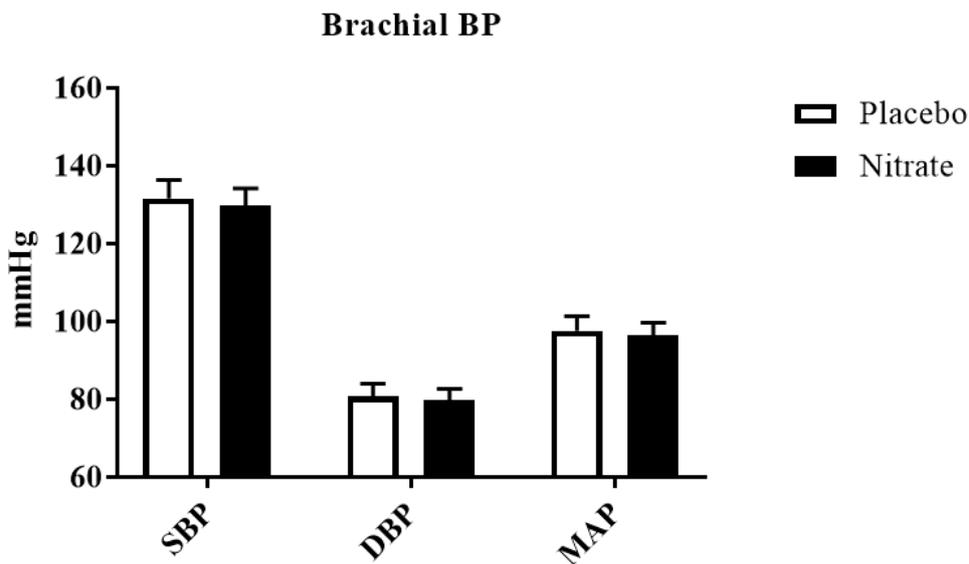


Figure 4.5 Effect of nitrate supplementation on brachial BP

Data presented as mean \pm SEM. Abbreviations: DBP-diastolic blood pressure and MAP-mean arterial blood pressure, SBP-systolic blood pressure.

There were also no significant differences in either aortic BP or measures of central stiffness (Table 4.3).

4.3.5 Quality of Life and Health Status Questionnaires

There were no statistically significant differences in the MLHF questionnaire between the placebo (28 ± 5) and the nitrate group (24 ± 3). There were also no significant differences between any of the three areas of the SEES between the placebo and nitrate group (Positive Well-being: placebo 23 ± 1 , nitrate 21 ± 1 ; Psychological Distress: placebo 5 ± 1 , nitrate 8 ± 2 ; Fatigue: placebo 13 ± 1 , nitrate 11 ± 1).

Table 4.3 Effects of nitrate supplementation on aortic pressure and stiffness

Measurement	Placebo	Nitrate	Significance
AorSBP (mmHg)	122 ± 4	121 ± 4	0.64
AorDBP (mmHg)	82 ± 3	80 ± 3	0.51 †
AorMAP (mmHg)	96 ± 4	95 ± 3	0.71
AorPP (mmHg)	40 ± 2	40 ± 2	0.77
AorAP (mmHg)	15 ± 2	14 ± 2	0.74
AorAIX (%)	32 ± 3	35 ± 3	0.3 †

Cohen's *d* ES of the change between interventions is indicated as follows: † indicates small ES (>.3). Abbreviations: Aor- aortic, AP- augmentation pressure, AIX- augmentation index, DBP- diastolic blood pressure, MAP- mean arterial blood pressure, PP- pulse pressure, , SBP- systolic blood pressure.

There were no significant differences ($p>0.05$) in the resting brachial artery diameters between the nitrate ($3.92 \text{ mm} \pm 0.16$) and placebo ($4.0 \text{ mm} \pm 0.13$) interventions. There were also no significant differences ($p=0.062$) in the BAFMD peak reactive hyperaemic response between nitrate ($5.7\% \pm 1.1$) and placebo ($4.1\% \pm 0.7$) (**Figure 4.6**). However, the percent change had a medium ES (ES=0.607).

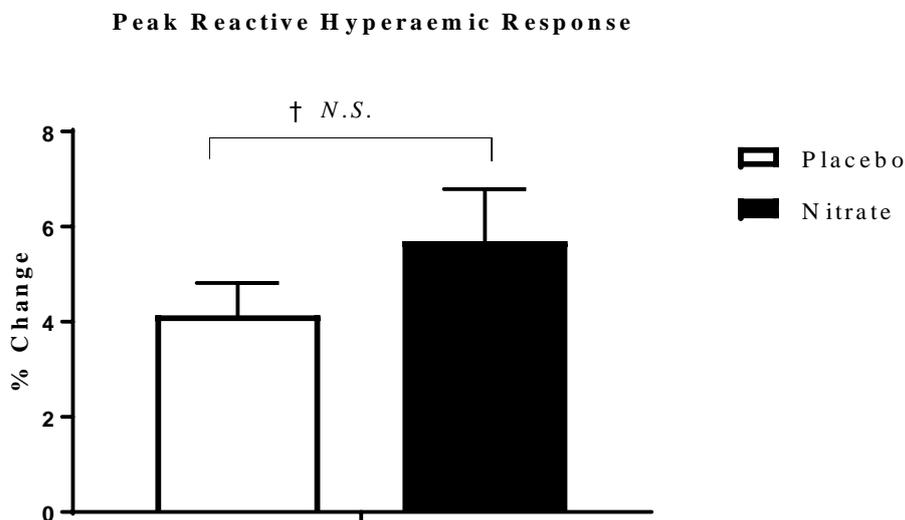


Figure 4.6 Effects of nitrate supplementation on BAFMD peak reactive hyperaemic percent change

Of the 12 participants who underwent BAFMD testing, three participants were excluded due to unclear intimal layers, and one due to a corrupted image file. Cohen's *d* ES of the change between interventions are indicated as follows: † indicates a medium ES (>.5).

4.4 Discussion

This is the largest trial to date examining the effects of inorganic nitrate supplementation on aerobic capacity in patients with HFrEF and the only one to utilise treadmill exercise. Finally, it is the only study to assess measures of vascular health and function following nitrate supplementation in patients with HFrEF. However, despite a significant increase in circulating levels of both plasma nitrate and nitrite, we observed no significant differences in $\dot{V}O_{2peak}$, TTE, vascular function or BP.

It was previously reported that inorganic nitrate supplementation is an effective method to increase plasma nitrate and nitrite in patients with HFrEF (Coggan et al., 2018; Coggan et al., 2015). Similarly, herein we report that > six days of supplementation resulted in significant increases in plasma nitrate (~933%) and smaller, but still significant, increases in plasma nitrite (~94%). The magnitude of the increase in circulating plasma nitrite in the present study is also similar to those reported in other HFrEF studies (Coggan et al., 2018; Hirai et al., 2017).

Previous studies have examined the effect of nitrate supplementation on exercise performance in patients with CHF using a cycle ergometer (Coggan et al., 2018; Hirai et al., 2017). In contrast, we utilised a treadmill and added the measurement of recovery oxygen kinetics. This was incorporated because it better reflects the patients' ADLs and also requires a higher metabolic demand as compared to testing on a cycle ergometer. However, despite seeing significant increases in plasma nitrate and nitrite, we report that dietary nitrate supplementation did not improve any parameters of either peak exercise capacity or submaximal oxygen consumption. These findings are in agreement with a previous smaller cycle study in HFrEF which also found no improvements in exercise tolerance following a

chronic dose of inorganic nitrate (Hirai et al., 2017). The present study included the collection of more comprehensive gas exchange measures, including GET and oxygen recovery kinetics following exercise cessation. However, these measures were also unaffected by the intervention. We have previously proposed that 400nM could serve as a threshold value required to see improvements in aerobic capacity (Woessner et al., 2018c). However, despite utilising a chronic dosing protocol and the highest concentration of daily nitrate, only seven of the 16 participants reached this threshold. Furthermore, subgroup analysis indicated no significant effect of supplementation. There were large variations in the individual response data for TTE (as indicated by a high SEM), and we propose that the differences seen within the subgroup analysis are a result of these variations rather than the intervention.

Two previous studies have reported beneficial effects of nitrate supplementation on exercise performance in patients with HFrEF (Coggan et al., 2018; Kerley et al., 2016). However, these studies may contain several limitations which make it difficult to interpret the results. First, Coggan et al. demonstrated an ~8% increase in $\dot{V}O_{2peak}$, but the sample (n=8) was a mixture of both patients with HFrEF and those with HFmrEF. While HFmrEF is a relatively new (2016) classification of CHF, these patients have a profile that is intermediary between HFrEF and HFpEF. Additionally, this study utilised a recumbent cycle, complicating the interpretation of the efficacy of nitrate supplementation since the supine position likely artificially enhanced venous return, lessening the impact of the reduced \dot{Q} in the patients (Coggan et al., 2018). The single other study in HFrEF found a significant improvement in aerobic capacity following an acute dose (12.9 mmol) of nitrate. However, the testing modality (an incremental shuttle walk test) is not widely used, and recruitment in the trial was limited to only those patients with CHF of a non-ischaemic aetiology, lessening the broader

applicability. They also used an EF percentage cut-off inclusive of HFmrEF (<45%). Thus, it is possible that the positive results in these two studies could have been mediated by methodological factors, including the use of supine position and the inclusion of patients with HFmrEF. This could explain the discord between our findings and the previous studies.

Our group has previously reported that nitrate supplementation can improve peripheral tissue oxygenation, as measured by NIRS, in clinical populations (Kenjale et al., 2011; Woessner et al., 2018a). However, we reported no changes in peripheral muscle tissue oxygenation despite the increase in circulating levels of nitrate and nitrite. The reason why there was no change in tissue oxygenation is not entirely clear. It is plausible that the perfusion/extraction is relatively high in muscle tissues of HFrEF already during exercise, leaving less room for improvement. Indeed, a recent invasive study identified significant elevations in oxygen extraction in HFrEF (via increased $a\text{-VO}_2\text{diff}$) during exercise (Dhakal et al., 2015). This increased oxygen extraction is likely compensatory to the decrease in oxygen delivery and may explain the lack of an effect of nitrate supplementation at further increasing this capacity. Future studies could seek to more directly assess the effect of nitrate supplementation on $a\text{-VO}_2\text{diff}$ in HFrEF.

One of the most reported benefits of nitrate supplementation in healthy and clinical populations is a reduction in SBP (Kapil et al., 2015). In the current study, however, we report no significant effects on peripheral or central BP, as well as no effect on vascular stiffness (AIX and AP). The only vascular parameter that demonstrated a trend toward significance was the percent change in brachial artery diameter following reactive hyperaemia. While not statistically significant, the change did have a medium ES, suggesting that the improvement could be clinically relevant. The literature is fairly positive regarding

the potential efficacy of nitrate supplementation for improving BAFMD. Studies in HTN and hypocholesteremia indicated improvements after four and six weeks of daily dosing (~6 mmol nitrate) (Kapil et al., 2015; Velmurugan et al., 2016). Another study that was conducted in a high altitude condition reported that nitrate supplementation appeared to have abolished the negative impact of altitude-induced hypoxia on endothelial function (Bakker et al., 2015). While these results seem to suggest the potential beneficial effects of nitrate on endothelial function, specifically in CVD/hypoxic tissues, the findings in this study did not reach a statistical significance level to confirm that, potentially due to a relative low sample size. Still, the percentage increase of BAFMD in the present study (37%) is higher than the aforementioned studies in hypercholesterolemia (24%) and HTN (20%) (Kapil et al., 2015; Velmurugan et al., 2016). This could be the result of either the higher dosing regimen utilised, or that the patients with HFrEF could have more substantial dysfunctions in the endothelium, thus potentiating the effects of nitrate supplementation. Future studies could consider the addition of BAFMD to their designs and utilise larger sample sizes to further explore this potential effect.

The current study has some potential limitations. While it is the largest to date in this population (60% more participants), it was still a relatively small sample size, which could have an impact on the results. The patient population was primarily male (n=15). While HFrEF is a condition more prevalent in men (60% of those with HFrEF are male), the population prevalence was not reflected in our study enrolment. This was not intentional as recruitment was open to both men and women, but the patient profile does align with other studies in HFrEF which have been predominantly male. While the lack of female participants does limit the applicability of the findings, to our knowledge no previous studies have suggested a differential response to nitrate supplementation in men and women.

In conclusion, targeting NO bioavailability in HFrEF via nitrate supplementation appears to be ineffective at improving aerobic capacity or vascular function in patients with stable HFrEF. The potential benefit to BAFMD warrants further investigation in larger trials. Whether the effect will be greater if combined with other interventions, such as exercise training, should also be explored in future studies.

5 The effect of dietary inorganic nitrate supplementation on cardiac performance during submaximal exercise in men with heart failure with reduced ejection fraction: a pilot study

5.1 Introduction

CHF is a common syndrome experienced by nearly 500,000 people in Australia and 26 million worldwide (Chan et al., 2016; Sahle et al., 2016; Savarese et al., 2017). Hallmark symptoms of this syndrome are a decrease in aerobic capacity (as measured by $\dot{V}O_{2\text{peak}}$), and increased fatigue during submaximal and peak exercise. HFrEF is characterised by a significant reduction in \dot{Q} at rest and during exercise compared to healthy individuals (Dhakal et al., 2015). Although patients with HFrEF display significant maladaptations in the peripheral tissues which contribute to a reduced exercise capacity, the precedent and major driver of the clinical disability is predominantly the marked reductions in cardiac systolic function. Thus, therapies that improve cardiac performance are likely to contribute to improved clinical outcomes and increased exercise tolerance in patients with HFrEF.

NO is a free radical produced by nearly every cell in the human body. It functions as a potent vasodilator synthesised by vascular and endocardial eNOS (Rastaldo et al., 2007). NO can increase coronary vasodilation thereby decreasing total peripheral resistance and afterload (Cotton et al., 2002; Zamani et al., 2015). Conversely, the lack of NO bioavailability due to the endothelial dysfunction in HFrEF has been linked to a worsening of the syndrome via the over production of ROS and consequential increase in oxidative stress (Ali et al., 2017; Bauersachs et al., 2008; Couto et al., 2015). The lack of eNOS function may be particularly consequential during exercise stress as increased NO bioavailability is critical to achieving an

adequate vascular response to the increased demand for blood flow during exercise (Hambrecht et al., 2000). Thus it has been suggested that interventions targeting increases in NO bioavailability could assist in improving the cardiac performance in patients with HFrEF (Bauersachs et al., 2008).

Inorganic nitrate supplementation has emerged as a novel means of increasing NO bioavailability in a variety of healthy and diseased populations with mixed results on increasing exercise capacity (Woessner et al., 2018c). In CHF studies, the results are further complicated by inconsistencies in classification, the limited modes of exercise employed, varied dosages and others. Studies examining the effect of nitrate on cardiac performance in CHF have been limited and have produced mixed results (Hirai et al., 2017; Zamani et al., 2015). The single study in HFpEF demonstrated decreases in TPR concomitant with increases in SV and Q during exercise following an acute dose (12.9mmol) of nitrate, while no effect was seen in a small sample of patients with HFrEF following a chronic dose (12.9mmol/day for 9 days). Of note, however, the study in HFrEF used an impedance cardiography device, whereas the HFpEF study utilised Doppler echocardiography. The differences between the two studies could be due to the dosing regimen, the different devices, or due to condition specific physiological distinctions not yet characterised. Given the significant implications of improving measures of cardiac performance in this population, future studies should continue to explore the potential efficacy of this supplement in CHF.

As such, the aim of this pilot study was to test the hypothesis that chronic inorganic nitrate supplementation would improve cardiac performance during submaximal exercise in patients with HFrEF.

5.2 Methodology

5.2.1 Study design

See General Methodology **Section 3.1**

5.2.2 Participant recruitment

Participants recruited for study 1 were invited to complete this additional visit. In total, 5 participants consented to the submaximal exercise visit.

5.2.3 Randomisation and supplementation

See General Methodology **Section 3.1.2.**

5.2.4 Submaximal exercise echocardiograph

All participants were asked to abstain from alcohol or exercise for the 24 hours preceding the test and to refrain from caffeine on the day of the test. All testing visits were conducted by the same tester at the same time of day to minimise the effects of both inter-tester variation and to account for diurnal variations in BP and cardiac response to the medications used.

On arrival, participants were connected to a 12-lead ECG and fitted on an echo-compatible recumbent cycle ergometer. The design of this cycle places the participant in an ideal position to capture echocardiograph images during exercise, allowing for a more accurate assessment of cardiac performance (Q) under stress (**Figure 5.1**). Imaging was completed with a Vivid 7 echocardiographic machine (GE, Milwaukee, Wisconsin, USA).



Figure 5.1 Echo-compatible recumbent cycle ergometer

The protocol was discontinuous (five minutes rest in-between each stage) and consisted of three low-moderate intensity workloads (15-25W, 25-40W and 35-60W). The intensities were individualized and were selected to elicit BORG RPE values of approximately 9 (very light), 11 (Fairly Light), and 13 (somewhat hard). Each stage was designed to last approximately five minutes, with imaging commencing at two and a half minutes into the stage. The stages ended when the imaging was complete. HR was continuously monitored through a 12-lead ECG. BP was recorded at rest, two and a half minutes into each stage and halfway through each rest period between stages. RPE was recorded in the final minute of each exercise stage.

\dot{Q} and SV were derived using Doppler velocity time integral from blood exiting the left ventricle during systole, according to the Huntsman method (Huntsman et al., 1983). This method utilises measures of left ventricular outflow tract diameter (LVOT) and LVOT subvalvular velocity time integral (VTI) to estimate Q and SV utilising the following equations:

$$SV = \pi \times \left(\frac{LVOT}{2}\right)^2 \times LVOT \times VTI$$

$$CO = \frac{SV \times HR}{1000}$$

MAP was calculated as:

$$MAP = \left[\frac{(SBP - DBP)}{3} \right] + DBP$$

TPR was calculated from the formula (Bond et al., 2014):

$$TPR \text{ (dyne} \cdot \text{sec} \cdot \text{cm}^{-5}\text{)} = \frac{80 \times MAP \text{ (mmHg)}}{Q \text{ (L} \cdot \text{min}^{-1}\text{)}}$$

5.2.5 Statistical analysis

Statistical analysis of the demographics and plasma nitrate/nitrite values were performed using Statistical Package for the Social Sciences (version 22, SPSS Inc. Chicago, IL, USA). Paired samples t-test were used to analyse the plasma nitrite/nitrate values and the dosing days between the placebo and nitrate interventions. The t-tests were 2-sided, with $p < 0.05$ considered statistically significant. The magnitude of change was assessed for all measures of cardiac function using Cohen's *d* ES, which was defined as small, 0.2; moderate, 0.5 and large 0.8 or very large 1.2 (Cohen, 1988). ES was the primary analysis methodology as this value has been established as being more representative of the clinical relevance of the results of smaller clinical trials than the p-value alone (Citrome, 2014). Secondary analysis on the main outcome variables included a two-way repeated measures ANOVA to assess the difference in cardiac function during each stage of exercise between the placebo and nitrate interventions. Graphs and figures were created utilising GraphPad Prism Version 7.00 for

Windows (GraphPad Software, La Jolla California USA). Unless otherwise indicated, all results are presented as mean \pm SEM.

5.3 Results

Patient characteristics are reported in **Table 5.1**. There were no significant differences between the number of days of dosing in either treatment intervention (13 ± 2 days and 12 ± 3 days, $p=0.74$). The duration of each exercise stage was designed to be equal but timing was dependent upon the technical difficulties during the initial echo image collection. As such, there were no differences in the exercise duration of stages two or and three between treatments. However, stage one took slightly longer during the inorganic nitrate treatment arm (nitrate 386 ± 23 sec, placebo 364 ± 20 sec, $p=0.04$).

Table 5.1 Participants' demographics

Variable	Value
Age, mean \pm SEM, y	61.4 \pm 3.6
Height, mean \pm SEM, cm	172.0 \pm 3.0
Mass, mean \pm SEM, kg	91 \pm 5.8
BMI	30.9 \pm 2.0
$\dot{V}O_{2peak}$ ml \cdot kg ⁻¹ \cdot min ⁻¹	17.2 \pm 2.1
Male, n (%)	5 (100)
eGFR <60, n (%), ml \cdot min ⁻¹ \cdot 1.73m ²	1 (20)
EF (%)	32 \pm 2.2
Aetiology	
Ischaemic	3
Non-Ischaemic Dilated Cardiomyopathy	1
Idiopathic Heart Disease	1
NYHA Class, n (%)	
Class I	2 (40)
Class II	3 (60)
Comorbidities, n (%)	
Diabetic	3 (60)
HTN	1 (20)
Obese	4 (80)
Drug therapy, n (%)	
Metformin	1 (20)
B-Blockers	5 (100)
ACE Inhibitor	2 (40)
Statin	3 (60)
Aspirin	4 (80)
Diuretics	3 (60)

Abbreviations: ACE inhibitor-angiotensin-converting-enzyme inhibitor, COPD-chronic obstructive pulmonary disease, EF-ejection fraction, HTN-hypertension, and NYHA-New York Heart Association. .

Plasma nitrate and nitrite values were measured at the CPX treadmill visit (day six of supplementation). All five participants demonstrated increases in both plasma nitrate and nitrite as compared to placebo treatment with group means increases of ~750% (p=0.004) and ~77% (p=0.01) respectively (**Figure 5.2**).

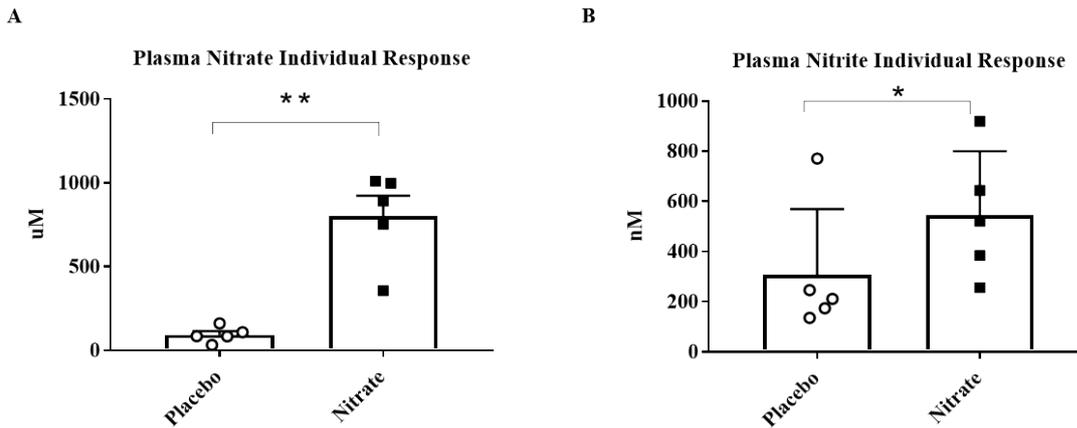


Figure 5.2 Individual and mean responses of plasma nitrate and plasma nitrite

There were significant increases in both plasma nitrate (A) and nitrite (B) following nitrate supplementation as compared to the placebo intervention. * indicates $p < 0.05$, ** indicates $p < 0.01$

At baseline, as expected, there were no differences for treatment groups in \dot{Q} , SV or TPR (**Figure 5.3**). However, the increase in resting HR following nitrate supplementation had a medium ES (mean= $\sim 5\%$ increase, $ES=0.59$). This difference in HR remained at all stages of exercise: stage one (mean= $\sim 9\%$ increase, $ES=0.59$), stage two (mean= $\sim 3\%$, $ES=0.56$), and stage three (mean= $\sim 5\%$ increase, $ES=0.92$) (**Figure 5.3 C**). Following nitrate supplementation, SV and \dot{Q} were both increased at stage two (SV mean increase = $\sim 12\%$, $ES=0.97$; \dot{Q} mean increase = $\sim 19\%$, $ES=0.51$) and stage three (SV mean increase = $\sim 16\%$, $ES=0.57$; \dot{Q} mean increase = $\sim 17\%$, $ES=0.50$) (**Figure 5.3 A and 5.3 B**).

TPR was not different at rest, but had reductions following nitrate supplementation at all stages of exercise: stage one (mean increase= $\sim 16\%$, $ES=.45$), stage two (mean increase = $\sim 19\%$, $ES=1.92$) and stage three (mean increase = $\sim 23\%$, $ES=.81$) (**Figure 5.3 D**).

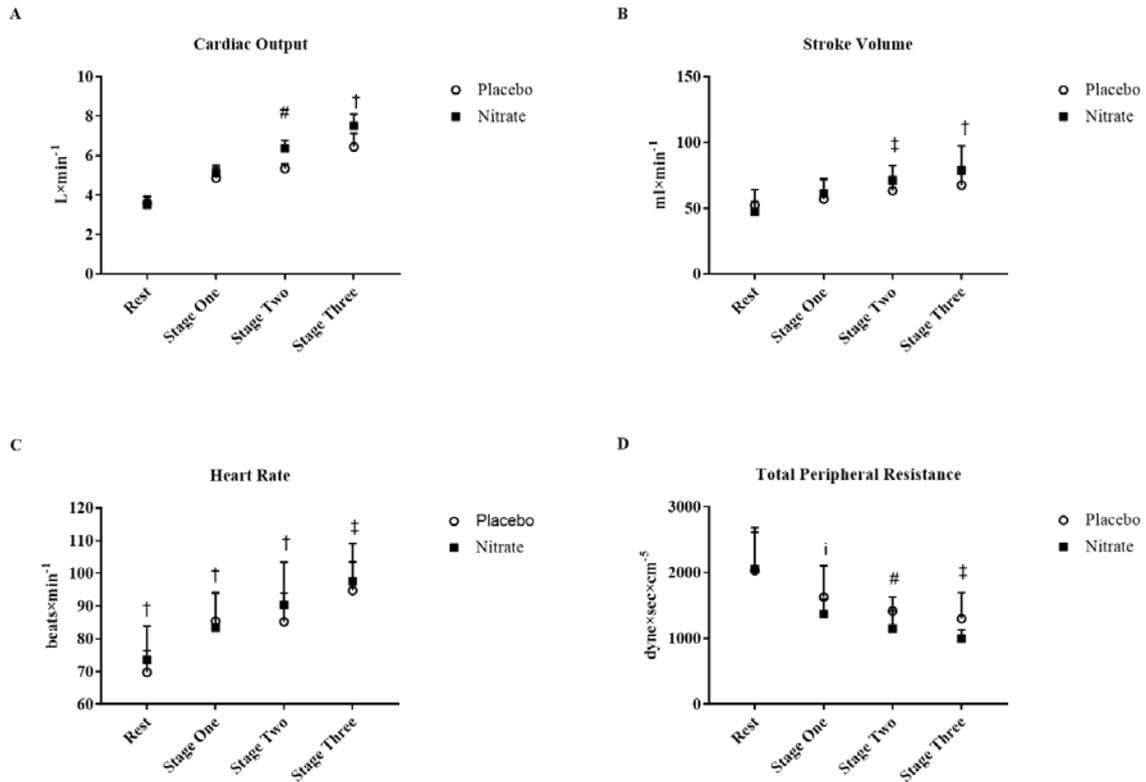


Figure 5.3 Resting and submaximal exercise responses of \dot{Q} , SV, HR and TPR

The improvement in \dot{Q} (A) and SV (B) had medium to very large ES in stages two and three of exercise (A and B). The increase in HR had medium to large ES at rest and all stages of exercise (C). TPR (D) had small, very large and large ES changes during stages one, two and three respectively. Cohen's *d* ES of the change between interventions is indicated as follows: † indicates medium ES (>.5), ‡ indicates large ES (>.8), and # indicates very large ES (>1.2). Abbreviations: \dot{Q} - cardiac output, HR- heart rate, SV- stroke volume and TPR- total peripheral resistance.

The changes in \dot{Q} and SV in stages two and three were accompanied by reductions in TPR.

When examining individual response data of \dot{Q} and TPR for stage two (**Figure 5.4**), it appears that the large effect size for \dot{Q} in favour of nitrate is potentially mediated by a decrease in TPR (ES=-1.62). While the two-way repeated measures ANOVA showed no significant main effect of nitrate at any stage for all variables ($p>0.27$), likely due to the underpowered sample size, the medium to very large ES suggests a potential clinical relevance to the changes.

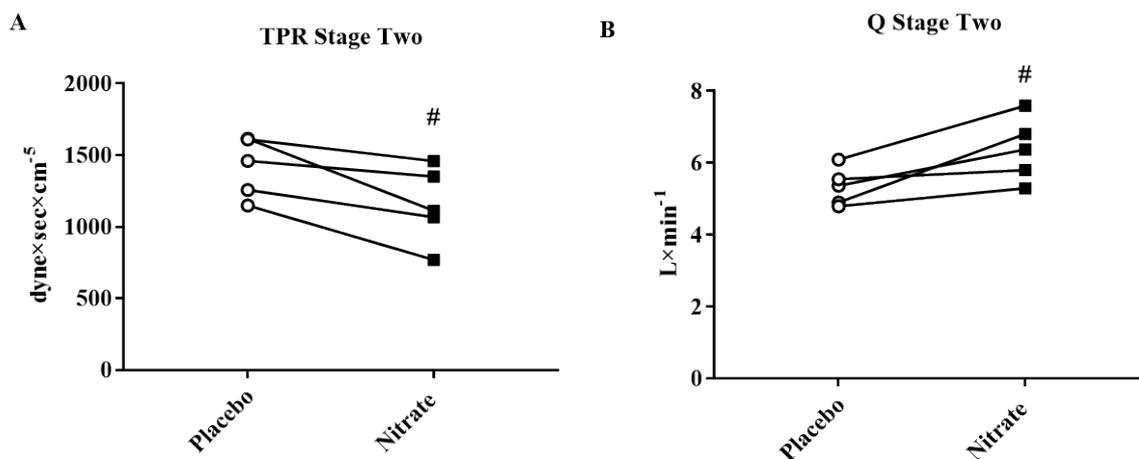


Figure 5.4 Individual responses for TPR and \dot{Q} during stage two

There were very large ES for both the reduction in TPR (A) and the increase in \dot{Q} (B) during stage two. Abbreviations: \dot{Q} - cardiac output and TPR- total peripheral resistance.

There were medium to large Cohen's *d* ES changes in DBP, MAP and RPP during all stages of exercise (Table 5.2).

Table 5.2 BP response at rest and during submaximal exercise

Variable	Supplementation	Rest	Stage 1	Stage 2	Stage 3
SBP	Placebo	116 ± 8	125 ± 2	126 ± 4	134 ± 8
	Nitrate	112 ± 6	124 ± 6	123 ± 7	136 ± 8
DBP	Placebo	74 ± 6	78 ± 8	78 ± 4	81 ± 5
	Nitrate	72 ± 7	67 ± 5 †	73 ± 4 ‡	71 ± 5 †
MAP	Placebo	88 ± 5	93 ± 6	94 ± 3	99 ± 4
	Nitrate	85 ± 6	86 ± 4 †	89 ± 5 ‡	93 ± 6 †

No changes were noted for SBP at rest or any of the exercise stages. There were large and medium ES changes in DBP during stage two and stage three respectively. There were medium to large ES changes in MAP at all stages of exercise. All data is presented as mean ± SEM. Cohen's *d* ES of the change between interventions are indicated as follows: † indicates medium ES (>.5), ‡ indicates large ES (>.8). Abbreviations: DBP- diastolic blood pressure, MAP- mean arterial blood pressure and SBP- systolic blood pressure.

5.4 Discussion

The results of this underpowered pilot study suggest that short-term inorganic nitrate supplementation may lead to potentially physiologically beneficial increases in \dot{Q} and SV

during submaximal exercise. These changes were concomitant to reductions in TPR, DBP and MAP. Although not statistically significant, these data suggest that inorganic nitrate supplementation could potentially induce beneficial changes to \dot{Q} -likely secondary to reductions in TPR in the peripheral tissues. Future studies with a larger sample size are needed to substantiate these results and identify potentially long-term clinical benefits to patients with HFrEF.

A reduction in \dot{Q} during exercise is a major characteristic of patients with CHF and contributes to the reduction in exercise capacity (Sullivan et al., 1989). As such, an increase in \dot{Q} during submaximal exercise is both clinically and functionally important in this population. In the current study we reported a potentially clinically relevant increase in \dot{Q} of 19% and 17% in stages two and three following inorganic nitrate supplementation. This finding is in agreement with an earlier study by Zamani et al. in patients with HFpEF who found an increase in \dot{Q} following a single acute dose of 12.9mmol inorganic nitrate three hours prior to testing. They concluded that the increased \dot{Q} response was likely mediated by an increase in SV in response to reduced TPR (Zamani et al., 2015). The current study did not directly measure TPR or SNS activity; however, indirect calculations of TPR indicated reductions with medium to very large ES during submaximal exercise, similarly indicating that the increase in \dot{Q} was potentially mediated by reductions in TPR during exercise.

The only other study to measure \dot{Q} in CHF following inorganic nitrate and during exercise stress found no changes in SV or \dot{Q} (Hirai et al., 2017). A limitation in the study was the use of bioelectrical impedance which is an indirect measurement technique that relies on a stable ECG. It is therefore often less accurate in subjects with cardiac abnormalities (Kemps et al., 2008)

Our suggestion that reductions in TPR may have contributed to the increased SV and \dot{Q} is further supported by the potential reductions in MAP and DBP at rest and exercise following inorganic nitrate treatment (**Table 5.3**). Given that decreases in DBP of just 5-6 mmHg are associated with a 38% reduction in the 5-year risk of stroke, the average reduction of 8 mmHg observed in this trial is a potentially clinically important finding. Two previous studies in HFrEF also failed to show significant reductions in BP during exercise following inorganic nitrate supplementation. (Coggan et al., 2018; Hirai et al., 2017). These studies did not report ES and had relatively small sample sizes.

This study has several limitations. The relatively small sample size comprised of only male participants limits the applicability to the broader population. While the results are encouraging, future investigations should seek to recruit a larger and more heterogeneous sample size. This study also employed a non-invasive design, thus requiring the indirect calculation of the central measures of cardiac performance. Despite numerous studies showing good congruity between echo and direct measures of cardiac performance during exercise, the addition of invasive direct measurement of cardiac performance should also be considered in future trials (Dericbourg et al., 1990; Mercado et al., 2017). Another limitation is that the study's primary focus was on measures of cardiac function known to be linked to exercise performance. It is possible that inorganic nitrate supplementation improved SV and \dot{Q} via changes in contractile function or even structural modification which were undetectable in the current approach (Pironti et al., 2016). A recent study showed that long term (six months) dietary nitrate supplementation induced beneficial cardiac remodeling resulting in a 5% reduction of left ventricular volume in individuals with or at risk of type II diabetes mellitus (Faconti et al., 2019). Future studies should seek to include measures of structure in

order to tease out the potential effects of nitrate on cardiac structure and further the understanding of how these changes could subsequently affect cardiac performance.

This study is the first to report potential improvements in \dot{Q} and SV and reductions in BP during submaximal exercise following dietary nitrate supplementation in patients with HFrEF. The results are encouraging and suggest the potential for nitrate supplementation to have clinical beneficial effects on cardiac performance and BP during submaximal exercise in this population. However, the contributors to exercise performance are multifactorial, and thus improvements in exercise hemodynamics or even cardiac performance alone may not directly translate to improvements in aerobic capacity in HFrEF. If future studies can confirm these benefits in cardiac performance during acute exercise, coupling nitrate supplementation with another intervention targeting improved function of the peripheral skeletal muscle tissues (such as exercise training) could have a synergistic effect exceeding that of either individual intervention.

6 The effect of dietary nitrate supplementation on mitochondrial respiration in men with heart failure with reduced ejection fraction

6.1 Introduction

Patients with CHF are characterised by reduced exercise capacity and early fatigue due, in part, to impairments in the structure and function of the mitochondria (Drexler et al., 1992; Rosca et al., 2013; Schrepper et al., 2012). Mitochondria are dynamic cellular organelles involved in the regulation of a wide range of metabolic processes, including the production of ATP via oxidative phosphorylation. This energy production pathway is reliant on the efficiency of electron transport across a membrane-bound complex (Rosca et al., 2009; Rosca et al., 2013). Dysfunctions in the amount, structure and/or function of either the mitochondrial complexes or associated proteins that regulate mitochondrial biogenesis and mitochondrial function can have profound impacts on effective cellular respiration and metabolism.

NO, a free radical produced by NOS, including nNOS and eNOS, has been established as a regulator of mitochondrial function and a stimulator of mitochondrial biogenesis via the activation of various cellular signaling pathways (Caballano-Infantes et al., 2017; Nisoli et al., 2004). Reductions in NO bioavailability and impaired mitochondrial function is associated with the development and progression of several CVDs, including CHF (Fischer et al., 2005; Nisoli et al., 2004). Studies in both animals and humans with CHF have reported decreases in the function of several individual mitochondrial ETS complexes following the development of CHF in cardiac and skeletal muscle tissues (Buchwald et al., 1990; Ide et al.,

1999; Rosca et al., 2009; Sparagna et al., 2007). NO is known to play a regulatory role in Complex IV, and mouse models have shown that reductions in eNOS derived NO results in significant decreases in both mitochondrial number and overall energy expenditure (Nisoli et al., 2003). Given the importance of mitochondrial function to overall skeletal muscle oxidative capacity, these dysfunctions could play a critical role in limiting exercise capacity in patients with CHF. As such, novel strategies to improve mitochondrial function via increases in NO bioavailability are potentially clinically important.

The cellular processes governing mitochondrial form and function are largely controlled by PGC-1 α , which is often referred to as the ‘master regulator’ of mitochondrial biogenesis (Puigserver et al., 1998; Wu et al., 1999). The transcriptional co-activator activity of PGC-1 α is responsible for coordinating a number of transcription factors that are involved in the regulation of mitochondrial respiratory function and content, and its increased expression is often viewed as a precursor to mitochondrial biogenesis (Puigserver et al., 1998; Safdar et al., 2011; Wu et al., 1999). Previous studies have demonstrated that over expression of PGC-1 α in cardiac myocytes leads to an increase in both mitochondrial content and respiratory function (Lehman et al., 2000). Nisoli and colleagues demonstrated that NO was involved in the induction of increased PGC-1 α expression in cell culture models of various animal tissues (cardiac, muscle and liver) and that PGC-1 α is significantly reduced in eNOS knock-out mice models (Nisoli et al., 2003; Nisoli et al., 2004). Humans with CHF are also characterised by a reduction or inhibition of eNOS and repressed expression of PGC-1 α (Riehle et al., 2012; Tobar et al., 2018). Together, these data suggest that increasing NO availability may upregulate PGC-1 α and provide downstream improvements for mitochondrial content and function.

In addition to PGC-1 α , other proteins that are linked to the regulation of mitochondrial content and function, and whose activities are potentially modulated by NO, include mTORC1, Akt and p38MAPK. mTORC1 is a master regulator of cell growth that can also positively regulate oxygen consumption and the transcription and translation of mitochondrial genes, including PGC-1 α (Cunningham et al., 2007; Gandin et al., 2016). Furthermore, there is evidence that, under certain circumstances, mTORC1 may be regulated by NO-dependent mechanisms (Aguiar et al., 2017; Ito et al., 2013). Akt is an upstream activator of mTORC1 whose activity is inhibited by NO-mediated S-nitrosylation (Yasukawa et al., 2005). Thus, increased NO availability also has the potential to inhibit the Akt/mTORC1 signaling pathway. p38MAPK is a stress-activated kinase that positively regulates PGC-1 α transcription and may also be negatively regulated by NO (Akimoto et al., 2005; Kinugawa et al., 2005). To date, however, the effect of increasing NO availability via sodium nitrate on these regulators of mitochondrial number and/or function remains to be determined.

As such, we tested the hypothesis that increased NO bioavailability via supplementation with inorganic nitrate will improve mitochondria respiration and protein expression in men with HFrEF.

6.2 Methodology

6.2.1 Study design

See General Methodology **Section 3.1**

6.2.2 Participant recruitment

See General Methodology **Section 3.1.1**. Participants recruited for study 1 were invited to complete this additional visit. In total, 7 participants consented to the muscle biopsy visit.

6.2.3 Randomisation and supplementation

See General Methodology **Section 3.1.2**.

6.2.4 Muscle Biopsies

All biopsies were performed at Victoria University, Footscray campus by an experienced medical physician. The biopsy was conducted in the same method previously described (Levinger et al., 2010; Levinger et al., 2011). In brief, biopsies were performed in the morning with the participant in a fasted state, with the exception of the beetroot juice supplementation that was taken two and a half hours prior to the biopsy. Upon arrival, participants were placed in a supine position. An injection of a local anaesthetic (1% Xylocaine) was made into the skin and fascia of the VL. After a period of five to ten minutes, the site was cleaned and a small incision was made. A muscle sample was taken using a Bergström biopsy needle with manual suction applied. One portion (10-20 mg) was immediately immersed in a 5-ml tube containing ~3 ml of biopsy preserving solution kept on ice and used for *in-situ* measurements of mitochondrial respiration, while the other portion was immediately frozen in liquid nitrogen and stored at -80°C for subsequent analyses.

6.2.5 Fibre preparation and high-resolution respirometry

Procedures for the following protocol have been previously published (Granata et al., 2016; Pesta et al., 2012). Muscle fibres were separated with forceps and immediately placed in ice-cold preserving solution BioPS (containing 2.77 mM CaK₂EGTA, 7.23 mM K₂EGTA, 5.77 mM Na₂ATP, 6.56 mM MgCl₂, 20 mM taurine, 50 mM 2 (Nmorpholino) ethanesulfonic acid

(MES), 15 mM Na₂-phosphocreatine, 20 mM imidazole, and 0.5 mM DTT adjusted to pH 7.1). The plasma membrane was permeabilised by agitation for 30 minutes at 4°C in BioPS containing 50 µg/ml saponin. Subsequently, three washes were performed in MiR05, a respiration medium containing 0.5 mM EGTA, 3 mM MgCl₂, 60 mM potassium-lactobionate, 20mM taurine, 10 mM KH₂PO₄, 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 110 mM sucrose, and 1 g/L bovine serum albumin (BSA), essentially fatty acid-free (pH 7.1).

6.2.6 Mitochondrial respiration

Mitochondrial respiration was measured in duplicate (from 2–4 mg wet weight of muscle fibres) in MiR05 at 37°C by using the high resolution respirometry (Oxygraph-2k, Oroboros, Innsbruck, Austria). A substrate-uncoupler-inhibitor titration (SUIT) protocol was utilised (Granata et al., 2016; Trewin et al., 2017; Wyckelsma et al., 2017). The SUIT sequence was as follows: malate (2 mM) and pyruvate (5 mM) in the absence of adenylates were added for measurement of leak respiration (CI)_L. ADP (5 mM) was added for measurement of oxidative phosphorylation capacity (P) (CI)_P. Succinate (10 mM) was added for the measurement of oxidative phosphorylation capacity (P) through complex 1 and 2 combined (CI+II)_P. Cytochrome c (10 mM) was then added to test for outer mitochondrial membrane integrity (an oxygen flux increase of <15% from (CI+II)_P was considered acceptable). This was followed by a series of stepwise carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone titrations (0.75–1.5 mM) for measurement of ETS capacity (E) through (CI+II)_E. Rotenone (0.5 mM), an inhibitor of CI, was added to determine E through CII (CII)_E. Finally, the addition of antimycin A (2.5 mM), an inhibitor of CIII, allowed measurement and correction of residual oxygen consumption, indicative of non-mitochondrial oxygen consumption. Reoxygenation during the protocol was by direct syringe injection of O₂ was necessary to

maintain O₂ levels between 275 and 450 nmol/ml and to avoid potential oxygen diffusion limitation. Oxygen concentration (in nanomoles per milliliter) and flux (in picomoles per second per milligram) were recorded with DatLab software (O2k, OROBOROS Instruments, Innsbruck, Austria). Mitochondrial specific respiration (pmol O₂·s⁻¹·citrate synthase activity⁻¹) was calculated by normalising mass-specific respiration (pmol O₂·s⁻¹·mg⁻¹) by the citrate synthase activity (CS) (mol·h⁻¹·kg protein⁻¹).

6.2.7 Whole-muscle lysates

Frozen muscle (10–20 mg) was homogenized in an ice-cold Auwerx lysis buffer (1:20 w/v) containing 50 mM Tris, 150 mM sodium chloride 1 mM EDTA, 1% IGEPAL, deionised water and a protease/phosphatase inhibitor cocktail (Cell Signalling Technology, Danvers, MA, USA), adjusted to pH 7.4. Metal beads were added to all samples and run at 30 Hz for 2 minutes in a homogeniser (Tissue Lyser II, Qiagen, Valencia, CA, USA) before samples were turned, and homogenisation was repeated for a further two minutes at 30 Hz. Muscle homogenates were rotated at 4°C for 60 minutes and centrifuged at 15,000 g at 4°C for 20 min, and the supernatant was used for western blot analysis and enzyme activity assay. Protein concentration was determined in triplicate with a commercial colorimetric assay (Protein Assay kit-II; Bio-Rad, Gladesville, New South Wales, Australia), against bovine serum albumin standards (BSA, A9647; Sigma-Aldrich).

6.2.8 Western blotting

Muscle homogenate was diluted in 4X Laemmli buffer (0.25 M Tris, 4% SDS, 20% glycerol, 0.015% bromophenol blue and 10% 2-mercaptoethanol), and equal amounts of total protein (15 or 20 µg) were loaded in different wells on Criterion™ 4-20% TGX Stain-Free™ Precast Gels (Bio-Rad). Samples from each participant were placed in adjacent lanes of the wells and

each gel plate included four to six internal standards of varying dilutions made from a mixed homogenate. These standards were used to form a calibration curve, with density plotted against protein content. Protein abundance was then calculated from the measured band intensity for each sample on the gel, using the linear regression equation from the calibration curve (Murphy et al., 2013).

Gel electrophoresis was run for 20 minutes at 80 V and then for a further 60-90 minutes at 80 – 150 V. Transfer of proteins from the gel to 0.2 μ m PVDF membrane at 25 V for 10 minutes, was done via turbo transfer (Bio-Rad). Membranes were then blocked in 5% non-fat dry milk diluted in Tris-buffered saline with 0.1% Tween-20 (TBST) for 60 minutes. Membranes were then washed in TBST and incubated overnight at 4°C with the appropriate primary antibody and prepared in TBST with 5% BSA and 0.02% sodium azide. The primary antibodies used were from Cell Signaling Technology and included Akt (#9272), p-Akt ser473 (#9271), p38MAPK (#9212), p-p38MAPK Thr180/Tyr182, #9211), mTORC1 (#2983), p-mTORC1 ser2448 (#5586). One antibody from Calbiochem for PGC-1 α (#st1202) was also utilised. Following TBST washes, samples were incubated at room temperature with the appropriate host species-specific secondary antibody for 60 minutes, before being exposed to a chemiluminescence solution. Images were taken with a ChemiDoc Imaging System fitted (Bio-Rad). Densitometry was performed with Image Lab 5.0 software (Bio-Rad). Images were displayed with a minimum of five bandwidths above and below the band of interest.

6.2.9 Citrate synthase activity analysis

CS activity was determined in triplicate on a 96-well microtiter plate by adding 5 μ L of a 6 mg·mL⁻¹ muscle homogenate (freeze thawed in liquid nitrogen twice), 40 μ L of 3mM acetyl

CoA, 25 μ L of 1mM 5,59-dithiobis(2-nitrobenzoic acid), 165 μ L of 100 mM Tris buffer (pH 8.3, kept at 30 °C). After addition of 15 μ L of 10 mM oxaloacetic acid, the plate was immediately placed in an xMark-Microplate spectrophotometer (Bio-Rad) at 30°C, and after 30 s of linear agitation, absorbance at 412 nm was recorded every 15 seconds for three minutes. CS activity is reported as moles per hour per kilogram protein.

6.3 Statistical analysis

Statistical analysis of the demographics and plasma nitrate/nitrite values were performed using Statistical Package for the Social Sciences (version 22, SPSS Inc. Chicago, IL, USA). One-way repeated measures analysis of variances (ANOVAs) were used to assess the changes in plasma nitrate/nitrite as well as changes in the primary outcome measures of mitochondrial respiration, and protein content of PGC-1 α , mTORC1, p38MAPK and Akt following placebo and nitrate supplementation. The t-tests were two-sided, with $p < 0.05$ considered significant. Secondary analysis on the main outcome variables included a one-way repeated measures ANOVA to assess the difference in parameters of mitochondrial respiration and PGC-1 α expression following placebo and nitrate supplementation. Pearson's product moment correlation coefficients were calculated where indicated. Graphs and figures were created utilising GraphPad Prism Version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA). Except where otherwise indicated, all results are presented as mean \pm SEM.

6.4 Results

Patient characteristics, disease pathology and relevant medications are described in **Table 6.1**. There were no significant differences between the dosing days prior to the placebo or nitrate visits (15.0 ± 2 days and 15.1 ± 2 days respectively, $p=0.72$).

Table 6.1 Participant's demographics

Variable	Value
Age, mean \pm SEM, y	61.7 \pm 1.7
Height, mean \pm SEM, cm	173.2 \pm 2.6
Mass, mean \pm SEM, kg	90 \pm 6.3
BMI	30.3 \pm 2.8
Male, n (%)	7 (100)
EF	32.1 \pm 3.2
Aetiology	
Ischaemic	3
Non-Ischaemic Dilated Cardiomyopathy	4
NYHA Class, n (%)	
Class I	1 (14.3)
Class II	4 (57.1)
Class III	2 (28.6)
Comorbidities, n (%)	
Diabetic	2 (28.6)
COPD	2 (28.6)
HTN	3 (42.9)
Current Smoker	3 (42.9)
Obese	3 (42.9)
Drug therapy, n (%)	
Metformin	2 (28.6)
β -Blockers	7 (100)
ACE Inhibitor	5 (71.4)
Statin	4 (57.1)
Aspirin	3 (42.9)
Diuretics	7 (100)

Abbreviations: ACE inhibitor- angiotensin-converting-enzyme inhibitor, COPD- chronic obstructive pulmonary disease, EF-ejection fraction, HTN- hypertension, NYHA- New York Heart Association.

Plasma nitrate (**Figure 6.1 A**) increased by 831% (nitrate: 1177.2 \pm 101.2uM, placebo: 126.5 \pm 44.8 uM, p<0.001) following nitrate supplementation. Plasma nitrite (**Figure 6.1 B**) also increased by 100%, but this did not reach statistical significance (nitrate: 312.1 \pm 227.6, placebo: 155.5 \pm 53.5 nM, p= 0.22).

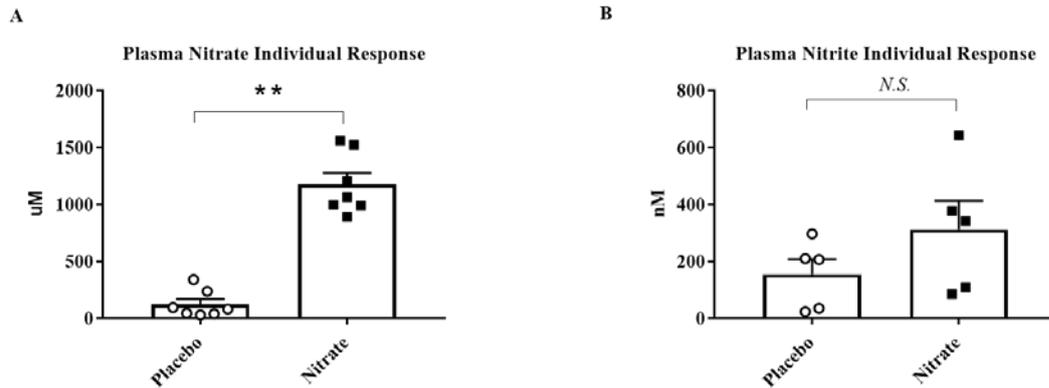


Figure 6.1. Effects of nitrate supplementation on plasma nitrate and nitrite response

There were significant increases in plasma nitrate and a trend toward significant increases in plasma nitrite. Nitrite data is displayed for n=5. Two participants were excluded due to their data being more than two standard deviations above the mean. They were included in all other outcome analyses as both their nitrite and nitrate values increased following nitrate supplementation. ** indicates $p < 0.001$. Abbreviations uM- micromolar, nM- nanomolar.

As illustrated in **Figure 6.2**, there were no changes in mitochondrial content, as assessed by CS activity, following supplementation (nitrate: $3.2 \pm 0.3 \text{ mol}\cdot\text{h}^{-1}\cdot\text{kg protein}^{-1}$, placebo: $3.3 \pm 0.1 \text{ mol}\cdot\text{h}^{-1}\cdot\text{kg protein}^{-1}$, $p=0.73$). There was no correlation ($r=0.11$, $p=0.70$) between CS and peak aerobic capacity.

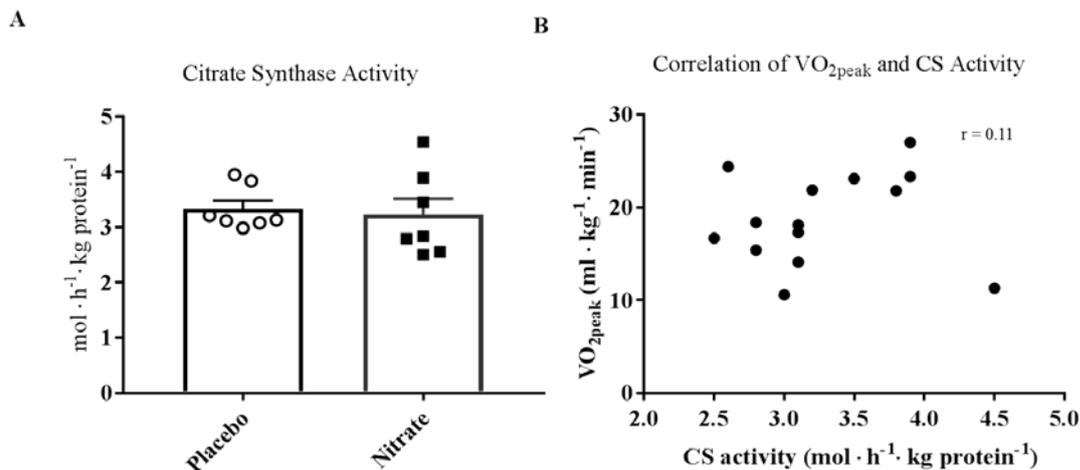


Figure 6.2 Effect of nitrate supplementation on CS activity

There was no change in CS activity (A) and no correlation was found between CS activity and $\dot{\text{V}}\text{O}_{2\text{peak}}$ (B). Both mean \pm SEM and individual values are displayed. Abbreviations: CS- citrate synthase.

Absolute values for both mass specific ($\text{pmol O}_2 \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$) and mitochondrial specific ($\text{pmol O}_2 \cdot \text{s}^{-1} \cdot \text{CS}^{-1}$) mitochondrial respiration are presented in **Table 4.2**. None of the examined parameters were significantly changed following supplementation (all $p > 0.05$).

Table 4.2 Mitochondrial respiration

Measure	Placebo (n=7)	Nitrate (n=7)
Mass-specific ($\text{pmol O}_2 \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$)		
CI _L	5.6 ± 4.4	5.6 ± 2.1
CI _P	37.3 ± 8.1	43.6 ± 2.6
(CI + II) _P	61.1 ± 5.4	61.9 ± 3.5
(CI + II) _E	69.1 ± 7.2	75.6 ± 3.5
CII _E	33.8 ± 5.9	32.0 ± 1.8
Mitochondrial-specific ($\text{pmol O}_2 \cdot \text{s}^{-1} / \text{CS}^{-1}$)		
CI _L /CS		
Activity	1.8 ± 1.3	1.9 ± 0.8
CI _P /CS		
Activity	11.7 ± 2.5	14.2 ± 4.1
(CI + II) _P /CS Activity	18.8 ± 2.0	20.0 ± 1.9
(CI + II) _E /CS Activity	21.3 ± 2.5	24.6 ± 2.3
CII _E /CS		
Activity	10.3 ± 0.9	10.6 ± 1.3

Data are displayed as mean ± SEM. Mitochondrial specific (Mito-specific) values were calculated by normalising mass-specific mitochondrial respiration values to CS activity (values expressed per kilogram of protein). No changes reached statistical significance at the $p < 0.05$ level. Abbreviations: (CI)_L, leak respiration through CI; (CI)_P, oxidative phosphorylation through CI; (CI+II)_P, maximal oxidative phosphorylation through CI+II; (CI+II)_E, Electron transport system (ETS) capacity (E) through CI+CII (uncoupled mitochondrial respiration); (CII)_E, ETS capacity (uncoupled mitochondrial respiration) through CI.

There were no changes noted in maximal oxidative phosphorylation (**Figure 6.3**) following nitrate supplementation ($p > 0.05$). There were no significant correlations between any of the mass-specific (absolute) or mitochondrial-specific (normalized for CS activity) respiration values and $\dot{V}O_{2\text{peak}}$ (all correlations > 0.1). Consistent with these findings, there was no change ($p = 0.75$) in $\dot{V}O_{2\text{peak}}$ (placebo: 18.7 ± 2.1 , nitrate: $19.0 \pm 1.8 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$).

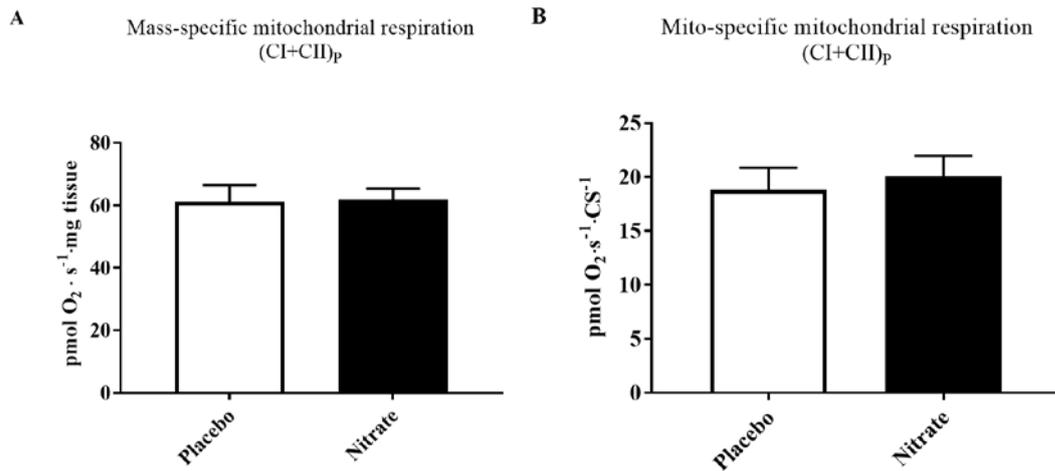


Figure 6.3 Mass specific and mitochondrial-specific respiratory function for maximal oxidative phosphorylation capacity

Data is displayed as mean \pm SEM of complex I and complex II (CI +CII)_p oxidative phosphorylation capacity in both the placebo and nitrate conditions. Abbreviations: mito- mitochondria.

No significant changes following supplementation were observed in mTORC1 (mTORC1:

p=0.31, **Figure 6.4 A**; pmTORC1: p=0.11, **Figure 6.4 B**; pmTORC1/mTORc1: p= 0.58,

Figure 6.4 C), Akt (Akt: p=0.18, **Figure 6.4 E**; p-Akt: p=0.36, **Figure 6.4 F**; p-Akt/Akt: p=

0.29, **Figure 6.4 G**) p38MAPK (p38MAPK: p=0.56, **Figure 6.4 I**; p- p38MAPK: p=0.54,

Figure 6.4 J; p-p38MAPK/ p38MAPK: p= 0.48, **Figure 6.4 K**) or. Representative blots for

mTORC1 (**Figure 6.4 D**), p38MAPK (**Figure 6.4 H**) and Akt (**Figure 6.4 L**) are displayed

below. There were also no significant changes in total PGC-1 α (p=0.92, **Figure 6.5**)

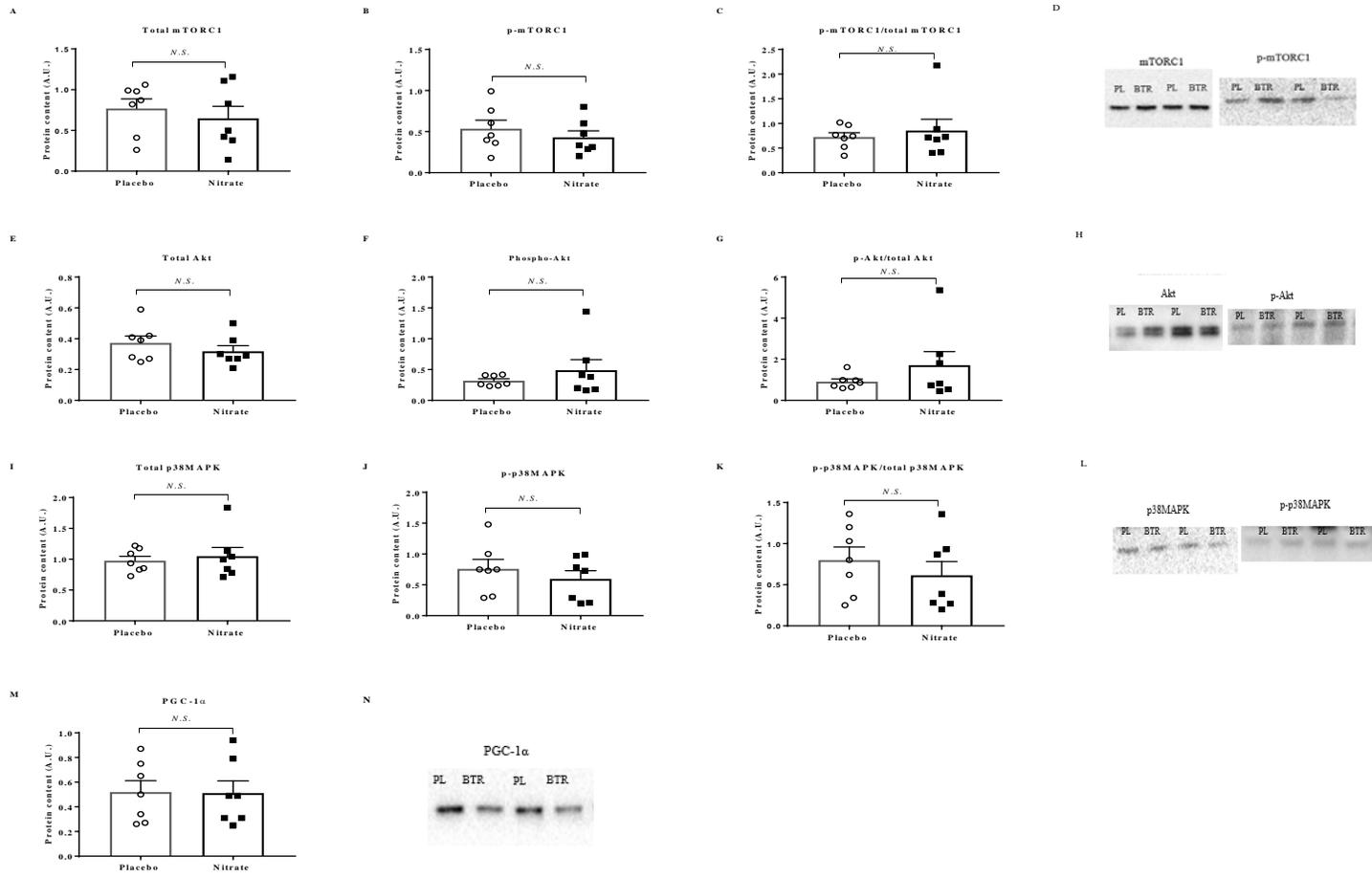


Figure 6.4 Effect of nitrate supplementation on mitochondrial protein concentration

Relative protein concentrations of total and phosphorylated mTORC1, p38MAPK and Akt and calculated phosphorylated to total ratios. Data is displayed as mean ± SEM and individual values for all proteins. Abbreviations: p- phosphorylated, mTORC1- mechanistic target of rapamycin complex 1, MAPK- mitogen-activated protein kinase, Akt- protein kinase

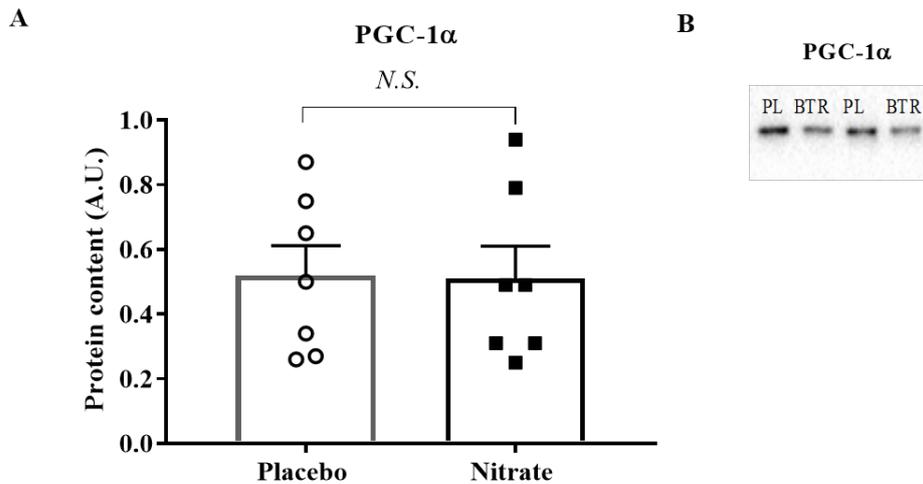


Figure 6.5 Effect of nitrate supplementation on PGC-1 α and a representative blot

Abbreviations: PGC-1 α - Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha, PL- placebo, BTR- nitrate

6.5 Discussion

We report that two weeks of nitrate supplementation had no beneficial effect on mitochondrial respiration or any of the mitochondrial proteins examined, including mTORC1, p38MAPK, Akt, and PGC-1 α , in patients with HFrEF despite an increase in circulating levels of nitrate. Future studies should explore the efficacy of a longer course of supplementation and/or combining nitrate supplementation with other interventions, such as exercise to further explore its potential to improve mitochondrial function in CHF.

NO is a regulator of mitochondrial function and biogenesis. There are substantial reductions in NO bioavailability and mitochondrial function in CHF which contribute to the development and progression of the syndrome (Fischer et al., 2005; Nisoli et al., 2004). In the current study, we have used two weeks of daily inorganic nitrate supplementation to exogenously increase NO bioavailability and potentially improve mitochondrial function. This supplementation is known to increase circulating levels of nitrate and nitrite in HFrEF

(Hirai et al., 2017). In the current study, despite a significant increase in plasma nitrate (831%), the change in plasma nitrite was only in the order of 100%, which did not reach statistical significance. It is not clear why we did not observe a significant increase in plasma nitrite as the previous studies reported (Coggan et al., 2018; Hirai et al., 2017). We hypothesise that the difference could be due to individual variation in response to nitrate dosing and the small sample size (typical in invasive studies). Yet, if nitrate and nitrite play a critical role with regards to mitochondrial respiration in this population, one would expect to potentially see changes in mitochondrial outcomes given the relative increases noted in circulating nitrate and nitrite. In particular, animal models suggest there is a relationship between decreased NO bioavailability and mitochondrial function (Nisoli et al., 2003; Nisoli et al., 2004). However, we did not find any correlation between plasma nitrite and any of the parameters of mitochondrial respiration. Studies in healthy humans have demonstrated inconsistent findings with regards to the effects of nitrate supplementation on mitochondrial function (Larsen et al., 2011; Whitfield et al., 2016). We hypothesised that nitrate supplementation could be particularly beneficial in patients with CHF due to the substantial dysfunctions present within the mitochondria. However, in the current study we observed only a limited to no effect of the nitrate supplementation on mass-specific and mitochondria-specific maximal oxidative phosphorylation $(CI+CII)_p$, indicating no effect on mitochondrial respiration.

It is not clear why the supplement had no effect on maximal respiration as this result is in contrast to previous studies in animal and cell cultures. However, the discrepancy could be due to different models used, animal and cell models as opposed to human fibres and perhaps also the relatively small sample size. Yet, our results are in line with the findings of a recent study that reported no significant changes in respiration, utilising the same technique,

following a much higher dose (26 mmol nitrate per day) for seven days in healthy active humans (Whitfield et al., 2016). It is also possible that despite the increase in circulating plasma nitrate, the local skeletal muscle concentrations were not similarly increased. A relatively recent study has hypothesised that the skeletal muscle tissue is a predominant reservoir of endogenous nitrate/nitrite stores. However, the measurement of nitrate/nitrite in muscle tissue has only been reported in animal models thus far, and no studies on the efficacy of exogenous supplementation have been published as of yet (Piknova et al., 2015).

Reductions in mitochondrial density (as assessed via CS activity) have been linked to the exercise intolerance experienced by patients with CHF (Toth et al., 2012). A recent review reported correlations between relative changes in CS activity and $\dot{V}O_{2peak}$ in healthy and HF cohorts (Vigelsø et al., 2014). However, we found no such correlation in our sample ($r=0.11$, $p>0.05$) and no change in $\dot{V}O_{2peak}$. We also report herein that mitochondrial density is unaffected by chronic (15 days) nitrate supplementation in men with HFrEF.

PGC-1 α is known to coordinate the molecular signaling pathways responsible for the induction of the cellular processes involved in mitochondrial biogenesis (Puigserver et al., 1998; Wu et al., 1999). In animals, NO bioavailability has a potential regulatory role on PGC-1 α protein content (Nisoli et al., 2003; Nisoli et al., 2004). However, herein we report no significant effect on PGC-1 α protein content following nitrate supplementation. This finding is the first to report no effect in CHF and supports the only other previous human trial which found no change in mitochondrial efficiency or messenger ribonucleic acid levels of PGC-1 α in healthy humans following nitrate supplementation (Larsen et al., 2011). It is also plausible that the nitrate supplementation could provide a benefit to mitochondrial protein

synthesis when combined with a stronger intervention stimulus, such as an exercise intervention.

Similar to PGC-1 α , we found no changes in the abundance or phosphorylation status of other potential regulators of mitochondrial number and function (e.g., mTORC1, Akt and p38MAPK). These results suggest that these proteins are not responsive to increases in NO availability under these conditions in these patients with HFrEF. It is also possible that, despite nitrate's ability to elevate plasma nitrate and nitrite, it did not elevate NO within the skeletal muscle sufficiently to affect NO-dependent signaling mechanisms.

The study has several potential limitations. Plasma nitrate/nitrite were only measured at one time point during the trial. This time point was on average nine days prior to the muscle biopsy (Woessner et al., 2018b). Thus, it is possible that the plasma nitrate/nitrite response was lower or higher on the day of the actual muscle biopsy. The study has a relatively small sample size due to its invasive protocol, which may affect the power and the ability to reach statistical significance. As discussed previously, we hypothesise that the lack of significant increase in plasma nitrite is due both to the small sample size and the large variation in the individual data. However, as all participants had increases in plasma nitrate following nitrate supplementation, thus the lack of significant increase in plasma nitrite could also be due to a dysfunction enterosalivary tract in some of these individuals. It is also plausible that nitrate supplementation is simply not a significant stimulus for mitochondrial function at this dosage. Future studies could explore differential dosing methodologies and volumes. However, the trial has several noteworthy strengths, including its double-blind design, the highly controlled supplementation regimen, as well as the invasive skeletal muscle measures completed in a highly complicated clinical population.

In conclusion, we report that two weeks of a nitrate supplementation had no effect on mitochondrial respiration and no effect on mTORC1, p38MAPK, Akt, and PGC-1 α expression in patients with HFrEF. Future studies should explore the efficacy of a longer course of supplementation and/or combining nitrate supplementation with other interventions, such as exercise, on mitochondrial function to explore it as a potential future treatment to improve mitochondria function in CHF.

7 General discussion and future recommendations

7.1 Major findings

Exercise intolerance and poor aerobic capacity are hallmark symptoms in patients with HFrEF. The reduced exercise capacity has been primarily linked to dysfunctions in the peripheral tissues, including skeletal muscle. A reduction in NO bioavailability has been implicated as a mediating, or concomitant, factor in the initiation, severity and/or progression of the peripheral dysfunctions in CHF. Thus, the primary purpose of this thesis was to assess the effectiveness of oral inorganic nitrate supplementation on exercise tolerance, resting vascular function, cardiac performance during submaximal exercise and mitochondrial respiration in patients with HFrEF.

The major findings of this thesis are illustrated in **Figure 7.1** and include:

1. A short-term inorganic nitrate supplementation had no significant effect on exercise tolerance (*Study 1*), peripheral tissue oxygenation (*Study 1*) or mitochondrial respiration (*Study 3*) in patients with HFrEF
2. A short-term inorganic nitrate supplementation has the potential to improve vascular function (*Study 1*), reduce TPR (*Study 2*) and reduce DBP and MAP during submaximal exercise (*Study 2*) in these patients.
3. A short-term inorganic nitrate supplementation regimen may have a meaningful clinical effect on \dot{Q} and SV during submaximal exercise (*Study 2*).

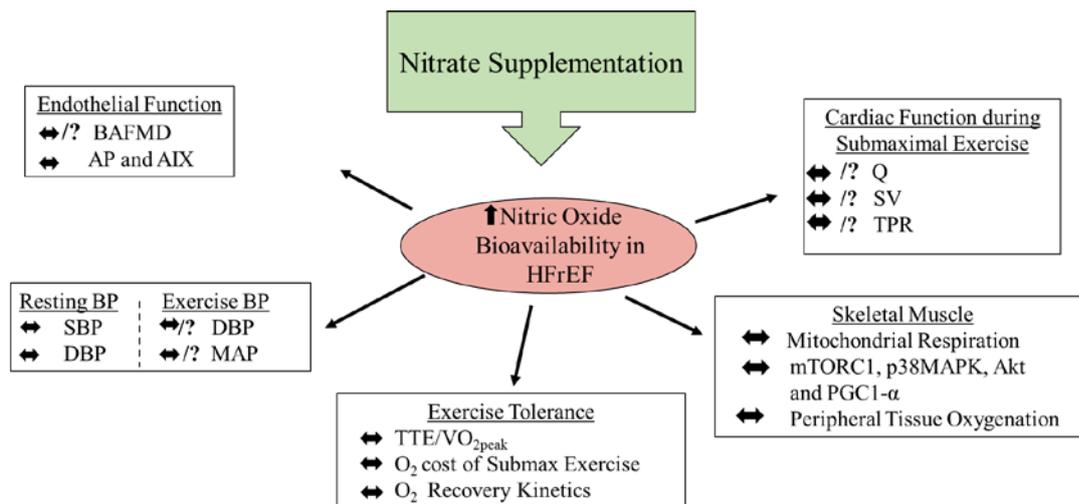


Figure 7.1 Summary of the thesis major findings: The effect of nitrate supplementation in HFrEF

Nitrate supplementation had no beneficial effect on exercise tolerance or skeletal muscle function. There were no significant effects on vascular function, but the change in BAFMD had a large Cohen's d ES, as did many of the parameters of cardiac performance (\dot{Q} and SV) during exercise, suggesting that these changes could be potentially important. ↔ Indicates no change, ? Indicates no significant change but medium to large Cohen's d ES. Abbreviations: mTORC1- mechanistic target of rapamycin complex 1, p38MAPK- mitogen-activated protein kinase, Akt- protein kinase B, TPR- total peripheral resistance, \dot{Q} - cardiac output, SV- stroke volume, BAFMD- brachial artery flow mediated dilation, AP- augmentation pressure, AIX- augmentation index, SBP- systolic blood pressure, DBP- diastolic blood pressure, MAP- mean arterial blood pressure, TTE- time to exhaustion and $\dot{V}O_{2peak}$ - maximal oxygen consumption.

7.1.1 Nitrate supplementation and effects on exercise tolerance

Based on previous studies, the hypothesis was that nitrate supplementation would improve aerobic capacity in patients with HFrEF. However, in contrast to the initial hypothesis, no significant improvements in any of the exercise tolerance parameters were observed following nitrate supplementation. It is not clear why changes were not observed as the project has several methodological strengths. First, this trial was the largest to examine the effect of nitrate supplementation in the HFrEF population. Second, the trial was the first to use aerobic exercise response during treadmill exercise, in contrast to others that used a cycle ergometer. The increase in metabolic demand during treadmill exercise, in theory, was supposed to elicit a greater response to the supplement. Third, we used the highest daily concentration of nitrate in the CHF literature over a chronic period to maximise the potential

effect. Lastly, we recruited patients using strict inclusion and exclusion criteria to ensure that only those with HFrEF would be recruited. Altogether, the study was robust and tightly controlled through the use of double-blinding and randomisation. These methodological strengths were designed on the back of methodological limitations of other studies. For instance, some studies that tested the effect of nitrate on patients with “HFrEF” also included patients which would now be classified as HFmrEF (Coggan et al., 2018; Coggan et al., 2015; Kerley et al., 2016). As all of the HFpEF studies have had some positive aerobic benefits following nitrate supplementation, it is perhaps unsurprising that the studies with the diluted HFrEF samples are also the only HFrEF studies indicating increases in aerobic performance.

The lack of effect on both the submaximal oxygen cost of exercise as well as maximal exercise capacity outcomes (measured by TTE and $\dot{V}O_{2peak}$) during treadmill exercise is consistent with the previous largest trial (n=10) in HFrEF which also indicated no benefits to aerobic performance during cycle exercise (Hirai et al., 2017). These non-significant findings were seen despite significant increases in plasma nitrate and nitrite in both the past and present study. Thus, increasing NO bioavailability as a means of improving exercise performance as an independent intervention does not appear to be a promising approach in HFrEF management. Previous studies in other clinical populations have reported inconsistent findings among patients with different CVDs. While supplementation appears effective for increasing aerobic performance in patients with PAD, results from human trials in COPD are mixed, and those utilising patients with T2DM indicated no beneficial effect (Gilchrist et al., 2013; Kenjale et al., 2011; Kerley et al., 2015; Leong et al., 2015).

Our group has previously reported significant increases in peripheral tissue oxygenation during exercise in patients with PAD after both an acute dose of inorganic nitrate and following a chronic dose administered in combination with aerobic exercise training (Kenjale et al., 2011; Woessner et al., 2018a). In contrast to these promising findings, we found no significant changes to tissue oxygenation during treadmill exercise following nitrate supplementation (**Table 4.2** and **Figure 4.4**). This suggests that nitrate is ineffective at improving tissue oxygenation during exercise in patients with HFrEF. Healthy controls were not included in this investigation; therefore, it is unknown whether the current participants had a reduced tissue oxygenation compared to controls. It is possible that the response to supplement was limited due to the current participants not having a reduced peripheral tissue oxygenation at baseline. Further studies with more invasive means to measure tissue perfusion and oxygen extraction at rest and during exercise are needed to explore this hypothesis. It is also possible that a greater benefit could be achieved from supplementation if it were combined with another intervention (such as exercise training) (Woessner et al., 2018a).

Another suggested mechanism for the potentiation of nitrate supplementation is improved mitochondrial function. In healthy individuals, three days of supplementation reduced the oxygen cost during submaximal cycling, in part, by increased mitochondrial efficiency via decreases in LEAK respiration (Larsen et al., 2011; Larsen et al., 2007). However, in the current thesis (*Study 3*), we reported no significant changes in the primary outcome measure of maximal mitochondrial oxidative phosphorylation in HFrEF, and no changes were noted in the oxygen cost of submaximal exercise (*Study 1*). Furthermore, no changes in absolute or phosphorylated mTORC1, p38MAPK, Akt or PGC-1 α muscle protein content were observed (*Study3*). As plasma nitrate was significantly increased following the supplement, it is

possible that local impairments within the muscle are present which prevent the action of NO. Future studies should explore whether nitrate supplementation could have a synergistic effect when combined with other interventions known to improve mitochondrial function, such as exercise.

7.1.2 Nitrate supplementation effects on BP and vascular function

The present study indicated potentially clinically relevant changes in vascular function following nitrate supplementation including, improved endothelial function (*Study 1*), decreased TPR (*Study 2*) and reductions in BP during exercise (*Study 2*). There were no effects on measures of aortic pressure or stiffness (*Study 1*).

Vascular function is an important regulator of blood flow at rest and during exercise, and patients with CHF are characterised by vascular endothelial dysfunction, in part due to reduced NO bio-availability. Our findings, *Study 2*, indicate the possibility of a clinically important improvement in endothelial function (BAFMD) but this would need to be confirmed in future studies with a larger sample size. To our knowledge, no previous study has assessed the effect of nitrate supplementation on endothelial function in HFrEF. Previous studies in other clinical populations have demonstrated inconsistent findings with regards to the efficacy of nitrate supplementation on measures of FMD. Studies recruiting individuals with HTN or hypercholesterolemia have indicated improved FMD following chronic doses (>2 weeks) of nitrate supplementation (Kapil et al., 2015; Velmurugan et al., 2016). However, similar supplementation regimens in individuals with T2DM have had no beneficial effect on endothelial function (Gilchrist et al., 2013). The lack of effect in individuals with T2DM could be due to the myriad of biochemical perturbations (hyperglycemia, dyslipidemia and increased oxidative stress) present, many of which have been noted as attenuators of NO bioactivity (Velmurugan et al., 2016). The current study included patients with HFrEF with

and without T2DM, but was not designed nor powered to assess the potential effects of hyperglycemia (T2DM).

Herein we report a 37% increase in BAFMD, which is similar to previous reports in people with hypercholesterolemia (24%) and hypertension (20%) (Kapil et al., 2015; Velmurugan et al., 2016). The slightly higher magnitude of change in the current study could be due to dosing differences, 6.4mmol nitrate/day in previous studies compared to 16mmol nitrate/day in the current study. The mechanisms mediating the beneficial changes to BAFMD were outside the scope of the current study. However, a previous study in patients with hypercholesterolemia suggested that the improved FMD response was mediated through the increase in NO bioavailability (supplied via nitrate supplementation) offsetting the inflammation-induced oxidative stress and consequential excess NO scavenging (Velmurugan et al., 2016). Oxidative stress and inflammation are established consequences of CHF and thus it is possible the changes noted in *Study 2* could be explained by similar mechanisms. Future studies should seek to expand upon these positive initial findings in larger samples of HFrEF and potentially seek to achieve a sample size large and diverse enough to conduct sub-analyses to assess the effect of common comorbidities (e.g., T2DM).

Improved endothelial function following nitrate supplementation suggests an enhanced vasodilatory response to shear stress (Moncada, 1997; Nosarev et al., 2015). Thus, it would be expected that there would be reductions in TPR and BP following nitrate supplementation. While previous studies in other clinical cohorts have indicated that nitrate supplementation can reduce resting and exercise BP in those with PAD or HFpEF, no studies to date have reported similar reductions in patients with HFrEF (Eggebeen et al., 2016; Kenjale et al., 2011; Kerley et al., 2015; Zamani et al., 2017). In this thesis, neither the resting SBP nor

DBP in *Study 1*, nor in *Study 2* were significantly changed by nitrate supplementation. Similarly, no significant effects of nitrate dosing on SBP was observed at any stage of the CPX test (*Study 1*) nor during submaximal cycle exercise (*Study 2*). However, during submaximal exercise (*Study 2*) reductions in both DBP and MAP had medium to large ES following nitrate supplementation. The reduction in both DBP and MAP suggest a likely decrease in TPR, and, indeed, while TPR was not measured directly in *Study 2*, predictive equations suggest nitrate reduced TPR during submaximal exercise. Reductions in the BP at rest and during exercise can reduce the risk of future stroke, increase SV and assist in promoting the regression of LVH.

Endothelial function is an independent predictor of mortality and reductions in resting BP have significant clinical implications (Fischer et al., 2005). For instance, a 10 mmHg reduction in SBP or 5 mmHg reduction in DBP have been associated with a 22% lower risk of CAD events and 41% lower risk of stroke (Law et al., 2009). While the sample sizes for the vascular function and BP studies were relatively small, the changes following nitrate supplementation (BAFMD: 37% increase, DBP Δ : stage 1= 9 mmHg, stage 2=5 mmHg, stage 3= 10 mmHg) were of potential clinical significance and warrant further investigation in larger studies

7.1.3 Nitrate supplementation and \dot{Q} and SV during exercise

Inorganic nitrate supplementation may lead to clinically important improvements in cardiac performance (\dot{Q} and SV). The small sample size in this exploratory arm of the overall study is likely a contributing factor to the changes being non-significant; however, the results suggest a potentially clinically beneficial effect.

Previous studies have indicated inconsistent findings regarding the efficacy of nitrate/nitrite supplementation at improving measures of cardiac performance during exercise in patients with CHF (Borlaug et al., 2015; Ormerod et al., 2015). Two studies, one in HFrEF and one in HFpEF, utilising a direct infusion of sodium nitrite reported significant increases in SV and \dot{Q} during exercise (Borlaug et al., 2015; Ormerod et al., 2015). The HFpEF trial also demonstrated significant increases for both measures at rest, but noted the beneficial effects were more pronounced during exercise (Borlaug et al., 2015). Infusions, however, often lead to supra-physiological levels of circulating nitrate/nitrite, and the effect is often short in duration which makes it not a viable strategy for chronic changes (Cosby et al., 2003).

Studies with oral inorganic nitrate supplementation are less clear, with previous publications indicating a beneficial effect in HFpEF but no effect in HFrEF. The study in HFpEF also found significant improvements in \dot{Q} , and suggested that the concomitant reduction in TPR mediated the change (Zamani et al., 2015). Similarly, we hypothesise that the reduction in TPR in the present study also facilitated the improvements in SV and \dot{Q} . The single previous study on inorganic nitrate supplementation and cardiac performance in HFrEF found no change in \dot{Q} and SV as measured by an electrical impedance technique, (Hirai et al., 2017). To our knowledge, this relatively new technique has only been assessed for accuracy in one previous study, which found it significantly over-estimates values of cardiac performance compared to the Fick method (Kemps et al., 2008).

This is the first study in HFrEF to indicate a potential beneficial effect of inorganic nitrate supplementation, as opposed to the nitrite infusions. The small number of published studies, as well as the variations in assessment techniques and intervention methodologies, make it difficult to draw firm conclusions on the efficacy of nitrate in patients with HFrEF. However,

the positive findings of the present study warrant further investigation in future studies with larger and more diverse samples.

7.2 Thesis limitations

The findings of this thesis should be considered in conjunction with the potential limitations of the project. While the sample size for *Study 1* makes this the largest trial to date to assess the effects of inorganic nitrate on aerobic capacity, the sample sizes for the other two studies are relatively small. Cohen's d ES were calculated for these studies in order to assess the potential clinical importance of the findings, but future studies with larger sample sizes should be conducted to confirm these findings.

Study recruitment was open to both males and females; however, the majority of the patients ($n=15$) enrolled were males. This is perhaps unsurprising given that while the overall incidence of CHF is similar between men and women, women are 65% less likely to develop HFrEF than their male counterparts (Ho et al., 2013). However, women with HFrEF are vastly underrepresented in the published clinical trials in nitrate supplementation (28 male participants versus 10 females in published trials to date) (Coggan et al., 2018; Coggan et al., 2015; Hirai et al., 2017; Kerley et al., 2016). The dearth of female participants makes it challenging to draw conclusions on the potential effect of the supplementation in women with HFrEF.

Under ideal conditions, supplementation days prior to each testing round of the trial would have been kept precisely consistent for all participants. Due to availability of medical staff as well as the participants' availability, this was not always practically possible. However, the dosing days prior to each testing visit were tightly controlled between the testing rounds for

each individual participant, and there were no significant differences noted between the mean dosing days prior to each visit on the placebo versus nitrate intervention (Coggan et al., 2018; Zamani et al., 2015).

7.3 General conclusions and suggestions for future research

Overall this thesis suggests that nitrate supplementation does not provide a clinical benefit to measures of submaximal or peak exercise performance on a treadmill in HFrEF. There were some potential effects on vascular function and exercise hemodynamics, and the improvements in cardiac performance during exercise have some clinical implications. However, larger sample sizes would be required to fully elucidate this potential. Future investigations are warranted to further explore the following research questions:

7.3.1 What is the optimal dosing strategy in HFrEF?

As alluded to previously, there is no firm consensus on the optimal dosing strategy for nitrate supplementation in HFrEF, CHF or any other population. While the previous literature tends to suggest a higher chronic dose is more efficacious, results from this study potentially contradict this. The present study utilised a chronically high dose (16 mmol a day), but the dose provided two and a half hours prior to each testing visit was relatively small in comparison to other studies (6.4mmol). This could suggest that a higher acute dose two and a half hours prior to the testing visit might still be required even in a chronic-dosing regimen. There have been no studies in HFrEF assessing the differential effects of acute and chronic dosing.

7.3.2 What are the normative values for plasma nitrate and nitrite in HFrEF?

The findings of this thesis challenge the original assertion that increases in NO bioavailability could improve the aerobic and vascular function of individuals with HFrEF. While previous

studies have established the deleterious effects of vascular dysfunction in CHF, improving NO bioavailability in HFrEF has not resulted in significant improvements in the vascular function. It is therefore plausible that alterations in NO bioavailability offer little clinical benefits in this classification of CHF. No studies to date have sought to determine normative values for circulating plasma nitrate and nitrite in CHF and as there are varying methodologies for quantifying these measures, it is challenging to compare values between studies. Thus future studies should consider

7.3.3 What is the effectiveness of nitrate supplementation in decompensated HFrEF?

The exercise tolerance results suggest that enhancing NO bioavailability in HFrEF does not significantly improve maximal aerobic exercise performance on a treadmill. However, future studies could explore the potential effects of this intervention in patients with either decompensated or acute HFrEF. While outside the scope of this individual study, there is some indication from the literature that these other patient populations in particular have reductions in resting plasma nitrite coupled with high levels of eNOS inhibitor, making them prime candidates for interventions aiming to increase NO bioavailability (Falls et al., 2017; Saitoh et al., 2003).

7.3.4 Are there other interventions targeting improvements in peripheral tissue function that could be efficacious when combined with nitrate supplementation in HFrEF?

While targeting reductions in NO bioavailability in HFpEF seems to hold promise, nitrate supplementation appears ineffective at improving aerobic or vascular function in patients with stable HFrEF. Future studies could consider exploring the efficacy of alternative interventions, such as adapted exercise programs, which specifically target improvements in the function of the peripheral skeletal muscle tissue.

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APPENDIX A- Recruitment cover letter



Date:

Insert Name and address

Dear ,

Thank you for expressing an interest to a staff/volunteer member at One Heart Cardiology in receiving information about a research trial. The project is currently being run by Western Health and Victoria University, and we are testing the effects of beetroot juice supplementation on exercise tolerance in patients with heart failure. We would like to invite you to participate in the project if you are interested.

You will find an information flier enclosed in this envelope that provides a brief overview of the project. Knowing what is involved will help you decide if you want to take part in the research.

The study will be conducted at Sunshine Hospital at Western Health. Please note, if you do not have transport, we can provide this for you at no cost. You will also be reimbursed for your time at the completion of the study with \$100 Coles/Meyer gift card.

If you are interested in participating (or would like some more information to help you decide), please contact the trial coordinator, Mary Woessner at 04 2169 2161. Please know that you are under no obligation to be involved if you do not wish to.

Kind Regards,

Professor Jason Allen

Principal Investigator

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APPENDIX B- Informed consent



Western Health



**Participant Information Sheet/Consent Form
Interventional Study**

Title

Effects of Dietary Inorganic Nitrate
Supplementation on Exercise Performance in Heart
Failure

Protocol Number

2015.136

**Coordinating Principal Investigator/
Principal Investigator**

Professor Jason David Allen

Associate Investigator(s)

A/Prof Christopher Neil, A/Prof Itamar Levinger,
Ms Mary Woessner,

Location

Western Health (Sunshine Hospital)/ Victoria
University

Part 1 What does my participation involve?

1 Introduction

You are invited to take part in this research project because you have heart failure.

This Participant Information contains detailed information about the research project. Its purpose is to explain to you as openly and clearly as possible all the procedures involved in this project before you decide whether or not to take part in it.

Please read this Participant Information carefully. Feel free to ask questions about any information in the document. You may also wish to discuss the project with a relative or friend or your local health worker. Feel free to do this.

Once you understand what the project is about and if you agree to take part in it, you will be asked to sign the Consent Form. By signing the Consent Form, you indicate that you understand the information and that you give your consent to participate in the research project.

You will be given a copy of the Participant Information and Consent Form to keep for your personal records.

2 What is the purpose of this research?

The purpose of this study is to determine if beetroot juice supplementation can improve exercise tolerance in patients with heart failure. Furthermore, this study will act as a pilot study for a planned larger project incorporating both Beetroot juice supplementation and exercise training.

A total of **30** people will participate in this project.

Previous experience has shown that beetroot juice supplementation improves exercise capacity in patients with heart failure, as well as patients with peripheral artery disease.

You are invited to participate in this research project because you have a diagnosis of heart failure. The results of this research may be used to help researcher Ms Mary Woessner to obtain her PhD and Ms Cassandra Smith obtain her Masters' degree. This project is investigator-initiated by Professor Jason Allen and it is funded by a Central Research Grant Scheme award.

3 What does participation in this research involve?

You will be participating in a randomised controlled research project. Sometimes we do not know which treatment is best for treating a condition. To find out we need to compare different treatments. We put people into groups and give each group a different treatment. The results are compared to see if one is better. To try to make sure the groups are the same, each participant is put into a group by chance (random).

Participation in this project will involve your attendance at a total of 7 appointments, for approximately 1-2 hours each. You will have one screening appointment, and then two separate testing rounds (Round 1 and Round 2), each consisting of three appointments. The purpose of these two testing rounds is to assess your body's physiological response to the consumption of

beetroot juice versus a placebo. The three appointments in each testing round will be identical in procedure with the only difference being that you will consume beetroot juice for a minimum of 5 days prior to one round and a placebo (a drink manufactured to look and taste the same, but with the active ingredient-nitrate-removed) prior to the other.

4 What do I have to do?

During the three appointments we will ask you to participate in exercise testing, vascular testing (blood flow), blood draws and muscle biopsies (optional). If you do not wish to complete the muscle biopsy visit, please indicate this preference on the consent form by checking the appropriate opt out box. An exact schedule of appointments will be provided to you on enrolment. Included in this schedule will be the location of appointments. All exercise testing will be conducted at Sunshine Hospital and supervised by a medical doctor. Other testing visits will occur either at Sunshine Hospital or Victoria University.

The initial screening visit will consist of an exercise test which will last no longer than 15-20 minutes. The entire visit will be supervised by a medical doctor. The test involves walking on a treadmill, with gradual increases to the speed and incline every minute until you reach exhaustion. You will be asked to breathe through a special mouthpiece to record your oxygen usage. During this test we will monitor the electrical activity in your heart with a 12 lead electrocardiogram (ECG), as well as with a PhysioFlow® device. The ECG measures the electrical conductance of the heart (for safety purposes), while the PhysioFlow® measures the electrical impedance (or the resistance in the signals) and uses this to predict functional measures of how your heart is working.

For the ECG monitoring, 10 electrodes will be used. The site preparation for the ECG placement includes shaving the sites with a razor, and cleansing the skin with alcohol prep pads. Following this a total of 10 electrodes will be placed on the chest, the arms and your hips. Once the electrodes are placed, the corresponding wires will be attached and the machine will begin to measure the electrical activity in the heart.

The procedures for the PhysioFlow® device setup include shaving the placement sites with a disposable razor and cleaning each site with an alcohol prep pad. The sites will also be prepped by using Nuprep® abrasive gel with a paper towel. Following this, a total of 6 electrodes will be placed on the neck, and either on your chest or your back.

Both the ECG and the Physioflow® are non-invasive tools. They will record the electrical impulses that make your heart beat. The ECG will generate waves based on this activity that will be monitored throughout the test by medical staff to ensure your safety. The Physioflow® will use this electrical activity combined with your heart rate and body surface area to predict the function of your heart.

Before your first appointments for either visit one or visit two, you will be asked to consume 210mL of either the active treatment (beetroot juice) or the passive treatment (placebo) twice daily, for a minimum of 5 days. The time that you drink these prior to your appointments will be important and further information will be given to you regarding the timing when the appointments are scheduled.

For each testing round (round 1 and 2), you will be asked to complete three appointments.

During appointment one for each round, you will complete a maximal exercise test on a treadmill similar to the one performed at the screening visit. At this testing visit there will be a six-minute

low-intensity stage for you to complete on the treadmill before starting the test. We will also be collecting blood at rest, during exercise and following exercise at this visit. Similar to the screening visit, we will monitor your heart's activity via ECG and the PhysioFlow®. During appointment two, you will be asked to arrive fasted (having not eaten or drank anything aside from water or the treatment beetroot juice for 8 hours). We will perform four non-invasive measures of vascular function (blood flow) while you are lying down resting. The assessments involve the use of multiple blood pressure cuffs and an ultrasound machine. While none of the procedures should be painful, the pressure on the blood pressure cuffs can sometimes be slightly uncomfortable.

Following these measures, you will be offered a snack prior to completing three bouts of six-minute low-intensity exercise test on a bicycle. You will have 5 minutes of rest between each exercise bout. During each stage a trained technician will perform an echocardiography. An echocardiography is a painless, non-invasive assessment that uses an ultrasound to capture moving pictures of your heart.

Appointment three consists of a muscle biopsy (optional).

You will be asked to arrive in the morning (between 7-9am) after an overnight fast and then rest on a bed in the laboratory. A blood sample will be taken from a blood vessel in your arm to check your blood sugar, insulin levels, blood cholesterol levels and liver function. Also we will measure some markers of metabolic health and inflammation. The blood sample will be drawn by a person qualified in the technique using clean materials. The amount of blood that will be drawn is about 15 ml.

The muscle biopsy procedure is used to obtain small samples of your muscle tissue for analysis of proteins, genes and energy sources. Before the test we will ask you to wear shorts in order for the doctor to have access to your thigh muscles.

An injection of a local anaesthetic is made in the skin overlying the muscle in your thigh, and then a small incision (approx. 0.6 cm long) is made in the skin. The biopsy needle is then inserted into your muscle and a small piece of tissue removed from the muscle. During this part of the procedure you will feel pressure and this will be quite uncomfortable and you may also experience some pain, but will last for only about 2-3 seconds. When the small piece of muscle is removed you may also experience a mild muscle cramp, but this only persists for a few seconds. The size of muscle removed by the biopsy needle is similar to 3-4 grains of rice. This poses no long-term effects for your muscle and will not be noticeable to others apart from a small scar on the skin for a few months.

Following the biopsy the incision will be closed using a steri-strip and covered by a transparent waterproof dressing. Then a pressure bandage will be applied which should be maintained for 24-48 hours. Steri-strip closure should be maintained for a few days. It is common for participants to experience some soreness in the muscle over the next 2-3 days, however this passes and does not restrict movement. In some rare cases mild haematomas (a swelling of blood under the skin) have been reported, but these symptoms disappear within a week. A medical practitioner will perform the whole procedure under sterile conditions.

On very rare occasions, some people have reported altered sensation (numbness or tingling) in the skin near the site of the biopsy. This is due to a very small nerve being cut, but this sensation disappears over a period of a few weeks-to-months. Although the possibility of infection, significant bruising and altered sensation is quite small, if by chance it does eventuate, please inform us immediately and we will immediately consult the doctor who performed the biopsy to review the reported problems and recommend appropriate action.

At two of your appointments (the maximal treadmill tests), blood samples will be taken from a catheter placed in your arm. No more than 50 mL of blood (3.5 tablespoons - or roughly 1/10 of the volume collected during blood donation) will be collected.

5 Other relevant information about the research project

You will not be paid for your participation in this project. However, you will be reimbursed \$100 in the form of a Coles voucher at the completion of the study. Additionally, if you are unable to drive, we will provide cab vouchers to cover the cost of your transport to and from the testing facilities on testing days.

6 Do I have to take part in this research project?

Participation in any research project is voluntary. If you do not wish to take part, you are not obliged to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage.

Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect your routine treatment, your relationship with those treating you or your relationship with Sunshine Hospital or Victoria University.

Before you make your decision, a member of the research team will be available to answer any questions you have about the research project. You can ask for any information you want. Sign the Consent Form only after you have had a chance to ask your questions and have received satisfactory answers.

If you decide to withdraw from this project, please notify a member of the research team before you withdraw. This notice will allow that person or the research supervisor to inform you if there are any health risks or special requirements linked to withdrawing.

7 What are the alternatives to participation?

You do not have to take part in this research project to receive treatment at Sunshine hospital.

8 What are the possible benefits of taking part?

We cannot guarantee or promise that you will receive any benefits from this project. We hope that in the future, the information gathered from this study will be used to help design new treatments to improve exercise capacity in patients with heart failure.

9 What are the possible risks and disadvantages of taking part?

There may be side effects that the researchers do not expect or do not know about and that may be serious. Tell your doctor immediately about any new or unusual symptoms that you get. Many side effects go away shortly after treatment ends. However, sometimes side effects can be serious, long lasting or permanent. If a severe side effect or reaction occurs, your study doctor may need to stop your treatment.

Your study doctor will discuss the best way of managing any side effects with you. Medical treatments often cause side effects. You may have none, some or all of the effects listed below, and they may be mild, moderate or severe. If you have any of these side effects, or are worried

about them, talk with your study doctor. Your study doctor will also be looking out for side effects.

Possible risks, side effects and discomforts include general feelings of muscle soreness (like those felt after exercise). We anticipate that if you experience this, that it will be short lived. Exercise tests will be performed under the supervision of medical staff, and those unsafe to exercise will not be asked to.

Risks of Exercise Test:

The risks of performing an exercise test are small, and complications from the test are rare. They include but are not limited to fainting, falling, irregular heartbeats, wheezing and shortness of breath, and, very rarely, heart attack or death (less than 1 in 10,000 cases). The exercise test will be performed under the supervision of medical staff equipped to deal with emergencies.

Risks of ECG:

There may be some irritation or redness where the electrodes (stickers) are placed on the subject's chest to monitor the heart's activity.

Risks of Drawing Blood/Biopsy:

Risks associated with drawing blood from the arm include momentary discomfort and/or bruising. Infection, excess bleeding, clotting, or fainting are also possible, although unlikely. Sterile techniques, similar to those used in a doctor's office, are used to minimize these risks. Having a tissue sample taken can also cause some discomfort, bruising, minor infection or bleeding. All of these are easily treatable. You will be monitored during the procedure and a study team member will contact you after the procedure to ensure that you are recovering as expected.

Blood pressure cuff inflation:

The blood pressure cuff will squeeze the arm tightly. However, any discomfort will be alleviated as soon as the pressure in the cuff is released. This process can be uncomfortable but should not be painful.

Risks of ingesting beetroot juice concentrate:

There are no known risks associated with consumption of beetroot juice concentrate in the doses used in this study. In rare instances, people may have allergic reactions to product ingredients. One common side effect is urine discolouration following beetroot juice consumption (it may turn pink for a day or two), but this is not indicative of anything more serious, and you should not be alarmed.

10 What will happen to my test samples?

This study involves mandatory blood tests, which will provide valuable information about the amount of nitrate circulating in your body.

The muscle biopsies are optional, and samples will be analysed to determine the muscle's ability to use oxygen (an important component of physical fitness).

The tissue samples will be coded with a re-identifiable ID number. They will be stored in a specialised freezer at Victoria University, which will be accessible only to research staff involved in this study. While the tissue will not necessarily be destroyed (unless you request for

it to be) it will not be used for further research projects without your express permission and approval from the appropriate research ethics committee.

You will be asked to provide additional consent for the collection of your blood and muscle tissue during the research project.

11 What if new information arises during this research project?

During the research project, new information about the risks and benefits of the project may become known to the researchers. If this occurs, you will be told about this new information. This new information may mean that you can no longer participate in this research. If this occurs, the person(s) supervising the research will stop your participation. In all cases, you will be offered all available care to suit your needs and medical condition.

12 Can I have other treatments during this research project?

Whilst you are participating in this research project, you may not be able to take some or all of the medications or treatments you have been taking for your condition or for other reasons. It is important to tell your study doctor and the study staff about any treatments or medications you may be taking, including over-the-counter medications, vitamins or herbal remedies, acupuncture or other alternative treatments.

You should also tell your study doctor about any changes to these during your participation in the research project. Your study doctor should also explain to you which treatments or medications need to be stopped for the time you are involved in the research project. Whilst you are participating in this research project, you may not be able to take some or all of the medications or treatments you have been taking for your condition or for other reasons. It is important to tell your study doctor and the study staff about any treatments or medications you may be taking, including over-the-counter medications, vitamins or herbal remedies, acupuncture or other alternative treatments

13 What if I withdraw from this research project?

If you decide to withdraw from this project, please notify a member of the research team before you withdraw. This notice will allow that person or the research supervisor to inform you if there are any health risks or special requirements linked to withdrawing.

14 Could this research project be stopped unexpectedly?

This research project may be stopped unexpectedly for a variety of reasons. One possible reason could be unacceptable side effects of the supplement. If the research is stopped you will be immediately notified.

15 What happens when the research project ends?

We hope that results from this study will be published in a medical journal. Information will be reported as a group data and you will not be individually identified. We will also be happy to provide you, upon request, with the results of the project and/or relevant publications.

Part 2 How is the research project being conducted?

16 What will happen to information about me?

By signing the consent form you consent to the study doctor and relevant research staff collecting and using personal information about you for the research project. Any information obtained in connection with this project and that can identify you will remain confidential. It will only be disclosed with your permission, except as required by law.

If you give us your permission by signing the Consent Form, we plan to publish the results in scientific medical journals. In any publication, information will be provided in such a way that you cannot be identified. Any information obtained in connection with this project and that can identify you will be stored in a password locked computer and will remain accessible only to the research team responsible for this project. Upon enrolment in the study, all personal health information will be stored separate to research data collection sheets. All data collection will be conducted using coded subject ID's free of any identifying information.

Study investigators will also enter data manually into individual (paper) case report forms, but these do not contain identifying information (they each have unique study code).

Following completion of the study, you will be given the results from your testing appointments. In accordance with relevant Australian and/or Victorian privacy and other relevant laws you have the right to access the information collected and stored by the researchers about you. You also have the right to request that any information with which you disagree be corrected. Please contact one of the researchers named below if you would like to access your information.

17 Complaints and compensation

If you require further information or if you have any problems concerning this project (for example, any side effects), you can contact the principal researcher.
The researchers responsible for this project are:

Principle researcher: A/Prof Jason David Allen (03 9919 4264)

Co-ordinating researcher: Miss Mary Woessner (04 2169 2161)

If you suffer any injuries or complications as a result of this research project, you should contact the study team as soon as possible and you will be assisted with arranging appropriate medical treatment.

If you have any complaints about any aspect of the project, the way it is being conducted or any questions about your rights as a research participant, then you may contact:

Position:	Manager, Western Health Office for Research
Telephone:	(03) 8395 8073
Email:	ethics@wh.org.au

(You will need to tell the Manager the name of one of the researchers given in section 17 above.)

In the event of loss or injury, immediate medical care will be available through Sunshine hospital. However, the study team do not commit to providing monetary compensation or care for treatment of a study-related injury.

18 Who is organising and funding the research?

The research project is investigator initiated and sponsored through a Central Research Grant Scheme at Victoria University. The project is also now funded by a Vanguard grant through the National Heart Foundation of Australia.

19 Who has reviewed the research project?

This project will be carried out according to the *National Statement on Ethical Conduct in Human Research* (2007) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of people who agree to participate in human research studies. The ethical aspects of this research project have been approved by the Western Health/Melbourne Health Human Research Ethics Panel. Professor Jason David Allen, Director of Clinical Exercise Science and Rehabilitation at Victoria University, has given approval for parts of the assessments to be carried out at Victoria University's laboratories.



Western Health

Consent Form - *Adult providing own consent*

Title Effects of Dietary Inorganic Nitrate Supplementation on Exercise Performance in Heart Failure

Protocol Number 2015.136

Coordinating Principal Investigator/ Principal Investigator Professor Jason David Allen

Associate Investigator(s) A/Prof Christopher Neil, A/Prof Itamar Levinger, Ms Mary Woessner

Location Western Health (Sunshine Hospital)/ Victoria University

Declaration by Participant

I have read the Participant Information Sheet or someone has read it to me in a language that I understand.

I understand the purposes, procedures and risks of the research described in the project.

I have had an opportunity to ask questions and I am satisfied with the answers I have received.

I freely agree to participate in this research project as described and understand that I am free to withdraw at any time during the study without affecting my future health care.

I understand that I will be given a signed copy of this document to keep.

Name of Participant (please _____)

Signature _____ Date _____

Name of Witness* to Participant's Signature (please print) _____

Signature _____ Date _____

* Witness is not to be the investigator, a member of the study team or their delegate. In the event that an interpreter is used, the interpreter may not act as a witness to the consent process. Witness must be 18 years or older.

Declaration by Study Doctor/Senior Researcher[†]

I have given a verbal explanation of the research project, its procedures and risks and I believe that the participant has understood that explanation.

Name of Study Doctor/ Senior Researcher [†] (please print) _____
Signature _____ Date _____

[†] A senior member of the research team must provide the explanation of, and information concerning, the research project.

Note: All parties signing the consent section must date their own signature.

I understand that, if I decide to discontinue the study treatment, I may be asked to attend follow-up visits to allow collection of information regarding my health status. Alternatively, a member of the research team may request my permission to obtain access to my medical records for collection of follow-up information for the purposes of research and analysis.

Blood Samples

I consent to the storage and use of blood samples taken from me for use, as described in the relevant section of the Participant Information Sheet, for:

- This specific research project.

By signing this consent section, I agree to the use of my tissue and blood samples for testing, as outlined in the relevant Section of the Participant Information Sheet.

Name of Participant (please _____
Signature _____ Date _____

Name of Witness* to Participant's Signature (please print) _____
Signature _____ Date _____

* Witness is not to be the investigator, a member of the study team or their delegate. In the event that an interpreter is used, the interpreter may not act as a witness to the consent process. Witness must be 18 years or older.

Name of Study Doctor/ Senior Researcher [†] (please print) _____
Signature _____ Date _____

† A senior member of the research team must provide the explanation of and information concerning the research project.

Note: All parties signing the consent section must date their own signature.

Muscle Biopsy Sample

I consent to the muscle biopsy procedure as well as the storage and use of the muscle tissue samples taken from me for use, as described in the relevant section of the Participant Information Sheet, for:

- This specific research project.

Name of Participant (please _____ Signature _____ Date _____

Name of Witness* to Participant's Signature (please print) _____ Signature _____ Date _____
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* Witness is not to be the investigator, a member of the study team or their delegate. In the event that an interpreter is used, the interpreter may not act as a witness to the consent process.

Witness must be 18 years or older.

Name of Study Doctor/ Senior Researcher† (please print) _____ Signature _____ Date _____

† A senior member of the research team must provide the explanation of and information concerning the research project.

Note: All parties signing the consent section must date their own signature.



Western Health

Form for Withdrawal of Participation - Adult providing own consent

Title Effects of Dietary Inorganic Nitrate Supplementation on Exercise Performance in Heart Failure

Protocol Number 2015.136

Principal Investigator Professor Jason Allen

Associate Investigator(s) Dr. Christopher Neil, Associate Professor Itamar Levinger, Ms. Mary Woessner

Location Sunshine Hospital/ Victoria University

Declaration by Participant

I wish to withdraw from participation in the above research project and understand that such withdrawal will not affect my routine treatment, my relationship with those treating me or my relationship with Sunshine Hospital or Victoria University.

Name of Participant (please _____)
Signature _____ Date _____

In the event that the participant's decision to withdraw is communicated verbally, the Study Doctor/Senior Researcher will need to provide a description of the circumstances below.

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Declaration by Study Doctor/Senior Researcher[†]

I have given a verbal explanation of the implications of withdrawal from the research project and I believe that the participant has understood that explanation.

Name of Study Doctor/ Senior Researcher [†] (please print) _____
Signature _____ Date _____

† A senior member of the research team must provide the explanation of and information concerning withdrawal from the research project. Note: All parties signing the consent section must date their own signature

