Oxygen utilisation during exercise – a potential therapeutic target in chronic obstructive pulmonary disease

by

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Submitted for the degree of PhD

DECLARATION OF ORIGINALITY

The data present in this thesis are the result of my original work. Where appropriate the contribution made by other persons has been appropriately acknowledged.

STATEMENT OF CONTRIBUTION

The studies described in this thesis were performed between 2015 and 2017 during my PhD candidature at the National Heart and Lung Institute, Imperial College London. This thesis was written wholly by myself with the guidance of my supervisor's Dr Nicholas Hopkinson and Professor Michael Polkey.

Dr Hopkinson and Professor Polkey developed the original idea for the research studies encompassed within this thesis. With the assistance of Dr Hopkinson, I designed and wrote the trial protocols. With the assistance of Dr Banya, I designed the statistical analysis plan for both trials.

For the studies presented in Chapters three, four and five subject trial visits and data collection was undertaken by myself (including blood sampling, exhaled nitric oxide, flow mediated dilatation and spirometry). When this was not possible assistance was provided by Dr Adam Lewis (Research Physiotherapist) and Ms. Sara Buttery (Research Physiotherapist). I undertook trial outcome assessments including exercise parameters (including incremental shuttle walk tests and endurance shuttle walk tests).

The pulmonary rehabilitation teams at the Royal Brompton and Harefield NHS Foundation Trust, the Taunton and Somerset NHS Foundation Trust, Oxleas NHS Foundation Trust and

Maidstone and Tunbridge Wells NHS Foundation Trust provided the pulmonary rehabilitation intervention described in Chapter three. These teams also helped identify potential trial subjects and assisted with data collection when required.

Venous blood sampling was undertaken by myself as was the samples initial processing. Professor Feelisch, Dr Fernandez and Miss Mikus-Lelinsk (University of Southampton) undertook plasma nitrite and nitrate analysis.

All data within this thesis was analysed by myself. Dr Winston Banya assisted with the Hodges-Lehman estimator of shift parameters.

All sources of information have been referenced within the text.

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ABSTRACT

This thesis addresses the impact of nitrate supplementation in the form of beetroot juice as a potential therapeutic intervention to improve exercise capacity in people with chronic obstructive pulmonary disease (COPD). Dietary nitrate supplementation can enhance levels of plasma nitrite (NO_2^-), nitrate (NO_3^-) and nitric oxide (NO). Nitric oxide is a ubiquitous signalling molecule with impacts on systemic and pulmonary vasculature. It also effects skeletal muscle oxygen consumption.

Within this thesis, I set out the background and evidence so far for NO_3^- supplementation in healthy individuals and those with COPD. To date studies have been small, with some conflicting findings. Specific potential responder phenotypes have not been selected for trials.

I undertook a randomised controlled trial, comparing the effect of 140 mLs concentrated nitrate-rich beetroot juice (NR-BRJ) cut with organic lemon juice (2%) containing 0.8 g (12.9 mmol) NO_3^- (140 mL BEET-IT® SPORT Shot, James White Drinks, Ipswich, UK) against a placebo (PL-BRJ) beverage which was otherwise identical, except that the NO_3^- had been removed by passage through an ion exchange column. Participants with COPD consumed one or other beverage before each session of an eight-week pulmonary rehabilitation programme. Data were available for 122 study completers. The median (IQR) improvement in the ISWT distance following PR was statistically greater with NO_3^--rich NR-BRJ than PL-BRJ, increasing by 60 (10, 85)m compared to 30 (0, 70)m, an estimated treatment effect 30 m (95%CI: 10, 40); p = 0.027.

In a single dose cross–over study in individuals with COPD who were established on long term oxygen therapy, endurance shuttle walk time was statistically significantly longer with NO_3^-

supplementation compared to placebo 194.6 (147.5, 411.7)sec vs 159 (121.9, 298.5)sec, estimated treatment effect 62 sec (95%CI: 33, 106); p 0.0009.

I also demonstrated that NO_3^- supplementation was associated with a fall in blood pressure and an improvement in endothelial function, assessed using flow-mediated dilatation.

Dietary NO_3^- supplementation enhances exercise performance in hypoxic individuals with COPD and augments the effects of pulmonary rehabilitation. There may also be effects that are beneficial for cardiovascular co-morbidities which are extremely common in COPD.

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What can I say of the Muscle Laboratory? When walked through the doors of this ramshackle room which carries such historic significance, I was very conscious of the many a great researcher who originated here. For me, the Muscle Laboratory became not only my place of work but also became my second home with my second family. I have made lifelong friends here. Sara, you are an absolute star, we have laughed together, eaten, drank and cried. It has been amazing. I look forward to seeing your rise to fame. Adam, where to start, your dad-jokes, your choice in golfing trousers (dubious at best!), the never-ending puns (so many puns) and your unflinching friendship. This experience would have been less without you. Then there is the "Interventionalists" a band of talented misfits if I've ever seen one, J-Dog on piano, K-Rex

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It would be remis of me not to thank the Pulmonary Rehabilitation teams that allowed me to bother them throughout my time undertaking these studies. Bhav and Helen you have taught me a great deal about Pulmonary Rehabilitation and have ignited a passion of exercise in healthcare that I plan on pursuing throughout my career. On a similar note, I must say a big thank you to the respiratory physiology department you have all played a huge part in my time in the Muscle Laboratory and confirmed (much to my surprise) I truly love respiratory physiology.

To all the individuals who took part in the studies which make up this thesis I shall be always grateful. You have taught me more about COPD than any textbook, lecture or research paper ever could. I have met some truly remarkable people. Your teachings will always be with me.

Finally, I must thank those closest to me without whom I would not be in the position I am in today. Mum and Dad, I am a product of your love and upbringing. Everything I achieve you have made possible. Thank you for just being you. Yannie (the wife!) you have lived this thesis as much as I have. Without your love and patience and constant support I would not have even been able to start this let alone finish it. I love you and I cannot thank you enough. LogoDog, you have been my break in the wind and rain and a companion at my feet. Thank you fur-baby. Finally, Emilia. We thought you would never come but here you are. I would not bother reading this baby girl. Just know (to quote Bryan Adams) “everything I do I do it for you”.

Dedication

I dedicate this thesis to Emilia. I hope as your dad I can make you proud.

ABBREVIATIONS

ATP	Adenosine Triophosphate
ATS	American Thoracic Society
BIA	Bioelectrical Impedance Analysis
BMI	Body Mass Index
BP	Blood Pressure
BRJ	Beetroot juice
CAGs	Circulating Angiogenic Cells
CAT SCORE	COPD Assessment Test Score
CO	Carbon Monoxide
COPD	Chronic Obstructive Pulmonary Disease
DASH	Dietary Approaches to Stop Hypertension
DBP	Diastolic Blood Pressure
DH	Dynamic Hyperinflation
DL_{CO}	Diffusion Cappacity for Carbon Monoxide
EELV	End Expiratory Lung Volume
EDEN-OX	Effect of Dietary Nitrate supplementation on Exercise Performance in Hypoxia
EDRF	Endothelial Derived Relaxing Factor
EF	Endothelial Function
ENO	Exhaled Nitric Oxide
ERS	European Respiratory Society
ESWT	Endurance Shuttle Walk test
F_ENO	Fractional Exhaled Nitric Oxide
F_ENO_{50}	Fractional Exhaled Nitric Oxide at a Flow Rate of 50 mL
FEV1	Forced Expiratory Flow in 1 second
FFM	Free Fat Mass
FFMI	Free Fat Mass Index
FVC	Forced Vital Capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
eGFR	estimated Glomerular Filtration Rate
HADs	Healthcare Anxiety and Depression Scale

HADS A	Hospital Anxiety and Depression Scale Anxiety
HADS D	Hospital Anxiety and Depression Scale Depression
HPLC	High Performance Liquid Chromatography
ICS	Inhaled Corticosteroid
ISWT	Incremental Shuttle Walk Test
ITP	Intrathoracic Pressure
KNO_3	Potassium Nitrate
LABA	Long-acting Beta-agonist
LAMA	Long-acting Muscarinic-agonist
MAP	Mean Arterial Pressure
MET	Metabolic Equivalent of Task
MCID	Minimal Clinical Important Difference
MID	Minimal Important Difference
MRC	Medical Research Council
N_ANO_3	Sodium Nitrate
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric Oxide Synthase
NEM	N-Ethylmaleimide
cNOS	Constrictive Nitric Oxide Synthase
NOS	Nitric Oxide Synthase
NOS1	Neuronal NOS
NOS2	Inducible NOS
NOS3	Endothelial NOS
O_2	Oxygen
ON-EPIC	Oral Nitrate Supplementation to Enhance Pulmonary Rehabilitation in Chronic Obstructive Pulmonary Disease
PA	Physical Activity
PAL	Physical activity level

PPB	Parts per Billion
PBS	Phosphate Buffered Saline
PR	Pulmonary Rehabilitation
QoL	Quality of Life
REC	Research Ethics Committee
RV	Residual Volume
sBP	Systolic Blood Pressure
SD	Standard Deviation
TLC	Total Lung Capacity
UK	United Kingdom
$\dot{V}O_2$	Oxygen Uptake
$\dot{V}O_{2peak}$	Peak Oxygen Uptake
W	Workload
WHO	World Health Organisation

PRIZES ARISING FROM THIS THESIS

- The Royal College of Physicians: Turner-Warwick Lecturer Winner. Kent, Surrey and Sussex 2019 – 'Oral dietary nitrate supplementation to enhance pulmonary rehabilitation in COPD: a multicentre, double-blind, placebo- controlled study"

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PUBLICATIONS ARISING FROM THIS THESIS

- Oral nitrate supplementation to enhance pulmonary rehabilitation in COPD: ON-EPIC a multicentre, double-blind, placebo-controlled, randomised parallel group study

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CHAPTER 1: INTRODUCTION

1.1 Background to Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is a highly heterogeneous disease with variable combinations of destruction of lung parenchyma and pulmonary vasculature and dysfunction of the large and small airways. COPD is defined by airflow limitation which is progressive and not fully reversible. COPD is a preventable disease and is caused by an inflammatory response to exogenous noxious particulates, most commonly cigarette smoke. The American Thoracic Society (ATS)/European Respiratory Society (ERS) and Global Initiative for Chronic Obstructive Lung Disease (GOLD) provide us with the following definitions:

"A preventable and treatable disease state characterised by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases, primarily caused by cigarette smoking. Although COPD affects the lungs, it also produces a significant systemic consequences." Source: ATS/ERS (Celli, MacNee et al. 2004)

"A common, preventable and treatable disease that is characterised by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases." Source: GOLD (https://goldcopd.org/)

The airflow limitation found in COPD, is caused by a composite of small airways disease (obstructive bronchiolitis) and parenchymal destruction (emphysema). It was in the 17th century

that emphysema was first described by Bonet (T. 1679) who attributed breathlessness to *"large voluminous lungs".* It was not until the 1960s that the term "COPD" was coined as a term for the spectrum of airways disease, chronic bronchitis and emphysema that result in airflow limitation (Briscoe and Nash 1965). As early as the 1970s it was recognised by Fletcher and colleagues (Fletcher and Peto 1977) that once an individual stops smoking the progression of COPD would slow and continuing smoking would accelerate disease progression.

To diagnose COPD, individuals should have the presence of cough, sputum production, dyspnoea or a history of exposure to risk factors of the disease. Confirmation of the diagnosis of COPD is based on spirometry and the presence of post-bronchodilator airflow obstruction, defined as a ratio of the forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) of less than 0.70 (https://goldcopd.org/). The severity of COPD is defined by the reduction in percentage predicted of FEV1, commonly described as the Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage (Table 1.1).

Table 1.1: Global Initiative of Chronic Obstructive Lung Disease (GOLD) Classification of Disease Severity (https://goldcopd.org/)

Patients with FEV1/FVC < 0.70		
GOLD 1:	Mild	FEV1 ≥ 80% predicted
GOLD 2:	Moderate	50% ≤ FEV1 < 80% predicted
GOLD 3:	Severe	30% ≤ FEV1 < 50% predicted
GOLD 4:	Very Severe	FEV1 < 30% predicted

Abbreviations: GOLD – Global Initiative for Chronic Obstructive Lung Disease; FEV1 – Forced Expiratory Volume in one second; FVC – Forced Vital Capacity

COPD is a significant health care burden and in 2016 the World Health Organisation (WHO) (2019) recognised COPD as the third leading cause of death worldwide. With approximately 41 deaths per 100,000 population. In the United Kingdom (UK) data from the Quality Outcomes Framework (QOF) identify 1.3 million people as having a diagnosis of COPD.

1.2 Exercise limitation in COPD

The sensation of dyspnoea (or breathlessness), commonly described as “air hunger, gasping, chest heaviness or an increased effort to breathe” (Elliott, Adams et al. 1991) is common in people with COPD (https://goldcopd.org/). Dyspnoea is a major influence on these individuals’ ability to undertake physical activity or exercise. Exercise and activities of daily living are commonly abandoned once the sensation of dyspnoea is no longer tolerable. The mechanistic

cause of this dyspnoea is likely multifactorial (Figure 1.1) encompassing: an imbalance between ventilatory capacity and demand; an imbalance between oxygen supply to respiratory and skeletal muscle and skeletal muscle dysfunction.

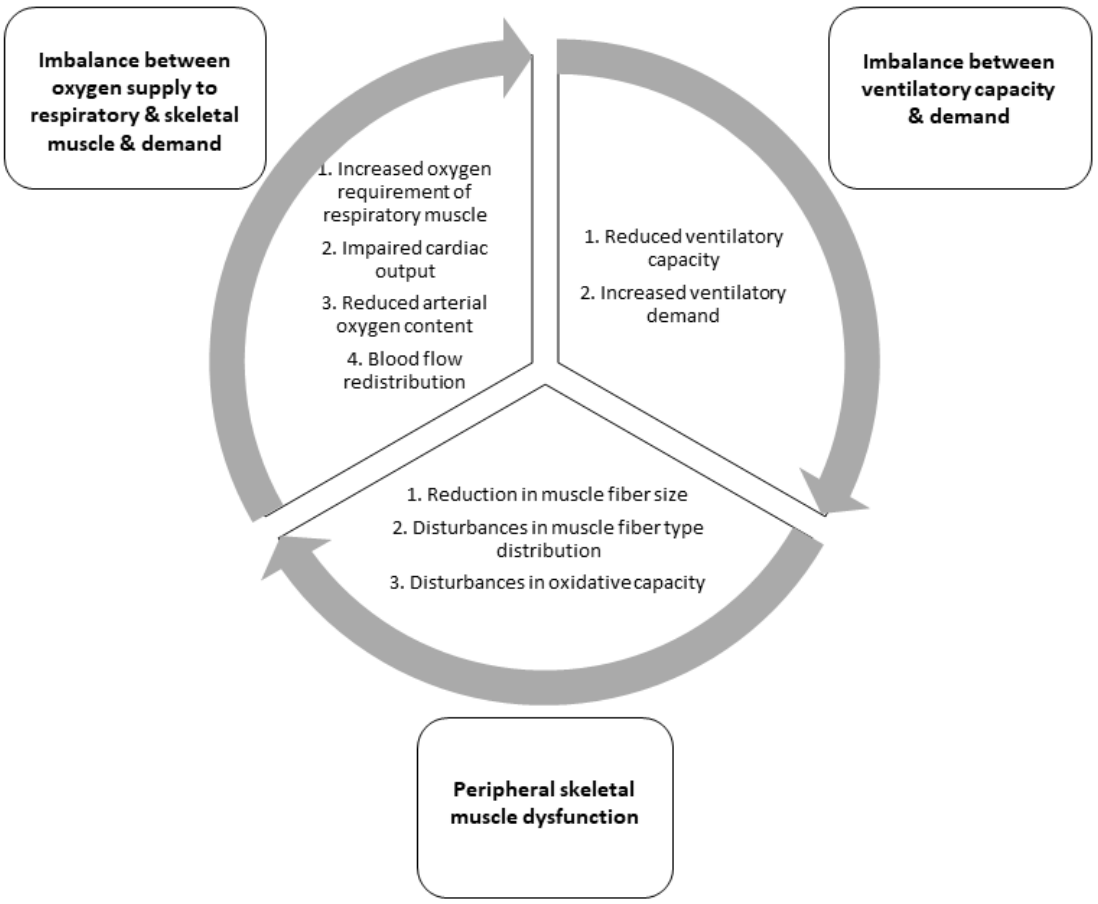


Figure 1.1 Mechanisms of dyspnoea in COPD

1.2.2 Imbalance between ventilatory capacity and demand

People with COPD develop ventilatory capacity limitation and increased ventilatory demand. Expiratory airflow limitation, the hallmark feature of COPD, results in the inability to reduce end-expiratory lung volumes (EELV) during exercise resulting in dynamic hyperinflation (DH) (O'Donnell, Revill et al. 2001, O'Donnell, Elbehairy et al. 2017). The sequelae of DH is a reduction

in elastic recoil and ultimately increasing the elastic loading of the inspiratory muscles (O'Donnell, Revill et al. 2001), a shortening of the diaphragm and inspiratory muscles resulting in a mechanical disadvantage (Mador, Kufel et al. 2000) and the generation of intrinsic positive airways pressure which must be overcome to initiate inhalation (Pepe and Marini 1982).

During exercise, people with COPD, also experience an increased ventilatory demand. In an attempt to increase ventilation these individuals tend towards an increase in minute ventilation expressed as a pattern of rapid and shallow breathing at rest and during exercise (O'Donnell, Bertley et al. 1997). This increased respiratory rate has the resultant effect of a reduction in expiration time which in turn compounds DH. This increase in ventilatory demand is similarly driven by increased alveolar dead space ventilation, increased ventilation/perfusion mismatch, impaired gas exchange, alveolar hypoxia and hypoxaemia.

1.2.3 Imbalance between oxygen supply and demand

Because of the physiological adaptations that occur as a result of COPD, it is not possible to meet the oxygen requirement of respiratory and skeletal muscles during exercise. Individuals with COPD develop not only DH but also high expiratory intrathoracic pressure (ITP), both of which synergistically result in several haemodynamic effects. High ITP decreases venous return to the right ventricle thus reducing right ventricular stroke volume (Pinsky 2005). High intrinsic positive airway pressures and ITP compress the pulmonary vascular bed which leads to increased pulmonary vascular resistance and subsequently an increase in the right ventricular afterload (Pinsky 2005). The respiratory mechanics of COPD also cause haemodynamic changes to the left ventricle with decreased preload (Slutsky, Hooper et al. 1981), increased cardiac transmural pressure and left shift of the interventricular septum with the consequence of left ventricular diastolic dysfunction (Morrison, Adcock et al. 1987, Vizza, Lynch et al. 1998).

It is not uncommon for people with COPD to display hypoxaemia. This hypoxaemia relates to emphysema generating an increased physiological dead space (areas of lung with high ventilation/perfusion ratios) and increased physiological shunt (areas of lung with low ventilation/perfusion ratios) found in the bronchitis phenotypical COPD individuals (West 2016). The importance of this hypoxaemia is as a factor that limits exercise tolerance. In those people with COPD and exercise-induced hypoxaemia supplemental oxygen improves exercise capacity (distance, time and number of steps) (Jarosch, Gloeckl et al. 2017, Suntharalingam, Wilkinson et al. 2017).

1.2.4 Peripheral skeletal muscle dysfunction

Skeletal muscle dysfunction is a common feature in COPD, driven largely by disuse, affecting principally the lower limbs because of reduced locomotion (Man, Hopkinson et al. 2005). Skeletal muscle weakness and reduction in muscle bulk can be identified even in early disease (Shrikrishna, Patel et al. 2012). Quadriceps weakness is associated with reduced survival in COPD, independent of lung function (Swallow, Reyes et al. 2007). As well as a reduction in bulk there is also a fibre type switch away from endurance fibres towards a faster twitch, less oxidative phenotype (Swallow, Gosker et al. 2007, Natanek, Gosker et al. 2013, Natanek, Gosker et al. 2013, Maddocks, Shrikrishna et al. 2014).

1.2.5 Affective component of dyspnoea

Breathing is an unconscious process; however, it is possible to voluntarily become aware of breathing or automatically become aware of breathing if it requires attention. The respiratory afferent input into the cerebral cortex may result in a somatosensory cognitive awareness of breathing and therefore generate a resultant affective response (Davenport and Vovk 2009). It

is possible therefore, that if there is a disruption in the afferent respiratory information these will be brought into consciousness resulting in the sensation of dyspnoea (O'Donnell, Banzett et al. 2007). Secondly affective processing of a new sensation and labelling it as unpleasant may also result in dyspnoea (Davenport and Vovk 2009). It is possible that the different sensations of dyspnoea have separate underlying mechanisms for example, the sensation of "air hunger" may relate to brainstem respiratory centres being exposed to increased stimulation which is not matched by an appropriate ventilatory response (el-Manshawi, Killian et al. 1986). Cortical pathways are thought to be involved in the processing of respiratory sensations with the response related to the type and quality of the respiratory afferent stimuli (Davenport and Vovk 2009). Lansing *et al* (Lansing, Gracely et al. 2009) described a two-stage model for the affective component of dyspnoea where an immediate unpleasantness results in withdrawal, and an emotional component leads to long-term adaptations.

1.2.6 COPD and health the experience of dyspnoea in relation to exercise

Whilst exercising there is tight coupling between muscular and cardiovascular requirements. When a healthy individual exercises the challenge falls mainly to the cardiovascular and locomotor systems than the respiratory system (Dempsey 1986). This is why, in healthy individuals, the most commonly reported cause of exercise cessation is limb fatigue (Hamilton, Killian et al. 1996). In healthy individuals the sensation of dyspnoea increases in proportion to increasing ventilation and respiratory contractile effort.

Conversely in individuals with COPD, respiratory limitation is commonly encountered. Dyspnoea in individuals with COPD is significantly increased for a given work rate when compared to age-matched healthy individuals (O'Donnell, Ora et al. 2009). When compared to age-matched

controls, individuals with COPD report a qualitatively different experience of dyspnoea with reports of “unsatisfied inspiration” and “inspiratory difficulty” (O'Donnell, Bertley et al. 1997).

1.3 Pulmonary Rehabilitation

The GOLD 2020 strategy document (https://goldcopd.org/) and the pyramid of value for COPD management (Zoumot, Jordan et al. 2014) includes smoking cessation, influenza vaccination and pulmonary rehabilitation (PR). The ATS/ERS task force define PR as:

“A comprehensive intervention based on a thorough patient assessment followed by patient-tailored therapies that include, but are not limited to, exercise training, education and behaviour change, designed to improve the physical and psychological condition of people with chronic respiratory disease and to promote the long-term adherence to health-enhancing behaviours”

Source ATS/ERS Task Force (Spruit, Singh et al. 2013)

PR is an effective management strategy for individuals with COPD with high grade evidence it improves exercises performance, reduces dyspnoea, reduces the risk of exacerbation, reduces hospitalisation, reduces healthcare visits and improves health related quality of life (QoL) (Rochester, Vogiatzis et al. 2015). PR has also been found to be effective following an acute exacerbation of COPD with benefits in symptoms, health related QoL, exercise tolerance and hospital readmission rates and survival (Puhan, Gimeno-Santos et al. 2016). PR is recommended for individuals with GOLD grade II to IV COPD with a Medical Research Council (MRC) dyspnoea score of 3-5 or those who are functionally limited by breathlessness. PR consists of a structured

intervention of supervised and individually tailored exercise training and education with the goal to reverse skeletal muscle deconditioning. PR has been shown to result in skeletal muscle adaptation, even within those individuals with severe COPD (Maltais, LeBlanc et al. 1996, Whittom, Jobin et al. 1998). The improvements in skeletal muscle function following PR are associated with improvements in exercise capacity (Franssen, Broekhuizen et al. 2004, Porszasz, Emtner et al. 2005). Skeletal muscle loss is not isolated to those individuals with COPD who have a low body mass index but it is now recognised that there is hidden obesity in normal weight individuals with COPD due to a proportionally low muscle mass (Schols, Soeters et al. 1993, Rutten, Breyer et al. 2010). This is commonly termed sarcopenic obesity where there is low muscle mass (sarcopenia) and high body fat (obesity) (Baumgartner 2000). Obesity impairs the muscle quality and decreases physical ability. Obese individuals with COPD who undertake a programme of PR and weight reduction experience both weight loss and preserved skeletal muscle mass (McDonald, Gibson et al. 2016). The PR programme should be a minimum of twice-weekly sessions over a period of 6-12 weeks, with a minimum attendance of 12 sessions (Bolton, Blakey et al. 2014).

Despite the evidence base for PR, inequalities exist in the provision and demand for pulmonary rehabilitation, with limitations in funding and lack of healthcare professionals, patient and caregiver awareness (Keating, Lee et al. 2011, Jones, Green et al. 2014, Celli, Decramer et al. 2015). In the ATS/ERS statement on respiratory questions in COPD studies are required to evaluate strategies to maintain the benefits of pulmonary rehabilitation (Celli, Decramer et al. 2015).

1.4 The role of dietary supplements in improving exercise capacity in COPD

The exercise limitation experienced by individuals with COPD may well be influenced by malnutrition and reduced muscle mass. Not only is being underweight associated with reduced exercise capacity but it is also associated with increased mortality (Hirai, Zelt et al. 2017) with weight loss also a predictor of mortality and morbidity in chronic lung disease (Nici, Donner et al. 2006). It is recommended that the average protein requirement for adults is 0.7 g protein.kg^{-1}.day^{-1} and older adults is 1.2 to 1.5 g protein.kg^{-1}.day^{-1} (Deutz, Bauer et al. 2014) with this protein requirement being higher in individuals with COPD. There is potential for dietary supplementation to improve exercise capacity, improve muscle strength and decrease the risk of mortality (Efthimiou, Fleming et al. 1988, Schols 2003). A 2012 meta-analysis (Ferreira, Brooks et al. 2012) concluded that in individuals with COPD who are malnourished, dietary supplementation increased muscle mass and body weight with associated improvements in respiratory function and exercise tolerance. In addition to this a further meta-analysis (Collins, Elia et al. 2013) found that nutritional supplementation improved weight gain and handgrip strength in individuals with COPD.

Despite the potential for dietary supplementation improving exercise capacity in individuals with COPD the evidence is currently lacking. Clinical studies assessing the role of carbohydrates, vitamin D, creatine or L-carnitine in addition to PR had no statistical benefit compared to PR alone (Schols, Soeters et al. 1995, Menier, Talmud et al. 2001, Vermeeren, Wouters et al. 2001, Steiner, Barton et al. 2003, Broekhuizen, Creutzberg et al. 2005, Fuld, Kilduff et al. 2005, Borghi-Silva, Baldissera et al. 2006, Faager, Soderlund et al. 2006, Kubo, Honda et al. 2006, Deacon, Vincent et al. 2008, Laviolette, Lands et al. 2010, Hornikx, Van Remoortel et al. 2012, Collins, Elia et al. 2013, Gurgun, Deniz et al. 2013, Ahnfeldt-Mollerup, Hey et al. 2015, Paulin, Zagatto

et al. 2017, van de Bool, Rutten et al. 2017, Ogasawara, Marui et al. 2018, van Beers, Rutten-van Molken et al. 2020). Low body weight is also observed in approximately 27% of individuals with COPD patients (Vermeeren, Creutzberg et al. 2006). Of interest there was a noted gender disparity found within the COSMIC study (Vermeeren, Creutzberg et al. 2006) with nutritional depletion being more common in females than in males, this has been noted in other studies (Schols, Soeters et al. 1993, Engelen, Schols et al. 1994). There is an association between nutritional depletion and lower diffusion capacity (Engelen, Schols et al. 1999) and an increased inflammatory response (Di Francia, Barbier et al. 1994, de Godoy, Donahoe et al. 1996, Engelen, Schols et al. 1999). The increased prevalence of nutritional depletion in females may be due to a lower diffusion capacity and or increased inflammatory response. Following nutritional supplementation clinical studies have observed a significant increase in body weight (Schols, Soeters et al. 1995, Creutzberg, Schols et al. 2000, Creutzberg, Wouters et al. 2003, Steiner, Barton et al. 2003, Baldi, Aquilani et al. 2010, Sugawara, Takahashi et al. 2012, Gurgun, Deniz et al. 2013, van Beers, Rutten-van Molken et al. 2020).

Currently there is a lack of evidence to suggest dietary supplements improve exercise capacity in individuals with COPD (Aldhahir, Rajeh et al. 2020). This lack of evidence may well relate to the heterogeneity of the study designs, including outcome measures assessed, the method of dietary supplementation and small sample sizes. There needs to be a greater understanding of the impact of diet in the prevention and outcomes of COPD. At the time of writing this thesis there are no specific dietary recommendations in the management of COPD other than nutritional counselling for those individuals who are malnourished. The evidence base of diet and dietary interventions is currently no available, including basic science and interventional studies, but a pragmatic approach would be the recommendation of a healthy balanced diet. There is a current necessity for appropriately powered double-blind randomised controlled

trials of dietary supplements to investigate the effect of nutritional support in enhancing PR outcomes in individuals with COPD.

In the last 10 years there has been an explosion of studies suggesting an ergonomic benefit in dietary nitrate (NO_3^-) supplementation to reduce the oxygen cost of submaximal exercise in healthy individuals (Larsen, Weitzberg et al. 2007, Bailey, Winyard et al. 2009, Bailey, Fulford et al. 2010, Vanhatalo, Bailey et al. 2010, Lansley, Winyard et al. 2011, Muggeridge, Howe et al. 2013, Muggeridge, Howe et al. 2014, Whitfield, Ludzki et al. 2016). There has also been a small number of studies investigating the role of dietary NO_3^-supplementation to augment the exercise outcomes in individuals with COPD (Curtis, O'Brien et al. 2015, Kerley, Cahill et al. 2015, Leong, Basham et al. 2015, Shepherd, Wilkerson et al. 2015, Beijers, Huysmans et al. 2017, Friis, Steenholt et al. 2017, Behnia, Wheatley et al. 2018). Dietary NO_3^-supplementation has exciting potential to improve exercise outcomes in individuals in COPD and warrants further investigation.

1.5 Nitric oxide – an introduction

In 1772 Joseph Priestly first identified the clear colourless gas nitric oxide (NO). NO was initially thought of as an atmospheric pollutant produced by burning nitrogen. In the 1980s, three independent groups commenced research into the mechanism for blood vessel dilatation. In 1980, Robert F. Furchgott discovered that, without a factor produced by endothelial cells, the smooth muscle in blood vessels would not be able to vasodilate (Furchgott and Zawadzki 1980). He coined this factor the Endothelial Derived Relaxing Factor (EDRF) (Furchgott and Zawadzki 1980). In 1977, whilst investigating how nitroglycerin works, Ferid Murad discovered that it released NO resulting in the dilatation of smooth muscle cells (Murad, Mittal et al. 1978).

However, it was not until 1986 when the pieces of this puzzle were put together by Louis J. Ignarro who identified EDRF and discovered that it had identical properties to NO (Ignarro, Buga et al. 1987). These observations helped establish NO as a mammalian physiological signalling molecule. For their discoveries concerning NO as a signalling molecule Robert F. Furchgott, Louis J. Ignarro and Ferid Murad were awarded the Nobel Prize in Physiology or Medicine in 1998. NO was also awarded "molecule of the year" in 1992. Since this discoverie, NO has been the subject of more than 160,000 scientific papers and has been shown to have a role in multiple body systems including the immune, reproductive, cardiovascular and neurological systems.

Following on from the Nobel Prize winning work of Furchgott, Ignarro and Maurad, NO is now recognised as a ubiquitous free radical signaling molecule which is involved in a number of biological process at a tissue and cellular level including; mitochondrial and cellular respiration (Brown and Cooper 1994, Shiva, Huang et al. 2007), glucose uptake in skeletal muscle (Merry, Lynch et al. 2010), skeletal muscle contraction (Reid 1998, Viner, Williams et al. 2000, Stamler and Meissner 2001), regulation of vasodilatation (Moncada and Higgs 1993, Joyner and Tschakovsky 2003), neurotransmission (Garthwaite 2008) and fatigue development (Percival, Anderson et al. 2010). The functional importance of NO is apparent due to its formation arising via complementary pathways: the oxygen (O_2)-dependent pathway and the O_2-independent pathway. It is via these synergistic pathways working within tissues and skeletal muscle that NO, nitrate (NO_3^-) and nitrite (NO_2^-) can be continuously recycled (Lundberg, Weitzberg et al. 2008, Piknova, Park et al. 2015, Gilliard, Lam et al. 2018). These complementary pathways allow for increased NO bioavailability, thus bolstering its many outputs.

Through the administration of exogenous NO_3^-, it is possible to augment the O_2-independent formation of NO, which would in turn increase NO bioavailability. This augmentation would have the potential to enhance physiological function during exercise. In 2007, in a landmark study by Larsen *et al* (Larsen, Weitzberg et al. 2007), it was found that following the administration of sodium nitrate ($NaNO_3$) (NO_3^-; 0.1 $mmol.kg^{-1}.day^{-1}$) there was a significant reduction in the O_2 uptake ($\dot{V}O_2$) during submaximal exercise in healthy individuals. This finding was replicated by Bailey *et al* (Bailey, Winyard et al. 2009) who also found a reduction in the $\dot{V}O_2$ during submaximal exercise following supplementation with inorganic nitrate in the form of beetroot juice (BRJ) (NO_3^-; 5.5 $mmol.day^{-1}$), in healthy individuals. The fact that supplementation of exogenous NO_3^- resulted in a reduction in the O_2-cost of exercise (i.e. improved exercise efficiency) was unexpected. Prior to this work, a fundamental belief of exercise physiology was the tight coupling between $\dot{V}O_2$ and workload, with the $\dot{V}O_2$ at a given workload being identical between different individuals or in the same individual if measured at different times (Larsen, Weitzberg et al. 2007, Bailey, Winyard et al. 2009).

At the time of writing this thesis, despite intense research, the mechanism by which NO_3^- supplementation produces its physiological effects on exercise outcomes are unclear, mainly due to equivocal results. Potential candidate mechanisms are: improved efficiency of muscle contractility (Bailey, Fulford et al. 2010), redox signalling (Whitfield, Ludzki et al. 2016), improved mitochondrial efficiency (Larsen, Schiffer et al. 2011), improved blood flow in type II muscle fibres (Hernandez, Schiffer et al. 2012) and improved contractile function of muscle (Ferguson, Hirai et al. 2013). These mechanisms may work independently or in combination. Despite, the mechanistic basis of NO_3^- supplementation remaining under debate, it is possible to assess the efficacy of NO_3^- supplementation through the increase in plasma NO_2^- reflecting

NO bioavailability (Dreissigacker, Wendt et al. 2010, Wilkerson, Hayward et al. 2012, Porcelli, Ramaglia et al. 2015).

1.6 Nitric oxide

The production of NO is continuous and this production is increased under conditions of exercise (Balon and Nadler 1994). The half-life of NO is short, between 2 milliseconds (Liu, Miller et al. 1998) to seconds (Kelm 1999, Crawford, Isbell et al. 2006). Given this short half-life, continuous production of NO is imperative. This is achieved via two pathways: the O_2-dependent nitric oxide synthase (NOS) pathway and the O_2-independent NO_3^--NO_2^--NO pathway (Figure 1.2). The recycling of NO is also important, this is emphasised by the fact that endogenous NO produced from the NOS-dependent pathway and exogenous NO_3^- and NO_2^- can enter the NO_3^--NO_2^--NO pathway, allowing for continuous NO production (Lundberg, Weitzberg et al. 2008).

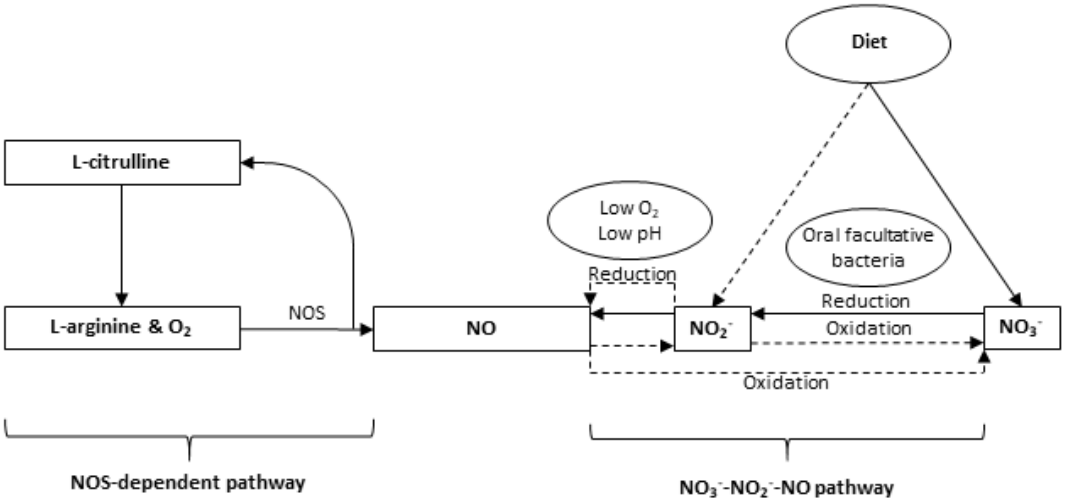


Figure 1.2. The pathways of nitric oxide (NO) production. Adapted with permission from Lundberg, 2004 (Lundberg and Govoni 2004)

Abbreviations: O_2 – oxygen; NOS – Nitric oxide synthase; NO – Nitric oxide; NO_2^- - Nitrite; NO_3^- - Nitrate.

1.6.2 NOS-dependent pathway

L-arginine and O_2 produce NO via a reaction catalysed by NOS enzymes. At the time of production of NO by NOS from L-arginine, L-citrulline is also produced and is recycled back into L-arginine. Following its production, NO can be oxidised to form NO_2^- and NO_3^- (dashed lines). This endogenously produced NO_3^- can be reduced by facultative bacteria in the mouth to NO_2^-, with a further reduction to NO.

1.6.3 NO_3^--NO_2^--NO pathway

Following the consumption of NO_3^- rich foodstuffs (such as green leafy vegetables and beetroot), NO_3^- can be reduced to NO_2^- which in turn, under acidic conditions (low pH) or hypoxic conditions (low O_2), can be further reduced to NO.

1.6.4 The nitric oxide synthase (NOS)-dependent pathway

In 1989, Moncada *et al* (Moncada and Higgs 1993) described the classical pathway for the formation of NO via the reduction of L-arginine to NO via NOS enzymes (Moncada and Higgs 1993, Bredt 1999) which is dependent on the presence of O_2 (Figure 1.2). There are three isoforms of NOS: Type 1 or neuronal NOS (NOS-1 or nNOS) (Nakane, Schmidt et al. 1993) are located in myocytes (Kobzik, Reid et al. 1994, Brenman, Chao et al. 1996); Type 2 or inducible NOS (NOS-2 or iNOS) (Williams, Brown et al. 1994) are located in macrophages (Williams, Brown et al. 1994, Park, Park et al. 1996); Type 3 or endothelial NOS (NOS-3 or eNOS) (Forstermann, Pollock et al. 1991) are located in vascular endothelial cells (Kobzik, Reid et al. 1994). This NOS-dependent reaction between L-arginine and O_2 is reliant on number of cofactors (Moncada and Higgs 1993) and if there is any limitation in the bioavailability of these cofactors the production of NO would be compromised. Within the L-arginine and O_2 reaction the binding of calmodulin/Ca^{2+} facilitates the donation of electrons from nicotinamide adenine dinucleotide phosphate to flavin adenine dinucleotide (Alderton, Cooper et al. 2001). Subsequent electrons react with haem iron and 5,6,7,8-tetrahydrobiopterin which catalyses the reaction between L-arginine and O_2 (Alderton, Cooper et al. 2001). The end products of this reaction are L-citrulline and NO (Alderton, Cooper et al. 2001). L-citrulline can be further recycled back into L-arginine (Alderton, Cooper et al. 2001). A number of disease states have been shown to result in the impairment of NOS these include COPD (Barnes and Kharitonov 1996), sarcopenia (Hall, Ma et

al. 2011), cerebral vascular disease (Forstermann 2010) and type 2 diabetes mellitus (Wu and Meininger 2009). The potential for NOS to be impaired by disease highlights the importance of NO production.

In individuals with COPD, endothelial dysfunction is results in alterations to the expression and release of endothelial derived vasoactive mediators which includes NO (Dinh-Xuan, Pepke-Zaba et al. 1993). The concentrations of the amino acids asymmetric dimethylarginine and symmetric dimethylarginine are increased in COPD and further increased in during an acute exacerbation of COPD (Ruzsics, Nagy et al. 2016). These amino acids suppress NOS function; asymmetric dimethylarginine a competitive inhibitor of L-arginine (Bode-Böger, Scalera et al. 2007) and symmetric dimethylarginine a competitor for L-arginine (Bode-Böger, Scalera et al. 2006). In stable and exacerbated COPD the serum L-arginine/ asymmetric dimethylarginine ratio is reduced and the serum symmetric dimethylarginine is increased which contributes to the impairment of vascular NO generation in individuals with COPD (Csoma, Bikov et al. 2019).

1.6.5 The NO_3^--NO_2^--NO pathway

Before the discovery of the NO_3^--NO_2^--NO pathway (Figure 1.2), NO_3^- and NO_2^- were considered to be stable and inactive end products of the metabolism of NO (Moncada and Higgs 1993). Endogenous NO_3^- and NO_2^- are generated via the oxidation of excess NO from the NOS-dependent pathway. Following the production of NO from the NOS-dependent pathway, excess NO is oxidised to NO_3^- (Lundberg, Weitzberg et al. 2008) via the reaction of NO with oxy-haem proteins (i.e. oxyhaemoglobin and oxymyoglobin) (Keszler, Piknova et al. 2008). NO also undergoes oxidation to NO_2^- (Ignarro, Fukuto et al. 1993) this reaction is catalysed by multi-copper oxidase and NO oxidase ceruloplasmin (Shiva, Wang et al. 2006). Exogenous NO_3^- and

NO_2^- comes from the consumption of NO_3^- rich foodstuffs, likes green leafy vegetables and beetroot. This pool of endogenous and exogenous NO_3^- and NO_2^- can undergo serial reduction to NO, a process that is upregulated by conditions of hypoxia and low pH (Lundberg and Weitzberg 2009). Under these same conditions the NOS-dependent production of NO is reduced (Lundberg and Weitzberg 2009). The NO_3^--NO_2^--NO pathway occurs in skeletal muscle (Piknova, Park et al. 2015), heart (Zweier, Wang et al. 1995) and liver (Gilliard, Lam et al. 2018).

1.6.6 Dietary nitrate

There is great variability in the quantity of dietary NO_3^- consumed by individuals due to the varied nature of our diets. The average European diet contains approximately 250 mg or 4 mmol of NO_3^- per 100 g weight consumed (Gangolli, van den Brandt et al. 1994, Hord, Tang et al. 2009, Lidder and Webb 2013). Foodstuffs rich in NO_3^- include green leafy vegetables (including kale, spinach, rocket and chard) and beetroot. A 140 mL BEET-IT® SPORT Shot (James White Drinks, Ipswich, UK) contains 0.8 g or 12.9 mmol of NO_3^- which is the equivalent of 400 g dry weight of green leafy vegetables. These foodstuffs comprise approximately 85% of dietary NO_3^- (Gangolli, van den Brandt et al. 1994). Of the dietary NO_3^- consumed approximately 60% is excreted in the urine (Wagner, Schultz et al. 1983, Lundberg and Govoni 2004). Following ingestion NO_3^- is absorbed from the gastrointestinal tract within one hour (Lundberg, Weitzberg et al. 2008). NO_3^- is relatively stable with a half-life of 5-8 hours (Wagner, Schultz et al. 1983). An NO_3^- transporter protein, sialin, found in the salivary glands actively transports 25% of the absorbed NO_3^- into the enterosalivary circulation (Qin, Liu et al. 2012, Petersson, Jadert et al. 2015).

1.6.7 The enterosalivary circulation

Following its entry into the enterosalivary circulation NO_3^- is concentrated approximately 20 fold (Govoni, Jansson et al. 2008). Following this, anaerobic facultative bacterium on the dorsal surface of the tongue reduce approximately 20% of the absorbed NO_3^- to NO_2^- via bacterial respiration (Duncan, Dougall et al. 1995). During this bacterial respiration NO_3^- reductases use NO_3^- as the terminal electron acceptor (Duncan, Dougall et al. 1995). Following ingestion, in the acid environment of the stomach, NO_2^- is reduced to NO (Benjamin, O'Driscoll et al. 1994, Lundberg and Govoni 2004). This process of reduction is enhanced by the presence of vitamin C and polyphenols (Gago, Lundberg et al. 2007). Some of the NO_2^- is rapidly absorbed into the circulation increasing the measurable plasma NO_2^- (Webb, Patel et al. 2008). The half-life of NO_2^- in plasma is approximately 1 to 5 minutes (Lundberg, Weitzberg et al. 2008). In the presence of spitting (Webb, Patel et al. 2008) or antibacterial alcohol based mouth wash (Govoni, Jansson et al. 2008) this rise in plasma NO_2^- is attenuated.

1.6.8 The final steps: nitrite to nitric oxide

The final stage of the NO_3^--NO_2^--NO pathway to produce NO from NO_2^- involves a one-step reduction which takes place in the blood and tissues. This process of reduction is catalysed by NO_2^- reductases; cytochrome P-450 (Kozlov, Dietrich et al. 2003), aldehyde oxidase (Li, Cui et al. 2008), xanthine oxidase (Zhang, Naughton et al. 1997), deoxyhaemoglobin (Cosby, Partovi et al. 2003, Nagababu, Ramasamy et al. 2003), deoxymyoglobin (Rassaf, Flogel et al. 2007, Shiva, Huang et al. 2007) and mitochondrial respiration complexes (Kozlov, Staniek et al. 1999). Crucially, this final stage of the NO_3^--NO_2^--NO pathway is greatly enhanced in conditions of hypoxia (Millar, Stevens et al. 1998, Castello, David et al. 2006) and low pH (Modin, Bjorne et al. 2001), both conditions which may be found in exercising skeletal muscle. Unlike the classical

NOS-dependent pathway (Giraldez, Panda et al. 1997, Ostergaard, Stankevicius et al. 2007, Lundberg, Weitzberg et al. 2008) which is down regulated under these conditions.

1.6.9 The role of the oral microbiome

The oral microbiome plays a pivotal role in the NO_3^--NO_2^--NO pathway (Hyde, Andrade et al. 2014). The type and quantity of bacteria in the oral cavity affect the initial reduction of NO_3^- to NO_2^- (Doel, Benjamin et al. 2005, Hyde, Andrade et al. 2014, Velmurugan, Gan et al. 2016). It is possible to interfere with these bacteria through the use of antiseptic and antibacterial alcohol based mouthwash (Govoni, Jansson et al. 2008, McDonagh, Wylie et al. 2015, Woessner, Smoliga et al. 2016). Similarly, if saliva is not swallowed, through the process of spitting, this would attenuate the elevation of NO_2^- (Webb, Patel et al. 2008). Initial absorption of NO_3^- from the gastrointestinal tract can also be hindered by concomitant ingestion of foodstuffs containing competing compounds such as thiocyanate (i.e. cruciferous vegetables) (Olea and Parras 1992).

1.6.10 Health concerns of nitrate ingestion

There have been several historic health concerns around the ingestion of NO_3^- and NO_2^- in relation to possible carcinogenic potential (McKnight, Duncan et al. 1999) and methaemoglobinaemia (Comly 1987). The evidence of harm following the ingestion of NO_3^- in humans is somewhat lacking. In a 1975 rodent study, the *in vivo* formation of N-nitrosamines (a carcinogenic substance) following the ingestion of NO_3^- was related to a carcinogenic effect (Mirvish 1975). It is important to recognise the dose of NO_3^- administered in this study was supra-physiological (Mirvish 1975). There have also been concerns of methaemoglobinaemia (otherwise known as 'Baby Blue Syndrome') where babies ingest water with high

concentrations of NO_3^- (Comly 1987) where NO_2^- mediates the oxidation of ferric iron in oxyhaemoglobin rendering haemoglobin unable to bind O_2, with the potential of hypoxia (Fan and Steinberg 1996, McKnight, Duncan et al. 1999). The current European Food Safety Authority target for acceptable daily intake of sodium and potassium salts of nitrite and nitrate, intentionally added to meat and other food products (e.g. fish and cheese) to hinder microbial growth, is 3.7 mg of NO_3^-.kg body weight^{-1}.day^{-1} which equates as 4.2 to 4.7 mmol of NO_3^- for a 70 to 80 kg individual ((EFSA) 2017). This target is easily exceeded by a single portion of spinach (containing NO_3^-: 400 mg per 100 g fresh weight) (Hord, Tang et al. 2009). The estimated NO_3^- intake from food is approximately 31-185 mg.day^{-1} in Europe and approximately 40-100 mg.day^{-1} in the United States (Hord, Tang et al. 2009). In the DASH study (Appel, Moore et al. 1997) participants consumed 1200 mg.day^{-1} of NO_3^- exceeding the acceptable daily intake by approximately 550%. NO_3^- is relatively inert, the harmful effects of NO_3^- ingestion are believed to be related to the reduction of NO_3^- to NO_2^-. The NO_2^- content of vegetables is lower than that of NO_3^- with concentrations of less than 100 mg NO_2^-.kg^{-1}. Given the low NO_2^- content of foodstuffs the average daily intake of NO_2^- is much lower than NO_3^- with estimates of between 0-20 mg.day^{-1} (J.A. 1998). NO_3^--rich BRJ at a dose of 140 ml (NO_3^-: 12.9 mmol or 0.8 g) is far less than that consumed in an average European diet.

1.6.11 Methods of nitrate supplementation

It is possible to supplement NO_3^- through various means. A vast number of research studies have employed NO_3^- salts in the form of sodium NO_3^- ($NaNO_3$) and potassium NO_3^- (KNO_3) (Larsen, Weitzberg et al. 2007, Kapil, Milsom et al. 2010, Bescos, Rodriguez et al. 2011). Other groups have opted to use beetroot juice BRJ, which is rich in dietary NO_3^-. Not only does BRJ contain NO_3^- it also contains several other substances which have potential positive benefits in mammals. BRJ contains betaine, which previously was thought to improve muscle strength,

power and endurance (Hoffman, Ratamess et al. 2011), however a 2017 literature review has found no evidence of this effect (Ismaeel 2017). BRJ has also been shown to potentially protect against exercise induced oxidative stress (Kanner, Harel et al. 2001) due to its high antioxidant content (Shepherd, Wilkerson et al. 2015). BRJ also contains polyphenols, quercetin and resveratrol which has been linked to mitochondrial biogenesis and increased aerobic capacity (Lagouge, Argmann et al. 2006, Davis, Murphy et al. 2009, Ganio, Armstrong et al. 2010). Given the variety of potential benefits of BRJ supplementation there is the potential of difficulty interpreting clinical studies when attempting to assess the NO_3^- effects of BRJ. This led to the creation of a NO_3^- deplete BRJ, generated by passing BRJ through an ion exchange column to remove the NO_3^- but retain all other constituents (Kelly, Vanhatalo et al. 2014). This NO_3^- deplete BRJ is identical in taste, colour and smell (Lansley, Winyard et al. 2011) and has allowed for the analysis of the NO_3^- effects of dietary NO_3^- supplementation in the form of BRJ against a robust placebo.

1.7 Effects of Dietary Nitrate Supplementation

1.7.1 Cardiovascular

There are well established pharmacological agents that work via NO signalling either by increasing bioavailability or reducing NO breakdown. Possibly the most well-known of these agents is glyceryl trinitrate which causes non-specific arterial and venous dilatation via the release of free radical NO which activates guanylate cyclase and increases synthesis of guanosine 3’ and 5’-monophosphate with resultant effects on phosphorylation of proteins in smooth muscle (www.medicines.org.uk 2017). A further example is that of selective inhibitors of cyclic guanosine monophosphate specific phosphodiesterase type 5 inhibitors (e.g. Sildenafil)

which enhance the relaxant effect of NO and are used in the treatment of pulmonary hypertension and erectile dysfunction (Ghofrani, Osterloh et al. 2006).

Evidence also exists for the role of dietary NO_3^- supplementation for the reduction of blood pressure (BP) with dietary approaches to stop hypertension (DASH) being a prime example (Appel, Moore et al. 1997, Sacks, Svetkey et al. 2001). This dietary plan consists of fruit, vegetables and nuts containing approximately 20 mmol of inorganic $NO_3^-.day^{-1}$ and has been shown to reduce BP (Hord, Tang et al. 2009). The evidence is building for both acute (Webb, Patel et al. 2008, Kapil, Milsom et al. 2010, Vanhatalo, Bailey et al. 2010) and chronic dosing with BRJ (Bailey, Winyard et al. 2009, Bailey, Fulford et al. 2010, Larsen, Weitzberg et al. 2010, Vanhatalo, Bailey et al. 2010). In 2006, Larsen *et al* (Larsen, Ekblom et al. 2006) found that in healthy normotensive individuals three days of supplementation with $NaNO_3$ (0.1 $mmol.kg^{-1}.day^{-1}$) resulted in a reduction of diastolic BP (dBP) of 3.7 mmHg ($p < 0.02$). These findings were emulated in healthy individuals by Webb *et al* when an acute 500 mL dose of BRJ (NO_3^- 22.5 mmol) reduced systolic BP (sBP) by 10.4 ± 3.0 mmHg ($p < 0.01$), reduced dBP by 8.1 ± 2.1 mmHg ($p < 0.01$) 2.3 h after ingestion and mean arterial pressure (MAP) by 8.0 ± 2.1 mmHg ($p <0.01$) 3 h after ingestion (Webb, Patel et al. 2008). This reduction in sBP, dBP and MAP correlated with an increase in plasma NO_2^- and was abolished by interrupting the enterosalivary circulation (Webb, Patel et al. 2008). The blood pressure lowering effects of dietary NO_3^- supplementation are not solely found in health but have been replicated in disease. In drug naïve grade 1 hypertensives acute dietary NO_3^- supplementation (NO_3^- 3.5 mmol) resulted in a reduction in both peak mean sBP and dBP of 11.2 ± 2.6 mmHg ($p <0.001$) and 9.6 ± 1.2 mmHg ($p <0.001$) respectively (Ghosh, Kapil et al. 2013). This reduction in BP parameters similarly correlated with increase plasma NO_2^- (Ghosh, Kapil et al. 2013). A further single-centre, double-blind, randomised, placebo-controlled study of dietary NO_3^- supplementation (NO_3^- 6.4 $mmol.day^{-1}$)

for six weeks in drug naïve hypertensives was associated with a reduction in clinic sBP 7.7 mmHg (95%CI, 4.1-11.2), $p < 0.001$ and clinic dBP 2.4 mmHg (95%CI, 0.0-4.9), $p = 0.05$ (Kapil, Khambata et al. 2015). Of great interest there was a similar reduction in 24 h ambulatory BP, with peak decreases occurring at six weeks with a reduction in ambulatory sBP of 8.1 mmHg (95%CI, 3.8-12.4), $p < 0.001$ and ambulatory dBP 3.8 mmHg (95%CI, 0.7-6.8), $p <0.01$ (Kapil, Khambata et al. 2015). Reductions in clinic and ambulatory BP parameters were once again associated with increases in plasma NO_3^- and NO_2^- (Kapil, Khambata et al. 2015). Not only are these studies statistically significant but they are clinically significant; a 1 mmHg increase in sBP is associated with an increased cerebrovascular mortality of 2% (Omar, Webb et al. 2016) and a 1 mmHg increase in dBP is associated with an increased stoke mortality 3% (Palmer, Bulpitt et al. 1992). The beneficial effect of dietary NO_3^- supplementation on blood pressure parameters has been shown to be attenuated in otherwise healthy smokers where there is no difference in sBP, dBP or MAP compared to non-smoking controls consuming a NO_3^- deplete BRJ (Bailey, Blackwell et al. 2016). The most likely mechanism of dietary NO_3^- BP lowering effects are mediated by the reduction of NO_3^- to NO_2^- and subsequently to NO. NO is an important endothelial relaxing factor via the secondary messenger cyclic guanosine monophosphate which results in smooth muscle relaxation and vasodilatation (Gruetter, Barry et al. 1979).

1.7.2 Endothelial function

Endothelial dysfunction is a key event in the development of arteriosclerosis. NO has an important signalling role in vascular biology (Furchgott and Zawadzki 1980) and if there is a deficit of NO it is associated with endothelial dysfunction (Heiss, Lauer et al. 2006). Prevention of atherosclerotic lesions relies on endothelial repair. The mobilisation of bone-marrow derived circulating angiogenic cells (CAGs) contribute to the endothelial repair mechanisms (Kondo,

Hayashi et al. 2004, Laufs, Werner et al. 2004). The mobilisation of these CAGs is via eNOS (NOS-3)-dependent process whereby NO mobilises CAGs via the activation of bone-marrow matrix metalloproteinase-9 (Aicher, Heeschen et al. 2003, Balligand, Feron et al. 2009). NO_3^- supplementation has been shown to increase the mobilisation of CAGs. Not only does a dose of $NaNO_3$ (NO_3^- 0.15 $mmol.kg^{-1}$) in healthy individuals result in an increase in the quantity of circulating CAGs but it also improves NOS-dependent flow mediated dilatation (FMD) (Heiss, Meyer et al. 2012) these effects are mediated by the formation of NO.

Endothelial dysfunction and reduced NO bioavailability are also linked to the initiation and progression of heart failure (Marti, Gheorghiade et al. 2012). Heart failure results in a state of increased oxidative stress which in turn reduces the production of NO (Ontkean, Gay et al. 1991, Vanhoutte, Shimokawa et al. 2009). The cytokines produced by this endothelial dysfunction down-regulate eNOS (Smith, Sun et al. 1996, Agnoletti, Curello et al. 1999). Ultimately these processes reduce NO bioavailability and worsen endothelial function which in turn worsens the development and progression of heart failure (Parodi, De Maria et al. 2007). Acute and chronic (7 days) of NO_3^- supplementation studies in heart failure have shown improved outcomes in heart failure with preserved ejection fraction (Zamani, Rawat et al. 2015, Eggebeen, Kim-Shapiro et al. 2016, Zamani, Tan et al. 2017). In a double-blind, randomised, cross-over study of an acute dose of dietary NO_3^- in the form of BRJ (NO_3^- 12.9 mmol) resulted in an increase in exercise duration (mean duration 0.8 ± 1.3 minutes, $p = 0.02$) (Zamani, Rawat et al. 2015). Chronic supplementation with dietary NO_3^- in the form of BRJ (NO_3^- 6.1 $mmol.day^{-1}$ for 7 days) in individuals with heart failure with preserved ejection fraction also resulted in an increased endurance time (BRJ: 449 ± 180 s vs PL: 363 ± 125; 24% increase, $p = 0.002$) (Eggebeen, Kim-Shapiro et al. 2016). When NO_3^- supplementation has been investigated in heart failure with reduced ejection fraction it has not conferred any benefit to exercise tolerance, central

haemodynamics, skeletal muscle oxygenation or the $\dot{V}O_2$ of exercise (Hirai, Zelt et al. 2017). The variation in response to dietary NO_3^- supplementation in heart failure with preserved ejection fraction and reduced ejection fraction on exercise outcomes may well be due to the accentuation of peripheral oxygen extraction abnormalities in heart failure with preserved ejection fraction compared to heart failure with reduced ejection fraction (Dhakal, Malhotra et al. 2015). The most likely mechanism of improved exercise efficiency in heart failure following the supplementation of NO_3^- is that of targeted delivery of NO resulting in decreased systemic vascular resistance and an increased delivery of O_2 to exercising muscle (Zamani, Rawat et al. 2015, Eggebeen, Kim-Shapiro et al. 2016, Hirai, Zelt et al. 2017, Zamani, Tan et al. 2017).

1.7.3 Platelet aggregation

It has long been understood that platelets play a pivotal role in the development and progression of coronary artery disease. NO_3^- supplementation has been found to exhibit an anti-platelet effect with reduced platelet aggregation (Richardson, Hicks et al. 2002, Webb, Patel et al. 2008). The mechanism behind this appears to be NO activation of cyclic guanosine monophosphate (Moro, Russel et al. 1996) and the formation of nitrotyrosine which inhibits platelet function (Beckman, Beckman et al. 1990). NO_3^- which is present in a readily ingested healthy diet may therefore relate to a reduction in the incidence of cardiovascular disease (Richardson, Hicks et al. 2002).

1.7.4 Exercise efficiency

The fuel of exercise is the terminal phosphate (P) of adenosine triphosphate (ATP). During steady state moderate intensity exercise the major source of ATP regeneration is oxidation of carbohydrates and fatty acids. At steady state the oxygen consumption ($\dot{V}O_2$) of all tissues,

including muscle, required to regenerate ATP is reflected by the alveolar $\dot{V}O_2$. Central to the understanding of exercise physiology is the measurement of alveolar oxygen uptake (measured by the collection of exhaled gases). It is by analysis of these exhaled gases that the alveolar $\dot{V}O_2$ can be used to provide an estimate of the systemic oxygen content for delivery to exercising muscles. The matching of the alveolar $\dot{V}O_2$ to the muscle $\dot{V}O_2$ is a reflection of the effectiveness of oxygen delivery by the circulation. During exercise at steady-state the alveolar $\dot{V}O_2$ should be reflective of the $\dot{V}O_2$ of all tissues. It is possible to define exercise efficiency as the ratio of mechanical energy output to metabolic energy output (Grassi, Rossiter et al. 2015). If the $\dot{V}O_2$ of a given exercise can be reduced, then it would be possible to improve the exercise efficiency and thus improve the performance of the exercise in question.

The dogma of exercise physiology was that the $\dot{V}O_2$ of exercise at a given workload (W) was fixed (with a 10 $mL.min^{-1}$ increase in $\dot{V}O_2$ required for every 1 watt increase in power output), and to be independent of factors such as age, health status and physical fitness (Jones 2005). This was until 2007, when Larsen *et al* (Larsen, Weitzberg et al. 2007) discovered that dietary NO_3^- supplementation could reduce the $\dot{V}O_2$ of a given exercise. In this randomised, double-blind, placebo-controlled crossover study in nine, healthy and well trained males, received 3-days of $NaNO_3$ (NO_3^- 0.1 mmol kg^{-1}.bodyweight.day^{-1}) or sodium chloride with a 10-day washout period (Larsen, Weitzberg et al. 2007). The headline finding being that following NO_3^- supplementation there was a statistically significant reduction of peak $\dot{V}O_2$ ($\dot{V}O_{2peak}$) compared to placebo ($\dot{V}O_2$ 0.16 $L.min^{-1}$, $p < 0.02$) (Larsen, Weitzberg et al. 2007). A further finding of interest, was a statistically and clinically significant reduction in dBP of 3.7 mmHg ($p < 0.02$) following NO_3^- supplementation (Larsen, Ekblom et al. 2006). This finding is suggestive of increased NO bioavailability following NO_3^- supplementation. The mechanism by which NO mediates a reduction in BP is unclear, but may related to vasodilatation (Cosby, Partovi et al.

2003, Larsen, Ekblom et al. 2006, Webb, Patel et al. 2008). It is also important to highlight that the equivalent dose of nitrate could be found in 150-250 g of NO_3^--rich vegetables (Lundberg and Govoni 2004) and is thus readily available to the general population (Larsen, Weitzberg et al. 2007, Hord, Tang et al. 2009).

As a follow up to the pivotal work by Larsen *et al* (Larsen, Weitzberg et al. 2007), a separate research group independently investigated a natural dietary source of NO_3^-, in the form of organic BRJ (Bailey, Winyard et al. 2009). In this randomised, double-blind, placebo-controlled crossover study, Bailey *et al* (Bailey, Winyard et al. 2009) recruited eight healthy and recreationally active males to consume 0.5 $L.day^{-1}$ of BRJ (NO_3^- 5.5 mmol.day-^{1}) for four to six days. The findings corroborated those of Larsen *et al* (Larsen, Ekblom et al. 2006) with a 19% reduction in the pulmonary $\dot{V}O_2$ during moderate-intensity cycling, following dietary NO_3^- supplementation (BRJ 521 ± 153 $ml.min^{-1}$ vs. placebo 640 ± 146 $ml.min^{-1}$, $p < 0.01$) (Figure 1.3). Once again, this study also demonstrated a statistically and clinically significant reduction in mean sBP of 6 mmHg (Bailey, Winyard et al. 2009).

The finding of a reduction in the oxygen cost of submaximal exercise by Larsen *et al* (Larsen, Weitzberg et al. 2007) and Bailey *et al* (Bailey, Winyard et al. 2009) has important implications for athletes in whom a high exercise efficiency (i.e. a low oxygen cost for a given power output) is a key determinant of exercise performance (Jones and Burnley 2009). Both studies were undertaken in healthy populations, but there are implications for other populations. In chronic respiratory conditions a reduction in the oxygen cost of the activities of daily living may significantly improve functional capacity and quality of life.

Following on from these early studies, the role of dietary NO_3^- supplementation on exercise efficiency has undergone a great deal of study. The results, however, have been variable. Dietary NO_3^- supplementation has shown to improve exercise efficiency in cycling (Larsen, Weitzberg et al. 2010, Vanhatalo, Bailey et al. 2010, Larsen, Schiffer et al. 2011, Cermak, Gibala et al. 2012, Wylie, Kelly et al. 2013, Muggeridge, Howe et al. 2014, Flueck, Bogdanova et al. 2016, Porcelli, Pugliese et al. 2016, Whitfield, Ludzki et al. 2016, Wylie, Ortiz de Zevallos et al. 2016, Thompson, Wylie et al. 2017, Thompson, Vanhatalo et al. 2018), walking (Lansley, Winyard et al. 2011), running (Lansley, Winyard et al. 2011), kayaking (Muggeridge, Howe et al. 2013, Peeling, Cox et al. 2015) and knee extension (Bailey, Fulford et al. 2010). Despite these positive outcomes a number of studies have also found no change to exercise efficiency (Bescos, Rodriguez et al. 2011, Peacock, Tjonna et al. 2012, Wilkerson, Hayward et al. 2012, Christensen, Nyberg et al. 2013, Boorsma, Whitfield et al. 2014, Muggeridge, Sculthorpe et al. 2015, Sandbakk, Sandbakk et al. 2015, Betteridge, Bescos et al. 2016, Ghiarone, Ataide-Silva et al. 2017, Nyakayiru, Jonvik et al. 2017, Balsalobre-Fernandez, Romero-Moraleda et al. 2018, Kocoloski and Crecelius 2018, Oskarsson and McGawley 2018, Wickham, McCarthy et al. 2019, Mosher, Gough et al. 2020). In a recent study Berry *et al* (Berry, Miller et al. 2020) seven days of dietary NO_3^- supplementation (NO_3^-: 9.9 $mmol.day^{-1}$) in well-trained middle to older age adults did not result in a statistically significant improvement in exercise tolerance compared to placebo. The finding of a 79 seconds increase in exercise time in the NO_3^- supplementation arm may well be meaningful for this population. There is also evidence to suggest that exercise economy and performance is increased following dietary NO_3^- supplementation in hypoxic conditions (Masschelein, Van Thienen et al. 2012, Pawlak-Chaouch, Boissière et al. 2016, Shannon, Duckworth et al. 2016). Cocksedge *et al* (Cocksedge, Breese et al. 2020) investigated if dietary NO_3^- supplementation would improve oxygen uptake and exercise tolerance in normoxia, hyperoxia and hypoxia following the acute consumption of dietary NO_3^- (NO_3^-: 18.6

mmol). Under hypoxaemic conditions there was an increase in peak oxygen uptake ($p < 0.05$) but not in normoxia or hyperoxia ($p > 0.05$). This suggests that the ergogenic potential of dietary NO_3^- supplementation may be augmented in conditions where there is skeletal muscle hypoxia.

The variability in outcomes in trials of dietary NO_3^- supplementation may well relate to differences in the dosing regimens, the doses of dietary NO_3^- supplementation and not using a robust placebo. When studying healthy and athletic populations it is also important to consider that there are responders and non-responders to NO_3^- supplementation (Hultström, Amorim de Paula et al. 2015, Porcelli, Ramaglia et al. 2015). The lack of response to dietary NO_3^- supplementation in athletes may in part be explained by intrinsic high levels of NO_3^- metabolites through both exercise induced upregulation of NOS activity (McConell, Bradley et al. 2007) and the selection of a diet rich in NO_3^-. These factors need to be considered when designing trials of dietary NO_3^- supplementation.

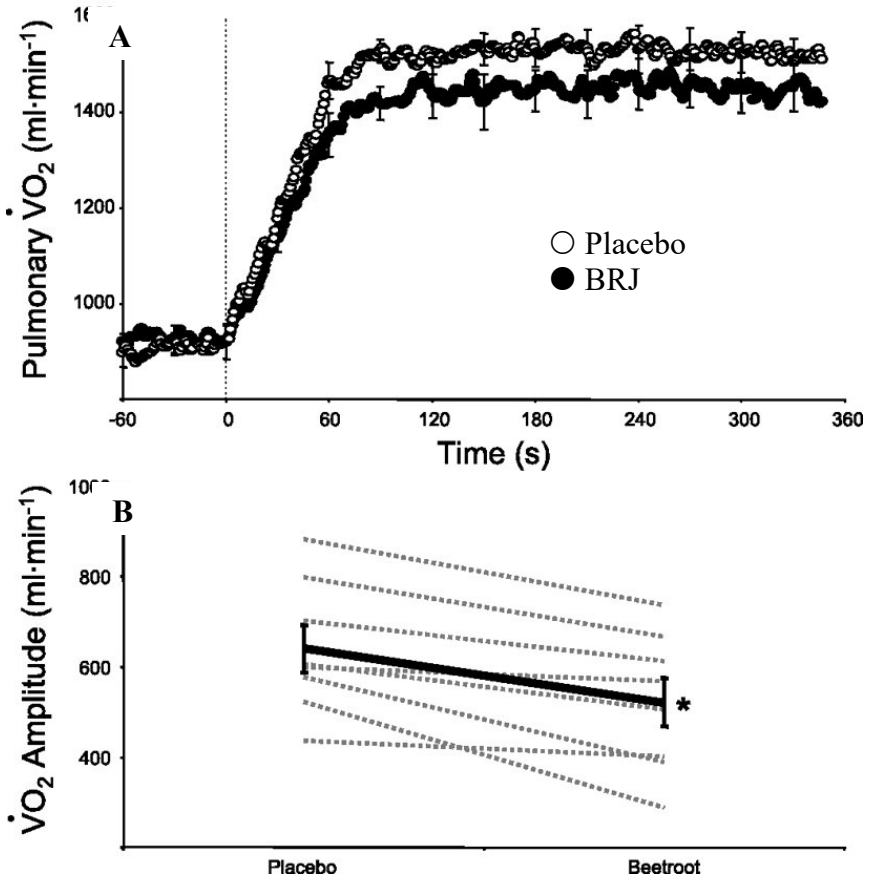


Figure 1.3. Pulmonary oxygen uptake response following NO_3^- and placebo supplementation during a step increment to a moderate-intensity work rate. Adapted with permission from Bailey *et al* (Bailey, Winyard et al. 2009).

Panel A. Data shown is mean pulmonary $\dot{V}O_2$ (with error bars) for every 30 s. Response to BRJ shown as solid circles and response to placebo shown as open circles. The dotted horizontal line represents the start of moderate-intensity work rate from a state of unloaded cycling. The pulmonary $\dot{V}O_2$ of moderate-intensity exercise was significantly reduced in the BRJ supplementation group (BRJ 521 ± 153 ml.min^{-1} vs. placebo 640 ± 146 ml.min^{-1}, $p < 0.01$).

Panel B. Data shown is individual changes in amplitude of $\dot{V}O_2$ response following BRJ supplementation (dotted lines) and the group mean (solid line)

Abbreviations: $\dot{V}O_2$ – Oxygen consumption; BRJ – Beetroot juice

1.8 Potential mechanisms by which dietary nitrate improves exercise efficiency

It is likely that the mechanisms by which dietary NO_3^- result in a reduction in the oxygen cost of exercise are either independent or a combination of improvements to mitochondrial function, muscle contractile efficiency and skeletal muscle blood flow. Given the ubiquitous nature of NO and the many and varied roles that NO plays on physiological parameters it is unsurprising that there are many possible molecular explanations for the reported systemic effects of NO_3^- supplementation.

1.8.1 Mitochondrial efficiency

Adenosine triphosphate (ATP) is the high energy molecule that provides the energy required to produce muscle contraction. During exercise there is an increase in energy demands, thus the supply of O_2 must also increase to support further production of ATP. It is possible to estimate the efficiency of ATP generation from oxidative phosphorylation from the ATP yield per O_2 molecule (or P/O ratio). With the higher the P/O ratio relating to a more efficient process of oxidative phosphorylation (Hinkle 2005). It is possible NO_3^- supplementation improves the efficiency of mitochondrial respiration. Potential mechanisms behind this improved mitochondrial efficiency are that NO_3^- increases the ATP yield per O_2 molecule thus increasing the P/O ratio via a reduction in the amount of ATP lost in the uncoupling process (e.g. mitochondrial proton pump leakage) (Echtay, Roussel et al. 2002, Brand, Pakay et al. 2005). Alternatively, NO acts as an alternative terminal electron acceptor which competes with O_2 for ATP production (Brown and Cooper 1994, Cleeter, Cooper et al. 1994, Bescos, Rodriguez et al. 2011).

In 2011, Larsen *et al* (Larsen, Schiffer et al. 2011) attempted to investigate how NO_3^- influenced mitochondrial efficiency. In this double-blind, placebo-controlled, randomised, cross-over study healthy individuals underwent three days of $NaNO_3$ supplementation (NO_3^- 0.1 mmol.kg-1.day-1) with muscle biopsies taken from the vastus lateralis to assess the mitochondrial P/O ratio. Following NO_3^- supplementation the mitochondrial P/O ratio increased by 19% which is suggestive that some of the improvement in exercise efficiency occurs at the mitochondrial level. The mechanistic basis for this improved mitochondrial P/O ratio may lie with a reduction in proton leak or slip from the inner mitochondrial membrane following NO_3^- supplementation. When the mitochondrial proteins (e.g. adenine nucleotide translocase) associated with proton conductance were studied the expression of these proteins was reduced (Larsen, Schiffer et al. 2011). There was a correlation between the detected increase in P/O ratio and whole-body steady-state exercise $\dot{V}O_2$. This correlation indicates NO_3^- supplementation in part lowers the O_2 cost of exercise through improved mitochondrial efficiency via the lowering of uncoupling protein expression (Larsen, Schiffer et al. 2011). Support for this mechanism has been shown in *vitro* where NO directly increases oxidative phosphorylation (Clerc, Rigoulet et al. 2007).

Contrary to the findings of Larsen *et al* (Larsen, Schiffer et al. 2011) an alternative mechanism for the NO_3^- supplementation mediated reduction in O_2 cost of exercise has been suggested by Whitfield *et al* (Whitfield, Ludzki et al. 2016) that of NO_3^- modifying mitochondrial bioenergetics. In this study NO_3^- supplementation conferred a reduction in the O_2 cost of submaximal exercise but showed no change in mitochondrial coupling P/O ratios or respiratory responses (Whitfield, Ludzki et al. 2016). In contrast to the findings of Larsen *et al* (Larsen, Schiffer et al. 2011) this study found no evidence to suggest NO_3^- supplementation altered the mitochondrial P/O ratio however it demonstrated an increase in reactive oxygen species (Whitfield, Ludzki et al. 2016) which act to induce cell signalling allowing skeletal muscle to generate increased force (Smith

and Reid 2006, Powers and Jackson 2008). Given these findings it is suggestive that changes to mitochondrial efficiency are not the only explanation for the improved exercise efficiency following NO_3^- supplementation.

The influence of NO_3^- supplementation on mitochondrial efficiency is somewhat controversial with the findings of Larsen *et al* (Larsen, Schiffer et al. 2011) not being replicated by Whitfield *et al* (Whitfield, Ludzki et al. 2016). The variation of these findings may well be influenced by the method of NO_3^- supplementation. Larsen *et al* choosing to use three days of sodium nitrate (Larsen, Schiffer et al. 2011) whereas Whitfield *et al* supplemented NO_3^- through seven days of NO_3^--rich beetroot juice (Whitfield, Ludzki et al. 2016). It may therefore be possible that these two supplements act through different mechanisms.

1.8.2 Muscle contractile efficiency

As previously discussed, ATP is required by skeletal muscle to perform work. The generation of ATP is via glycolysis and oxidative phosphorylation or regeneration from adenosine diphosphate via muscle stores of phosphocreatine. A potential alternative mechanism for the improved exercise efficiency seen following the supplementation of NO_3^- is that NO_3^- reduces the ATP cost of force production. This finding was first reported by Bailey *et al* (Bailey, Fulford et al. 2010). In this double-blind, randomised, placebo-controlled cross-over study of six days of NO_3^- supplementation in the form of BRJ (NO_3^- 5.1 $mmol.day^{-1}$) a reduction in the O_2 cost of was detected and by using ^{31}P magnetic resonance spectroscopy for non-invasive *in vivo* assessment of intramuscular metabolites a reduction in the ATP cost of force production for a given work rate was also detected (Figure 1.4) (Bailey, Fulford et al. 2010). The authors hypothesised that the mechanism behind this reduction of the ATP cost of force production relates to the

possibility that NO reduces calcium cycling (Hart and Dulhunty 2000, Viner, Williams et al. 2000) and slows cross-bridge cycling kinetics (Galler, Hilber et al. 1997, Heunks, Cody et al. 2001). The ATP cost of force production in skeletal muscle is the sum of ATP consumption via the interaction between actin and myosin (actomyosin-ATPase) and calcium pumping within the sarcoplasmic reticulum (Calcium-ATPase). By slowing cross bridge cycling, NO reduces calcium release into the sarcoplasmic reticulum and therefore slows ATP consumption.

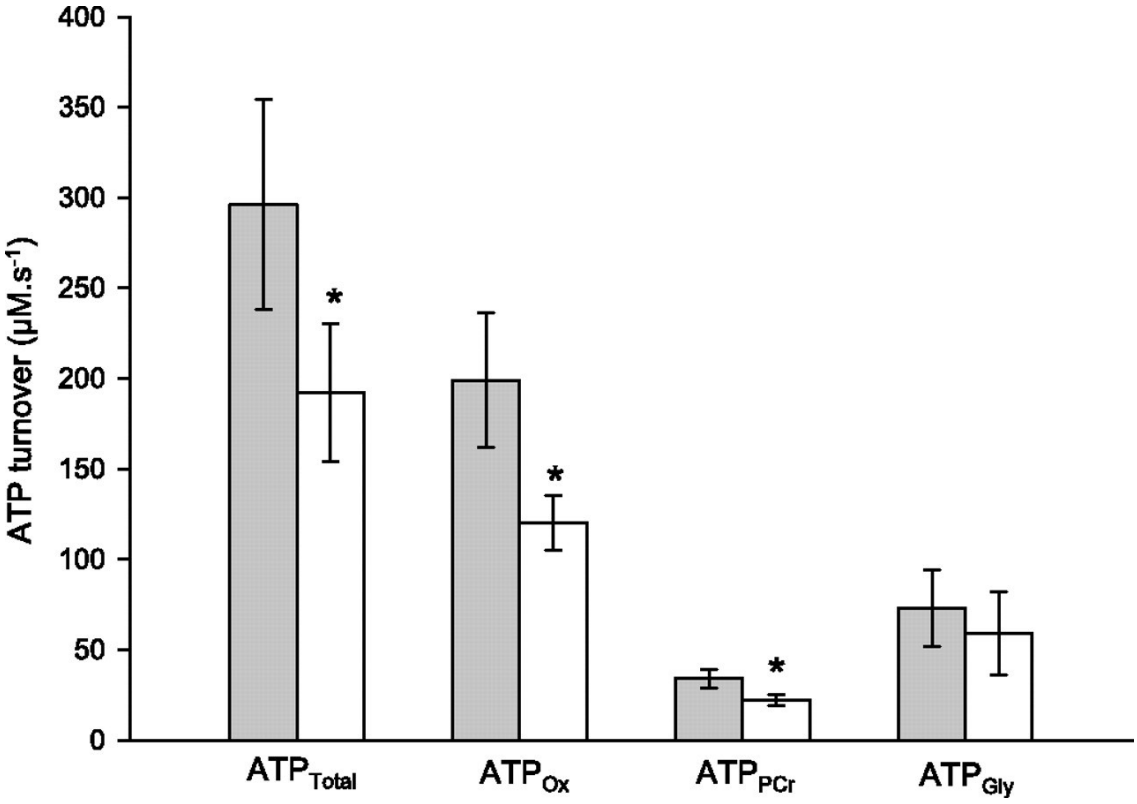


Figure 1.4. ATP resynthesis averaged over entire low-intensity knee-extension exercises. Adapted with permission from Bailey *et al* (Bailey, Fulford et al. 2010)

Data shown are group mean ± SE. Response to dietary NO_3^- are shown in white bars and response to placebo are shown in grey bars. Total ATP turnover (ATP_{Total}), ATP from oxidative phosphorylation (ATP_{Ox}), ATP derived from PCr splitting (ATP_{PCr}) and ATP from glycolysis (ATP_{Gly}) are shown. Reductions in ATP_{Total}, ATP_{Ox} and ATP_{PCr} were statistically significant (*$p < 0.05$)

Abbreviations: ATP – Adenosine triphosphate; ATP_{Total} – Total ATP turnover; ATP_{Ox} – ATP from oxidative phosphorylation; ATP_{PCr} – ATP derived from PCr splitting; ATP_{Gly} – ATP from glycolysis

1.8.3 Skeletal muscle blood flow

NO is an important endothelial relaxing factor via the secondary messenger cyclic guanosine monophosphate which results in smooth muscle relaxation and vasodilatation (Gruetter, Barry

et al. 1979). This is evidenced following supplementation of NO_3^- by a reduction in blood pressure parameters (Larsen, Ekblom et al. 2006, Webb, Patel et al. 2008). There is also evidence to suggest that NO_3^- supplementation results in increased blood flow to exercising skeletal muscle. In a Sprague-Dawley rat model NO_3^- supplementation in the form of BRJ (NO_3^- 1 $mmol.kg^{-1}.da^{-1}$ for 5 days) resulted in a reduction in exercising MAP and an increase in hind limb blood flow predominately to the fast twitch type IIb muscle fibres (Ferguson, Hirai et al. 2013). This suggests that NO_3^- has a fibre type selective effect. It has been possible using near-infrared spectroscopy of the gastrocnemius muscle during exercise to show that NO_3^- supplementation also results in an increase in tissue oxygenation with a reduction in tissue fractional O_2 extraction at maximal exercise (Kenjale, Ham et al. 2011).

1.9 Optimal dietary nitrate supplementation strategy

Various dosing regimens and dosing strategies have been assessed including acute (1-3 h dosing) and chronic (3-15 days) at doses between 5.2 mmol and 16.8 mmol. Various combinations of these strategies have been shown to reduce the O_2 cost of exercise. A reduction in the O_2 cost of exercise has been demonstrated following acute supplementation of NO_3^- (Vanhatalo, Bailey et al. 2010, Muggeridge, Howe et al. 2013, Wylie, Kelly et al. 2013). However, other groups have found that chronic dosing may be more effective (Muggeridge, Howe et al. 2013, Jones 2014) with 3-7 days NO_3^- supplementation showing improved mitochondrial efficiency (Larsen, Schiffer et al. 2011) and contractile function (Hernandez, Schiffer et al. 2012).

Wylie *et al (Wylie, Kelly et al. 2013)* set out to investigate the pharmacodynamics and pharmacodynamic effects of acute (2.5 h) NO_3^- supplementation in the form of BRJ. The NO_3^- doses investigated included 4.2, 8.4 and 16.8 mmol the equivalent of 70, 140 or 280 mL of

concentrated BRJ. The concentrations of plasma NO_3^- and NO_2^- increased in a dose-dependent fashion up to 16.8 mmol of NO_3^- with peak changes occurring at 2-3 h post dosing. There was no difference in the O_2 cost of moderate-intensity exercise at a dose of 4.2 mmol of NO_3^- and placebo. However, at the higher doses of 8.4 and 16.8 mmol of NO_3^- there was a reduction in steady state O_2 cost of moderate-intensity exercise (Wylie, Ortiz de Zevallos et al. 2016).

The same research group went on to investigate the different dosing strategies of NO3-supplementation on the O_2 cost of submaximal exercise: acute (1-3 h) vs chronic (3-30 days) (Wylie, Ortiz de Zevallos et al. 2016). The doses assessed were 3 mmol and 6 mmol of NO_3^- in the form of BRJ. Assessments were made at days 7 and 30, with the testing at day 30 being made without acute consumption of NO_3^- and on a separate occasions following acute consumption thus also assess the acute response of NO_3^- supplementation following chronic dosing. Despite resulting in an increased plasma NO_3^- and NO_2^- dosing for 7 and 30 days of 3 mmol did not result in a reduction in the O_2 cost of submaximal exercise. Whereas dosing of 6 mmol NO^{3-} was enough to reduce the O_2 cost of submaximal exercise at 7 and 30 days. At this dose there was also a trend to reduction of the O_2 cost of exercise following acute dosing (Wylie, Ortiz de Zevallos et al. 2016).

These two studies by Wylie *et al* (Wylie, Kelly et al. 2013, Wylie, Ortiz de Zevallos et al. 2016) suggest that there is no additional benefit of acutely dosing (1-3 h) NO_3^- supplementation at a dose greater than 8.4 mmol NO_3^-. They also suggest that chronic daily dosing at a dose of 6 mmol NO_3^- is also effective with evidence that the improved exercise efficiency persists even when a dose has not been consumed at 30 days.

Following a NO_3^- bolus, plasma NO_3^- concentration peak at approximately 1-2 h and plasma NO_2^- levels peak at 2-3 h (Wagner, Schultz et al. 1983), with both concentrations falling to baseline after 24 h (Webb, Patel et al. 2008). Studies have shown that between 2-6 days and up to 30 days of daily supplementation appear to elicit a favourable physiological response to exercise. The acute supplementation of NO_3^- on exercise is less consistent. The efficacy of NO_3^- supplementation will depend on several factors including age, health, diet, fitness and training status and the intensity and nature of the task being completed. The duration of continuous exercise for which NO_3^- is ergogenic ranges between 5 and 30 minutes with limited evidence for longer durations of exercise (> 40 minutes).

1.10 Studies of dietary nitrate supplementation on exercise efficiency and exercise performance in individuals with COPD

Following on from the finding of improved exercise efficiency and exercise performance in healthy individuals there has been interest in the potential role of dietary NO_3^- supplementation in individuals with COPD. At the onset of this thesis, there have been nine studies investigating the role of dietary NO_3^- supplementation on exercise efficiency and exercise performance outcomes in individuals with COPD (summarised in Table 1.2). To date the findings have been somewhat equivocal with four of the nine studies finding a beneficial outcome of NO_3^- supplementation in individuals with COPD.

Author	Participants	FEV1 % Pred	Primary Outcome	Supplementation	Placebo	Timing of Intervention	Protocol	NO Indices	Effect on Blood Pressure	Primary Outcome
(Kerley, James et al. 2019)	n = 8	55 ± 19	ISWT distance	140 mL BRJ (12.9 mmol NO_3^-.day^{-1}) for 14 days	140 mL NO_3^- deplete BRJ (0.002 mmol NO_3^-)	2.5 h	Double-blind, randomised, placebo-controlled, cross-over study	↑Plasma [NO_3^-] ↑Plasma [NO_2^-]	↔sBP ↔dBP	↑ISWT distance
(Behnia, Wheatley et al. 2018)	n = 25	N/A (GOLD Stage 2.4 ± 0.8)	SBDLCO & Cycle ergometry maximal cycle $\dot{V}O_{2peak}$	70 mL BRJ mixed with 140 mL Black currant juice (4.8 mmol NO_3^-.day^{-1}) 8 days	250 mL Black currant juice (no NO_3^-)	N/A	Double-blind, randomised, placebo-controlled, parallel group study	N/A	N/A	↔ SBDLCO ↔ $\dot{V}O_{2peak}$
(Beijers, Huysmans et al. 2017)	n = 18	69.2 ± 16.3	Cycle ergometer – submaximal (50% & 70%) $\dot{V}O_2$	140 mL $NaNO_3$ (~8 mmol NO_3^-.day^{-1}) for 7 days	140 mL NaCl (no NO_3^-)	2.5 h	Double-blind, randomised, placebo-controlled, cross-over study	↑Plasma [NO_3^-] ↔ Plasma [NO_2^-]	N/A	↔ 50% or 70% submaximal $\dot{V}O_2$
(Friis, Steenholt et al. 2017)	n = 15	44.7 ± 15.1	6MWT distance & cycle ergometer submaximal (50%) cycling $\dot{V}O_2$	140 mL BRJ (12.9 mmol NO_3^-.day^{-1}) for 6 days	140 mL NO_3^- deplete BRJ (0.002 mmol NO_3^-)	2.5 h	Double-blind, randomised, placebo-controlled, cross-over study	↑Plasma [NO_2^-]	↔sBP ↓dBP	↔6MWT distance ↔submaximal cycling $\dot{V}O_2$

(Curtis, O'Brien et al. 2015)	n = 21	50.1 ± 21.6	Cycle ergometer - constant work rate (70%), isotime $\dot{V}O_2$ & exercise endurance time	140 mL BRJ (12.9 mmol NO_3^-) single dose	140 mL NO_3^- deplete BRJ (mmol NO_3^-)	3 h	Double-blind, randomised, placebo-controlled, cross-over study	↑Plasma [NO_3^-] ↔Plasma [NO_2^-]	↔sBP ↓dBP ↔MAP	↔exercise endurance time ↓cycling isotime $\dot{V}O_2$
(Leong, Basham et al. 2015)	n = 19	62.0 ± 6.9	ESWT distance	70 mL BRJ (4.8 mmol NO_3^- twice.day^{-1}) for 3 days	140 mL NO_3^- deplete BRJ (0.002 mmol NO_3^-)	1 h	Double-blind, randomised, placebo-controlled, cross-over study	N/A	↔sBP ↔dBP	↔ESWT distance
(Shepherd, Wilkerson et al. 2015)	n = 14	57 ± 9	Cycle ergometer - submaximal (80%) & 6MWT distance	70 mL BRJ (6.77 mmol NO_3^- twice.day^{-1}) for 2 days	140 mL NO_3^- deplete BRJ (0.002 mmol NO_3^-)	2.5 h	Double-blind, randomised, placebo-controlled, parallel group study	↑Plasma [NO_3^-]	↔sBP ↔dBP	↔$\dot{V}O_2$ sub-maximal cycling ↔6MWT distance
(Kerley, Cahill et al. 2015)	n = 11	43.4 ± 18.9	ISWT distance	140 mL BRJ (12.9 mmol NO_3^-) & 200 mL blackcurrant cordial single dose	140 mL water & 200 mL blackcurrant cordial	3 h	Double-blind, randomised, placebo-controlled, cross-over study	↑Plasma [NO_2^-]	↓sBP ↓dBP ↓MAP	↑ISWT distance

(Berry, Justus et al. 2015)	n =15	61.8 ± 17.2	Cycle ergometer - submaximal (75%)	140 mL BRJ (7.58 mmol NO_3^-.day^{-1}) for 2 days	163 mL prune juice (0.01 mmol NO_3^-)	2.5 h	Single-blind, randomised, placebo-controlled, cross-over study	↑Plasma [NO_3^-] ↑Plasma [NO_2^-]	↓sBP (rest) ↓dBP (isotime)	↑exercise time

Table 1.2. Description of studies that have investigated the effect of dietary nitrate supplementation on exercise in individuals with COPD

Abbreviations and symbols: GOLD - Global Initiative for Chronic Obstructive Lung Disease; N/A – Not Applicable; ISWT – Incremental Shuttle Walk Test; SBDLCO – Single Breath Diffusion Capacity of the Lung for Carbon Monoxide; 6MWT – Six-metre Walk Test; ESWT – Endurance Shuttle Walk Test; NO_3^- - Nitrate; $NaNO_3$ – Sodium nitrate; NaCl – Sodium Chloride; $\dot{V}O_2$ – Oxygen Cost ↑ - statistically significant increase; ↓ - statistically significant decrease; ⇔ equivocal difference

The first study of NO_3^- supplementation in COPD to show benefits to exercise was performed by Berry *et al* (Berry, Justus et al. 2015). In this single-blind, randomised, placebo-controlled, cross-over study 15 people with GOLD grade 2 COPD (FEV1 % predicted 61.8 ± 17.2) were randomised to either 140 mL of NO_3^- rich BRJ (NO_3^- 7.58 mmol) or PL (163 mL Prune Juice, NO_3^- 0.01 mmol) consumed 2.5 h prior to undertaking a submaximal (75%) constant work rate exercise test on a cycle ergometer (Berry, Justus et al. 2015). Following NO_3^- consumption a median increase of 28.8 sec was found in the NO_3^- supplementation group (Berry, Justus et al. 2015). This study also demonstrated a statistically significantly difference in rest sBP in the favour of the NO_3^- supplementation group (NO_3^--rich BRJ 124.3 [115.2, 133.4] mmHg vs. PL 132.5 [121.7, 143.4] mmHg; p = 0.019) but no difference in iso-time or end exercise sBP (Berry, Justus et al. 2015). No difference was found in resting dBP but there was a statistically significant difference in isotime dBP (NO_3^--rich BRJ 79.9 [72.8, 86.9] mmHg vs. PL 86.3 [79.6, 92.9] mmHg; p = 0.001) and end exercise (NO_3^--rich BRJ 80.0 [72.9, 87.1] mmHg vs. PL 85.6 [79.7, 91.5] mmHg; p = 0.008) (Berry, Justus et al. 2015). Consumption of NO_3^--rich BRJ also resulted in a statistically significantly increase plasma NO_3^- (938%; $p < 0.001$) and NO_2^- (379%; p = 0.001) (Berry, Justus et al. 2015).

Following on from this work a research group from the University of Dublin have undertaken two studies which have demonstrated an increase in exercise efficiency in individuals with COPD following consumption of NO_3^--rich BRJ. The first of these two studies performed in 2015, a double-blind, randomised, placebo-controlled, crossover study, 11 individuals with GOLD grade 3 COPD (FEV1 % predicted 43.4 ± 18.9) were randomised to either 140 mL NO_3^--rich BRJ (NO_3^- 12.9 mmol) mixed with 200 mL of blackcurrant cordial or 140 mL of water mixed with 200 mL of blackcurrant cordial

consumed 3 h prior to undertaking an ISWT (Kerley, Cahill et al. 2015). The primary outcome in this study was a mean increase in ISWT following NO_3^- -rich BRJ supplementation (NO_3^--rich BRJ +24.5 m ± 31.1 vs. PL -13.6 m ± 16.3; p = 0.005) (Kerley, Cahill et al. 2015). Following NO_3^--rich BRJ the was a statistically significant reduction in mean sBP (NO_3^--rich BRJ -12 mmHg ± 19 vs. PL +3 mmHg ± 15; p = 0.03), dBP (NO_3^--rich BRJ -1.6 mmHg ± 16 vs. PL +9 mmHg ± 15; p = 0.045) and MAP (NO_3^--rich BRJ -5 mmHg ± 4 vs. PL +7 mmHg ± 15; p = 0.0018) (Kerley, Cahill et al. 2015). Consumption of NO_3^--rich BRJ also resulted in a statistically significant increase in plasma NO_3^- (NO_3^--rich BRJ 452 uM ± 167 vs. PL 3.4 uM ± 13; p = 0.0000044) and NO_2^- (NO_3^--rich BRJ 612 uM ± 603 vs. PL -3.7 uM ± 54.3; p = 0.009) (Kerley, Cahill et al. 2015). Kerley *et al* (2018) undertook a follow up study, which to date is the longest period of supplementation of NO_3^--rich BRJ (Kerley, James et al. 2019). In this double-blind, randomised, placebo-controlled, cross-over study, eight individuals with GOLD grade 2 COPD (FEV1 % predicted 55 ± 19) consumed either 140 mL of NO_3^--rich BRJ (NO_3^- 12.9 mmol) or a NO_3^--deplete PL (NO_3^- 0.5 mmol) at 9 am for a total of 14 days with the primary outcome measure of a change in ISWT distance (Kerley, James et al. 2019). As in the previous study by this research group (Kerley, Cahill et al. 2015) there was a statistically significant increase in the ISWT in the NO_3^--rich BRJ supplementation group (NO_3^--rich BRJ 440 m ± 161.2 vs. PL 396 m ± 115.6; p = 0.0004) and a statistically significant increase in plasma NO_3^- (NO_3^--rich BRJ 1045.5 µM ± 1201 vs. PL 35.4 µM ± 10.4; p = 0.015) and NO_2^- (NO_3^--rich BRJ 713.2 µM ± 576.5 vs. PL 182.8 µM ± 106.3; p = 0.02) (Kerley, James et al. 2019). On this occasion no difference was found in blood pressure parameters (Kerley, James et al. 2019).

Our own research group undertook a double-blind, randomised, placebo-controlled, cross-over single dose study of dietary NO_3^- supplementation in the form of NO_3^--rich BRJ

(NO_3^- 12.9 mmol) in 21 individuals with GOLD grade 2 COPD (FEV1 % predicted 50.1 ± 21.6) with the primary outcome of the O_2 cost of submaximal exercise (Curtis, O'Brien et al. 2015). No difference was detected in median endurance time (NO_3^--rich BRJ 5.65 minutes (3.90-10.40) vs PL 6.40 minutes (4.01-9.67), p = 0.50) (Curtis, O'Brien et al. 2015). However, there was a reduction in isotime oxygen consumption following NO_3^- supplementation (NO_3^--rich BRJ 16.6 ± 6.0 $ml.min^{-1}.kg^{-1}$ vs PL 17.2 ± 6.0 $ml.min^{-1}.kg^{-1}$, p = 0.043) (Curtis, O'Brien et al. 2015).

The remaining five studies of dietary NO_3^- supplementation in individuals with COPD were unable to replicate these findings. With no improvement in the O_2 cost of submaximal exercise or six-minute walk test (6MWT) distance (Shepherd, Wilkerson et al. 2015, Beijers, Huysmans et al. 2017, Friis, Steenholt et al. 2017), endurance shuttle walk test distance (Leong, Basham et al. 2015) or maximal O_2 uptake (Behnia, Wheatley et al. 2018) following NO_3^- supplementation. It may well be that the variation in outcomes seen in these studies relates to a variation in the dose, dosing schedule employed and timing of assessment.

The studies of dietary NO_3^- in individuals with COPD which failed to detect an improvement in exercise parameters may have done so due to dosing level, dosing duration and then endpoints selected. Shepherd *et al* (Shepherd, Wilkerson et al. 2015) assessed the effect of 2.5 days of dietary NO_3^- supplementation on the oxygen cost of submaximal cycling (n=14). The individuals in this trial had moderate COPD (FEV1 57 ± 9%) and a BMI of (BMI 29 ± 8 $kg.m^{-2}$). Participants consumed 70 mL NO_3^--rich BRJ (NO_3^-:6.77 mmol) once a morning and once an evening for two days and then once in the morning

on the day of testing. This study failed to show a reduction in the oxygen cost of submaximal cycling. Beijers *et al* (Beijers, Huysmans et al. 2017) assessed the acute and seven day effects of sodium NO_3^- (NO_3^-: 8 mmol.day^{-1}) on submaximal cycling endurance time (n=18). Individuals studied had moderate COPD (FEV1 69.2 ± 16.3%) and a BMI of (25.9 ± 3.4 kg.m^{-2}). No improvement of submaximal endurance cycling time was detected. Friis *et al* (Friis, Steenholt et al. 2017) assessed the effect of seven days of dietary NO_3^- supplementation on exercise performance using the 6-minute walk test (n=15). The supplementation protocol was 140 mL of NO_3^--rich BRJ (NO_3^-: 12.9 mmol.day^{-1}). Individuals studied had severe COPD (FEV1 44.7 ± 15.1%) and a BMI of (25.5 ± 4.8 kg.m^{-2}). No difference was detected in 6-minute walk test distance. Leong *et al* (Leong, Basham et al. 2015) assessed the effect of three days of dietary NO_3^- supplementation in the form of twice daily 70 ml of NO_3^--rich BRJ (NO_3^-: 4.8 mmol) on a submaximal walk test in the form of the ESWT (n=19). Individuals studied had moderate COPD (FEV1 62.0 ± 6.9%) and BMI (29 ± 6,5 kg.m^{-2}). No improvement in submaximal exercise endurance was detected. Behnia *et al* (Beijers, Huysmans et al. 2017) assessed the effect of 7 days of dietary NO_3^- supplementation in the form of 70 ml of NO_3^--rich BRJ (NO_3^-: 4.8 mmol) on pulmonary gas exchange with the secondary outcome of oxygen cost of maximal exercise via cycle ergometry (n=25). Individuals studied had moderate COPD (GOLD grade 2.4 ± 0.8) and a BMI of (28 ± 6 kg.m^{-2}). Not only did dietary NO_3^- supplementation fail to modulate pulmonary gas exchange it also failed to show a reduction in the oxygen cost of maximal exercise.

The five studies which failed to show an improvement in exercise parameters in individuals with COPD may have done so for several reasons. Various dosing strategies were employed with possible suboptimal dosing with dietary NO_3^-, only one study

employing 12.9 mmol of NO_3^- supplementation. COPD is a heterogenous population and there may well be responders and non-responders to NO_3^- supplementation. With physiological alteration in COPD meaning a dose below 12.9 mmol may be too low to result in benefit. Secondly the choice of cycle ergometry may not have been the correct exercise outcome. Individuals with COPD spend more time walking than cycling and thus walking tests may well be a more appropriate choice in this group. The number of participants in these trials were low, with some trials having a high attrition rate, potentially contributing to these negative findings. The severity of COPD was mild to moderate in all five studies and it may be that more severe disease would have a greater benefit. It is noted within these five studies that the participants had a high BMI. The effect of adiposity on the NO_3^- supplementation effect on exercise outcomes is yet to be defined.

It is now known from the work of Wylie *et al* (Wylie, Kelly et al. 2013, Wylie, Ortiz de Zevallos et al. 2016) that the optimum dose for NO_3^- supplementation is 8.4 mmol with acute effects apparent at 2.5 hours and an evidence basis for chronic dosing.

1.11 Summary

In summary, COPD is a common preventable and treatable heterogeneous condition characterised airflow limitation that is progressive, caused by exposure to noxious particles resulting in an abnormal inflammatory response affecting the lungs and producing a systemic sequela. Dyspnoea is a common finding in individuals with COPD and is a result of an imbalance between oxygen supply to respiratory and skeletal muscle, an imbalance between ventilatory capacity and demand and peripheral skeletal muscle

dysfunction. As well as the physiological mechanisms underlying dyspnoea, there is also an affective component which undoubtably contributes to the development of dyspnoea.

Interventions for COPD are both pharmacological and non-pharmacological. . Poor adherence to pharmacological and non-pharmacological interventions, are of significant concern. The adherence to pharmacological-therapy in individuals with COPD is generally poor, with reports citing adherence rates to varied treatments at approximately 50% (Make 2003). The adherence to non-pharmacological therapies, including a healthy lifestyle and regular exercise programmes are crucial to the management of COPD. Pulmonary rehabilitation is a cost effective and effective intervention in the management of COPD. The impact of PR can be expressed in patient centred outcome measures including improvement in symptoms (including dyspnoea), exercise performance, physical activity, and quality of life measures. Suboptimal responses to PR are still encountered and can be broadly related to the individual, the programme and external factors. Individual reasons for suboptimal response to PR maybe related to previous negative experience of exercise (e.g. fear of exercise) or PR and how valuable the individual perceives the programme to be. Programme related causes of suboptimal response to PR maybe related to the exercise intensity either being too low, resulting in a suboptimal response, or too high with the individual struggling to complete the programme. External factors resulting in suboptimal responses relate to transport barriers (distance and immobility) and social barriers (fear of group work). Augmentation of the benefits in exercise capacity achieved by PR would help enhance the benefits of PR.

One potential mechanism is dietary NO_3^- supplementation. NO is a ubiquitous free radical signaling molecule involved in several biological process at a tissue and cellular level. Supplementation with dietary NO_3^- results in elevated NO bioavailability. Following dietary NO_3^- supplementation exercise efficiency has been found to be improved in healthy individuals and in individuals with COPD. It is likely that the mechanisms for this improved exercise efficiency are a combination of improved mitochondrial efficiency, improved contractile function and increase skeletal muscle blood flow. It is through these potential mechanisms that dietary NO_3^- supplementation in combination with an exercise programme or in hypoxic conditions may well bridge the suboptimal response to exercise in individuals with COPD. Outside of exercise training, individuals with COPD when undertaking activities of daily living work close to their maximum oxygen consumption. Supplementation with dietary NO_3^- may allow for more efficient activities of daily living and energy conservation. Resulting in an improvement in exercise efficiency.

Therefore, the current thesis aims to further extend the knowledge of dietary NO_3^- supplementation on exercise capacity in individuals with COPD in combination to PR and in extreme situations of hypoxia.

1.12 Research questions and hypothesis

The purpose of this thesis was to investigate the effects of dietary NO_3^- supplementation, administered as concentrated NO_3^--rich beetroot juice, on exercise outcomes in individuals with COPD in conjunction with a programme of pulmonary rehabilitation or in the specific phenotype of individuals who are hypoxic during exercise. A secondary aim of this thesis was to investigate the potential effect of dietary nitrate supplementation on

exhaled nitric oxide and endothelial function. This thesis will address the following research questions in UK based individuals with COPD:

1. Does dietary nitrate supplementation in combination with a programme of pulmonary rehabilitation enhance the exercise capacity gains in people with COPD?

I hypothesised that dietary NO_3^- supplementation consumed prior to each exercise session during a programme of pulmonary rehabilitation would enhance the exercise capacity achieved by people with COPD. To address this a double-blinded, placebo-controlled, randomised controlled multicentre study was conducted, with the focus on exercise capacity.

2. Does dietary NO_3^- supplementation administered acutely to people with COPD who experience exercise induced hypoxaemia and require supplemental oxygen enhance exercise endurance?

I hypothesised that in individuals with COPD who experience exercise hypoxaemia who acutely consume a dietary NO_3^- supplement prior to undertaking a period of endurance exercise would enhance the endurance times comparatively to a placebo. To address this a double-blind, placebo-controlled, cross-over randomised study was conducted, with a focus on exercise endurance.

3. How does dietary NO_3^- supplementation effect endothelial function, exhaled nitric oxide and blood pressure?

I hypothesised that dietary NO_3^- supplementation would enhance endothelial function, increased exhaled nitric oxide and reduce blood pressure parameters. To address these secondary outcome measures were undertaken in both studies.

CHAPTER 2: DESCRIPTION OF METHODS

2.1 General methods

The two experimental studies that comprise this thesis (Chapters 3 to 5) involved 142 individuals with COPD undertaking between them 528 incremental shuttle walk tests and 60 endurance shuttle walk tests. These assessments of exercise assessments were undertaken at sea level.

Schematic diagrams of the study design, recruitment dates and statistical analysis plans are contained within relevant Chapters.

2.2 Ethical approval

The studies in this thesis were approved by the London Bridge Research Ethics Committee (REC reference 14/LO/1474) and the London Chelsea Research Ethics Committee (REC reference 15/LO/0975).

All participants involved in these studies provided written informed consent and research was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Table 2.1: Outcome Measures used in each Study

Outcome	ON-EPIC	EDEN-OX
Pulmonary Function Test		☑
Spirometry Only	☑	
MRC Dyspnoea Scale	☑	☑
Anthropometry	☑	☑
Incremental Shuttle Walk Test	☑	☑
Endurance Shuttle Walk Test		☑
CAT Score	☑	☑
HAD A & D Score	☑	☑
Pulmonary Rehabilitation	☑	
Plasma Nitrate and Nitrite Sampling		☑
Flow Mediated Dilatation	☑	☑
Exhaled Nitric Oxide		☑
Physical Activity Monitoring	☑	

Abbreviations: ON-EPIC – Oral Nitrate Supplementation to Enhance Pulmonary Rehabilitation in COPD; EDEN-OX – Effect of Dietary Nitrate Supplementation on Exercise Performance in Hypoxia; MRC – Medical Research Council; CAT – COPD Assessment Test; HAD – Hospital Anxiety and Depression

2.3 Subjects Studied

Participants recruited for the studies in this thesis have a diagnosis of COPD, as defined by the National Institute for Health and Care Excellence (NICE) guidance (www.nice.org.uk) as persons with a risk factor, symptoms suggestive of the diagnosis and post bronchodilator spirometry confirming the diagnosis. The Global Initiative in Obstructive Lung Disease (GOLD) classification was used to categorise the severity of COPD (Table 1.1) (https://goldcopd.org/).

Participants were recruited from outpatient clinics at the Royal Brompton and Harefield NHS foundation Trust, Taunton and Somerset NHS Foundation Trust, Oxleas NHS Foundation Trust and Maidstone and Tunbridge Wells NHS Trust and via referrals to Pulmonary Rehabilitation services and through public outreach events including World COPD Day and Research Engagement Events held at the Royal Brompton Hospital.

Exclusion was made of any participants who possessed comorbid factors that would limit their exercise capacity, significant renal impairment (defined by estimated Glomerular Filtration Rate (eGFR) < 50 ml/min) (significant renal impairment excluded due to supplementation with NO_3^- being associated with reduced renal blood flow secondary to blood flow redistribution resulting in reduced renal perfusion), use of nitrate-based medications (Table 2.3), other clinical reason to benefit from nitrates (ischaemic heart disease and peripheral artery disease), within one month of a pulmonary exacerbation, within one month pulmonary rehabilitation, hypotension (defined as a systolic blood pressure of < 100 mmHg) or pregnancy.

Table 2.3: List Nitrate-based Medications

Medication Class	Brand Name
Glyceryl Trinitrate	Nitro-Dur®
	Minitran®
	Transiderm-Nitro®
	Deponit®
	Percutol®
Isosorbide Dinitrate	
Isosorbide Mononitrate	Imdur®
	Ismo Retard®
	Isotard®
	Monomil® XL
	Chemydur® 60XL
	Modisal® XL
	Monomax® XL
	Isib® 60XL
	Monosorb® XL60
	Zemon®
	Isodur®
	Elantan® LA
Nicorandil	Ikorel®

2.4 Anthropometric Measurements

Height (cm) was measured to the nearest centimetre with the subject in bare feet, using a wall mounted measure. Weight (kg) was measured using standardised scales. Body Mass Index (BMI, $kg.m^{-2}$) was calculated using the following equation:

$$BMI = \frac{\text{Weight (kg)}}{\text{Height}^2\ (m^2)}$$

2.5 Blood Pressure Measurement

Blood pressure was measured with an automated blood pressure monitor (Omron M6, Omron Healthcare Europe, Hoofddorp, Netherlands). Prior to measurement participants were instructed to rest for 10 minutes, seated upright in a chair, with their arm supported at the level of the heart. An appropriate size cuff was selected for each individual. A mean of three measurements was recorded. Mean Arterial Pressure (MAP, mmHg) was calculated using the following equation:

MAP = 1/3 x Systolic Blood Pressure (mmHg) + 2/3 x Diastolic Blood Pressure (mmHg)

2.6 Body Composition

Bioelectrical impedance analysis (BIA) was used to estimate body composition. This technique measures the electrical impedance of the body by introducing an alternating electrical current into the body. The current flows through body fluids by the movement of ions (Foster and Lukaski 1996) and cell membranes act as incomplete capacitors. BIA was performed in a semi supine position using a Bodystat 4000 device (Bodystat, Isle of Man, United Kingdom). Prior to undertaking BIA the Bodystat 4000 device was calibrated as per the manufacture specifications. Two electrodes were placed on the right hand and foot (Figure 2.1) the right side of the body is chosen due to the increased resistance generated by the myocardium if place on the left. Measurements were taken prior to participants undertaking any exercise.

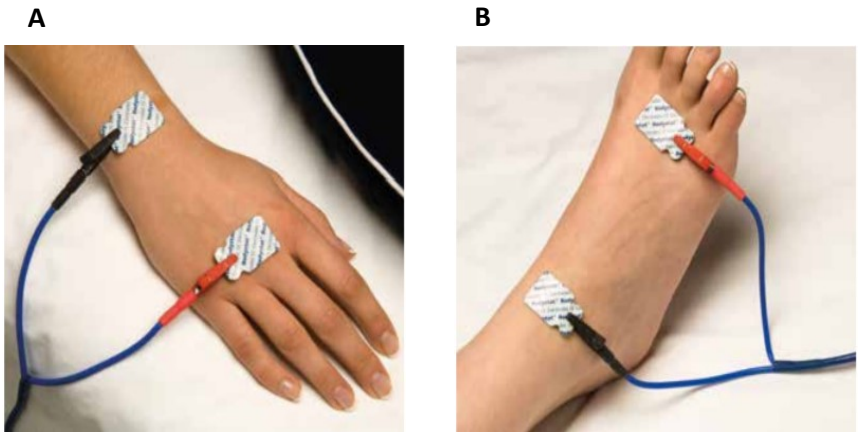


Figure 2.1. Electrode positioning for Bodystat 4000

Electrodes are positioned on the right and the right foot. The right side of the body is preferred over the left due to the resistance generated by the myocardium.

A disease specific regression equation, derived from a population of COPD patients, taking into account height, weight and gender was used to calculate Fat Free Mass (FFM) (Steiner, Barton et al. 2002).

COPD Males:

FFM (kg) = 8.383 + [0.465 x Height^2 (cm) / Resistance (ohm)] + [0.213 x Weight (kg)]

COPD Females:

FFM (kg) = 7.610 + [0.47 x Height^2 (cm) / Resistance (ohm)] + [0.184 x Weight (kg)]

Fat Free Mass Index (FFMI) was calculated by dividing FFM by height (m) squared (kg.m^{-2}). Those participants with a FFMI below 16 kg.m^{-2} in males and 15 km.m^{-2} in females were considered to have fat free mass depletion (Steiner, Barton et al. 2002).

2.7 Exercise Capacity

2.7.1 Incremental Shuttle Walk Test (ISWT)

The incremental shuttle walk test (ISWT) is an externally paced, progressive and maximal exercise test (Holland, Spruit et al. 2014, Holland, Spruit et al. 2015). The ISWT is widely used to assess exercise capacity as part of PR programmes in the UK. The ISWT is a valid measure of cardiopulmonary exercise capacity in COPD and provokes a similar physiological response to cardiopulmonary exercise testing (Palange, Forte et al. 2000, Hill, Dolmage et al. 2012, Holland, Spruit et al. 2014, Zainuldin, Mackey et al. 2015). The

minimal important difference (MID) of the ISWT is between 35.0 and 36.1 metres (Evans and Singh 2019).

The European Respiratory Society (ERS) and American Thoracic Society (ATS) have published guidelines on how to conduct the ISWT (Holland, Spruit et al. 2014). For this thesis, the ISWT was performed to these guidelines. The 10-metre course was laid out on a flat, non-slip surface. Cones were placed nine metres apart to prevent abrupt changes in direction (Figure 2.2) (Holland, Spruit et al. 2014, Holland, Spruit et al. 2015). Standardised instructions were played to participants from a calibrated CD (Glenfield Hospital, Leicester, UK), with the following instructions:

"Walk for as long as possible there and back along the 10 metre course keeping to the speed indicated by the beeps on the CD. You will hear theses beeps at regular intervals. You should walk at a steady pace aiming to turn around the cone at one end of the course when you hear the first beep and at the other end when you hear the next"(Singh, Morgan et al. 1992)

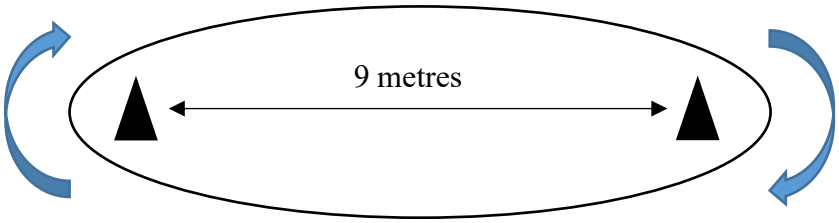


Figure 2.2: ISWT Course Layout

Cones are set at 0.5 metres from either end to avoid abrupt changes in direction.

The ISWT comprises 12 levels and the walking speed during the first level is set at 0.50 $m.sec^{-1}$ (20 seconds per shuttle), with an increment of 0.17 $m.sec^{-1}$ per minute (Holland, Spruit et al. 2014, Holland, Spruit et al. 2015). At the end of each level the participant is instructed to "*increase your speed* now" (Holland, Spruit et al. 2014, Holland, Spruit et al. 2015). The participant is given a warning if they do not walk within half a metre of the cone before the beep "*you are not going fast enough; try to make up the speed this time*" (Holland, Spruit et al. 2014, Holland, Spruit et al. 2015). There are three reasons for the test to be terminated 1) the participant indicates that they are unable to continue, 2) the operator determines that the participant is not fit to continue, or 3) the operator assess the patient will not be able to maintain the speed to the test, defined as the participant being greater than half a metre away from the cone on the second consecutive occasion (Holland, Spruit et al. 2014, Holland, Spruit et al. 2015). The maximum duration of the ISWT is 20 minutes. Prior to testing oxygen saturations, heart rate, dyspnoea and fatigue (using the Borg scale) are recorded. These parameters were recorded at completion of the test and after recovery. Participants were allowed to use prescribed ambulatory oxygen. Its use and method of carriage recorded to keep these factors constant for retesting. To mitigate for learned effect, two tests were performed with 30 minutes rest between tests (Holland, Spruit et al. 2014, Holland, Spruit et al. 2015). The furthest distance walked was used for analysis.

2.7.2 Endurance Shuttle Walk Test (ESWT)

The Endurance Shuttle Walk Test (ESWT) is a constant work-rate tests used to measure exercise endurance. It is conducted at 85% of the maximal walking speed achieved on the ISWT. It has been shown that there is no significant difference between test and retest for the ESWT (Revill, Williams et al. 2009, McKeough, Leung et al. 2011), for the studies in this

thesis a practice ESWT was performed allowing for familiarisation with the testing protocol and to assess the walking speed was correct. The MID of the ESWT is between 186 to 199 seconds (Altenburg, Duiverman et al. 2015) and the MCID of the ESWT is 177 seconds (Zatloukal, Houchen-Wolloff et al. 2015). In studies of bronchodilation a change of 65 seconds (95% CI 45-85 seconds) was found to be perceivable by patients (Brouillard, Pepin et al. 2008).

As with the ISWT the ERS/ATS guidelines were followed when conducting the ESWT (Holland, Spruit et al. 2014). The ESWT is conducted on the same course as the ISWT (Figure 2.2). All elements of the ESWT were performed with participants using their prescribed oxygen. Prior to testing resting heart rate, blood pressure, oxygen saturations and Borg dyspnoea and fatigue scores were recorded. Throughout the ESWT continuous monitoring of heart rate and oxygen saturations were recorded using a Pulseox-300i (Konica Minolta, Basildon, UK). Standardised instructions were played to participants from a calibrated CD (Glenfield Hospital, Leicester, UK), with the following instructions:

"The walking speed for the first two minutes is fairly slow, so don't go too fast. The test will start in 10 seconds so get ready at the start now. the test starts with a triple bleep after a four second countdown. At the next triple bleep increase your walking speed"

(Holland, Spruit et al. 2015)

The ESWT comprises a warm up period of one and a half minutes (Holland, Spruit et al. 2014, Holland, Spruit et al. 2015) following this a triple bleep is played indicating the start of test and an increase in the walking speed. During the test the only verbal cue given

was *"You need to increase your speed to keep up with the test*" (Holland, Spruit et al. 2014). Reasons for termination of the ESWT are the same as those for the ISWT. The outcome of the ESWT is recorded as the time walked in seconds. Peak heart rate, blood pressure and Borg scores were recorded, as was walk recovery time.

2.8 Pulmonary Rehabilitation (PR) Programme

Pulmonary rehabilitation (PR) is a key management strategy in people with chronic respiratory disease. The joint ATS/ERS taskforce defines PR as:

> *"Pulmonary rehabilitation is a comprehensive intervention based on a thorough patient assessment followed by patient-tailored therapies, which include, but are not limited to, exercise training, education, and behavioural change, designed to improve the physical and psychological condition of people with chronic respiratory disease and to promote long-term adherence of health-enhancing behaviours"* (Spruit, Singh et al. 2013)

PR is overseen by a multidisciplinary team, including physicians, physiotherapists and other healthcare professionals (Bolton, Bevan-Smith et al. 2013, Spruit, Singh et al. 2013). PR undertaken for the studies in this thesis was of eight weeks in duration, delivering two exercise sessions per week under direct supervision, with the expectation the participants will exercise at home. The programme was a circuit-style with individually prescribed exercises based on the participants' baseline exercise capacity. Exercise training was progressive in nature with frequent re-assessment and goal setting. Alongside the exercise component there was a regular educational component which included disease and lifestyle specific advice.

The circuit programme included aerobic exercise training, alongside strength training of the upper and lower limbs. Aerobic training included treadmill walking and cycle ergometry, prescribed at 60 to 80% of predicted peak oxygen consumption based on the ISWT performance (Revill, Morgan et al. 1999), with the aim of the participant exercising for 15 minutes with a Borg Dyspnoea Scale level three to four.

Lower and upper limb strength training comprised of resistance exercises based on the American College of Sports Medicine resistance training guidelines (Garber, Blissmer et al. 2011). These exercises include upper and lower limb resistance exercises with weights, sit-to-stand, knee lifts/extension, bicep curls and push ups.

The education component was delivered by a multidisciplinary team which included physicians, physiotherapists, psychologists, dieticians, nurses, occupational therapists, social workers, speech and language therapists and expert patients. This educational component promotes adaptive behaviour change and self-efficacy (Spruit, Singh et al. 2013).

2.9 Physical Activity Monitoring

In patients with COPD, reported physical activity level is related to lung function decline (Garcia-Aymerich, Lange et al. 2007) hospitalisations (Garcia-Aymerich, Farrero et al. 2003) and mortality (Garcia-Aymerich, Lange et al. 2006). The definition of physical activity is any energy expenditure that is a result of skeletal muscle movement (Thompson, Buchner et al. 2003).

To assess physical activity participants were asked to wear a physical activity monitor SenseWear Pro Armband (Bodymedia, Pittsburgh, USA) which incorporates a piezoelectric biaxial accelerometer measuring the body's acceleration in three axes. The device provides an evaluation of daily step count, galvanic skin response, heat flux and skin temperature. The SenseWear Pro Armband has been validated in COPD patients (Patel, Benzo et al. 2007). The armband was worn over the triceps of the right arm (Figure 2.3). The participant was asked to wear it for 24 hr/day (with the exception of when bathing or showering) for 8 days. Data were collected over a period of five days, including two weekend days. For a day to be considered valid for activity measurement, a threshold was set at 22.5 hours of wear time (94% wear time per day). Data were analysed using the SenseWear investigational software (SenseWear Professional version 8.0, Bodymedia, Pittsburgh, USA).



Figure 2.3. SenseWear Pro Armband positioning

The SenseWear Pro Armband (Bodymedia, Pittsburgh, USA) is positioned over the triceps of the right arm.

Data used for analysis included average daily step count, minutes spent above 3 metabolic equivalents (METs) and physical activity level (PAL) was calculated using the following equation:

$$PAL = \frac{\text{Daily Energy Expenditure}}{\text{Whole-night Sleeping Energy Expenditure}}$$

A physical activity level ≥ 1.70 defines an active person (Manini, Everhart et al. 2006), 1.40 – 1.69 defines a predominately sedentary person (Manini, Everhart et al. 2006), <1.40 defines a very inactive person (Black, Coward et al. 1996) and 1.2 defines a chair- or bed-bound person (Black, Coward et al. 1996).

2.10 Pulmonary Function Testing

Pulmonary function testing was performed at the respiratory physiology unit at the Royal Brompton and Harefield NHS Foundation Trust according to the ERS/ATS guidelines (Brusasco, Crapo et al. 2005, Culver, Graham et al. 2017). The following absolute and percentage predicted values recorded: forced expiratory volume in one second (FEV1), forced vital capacity (FVC), FEV1/FVC ratio, residual volume (RV), total lung capacity (TLC) and diffusion capacity for carbon monoxide (DL_{co}). The equipment used was the Spirostick™ PC spirometry system (Love Medical Ltd, Manchester, UK), Jaeger MasterScreen™ Pulmonary Function Laboratory (nSpire Health Ltd, Hertford, UK). Rigorous quality assurance was in place with daily equipment calibration. All pulmonary function tests were performed with participants taking their regular inhaled therapies.

2.10.1 Spirometry

At different PR sites, spirometry was performed using an MicroLab 3500 Spirometer MK 8 (MicroMedical Ltd, Kent, UK) according to the ATS/ERS guidelines (Brusasco, Crapo et al. 2005). Three manoeuvres were performed and data from the best of three reproducible were recorded. Participants were asked to take a deep inspiration to total lung capacity and then perform a maximal forced expiration. The spirometer was calibrated daily according to the manufacturer's specifications.

2.10.2 Lung Volumes and Body Plethysmography

Lung volumes and body plethysmography were performed to the ERS/ATS guidelines (Brusasco, Crapo et al. 2005). Lung volumes were derived via the method of body plethysmography, where the volume within a constant-pressure chamber is measured (Wanger, Clausen et al. 2005). Gas transfer was measured by the inhalation of a known concentration of carbon monoxide (CO), followed by a breath hold and the subsequent measurement of end expiratory CO (Culver, Graham et al. 2017).

2.10.3 Reference Equations

Standardised pulmonary function testing reference ranges were based on the European Coal and Steel Community reference values (Gibson 1993).

2.10.4 Fractional Exhaled Nitric Oxide (F_ENO)

The measurement of fractional nitric oxide (F_ENO) is a safe and non-invasive method to quantitatively measure airways inflammation (Dweik, Boggs et al. 2011). F_ENO was

measured as a steady breath at a flow rate of 50 mL.second^{-1} (F_ENO_{50}) with a NIOX Mino (Aerocrine Systems, Solna, Sweden). The NIOX Mino is a hand-held analyser that has been found to be reliable when compared to the 'gold standard' chemiluminescence F_ENO_{50} analyser (Maniscalco, Vitale et al. 2016) and has a good test-retest reliability thus only one measurement is required (Alving, Janson et al. 2006). The NIOX Mino measures F_ENO_{50} in parts per billion (ppb).

Participants undertook baseline measurements of F_ENO_{50} taken at the screening visit. On the intervention study visits a baseline F_ENO_{50} was measured prior to consuming intervention beverage. Following consumption of the intervention beverage F_ENO_{50} was measured six times at 30-minute intervals (intervals 0 minutes, 30 minutes, 60 minutes, 90 minutes, 120 minutes, 150 minutes and 180 minutes). Participants served as their own controls, study visits conducted at the same time of day and were asked to consume similar meals prior to testing days.

2.11 Health-related Quality of Life Assessments

2.11.1 The Medical Research Council (MRC) Dyspnoea Scale

Level of functional breathlessness in COPD participants was assessed using the MRC Dyspnoea Scale (Bestall, Paul et al. 1999). This is a 5-point scale where the participant subjectively grades their level of dyspnoea when performing activities of daily living (Table 2.4). The MRC dyspnoea score correlates well with lung function measurements and exercise capacity (Stenton 2008, Kelly, Bamsey et al. 2012).

Medical Research Council (MRC) Dyspnoea Scale	
Grade	**Degree of breathlessness related to activities**
1	Not troubled by breathlessness except on strenuous exercise
2	Short of breath when hurrying on the level or walking up a slight hill
3	Walks slower than most people on the level, stops after a mile or so, or stops after 15 minutes walking at own pace
4	Stops for breath after walking about 100 yds or after a few minutes on level ground
5	Too breathless to leave the house, or breathless when undressing

2.11.2 COPD Assessment Test (CAT)

The COPD Assessment Test (CAT) is an eight-item health status assessment for individuals with COPD (Jones, Harding et al. 2009). It is easy to complete independently, taking 2-3 minutes. It has been shown to be responsive to PR and has been validated in assessing health status in COPD (Dodd, Marns et al. 2012). The participants rate their response 0-5, the maximum score being 40, which relates to poor health status The MCID for the CAT is 2 points (Kon, Canavan et al. 2014).

2.11.3 The Hospital Anxiety and Depression Scale (HADS)

The Hospital Anxiety and Depression (HAD) Scale is widely used to assess symptoms of anxiety and depression. It can be completed independently taking 5-10 minutes. It comprises of two seven-item subscales (HADS-A and HADS-D). Scores range from 0 to 21.

Higher scores (≥8) being indicative of increased anxiety or depression. The MCID for the HADS is 1.5 points (Puhan, Frey et al. 2008).

2.12 Venous Blood Sampling and Processes

2.12.1 Venous Blood Sampling

At baseline (0 hours) and at three hours venous blood samples were taken. 6 mL of venous blood was collected into spray-coated EDTA vials. Upon collection the vials were gently inverted five times. Within five minutes of collection, the sample was then split into two 3 mL aliquots in separate centrifuge tubes. To one tube, 300 mL of stock N-Ethylmalemide solution was added. This stock solution had been pre-made and stored at -20^{o}C. The samples were centrifuged at 1,000 g for eight minutes at room temperature. Following centrifugation 1 mL of the supernatant was pipetted into freshly labelled cyro tubes and snap frozen with liquid nitrogen. Samples were stored at -80^{o}C prior to further processing.

2.12.2 Plasma Nitrate and Nitrite Measurement

Plasma nitrate and nitrite levels are determined by a method of combining a diazo coupling reaction (Griess reaction) with High Performance Liquid Chromatography (HPLC) (Eicom NOx analyser, ENO-20, San Diego, USA) (Figure 2.4). This analysis was performed in conjunction with Dr B. Fernandez and Ms. M Lelinska at the University of Southampton.

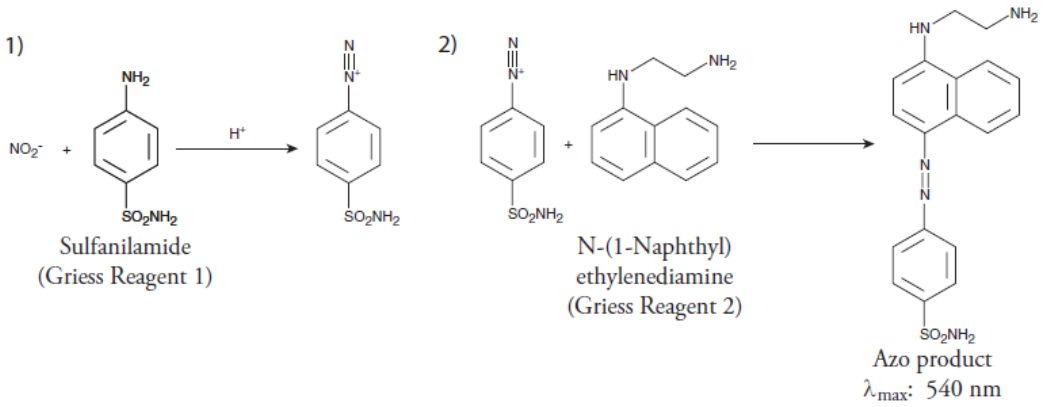


Figure 2.4. Chemistry of the Griess Reagents for Measurement of Plasma Nitrate Metabolites

Image provided by the University of Southampton with permission.

The pre-treated sample is injected on the HPLC system. Samples are first filtered through the pre-column and then the nitrite and nitrate are separated on the analytical column. With the nitrate being retained longer than the nitrite. From the separation column nitrite and nitrate elute into the reduction column. Nitrite passes through the reduction column without any reaction and is then mixed with the reactor solution (Griess reagent). Nitrite reacts with the Greiss reagent in the reaction coil and generates diazo compounds, which have a pink/red colour. The level of diazo is measured by absorbance at 540 nm using a spectrophotometric detector. Nitrate is reduced to nitrite by reaction with cadmium and reduced to copper. Nitrite derived from nitrate undergoes the same reaction as previously described. The concentrations of nitrite and nitrate in the sample are measured by assessing the peak area of absorption and comparing it to the peak area of the absorption for the standards of known concentration (Figure 2.5).

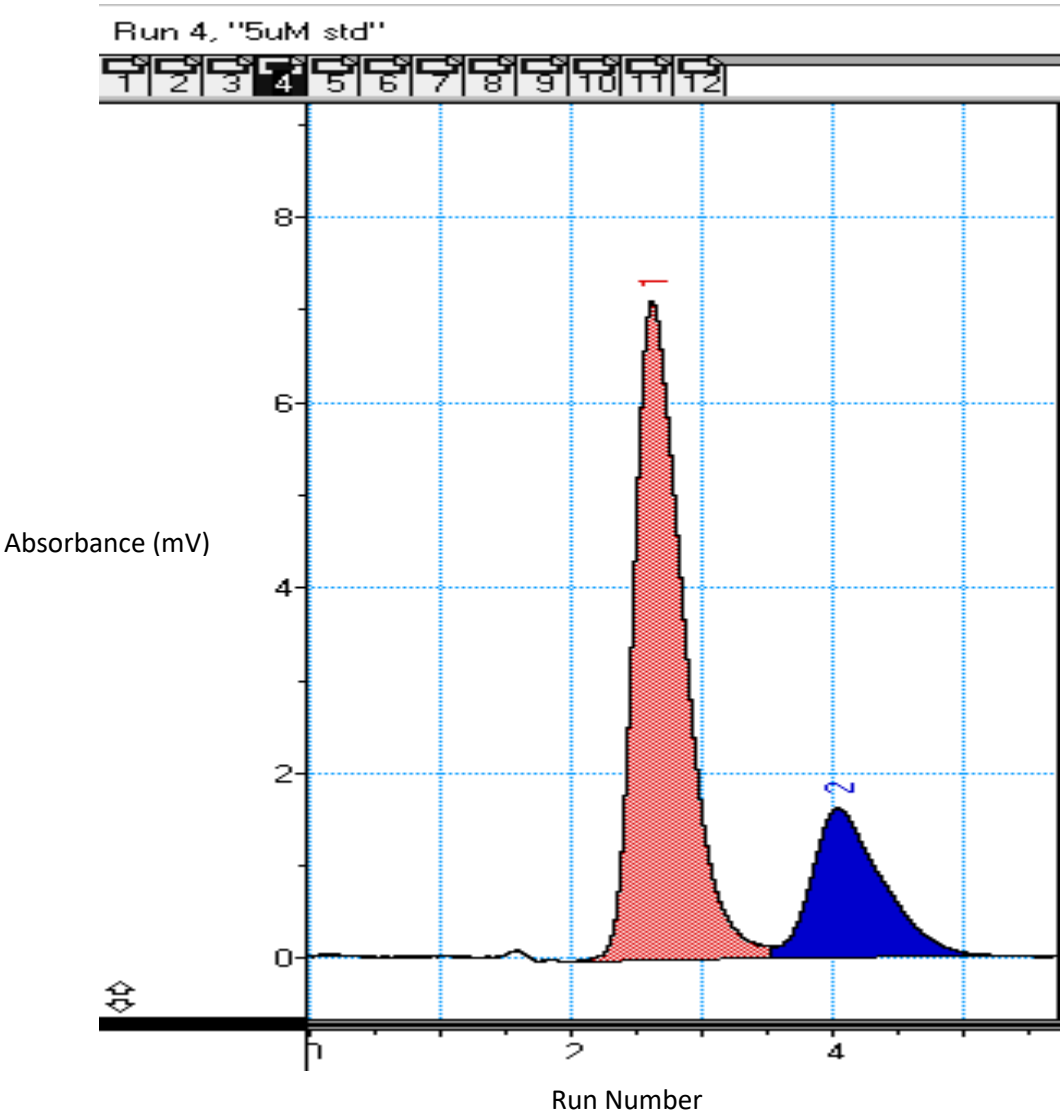


Figure 2.5. Example of chromatogram for 5 uM standard

Peak 1 is nitrite and peak 2 is nitrate. Image provided by the University of Southampton with permission.

2.13 Flow Mediated Dilatation (FMD)

Flow mediated dilatation (FMD) was developed in 1992 (Celermajer, Sorensen et al. 1992) and is a non-invasive method to assess vascular endothelial function. The measurement of FMD is a functional representation of endothelium-derived nitric oxide (NO)

bioavailability in humans (Green 2005). The typical sites of FMD assessment are large peripheral arteries (brachial, radial and femoral). During the test temporary limb occlusion is deployed, generating an ischaemia induced reactive hyperaemia (Pyke and Tschakovsky 2005). A resultant arterial dilatation occurs. FMD is expressed as a percentage of the baseline diameter. To calculate the FMD percentage the following equation is used:

$$FMD\ \% = \left[\frac{Peak\ Diameter - Baseline\ Diameter}{Baseline\ Diameter}\right] x\ 100$$

2.13.1 Participant Preparation

The participant is positioned supine for 20 minutes in a quiet, temperature-controlled (22-24^{o}C) room (Figure 2.6). Participants were assessed at a similar time at each visit and were requested to have a similar diet prior to each visit. On testing days caffeine consumption was restricted. All measurements were made prior to any exercise.

2.13.2 Peripheral Artery Selection

The brachial artery was selected for FMD image acquisition (Corretti, Anderson et al. 2002).

2.13.3 Equipment

FMD measurement is dependent upon identifying the arterial wall. A high resolution, multi-frequency linear ultrasound was used (Logiq 3, Freq 6.0 – 9.0 MHz, GE Medical Systems, Milwaukee, Wisconsin, USA). Image acquisition was undertaken using a linear probe carrying a 10-14 MHz resolution. Images are acquired in B-mode. External electrocardiogram gating was employed to allow for FMD measurement to be captured at end-diastole. A bespoke arm rest was used to support the arm and hold the ultrasound probe in situ (Figure 2.6).

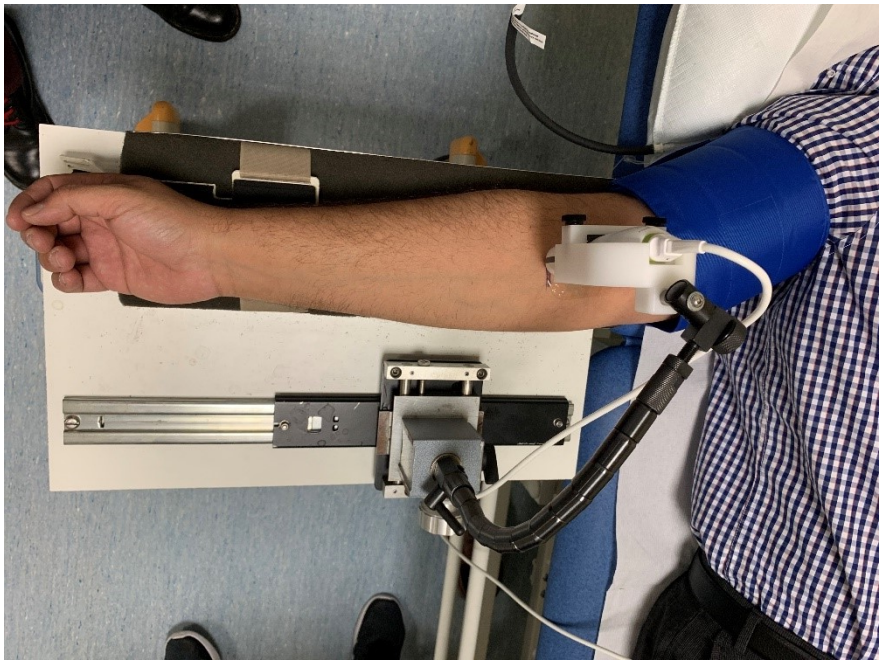


Figure 2.6. Positioning of linear probe over brachial artery

Linear ultrasound probe positioned over the brachial artery, fixed in place using a bespoke arm rest. Rapid Cuff Inflation system used to generate reactive hyperaemia and sheer stress.

2.13.4 Vascular Occlusion

The stimulus for FMD requires temporary vascular occlusion generating reactive hyperaemia and sheer stress. Vascular occlusion was achieved using a Rapid Cuff Inflation System (Hokanson Inc., Bellevue, USA). The cuff was appropriately sized for each participant. The cuff was positioned proximal to the ultrasound probe. This allows for instantaneous inflation and deflation of the cuff (0.3 seconds). The cuff was inflated for five minutes to suprasystolic pressure (200 – 250 mmHg) (Kooijman, Thijssen et al. 2008).

2.13.5 Reactive Hyperaemia Measurements

Following deflation of the cuff brachial artery diameter was acquired at 30 second intervals (30 seconds, 60 seconds, 90 seconds and 120 seconds) (Black, Cable et al. 2008). Image acquisition was for 10 seconds.

2.13.6 Image Analysis

The diameter of the brachial artery was measured from longitudinal images where the lumen-intima interface is visualised (Figure 2.7). The lumen-intima were identified as the boundaries for diameter measurements using ImageJ2 software (Rueden, Schindelin et al. 2017).

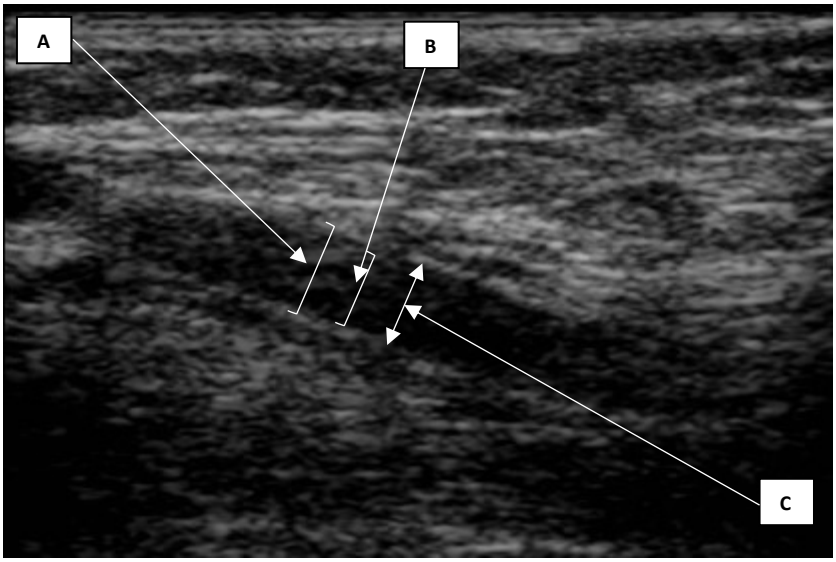


Figure 2.7. Example of B-mode imaging acquired of brachial artery

Brachial artery imaged under B-mode acquisition. A – media to media interface; B – Intima to intima interface; C – diameter measured

2.13.7 FMD Reproducibility

FMD is a reproducible measure of locally derived dynamic NO bioavailability when a standardised method of measuring is employed. The reproducibility of FMD assessment has been undertaken and in a large multicentre (seven centres) Italian study (Ghiadoni, Faita et al. 2012), involving 135 subjects, FMD measurements were not significantly different between sites ($p = 0.91$). The FMD intra-session coefficient of variation was 9.9 ± 8.4% (from 7.6 to 11.9% across centres) and the FMD inter-session coefficient of variation was 12.9 ± 11.6% (from 11.6 to 16.1% across centres) (Ghiadoni, Faita et al. 2012). The reproducibility of FMD was also assessed by Charakida *et al* (Charakida, de Groot et al. 2013) in a multicentre trial across 19 vascular imaging centres in six European Countries including 375 subjects with valid FMD measurements. This study found range

for absolute the technical error of measurement between sonographers was 0.5-1.1% and that there was a good correlation between FMD percentage values with an intraclass correlation coefficient of 0.74 (Charakida, de Groot et al. 2013). Charakida *et al* concluded that with optimal training and adherence to standardised protocols, endothelial function can be assessed serially using FMD (Charakida, de Groot et al. 2013).

Within this thesis, all FMD measurements were undertaken by myself to a set standardised protocol, following training in the technique of measuring FMD. No assessment of operator reproducibility was undertaken.

CHAPTER 3: ORAL NITRATE SUPPLEMENTATION TO ENHANCE PULMONARY REHABILITATION IN COPD – THE ON-EPIC TRIAL

3.1 Background

A cardinal feature of chronic obstructive pulmonary disease (COPD) is limited exercise capacity which impacts on individuals' ability to complete daily activities. Pulmonary rehabilitation (PR) is a multimodal therapy in which education, nutritional and psychological interventions are combined with exercise training. The evidence base for PR is well established (Rochester, Vogiatzis et al. 2015) and PR is recommended by all current international guidelines for the treatment of COPD. However, resources to deliver programmes are limited (Philip, Gaduzo et al. 2019) and approaches to optimise individuals' response and are needed.

One potential approach is via the supplementation of dietary nitrate (NO_3^-). Dietary NO_3^- supplementation results in enhanced levels of plasma nitrite (NO_2^-), plasma NO_3^- and ultimately increased nitric oxide (NO) bioavailability via the oxygen independent NO_2^--NO_3^--NO pathway. NO is a key regulator of skeletal muscle blood flow, contractility, glucose and calcium haemostasis and mitochondrial function (Stamler and Meissner 2001). Dietary NO_3^- supplementation has been shown to improve exercise efficiency in both healthy individuals and individuals with COPD.

In a landmark study of NO_3^-supplementation by Larsen *et al* (Larsen, Weitzberg et al. 2007) found that acute NO_3^-supplementation reduced the oxygen cost of submaximal exercise in healthy individuals, a finding confirmed by subsequent trials (Bailey, Winyard et al. 2009). In a prior study we showed that acute dietary NO_3^-supplementation reduced the oxygen cost of sub-maximal exercise assessed via cycle ergometry, but not exercise capacity, in people with COPD (Curtis, O'Brien et al. 2015). Research from independent groups has demonstrated that in COPD supplementation with dietary nitrate, acutely increases exercise capacity (Berry, Justus et al. 2015, Kerley, Cahill et al. 2015), after 14 days of dosing increased exercise capacity (Kerley, James et al. 2018), and decreased blood pressure (Berry, Justus et al. 2015, Kerley, Cahill et al. 2015, Leong, Basham et al. 2015). Conversely, other groups have found no effect on exercise capacity (Leong, Basham et al. 2015, Shepherd, Wilkerson et al. 2015, Beijers, Huysmans et al. 2017) or pulmonary kinetics (Shepherd, Wilkerson et al. 2015).

The mechanistic basis for the improvements in exercise efficiency seen in healthy individuals and those with COPD is likely related to a combination of improved mitochondrial efficiency, improved contractile function, and increased skeletal muscle blood flow. It is through these potential mechanisms that dietary NO_3^- supplementation in combination with a structured exercise programme (pulmonary rehabilitation) may enhance the improvements in exercise capacity achieved by PR. Individuals with COPD whilst undertaking activities of daily living work close to their maximum oxygen consumption. Supplementation with dietary NO_3^- may allow for more efficient exercise, energy conservation and enhanced adaptation to exercise training. With a resulting improvement in exercise efficiency.

3.2 Rationale and hypothesis

We therefore hypothesised that dietary nitrate supplementation, taken prior to each exercise session during a PR programme would enhance the gains in exercise capacity achieved by COPD patients.

3.3 Methods

3.3.1 Participant selection

All participants provided written, informed consent prior to enrolment in the study, which was approved by the London Bridge Health Research and Ethics Committee (REC reference 14/LO/1474) and conducted in line with the principles of the Declaration of Helsinki.

The study was registered prospectively on a publicly accessible database (www.isrctn.com/ISRCTN27860457).

Recruitment took place between 26th January 2015 and 13th November 2017, with last patient last visit completed on the 8th January 2018. Study inclusion and exclusion criteria can be reviewed in Chapter 2, section 3.4.1 Recruitment process.

Exclusion criteria included clinical instability (i.e. less than one month after an exacerbation), significant other comorbidity limiting exercise tolerance (including musculoskeletal disease limiting exercise, unstable cardiovascular disease, abdominal

aortic aneurysm greater than 5.5 cm and uncontrolled hypertension), significant renal impairment (estimated glomerular filtration rate <50 $ml.min^{-1}$), hypotension (systolic blood pressure <100 mmHg), use of nitrate based medications or phosphodiesterase V inhibitors or other reason to benefit from nitrate supplementation (i.e. ischaemic heart disease and peripheral arterial disease).

3.3.2 Study design and randomisation

This was a multi-centre, double-blind, placebo controlled, parallel group study, comparing dietary nitrate supplementation to a matched placebo. Participants were randomised (1:1) using computer-generated block randomisation list at each site, block size 10, produced by an independent statistician with consecutive numbers linked to either intervention or placebo. Both the participants and researcher were blinded throughout the study and during data analysis to treatment allocation. See Figure 3.1 for study design schematic.

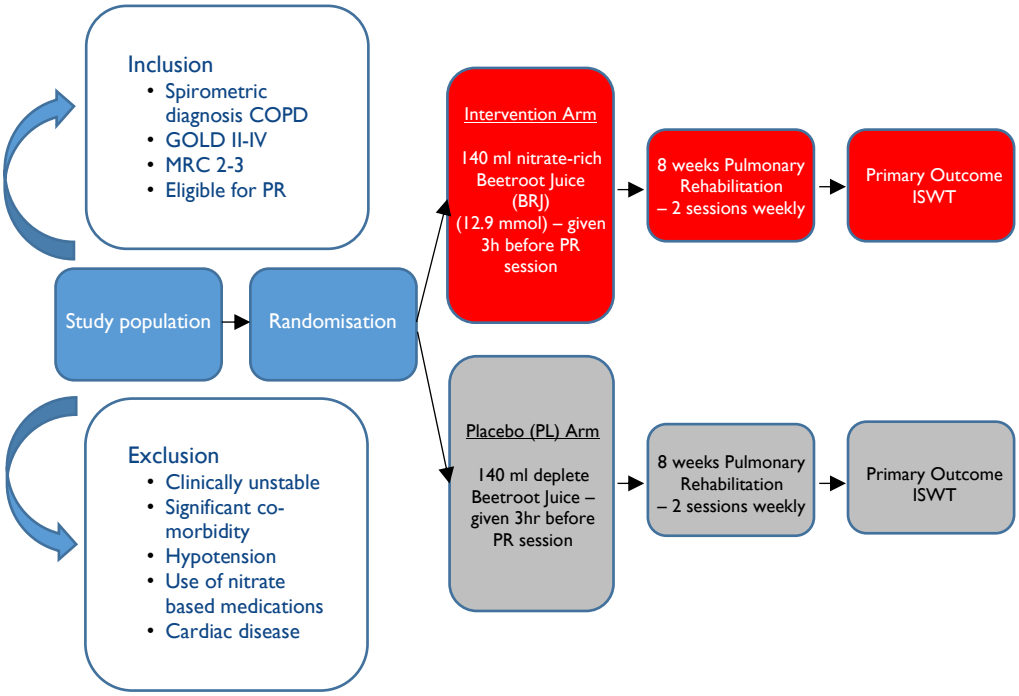


Figure 3.1. Study design schematic

Abbreviations: COPD – Chronic Obstructive Pulmonary Disease; GOLD – Global Initiative for Chronic Obstructive Lung Disease; MRC – Medical Research Council; PR – Pulmonary Rehabilitation; BRJ – Beetroot Juice; PL – Placebo; ISWT – Incremental Shuttle Walk Test

3.3.3 Intervention

The active intervention was concentrated beetroot juice (BRJ) cut with organic lemon juice (2%) containing 0.8 g or 12.9 mmol of NO_3^- (140 ml BEET-IT SPORT Shot, James White Drinks, Ipswich, UK). The placebo was 140 ml of the same beverage which had been run through an ion exchange column prior to pasteurisation to remove the nitrate. The placebo drink, provided by the same manufacturer, was identical in taste and appearance with both causing beeturia (Lansley, Winyard et al. 2011). Plasma nitrite (NO_2^-) increases in a dose dependent manner, 70 ml of BRJ has been found to not alter the physiological

response to exercise, whereas 140 ml and 280 ml reduce the steady state oxygen consumption, with no additional improvement in exercise at the higher dose (Wylie, Kelly et al. 2013). Thus a dose of 140 ml of BRJ was selected.

Study participants were instructed to consume the intervention 3 hours prior to attending each PR session exercise component, plasma nitrite (NO_2^-) reaches peak concentrations at three hours after ingestion (Webb, Patel et al. 2008). They were asked to avoid the use of antibacterial mouthwash and chewing gum throughout the trial period, which has been shown to reduce the oral facultative bacteria whose nitrate reductase activity is essential for the metabolism of an oral nitrate load (Woessner, Smoliga et al. 2016) and to avoid consuming food naturally high in nitrate 48 hours prior to testing (a dietary advice sheet was provided see Appendix 1). Prior to study visits, participants were asked to match food and caffeine consumption. Diaries of dietary intake were not employed in an attempt to have as minimal impact on participants day to day life. Participants were asked to return empty bottles to the PR sessions and this compliance measure was recorded by the PR team on a bottle receipt.

PR programmes were conducted according to the ATS/ERS PR statement (Spruit, Singh et al. 2013). The exercise component involved eight weeks of twice weekly supervised exercise as well as a programme of home-based exercises, with a mixture of aerobic and strength training. The intensity of exercise was increased according to the participant's symptom tolerance during the programme. The initial level of exercise was based on performance during a baseline ISWT. Endurance and resistance training were initiated and progressed as per the ATS/ERS statement on PR (Spruit, Singh et al. 2013). A continuous

training programme was employed for endurance training. Following the initial ISWT the intensity of endurance training was set at 60-70% of the peak work rate and workload was progressively increased by 5-10% as tolerated to attempt to reach 80-90% of peak work rate. Over the course of PR, the duration of endurance training initially was 10-15 minutes for the first three to four sessions and progressively increased to a duration of 30-40 minutes. During endurance training a perceived exertion on the 10-point Borg scale of 4 to 6 was targeted. Resistance training aimed to target local muscular exhaustion within a set number of repetitions for the major upper and lower limb muscle groups. Resistance exercises were performed in two to four sets of six to twelve repetitions. Resistance was selected initially as 50 to 85% of one repetition maximum and workload increased by 2 to 10% as able. Both endurance and resistance training were progressed at the discretion of the local PR teams. Data on this progression was not available. Completion of PR was defined as having attended at least 12 of the possible 16 sessions (Rochester, Vogiatzis et al. 2015).

3.3.4 Study protocol

Visit 1 (Screening):

- Review of inclusion and exclusion criteria
- Written consent taken
- Spirometry (FEV1, FVC and FEV1/FVC ratio)
- Blood drawn for renal function (if not tested in the previous 12 months)
- Intervention randomisation

Visit 2 (Baseline Phenotyping)

- Consent review

- Medical history review and examination including exacerbation history and previous PR attendance
- Anthropometric measurements
- Vital signs (resting blood pressure, heart rate, peripheral oxygen saturations and respiratory rate)
- Measure of body composition
- Exercise capacity assessment with practice ISWT and repeat ISWT after 30 minutes rest
- Quality of life assessments using the CAT Score, psychological status using the HADS and breathlessness using the MRC Dyspnoea score
- Physical activity monitoring for a period of one week using a triaxial accelerometer

Pulmonary Rehabilitation

- Participants complete eight-week programme of twice weekly PR sessions, consuming intervention drink three hours prior to session
- Empty bottles collected and PR attendance recorded

Visit 3 (Outcome Visit)

- Consent review
- Medical history review and examination including exacerbation history
- Trial compliance and PR attendance review
- Anthropometric measurements
- Vital signs (resting blood pressure, heart rate, peripheral oxygen saturations and respiratory rate)
- Measure of body composition
- Spirometry (FEV1, FVC and FEV1/FVC ratio)
- Exercise capacity assessment with practice ISWT and repeat ISWT after 30 minutes rest

- Quality of life assessments using the CAT Score, psychological status using the HADS and breathlessness using the MRC Dyspnoea score
- Physical activity monitoring for a period of one week using a triaxial accelerometer

The outcome visit took place after a minimum of seven days after consumption of the final intervention drink. A period of seven days following consumption of the final intervention beverage was chosen as this would allow for complete washout of the dietary NO_3^- given the short plasma half-life of 5-8 hours of dietary NO_3^- following supplementation. Exercise testing was conducted at the same time on each occasion.

Further details on study conduct can be found in Chapter 2 Description of Methods.

Table 3.1: Protocol for multi-centre, double-blind, placebo controlled, parallel group study, comparing dietary nitrate supplementation to a matched placebo

	Visit 1	Visit 2	8-week Pulmonary Rehabilitation Programme	Visit 3
Inclusion/Exclusion Criteria	☒			
Informed Consent	☒	☒	☒	☒
Spirometry	☒			☒
Blood for Urea and Electrolytes	☒			
Intervention Randomisation	☒			
Anthropometric Measurements		☒		☒
Vital Signs		☒		☒
Measure of Body Composition		☒		☒
Practice ISWT		☒		☒
Second ISWT		☒		☒
Questionnaires		☒		☒
Physical Activity Monitoring		☒		☒
Consumption of intervention drink			☒	
Compliance check			☒	
PR attendance recorded			☒	

3.3.5 Primary and secondary outcome measures

1. Change in exercise capacity, assessed using the ISWT according to ATS/ERS guidelines (Holland, Spruit et al. 2014, Rochester, Vogiatzis et al. 2015). A practice walk was performed and a rest of at least 30 minutes given between walks at each assessment to reduce learning effect. The best value of the two walks was used for analysis - **Primary Outcome Measure**
2. Change in health related quality of life assed using the CAT Score (Jones, Harding et al. 2009), psychological status using the HADS (Puhan, Frey et al. 2008) and breathlessness using the MRC dyspnoea score – **Secondary Outcome Measure**
3. Change in blood pressure parameters – **Secondary Outcome Measure**
4. Change in body composition – **Secondary Outcome Measure**
5. Change in daily physical activity level – **Secondary Outcome Measure**

3.3.6 Sample size, data analysis and statistics

Sample size

The change in ISWT distance from baseline was selected as the primary endpoint of the study, being well validated as an outcome in pulmonary rehabilitation (Singh, Jones et al. 2008). This was a superiority trial, with-nitrate rich BRJ as the intervention and nitrate-deplete BRJ as the control intervention. The Cochrane Meta-Analysis found mean ± standard deviation (SD) increase in the ISWT of 48 (16) metres (Lacasse, Brosseau et al. 2002) following PR. To have an 80% statistical power, with a significance level of 0.05, and assuming that a 20% improvement in ISWT response would be clinically relevant, 61 subjects in each arm would be needed to complete the study. A 20% improvement in the ISWT response was selected as likely to be clinically relevant given that nebulised

SABA/SAMA results in a mean (SE) increase in ISWT of 13.2 ± 4.8% 95%CI (-28.5-61.3%). Allowing for a 15% withdrawal rate a sample size of 140 participants was anticipated.

Data analysis and statistics

Data analysis was conducted on an intention to treat basis (i.e. per study arm allocation), including all participants from whom there was baseline and post PR data. Data are presented as mean(SD) or median(IQR) if not normally distributed and are reported alongside the accompanying *p* value. Differences in response between groups were assessed using unpaired t-test or Mann-Whitney U test as appropriate. Treatment effect was estimated using the Hodges-Lehmann estimates of shift parameters. Analysis was performed using SPSS Version 24 for Windows (SPSS Software, Chicago, Illinois, USA) and Stata version 16.1 (StataCorp 2019, Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC). Figures were prepared using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, California, USA). A p value of <0.05 was considered to be statistically significant.

3.4 Results

3.4.1 Recruitment process

Participants were recruited from 26th January 2015 and 13th November 2017, with last patient last visit completed on the 8th January 2018. 279 people were screened for eligibility, of which 52 participants had a comorbidity precluding entry, 20 had insufficiently severe COPD (GOLD grade I) or insufficiently limited to meet the criteria for PR and 38 declined to participate in the study. After enrolment in the study 4 participants withdrew consent. Eventually, 165 subjects were randomised to receive nitrate-rich BRJ

(n = 78) or placebo (n-87) (Figure 3.2). The imbalance in randomisation was due to the use of separate randomisation lists at each site. After randomisation, 21 subjects withdrew from the nitrate-rich BRJ group and 22 subjects withdrew from the placebo group. Table 3.2 shows trial completion and withdrawal rates by site. The only difference between trial completers and trial withdrawals was that of baseline exercise capacity, with the trial withdrawal group having a statistically significantly lower exercise capacity as assessed by the baseline ISWT 280 m (208, 318) vs trial completers 300 m (215, 460); p = 0.03. Otherwise trial completers and withdrawals were well matched. Individual reasons to withdraw from PR and the trial was not documented. The inference from the available data maybe that those with a reduced baseline exercise capacity maybe less likely to complete PR. No subjects had undertaken PR within six months of enrolling in the study. Table 3.3 shows the differences between trial completers and non-completers.

Table 3.2. Trial completion and withdrawal rates by site

	Combined	RBH	OHF	MPH	MTW
Trial Completers, n (%)	122 (73.9)	71 (79.8%)	24 (57.1%)	16 (84.2%)	11 (73.3%)
Trial Withdrawals, n (%)	43 (26.1)	18 (20.2%)	18 (42.9%)	3 (15.8%)	4 (26.7%)

Trial completion and withdrawal rates by site. Data shown are number (percentage, %). *Abbreviations: RBH – Royal Brompton and Harefield NHS Foundation Trust; OHF - Oxleas NHS Foundation Trust; MPH - Musgrove Park Hospital; MTW – Maidstone and Tunbridge Wells NHS Trust.*

Table 3.3. Trial completers vs non-completers

	Trial Withdrawals (n=43)	Trial Completers (n=122)	p-value
Gender (% Female)	35	43	0.4
Age (years)	66 (62, 71)	64 (60, 76)	0.2
Smoking (Pack years)	41 (29, 94)	45 (31, 61)	1.0
BMI (kg/m^2)	30.6 (24.4, 32.6)	26.3 (22.7, 29.1)	0.4
FEV1, L	0.95 (0.60, 1.92)	1.19 (0.88, 1.48)	0.3
FEV1 (% Predicted)	46 (31, 65)	52 (35, 64)	1.0
GOLD Stage			
2 (%)	50	50	0.6
3 (%)	31	34	0.6
4 (%)	19	16	1.0
FFMI (kg/m^2)	17.1 (15.1, 19.8)	18.1 (15.3, 20.2)	0.9
Baseline ISWT (m)	280 (208, 318)	300 (215, 460)	0.03
Inhalers			
LABA (%)	0	2	0.4
LAMA (%)	86	82	0.5
LABA-ICS (%)	72	63	0.3
LABA-LAMA (%)	9	7	0.7
MRC Dyspnoea Score	3.0 (2.8, 3.3)	3.0 (2.0, 3.0)	0.1
CAT Score	22 (19, 26)	18 (13, 26)	0.9
HADS A	9 (4, 13)	7 (4, 10)	0.2
HADS D	7 (6, 11)	5 (2, 9)	0.6
Baseline Systolic BP (mmHg)	125 (119, 145)	122 (114, 145)	0.5
Baseline Diastolic BP (mmHg)	78 (74, 90)	78 (71, 84)	0.4
Baseline MAP (mmHg)	99 (89, 104)	95 (86, 101)	0.7
Baseline PAL	1.2 (1.0, 1.6)	1.4 (1.2, 1.6)	0.5
Baseline Step Count	5627 (4238, 6979)	3747 (3087, 5980)	0.5

Data shown are median (IQR) or percentage (%). Data was not normally distributed thus between group analysis was undertaken using a Mann-Whitney U test. Spirometry was performed as per the ATS/ERS statement, with three efforts performed and the best result recorded. As per best practice for performing the ISWT, a practice and repeat walk was performed and the better of the two results is recorded. Blood pressure was recorded

at rest, seated for 10 minutes prior to recording, with three measures taken and the average reported. Physical activity data was gathered as per the described methods.

Abbreviations: BMI – Body Mass Index; FEV1 – Forced Expiratory Volume in 1 second; FVC – Forced Vital Capacity; GOLD – Global Initiative for Chronic Obstructive Lung Disease; FFMI – Fat Free Mass Index; ISWT – Incremental Shuttle Walk Test; LABA – Long acting Beta-agonist; LAMA – Long acting muscarinic-agonist; ICS – Inhaled Corticosteroid; MRC – Medical Research Council; CAT – COPD Assessment Test; HADS – Hospital Anxiety and Depression Scale; BP – Blood Pressure; MAP – Mean Arterial Pressure; PAL – Physical Activity Level.

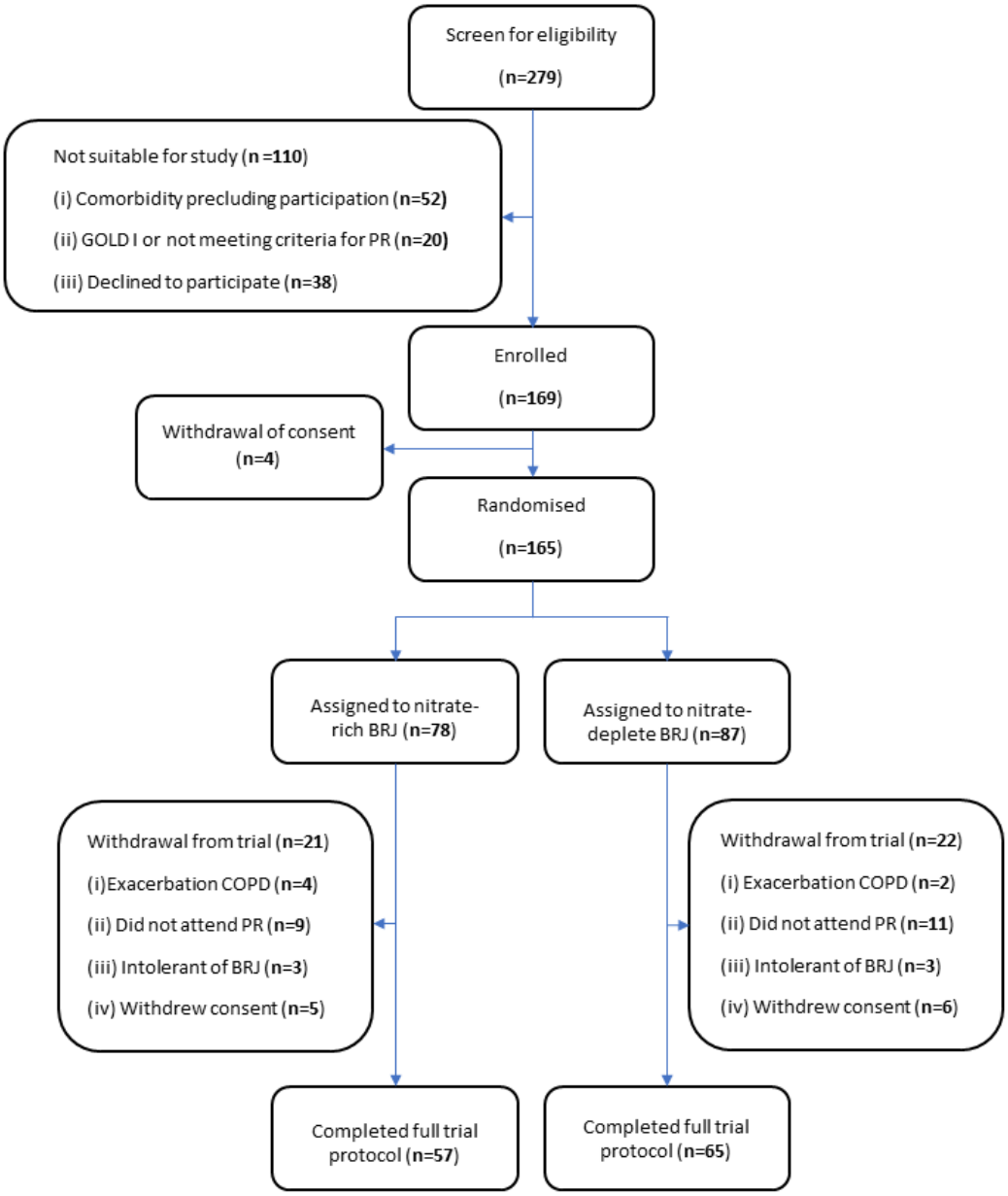


Figure 3.2. CONSORT diagram of enrolment and trial completion

Abbreviations: GOLD – Global Initiative for Chronic Obstructive Lung Disease; PR – Pulmonary Rehabilitation; BRJ – Beetroot Juice; COPD – Chronic Obstructive Pulmonary Disease

3.4.2 Baseline characteristics

One hundred and twenty-two participants completed the full study protocol, 57 in the nitrate-rich BRJ arm and 65 in the placebo arm. Table 3.4 shows the baseline characteristics for both subgroups, which were well matched, particularly for exercise capacity – median baseline ISWT for NO_3^--rich BRJ subgroup was 270 m (190, 360) vs placebo group 280 m (210, 395), p = 0.7. Six individuals (three in each arm) withdrew from the study due to intolerance of BRJ related to taste and gastrointestinal discomfort. All participants were 100% compliant with the dosing protocol as assessed by empty bottle count. There were no significant adverse effects reported. Beeturia was almost universally reported. Among those completing the study the mean (SD) number of PR sessions attended was 15 (2) sessions. No subjects had undertaken PR within six months of undertaking the study.

Table 3.4 Demographic and baseline clinical characteristics

	Placebo BRJ (n=65)	Nitrate-rich BRJ (n=57)	p-value
Gender n (% Female)	26 (41)	27 (42)	1
Age years	68 (62, 74)	70 (64, 78)	0.3
Caucasian (%)	100	100	1
Smoking Pack years	45 (29, 60)	45 (26, 60)	0.6
BMI (kg/m^2	26 (23, 31)	27 (24, 32)	0.6
FEV1, L	1.1 (0.8, 1.4)	1.2 (1.0, 1.6)	0.4
FEV1 (% Predicted)	48 (33, 63)	53 (37, 65)	0.3
GOLD Stage			
2 n (%)	27 (42)	31 (54)	0.4
3 n (%)	24 (37)	20 (35)	1.0
4 n (%)	14 (21)	6 (11)	1.0
FFM (kg)	48.4 (41.7, 57.4)	48.9 (43.2, 55.5)	0.8
FFMI (kg/m^2)	17.1 (15.5, 19.8)	17.5 (15.9, 19.2)	0.8
Baseline ISWT (m)	280 (210, 395)	270 (190, 360)	0.7
Inhalers			
LABA n (%)	2 (3)	0 (0)	0.2
LAMA n (%)	50 (77)	50 (88)	0.4
LABA-ICS n (%)	39 (60)	38 (67)	0.1
LABA-LAMA n (%)	7 (11)	2 (4)	0.1
MRC Dyspnoea Score	3 (3, 3)	3 (3, 3)	0.7
CAT Score	19 (13, 23)	20 (13, 24)	0.9
HADS A	6 (3, 10)	7 (3, 10)	1.0
HADS D	5 (3, 9)	5 (3, 8)	1.0
Baseline Systolic BP (mmHg)	130 (120, 145)	135 (124, 152)	0.2
Baseline Diastolic BP (mmHg)	80 (74, 87)	82 (75, 88)	0.5
Baseline MAP (mmHg)	96 (90, 106)	100 (95,107)	0.1
Baseline PAL	3.0 (2.6, 3.8)	3.0 (2.4, 4.3)	0.9
Baseline Step Count	3791 (2271, 6885)	3507 (1605, 5314)	0.3
Baseline Time > 3 METs, (min)	43 (13, 94)	17 (8, 36)	0.03

Data shown are median(IQR) or number(percentage,%). P values are for Mann-Whitney U test between groups.

Abbreviations: BMI – Body Mass Index; FEV1 – Forced Expiratory Volume in 1 second; FVC – Forced Vital Capacity; GOLD – Global Initiative for Chronic Obstructive Lung Disease; FFMI – Fat Free Mass Index; ISWT – Incremental Shuttle Walk Test; LABA – Long acting

Beta-agonist; LAMA – Long acting muscarinic-agonist; ICS – Inhaled Corticosteroid; MRC – Medical Research Council; CAT – COPD Assessment Test; HADS – Hospital Anxiety and Depression Scale; BP – Blood Pressure; MAP – Mean Arterial Pressure; PAL – Physical Activity Level.

3.4.3 Effect of dietary nitrate supplementation on incremental shuttle walk test distance

The change in ISWT distance following PR in the different intervention arms, nitrate-rich BRJ and placebo, are shown in Figure 3.3. Data for ISWT was non-parametric and thus a non-parametric test was used to assess the difference in ISWT distance. Data was available for all 122 study completers. The median (IQR) improvement in the ISWT distance following PR was statistically significant between NO3- -rich BRJ and PL, increasing by 60 m (10, 85) m compared to 30 m (0, 70), estimated treatment effect 30 m (95%CI: 10, 40); p = 0.027.

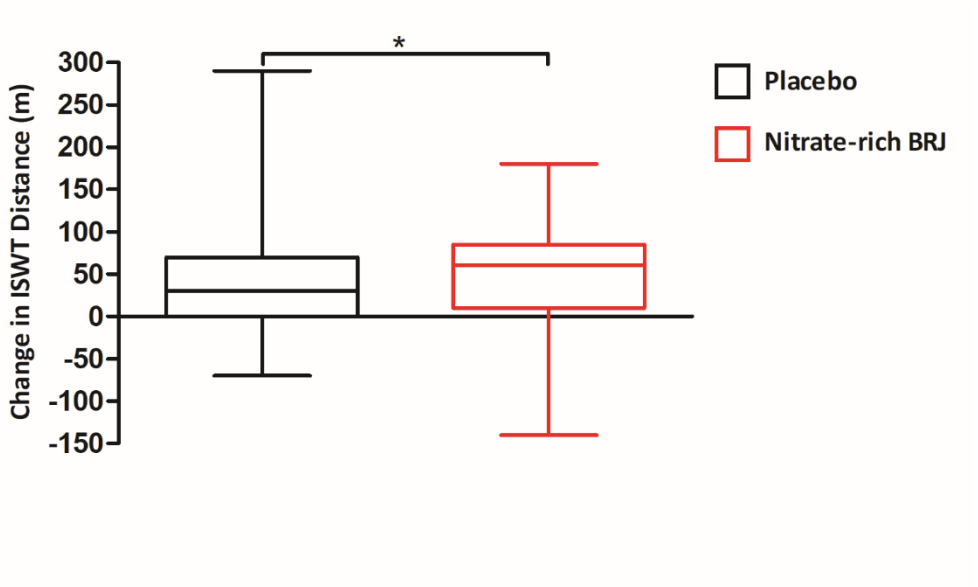


Figure 3.3. Effect of dietary nitrate supplementation on incremental shuttle walk test distance

Change ISWT distance (m) in the placebo and nitrate-rich BRJ dosing conditions. Data presented as interquartile range with the solid line representing the median value, and whiskers the minimum to maximum values. Mann-Whitney U test was used to compare change in ISWT distance between treatment conditions; nitrate-rich BRJ 60 m (10, 85) compared to placebo 30 m (0, 70); * p = 0.027

Abbreviations: BRJ – Beetroot Juice; ISWT – Incremental Shuttle Walk Test

3.4.4 Effect of dietary nitrate supplementation on change in blood pressure parameters

At baseline there was no statistical difference between baseline sBP, dBP and MAP between treatment conditions; p=0.167, p=0.469 and p=0.132 respectively. At end of study there was a statistically significant difference between sBP and MAP between

interventions; *p=0.044 and *p=0.015 respectively. There was no statistically significant difference in dBP; p = 0.052.

The nitrate-rich BRJ group displayed a statistically significant reduction in change in median(IQR) systolic blood pressure at the end of PR compared to placebo (Figure 3.4); a reduction of -5.0 mmHg (-5.0, -3.0) vs an increase of 6.0 mmHg (-1.0, 15.5) respectively, estimated treatment effect 7 mmHg 95%CI (-7, 20); p<0.0005. Similar results were seen for change in diastolic blood pressure; a reduction of -5.0 mmHg (-6.0, -2.0) vs an increase of 6.0 mmHg (-4.5, 7.5), estimated treatment effect 7 mmHg 95%CI (0, 14); p<0.0005 and for change in mean arterial pressure; a reduction of -5.0 mmHg (-5.0, -2.0) vs an increase of 6.0 mmHg (-3.0, 11.0); p<0.0005. Figure 3.5 shows the absolute values for baseline blood pressure parameters and end of study blood pressure parameters.

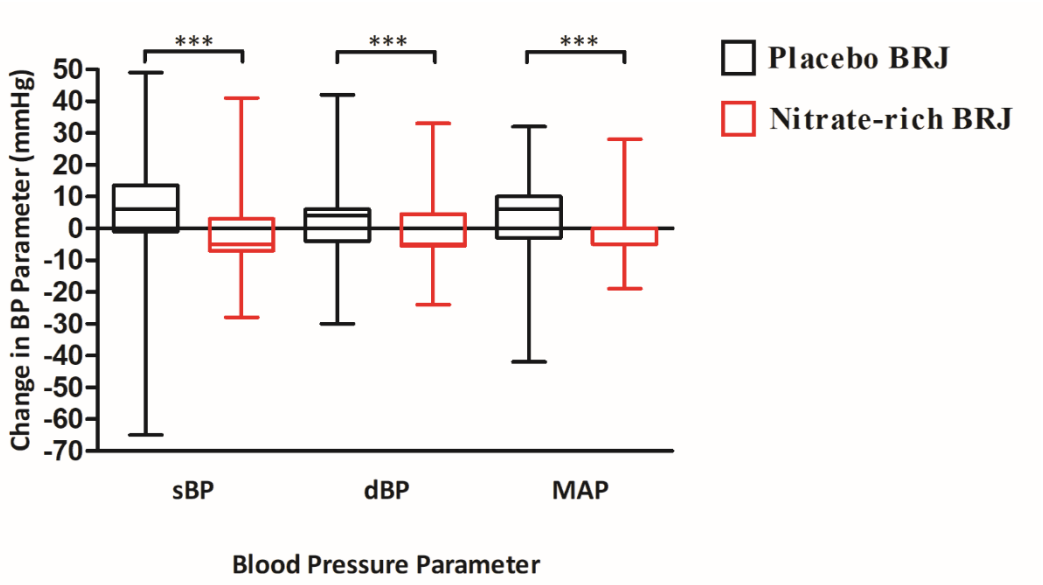
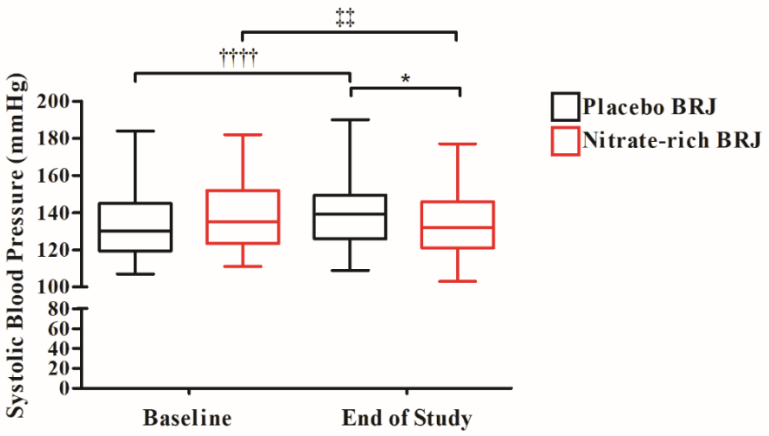


Figure 3.4. Effect of dietary nitrate supplementation on change in blood pressure parameters

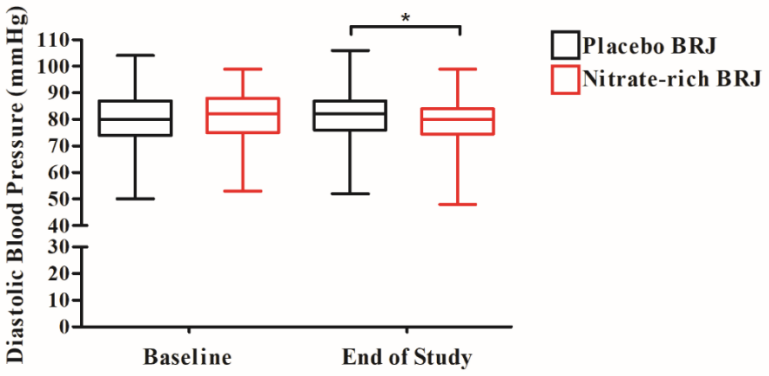
Change in blood pressure parameters (mmHg) in the nitrate-rich BRJ and placebo dosing conditions. Data are presented as IQR with the solid line representing the median value, and the whiskers the minimum to maximum values. Mann-Whitney U test was used to compare blood pressure parameters; *** $p<0.0005$. Blood pressure was measured at rest seated for 10 minutes, with an average of three values reported.

Abbreviations: BP – Blood Pressure; sBP – Systolic Blood Pressure; dBP – Diastoloic Blood Pressure; MAP – Mean Arterial Pressure

1A


‡‡
††††
*
Systolic Blood Pressure (mmHg)
200
180
160
140
120
100
80
60
40
20
0
Baseline
End of Study
Placebo BRJ
Nitrate-rich BRJ

1B


*
Diastolic Blood Pressure (mmHg)
110
100
90
80
70
60
50
40
30
20
10
0
Baseline
End of Study
Placebo BRJ
Nitrate-rich BRJ

1C

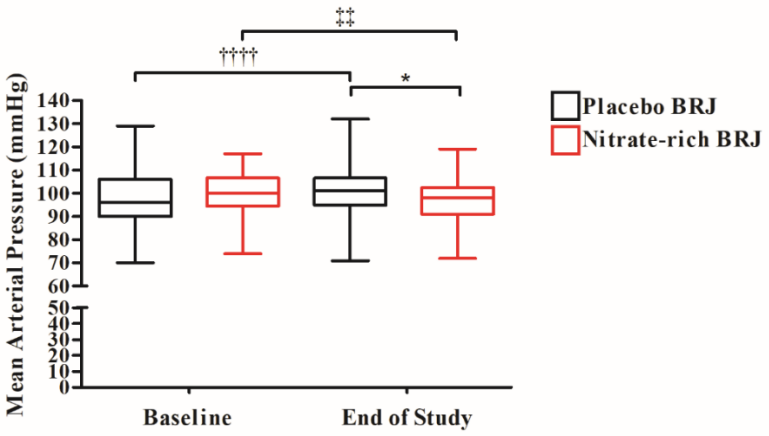

‡‡
††††
*
Mean Arterial Pressure (mmHg)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Baseline
End of Study
Placebo BRJ
Nitrate-rich BRJ

Figure 3.5. Effect of dietary nitrate supplementation on absolute blood pressure parameters

Absolute blood pressure parameters (mmHg) at baseline and end of study in either placebo BRJ (black) or nitrate-rich BRJ (red). Data are presented as IQR with the solid line representing the median value, and the whiskers the minimum to maximum values. Data was not normally distributed thus between group analysis was undertaken with a Mann-Whitney U test. Within group analysis was undertaken with a Wilcoxon Signed Rank test. Blood pressure was assessed using an automated blood pressure monitor (Omron M6, Omron Healthcare Europe, Hoofddorp, Netherlands) at baseline and outcome visits after 10 minutes of seated rest, with an average of three values reported.

Panel 1A. Absolute systolic blood pressure

Between treatment conditions there was no difference between baseline absolute sBP; nitrate-rich BRJ sBP 135 mmHg (124, 152) vs placebo BRJ 130 mmHg (120, 145); p = 0.167. Between treatment conditions at end of study there was a statistically significant difference in absolute sBP; nitrate-rich BRJ 132 mmHg (121, 146) vs placebo BRJ 139 mmHg (126, 150); * p = 0.044. Data described Median (IQR). Mann-Whitney U test. Within the treatment conditions in the nitrate-rich BRJ group there was a statistically significant reduction in absolute sBP; 135 (124, 152) mmHg to 132 (121, 146) mmHg; ‡‡ p=0.005. There was a statistically significant increase in absolute sBP in the placebo BRJ group; sBP 130 (120, 145) mmHg to 139 (126, 150) mmHg; †††† p=0.000075. Data described median (IQR). Wilcoxon Signed rank test.

Panel 1B. Absolute diastolic blood pressure

Between treatment conditions there was no difference between baseline absolute dBP; nitrate-rich BRJ dBP 82 mmHg (75, 88) vs placebo BRJ 80 mmHg (74, 87); p = 0.469. Between treatment conditions at end of study the difference in dBP approached significance; nitrate-rich BRJ 80 mmHg (75, 84) vs placebo BRJ 80 mmHg (76, 87); p = 0.052. Data shown Median (IQR). Data described Median (IQR). Mann-Whitney U test. Within the treatment conditions in the nitrate-rich BRJ group dBP approached a statistical significant reduction; 82 mmHg (75, 88) to 80 mmHg (75, 84); p=0.059. Within the placebo BRJ group there was no difference in baseline and post intervention dBP 80 mmHg (74, 87) and 80 mmHg (76, 87); p = 0.085. Data described median (IQR). Wilcoxon Signed rank test.

Panel 1C. Absolute mean arterial blood pressure

Between treatment conditions there was no difference between baseline absolute MAP; nitrate-rich BRJ MAP 100 mmHg (95, 107) vs placebo BRJ 96 mmHg (90, 106) p = 0.132. Between treatment conditions at end of study there was a statistically significant difference in MAP; nitrate-rich BRJ 98 mmHg (91, 103) vs placebo BRJ 101 mmHg (95, 107), p = 0.015. Data shown Median (IQR). Data described Median (IQR). Mann-Whitney U test. Within the treatment conditions in the nitrate-rich BRJ group there was a statistically significant reduction in MAP 100 mmHg (95, 107) to 98 mmHg (91, 103), ‡‡ p=0.002. Within the placebo BRJ group there was a statistically significant increase in MAP; MAP 96 mmHg (90, 106) to 101 mmHg (95, 107), †† p= 0.003. Data described median (IQR). Wilcoxon Signed rank test.

3.4.5 Effect of dietary nitrate supplementation on physical activity level

Because of issues with technical reliability and compliance relating to the use of SenseWear monitors only 65 paired sets of results were available (nitrate-rich BRJ n = 28 and placebo n = 37). Step count increased in the nitrate-rich BRJ group by median(IQR) 348steps/day (-94; +1629) and fell in the placebo group by 329 steps/day (-915; +640), estimated treatment effect 784 steps/day 95%CI (100, 1471), p = 0.023 (Figure 3.6). There was no difference in change in physical activity level (PAL) between groups; nitrate-rich BRJ 0.0 (0.0; 0.0) vs placebo BRJ 0.0 (-1.0; 1.0), estimated treatment effect 0.2 95%CI (-0.3, 0.7), p = 0.73, but there was a statistically significant difference in the change in time spent in activity > 3 METs; nitrate-rich BRJ increasing by 2.0minutes/day (-3.6; 9.5) vs placebo BRJ falling by 7.0 minutes/day (-30.0; +6.0), estimated treatment effect 13minutes/day 95%CI (2, 28), p = 0.0016 (Table 3.6 and Figure 3.6).

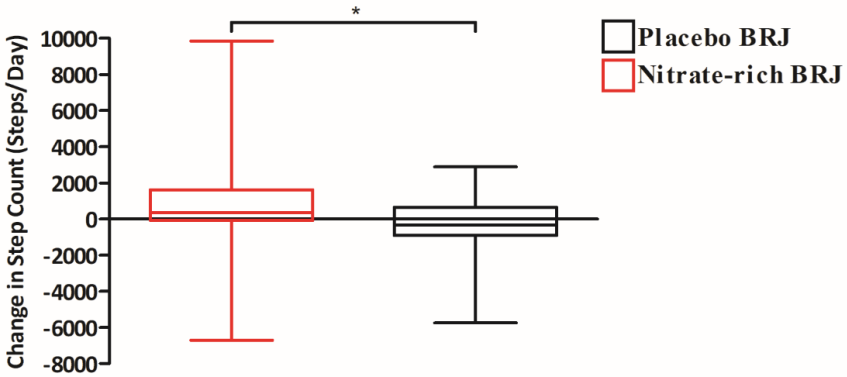
Table 3.6: Effect of dietary nitrate supplementation on physical activity levels from baseline to post pulmonary rehabilitation

Measure	Placebo (n=37)	Nitrate-rich BRJ (n=28)	p-value
Δ Daily Step Count	-329 (-915, 640)	348 (-94, 1629)	* 0.023
Δ PAL	0.0 (-1.0, 1.0)	0.0 (0.0, 0.0)	0.73
Δ METs >3 minutes	-7.0 (-30.0, 6.0)	2.0 (-3.6, 9.5)	* 0.016

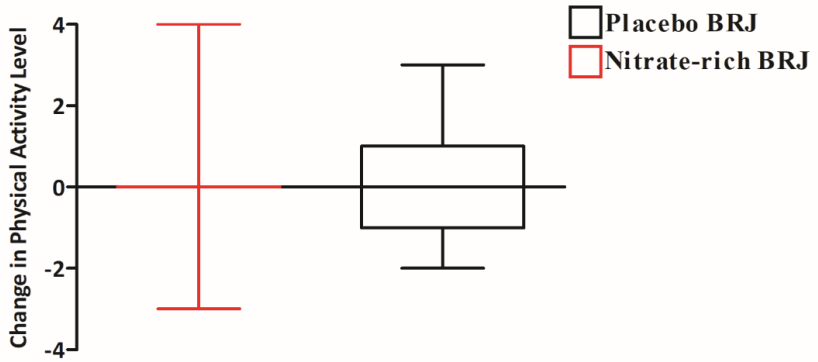
Change in physical activity levels (daily step count, PAL and METs > 3 minutes) in nitrate-rich BRJ and placebo conditions. Data is presented as median (IQR) and was analysed from 65 paired complete sets of data. Mann-Whitney test used to compare physical activity levels between conditions; change in daily step count * p=0.023, change in PAL p=0.73, change in METs > 3 minutes *0.016.

Abbreviations: PAL – Physical Activity Level; METs – Metabolic Equivalents of Task

1A


*
Placebo BRJ
Nitrate-rich BRJ
Change in Step Count (Steps/Day)
10000
8000
6000
4000
2000
0
-2000
-4000
-6000
-8000

1B


Placebo BRJ
Nitrate-rich BRJ
Change in Physical Activity Level
4
2
0
-2
-4

1C

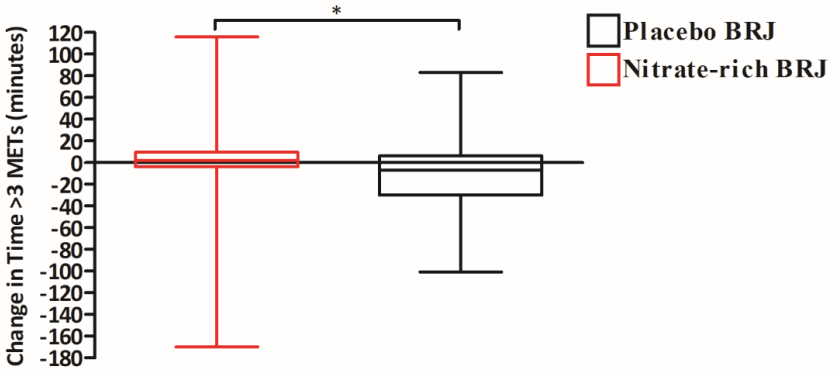

*
Placebo BRJ
Nitrate-rich BRJ
Change in Time >3 METs (minutes)
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
-180

Figure 3.6. Effect of nitrate supplementation on physical activity

Change in measures of physical activity (step count, physical activity level and time spent in physical activity at greater than 3 METs. Data are presented as IQR with the solid line representing the median value, and the whiskers the minimum to maximum values. Mann-Whitney U test was used to compare change in measures of physical activity.

Panel 1A. Change in step count (steps/day) in the nitrate-rich BRJ and placebo BRJ dosing conditions. There was a statistically significant difference in step count; nitrate-rich BRJ 348 (-94, 1629) steps.day^{-1} vs placebo BRJ -329 (-915, 640) steps.day^{-1}; *p = 0.023.

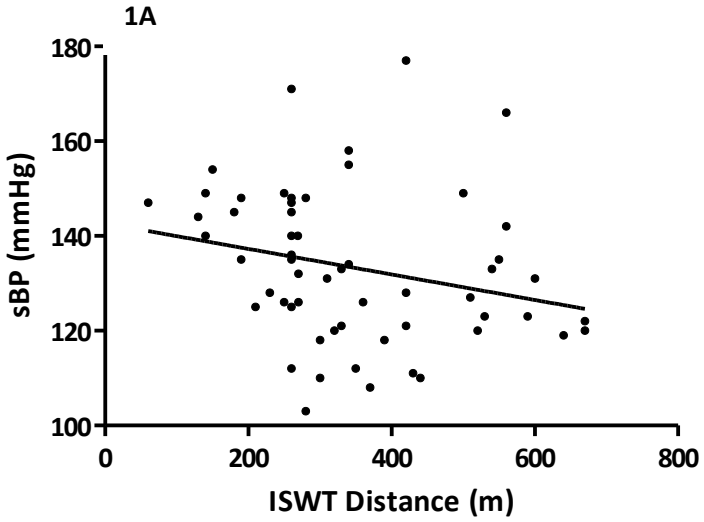
Panel 1B. Change in PAL in the nitrate-rich BRJ and placebo BRJ dosing conditions. There was no statistically significant difference in PAL; nitrate-rich BRJ 0.0 (0.0, 0.0) vs placebo BRJ 0.0 (-1.0, 1.0); p = -0.73 ns.

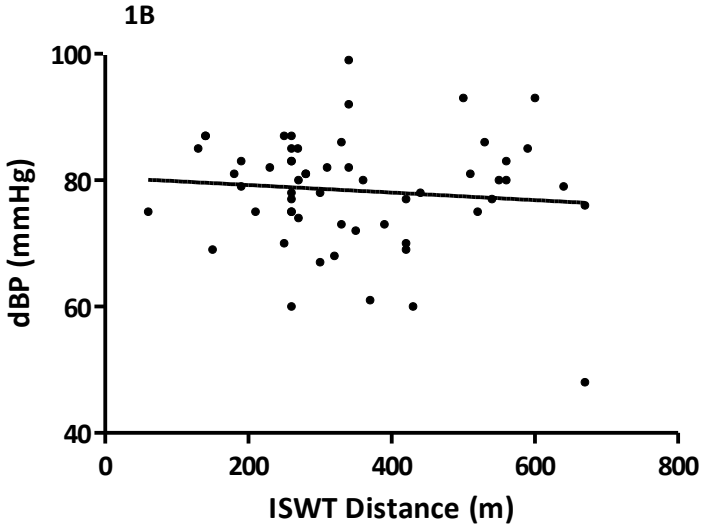
Panel 1C. Change in time spent in activity greater than 3 METs (minutes) in the nitrate-rich BRJ and placebo dosing conditions. There was a statistically significant difference in time spent in activity greater than 3 METs in the nitrate-rich BRJ 2.0 (-3.6, 9.5) minutes.day^{-1} vs placebo BRJ -7.0 (-30.0, 6.0) minutes.day^{-1}; p = 0.016.

Abbreviations: BRJ – Beetroot Juice; MET – Metabolic Equivalent of Task

3.4.6 Correlation of ISWT distance and absolute blood pressure parameters in nitrate-rich BRJ group

A Spearman's rank-order correlation was run to determine the relationship between the post intervention ISWT distance and the blood pressure parameters in the NO_3^--rich BRJ group (n=57). There was a weak negative correlation between the ISWT distance and the absolute sBP, which was statistically significant (r_s = -0.4 (95% CI: -0.6, -0.1); p = 0.006) (Figure 3.7 Panel 1A). There was no correlation between the ISWT distance and the absolute dBP (r_s = -0.1 (95% CI: -0.4, 0.2); p = 0.5) (Figure 3.7 Panel 1B). There was no correlation between the ISWT distance and the absolute MAP (r_s = -0.3 (95% CI: -0.5, 0.02); p = 0.06) (Figure 3.7 Panel 1C).


1A
sBP (mmHg)
180
160
140
120
100
0
200
400
600
800
ISWT Distance (m)


1B
dBP (mmHg)
100
80
60
40
0
200
400
600
800
ISWT Distance (m)

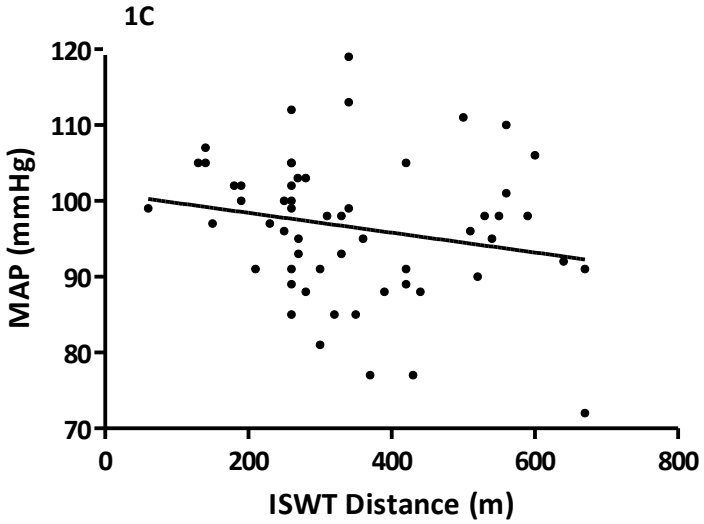

1C
MAP (mmHg)
120
110
100
90
80
70
0
200
400
600
800
ISWT Distance (m)

Figure 3.7. Correlation of post intervention ISWT distance and absolute blood pressure parameters in the nitrate-rich BRJ group

Panel 1A. Correlation of post intervention ISWT distance and absolute systolic blood pressure in the nitrate-rich BRJ group. There was a weak negative correlation between the ISWT distance and the absolute sBP, which was statistically significant (r_s = -0.4 (95% CI: -0.6, -0.1); p = 0.006).

Panel 1B. Correlation of post intervention ISWT distance and absolute diastolic blood pressure in the nitrate-rich BRJ group. There was no correlation between the ISWT distance and the absolute dBP (r_s = -0.1 (95% CI: -0.4, 0.2); p = 0.5).

Panel 1C. Correlation of post intervention ISWT distance and MAP in the nitrate-rich BRJ group. There was no correlation between the ISWT distance and the absolute MAP (r_s = -0.3 (95% CI: -0.5, 0.02); p = 0.06).

Abbreviations: sBP – systolic blood pressure; dBP – diastolic blood pressure; MAP – mean arterial pressure; ISWT – incremental shuttle walk test; BRJ – beetroot juice; CI – confidence interval; r_s – spearmen rank-order correlation coefficient.

3.4.8 Effect of dietary nitrate on health-related quality of life, psychological status and breathlessness

No differences were observed between active and placebo treatment in MRC dyspnoea score, CAT score and HAD score following PR (Table 3.7), although as expected both study groups experienced improvement compared to baseline. There was no statistically significant difference in the change in FFMI between intervention groups; median (IQR) nitrate-rich BRJ +0.7 (-1.7, 2.0) $kg.m^{-2}$ vs placebo -0.2 (-1.9, 2.3) kg/m^{-2}, estimated treatment effect 0 kg/m^{-2} 95%CI (-0.7, 0.6), p = 0.71 (Table 3.8).

Table 3.7 Impact of pulmonary rehabilitation on symptoms and quality of life

	Placebo BRJ (n=64)	**BRJ (n=53)**	**Treatment Effect**	**95% CI**	**p-value**
Δ MRC dyspnoea score	0.0 (-1.0, 0.0)	-1.0 (-1.0, -0.0)	0	0, 0	0.90
Δ HADS anxiety	-1.0 (-3·0, 0.8)	-1.0 (-2.0, 0.3)	0	-1, 1	0.58
Δ HADS depression	-1.0 (-2.8, 1.0)	-1.0 (-3.0, 1.0)	0	-1, 1	0.51
Δ CAT Score	-2.0 (-7.0, 1.0)	-2.5 (-5.0, 0.0)	0	-1, 3	0.74

There was no difference in the effect of pulmonary rehabilitation on quality of life or symptom scores between treatment arms. Data shown are median (IQR). P values are for Mann-Whitney U test between study arms. Treatment effect was tested using Hodges-Lehmann estimates of shift parameters.

Abbreviations: MRC – Medical Research Council; HADS – Hospital Anxiety and Depression Scale; CAT – COPD Assessment Test; BRJ – Beetroot Juice

Table 3.8 Effect of dietary nitrate supplementation on fat free mass

	Placebo (n=65)	Nitrate rich BRJ (n=57)	p-value	Whole Group (n=122)	95% CI	p-value
ΔFMM (kg)	0.50 (-7.20, 4.53)	-1.60 (-5.78, 4.70)	0.81	-0.92 (0.86)	-2.62, -1.07	0.27
ΔFFMI (kg/m^2)	-0.20 (-1.85, 2.30)	0.70 (-1.73, 2.03)	0.71	-0.24 (0.29)	-0.83, -0.34	0.413

There was no difference in the effect of pulmonary rehabilitation on body composition between treatment arms. Data shown are median (IQR). P values are for Mann-Whitney U test between study arms. Treatment effect was tested using Hodges-Lehmann estimates of shift parameters.

Abbreviations: FFM – Fat Free Mass; FFMI – Fat Free Mass Index; BRJ – Beetroot Juice; CI – Confidence interval

3.4.9 Effect of dietary nitrate supplementation on spirometry

No difference was observed between active and placebo groups in spirometry (Table 3.9). There was no significant significantly difference in spirometry for the whole group following pulmonary rehabilitation (Table 3.9)

Table 3.9 Effect of dietary nitrate supplementation on spirometry

Measurement	Placebo (n=58)	Nitrate-rich BRJ (n=45)	p-value	Whole Group (n=103)	p-value
Δ FEV1 (L)	0.00 (-0.07, 0.10)	0.03 (-0.03, 0.11)	0.37	0.00 (-0.40, 0.10)	0.84
ΔFVC (L)	0.00 (-0.16, 0.29)	-0.01 (-0.15, 0.26)	0.78	0.00 (-0.14, 0.26)	0.68

There was no difference in the effect of pulmonary rehabilitation on spirometry between treatment arms. Data shown are median (IQR). P values are for Mann-Whitney U test between study arms. Treatment effect was tested using Hodges-Lehmann estimates of shift parameters.

Abbreviations: FEV1 – Force Expiratory Volume in 1 second; FVC – Forced Vital Capacity; BRJ – Beetroot Juice

3.4.10 Effect of dietary nitrate supplementation on COPD exacerbation rate, supplement compliance and pulmonary rehabilitation attendance

Of those who completed the full trial protocol, there were 17 exacerbations of COPD in the placebo group and 9 in the nitrate-rich BRJ group, this was not a statistically significant difference (median IQR)): placebo 0.0 (0.0, 0.1) vs nitrate-rich BRJ 0.0 (0.0, 0.0); p = 0.0165 (Mann-Whitney U test).

All trial participants were 100% compliant with supplementation protocols, regardless of study arm as assessed by empty bottle collection monitoring.

Pulmonary rehabilitation per individual recruitment site can be reviewed in Table 3.10.

Table 3.10. Pulmonary rehabilitation attendance Compliance per study site

Centre		PR Session Attendance	p-value
All Centres	**Placebo (n=65)**	16 (12.5, 16.0)	0.13
	Nitrate-rich BRJ (n=57)	16 (14.0, 16.0)	
Royal Brompton	**Placebo (n=36)**	16.0 (15.0, 16.0)	0.70
	Nitrate-rich BRJ (n=35)	16.0 (15.0, 16.0)	
Oxleas	**Placebo (n=14)**	16.0 (14.3, 16.0)	0.51
	Nitrate-rich BRJ (n=10)	16.0 (15.0, 16.0)	
Musgrove Park	**Placebo (n=9)**	14.0 (12.0, 15.5)	*0.04
	Nitrate-rich BRJ (n=7)	16.0 (15.0, 16.0)	
MTW	**Placebo (n=6)**	12.0 (12.0, 12.0)	1.00
	Nitrate-rich BRJ (n=5)	12.0 (12.0, 12.0)	

There was no difference across between compliance in completion of pulmonary rehabilitation in either the placebo or nitrate-rich BRJ study arms. Apart from at the Musgrove Park site (p = 0.04). This difference is likely due to low numbers recruited at this site. Data shown are median (IQR). P values are for Mann-Whitney U test.

3.4.11 Adverse reactions

There were no serious adverse events or serious adverse reactions, as described in the study protocol (i.e. event is resultant in death, is life threatening, requires hospitalisation, results in persistent or significant disability) in either of the groups. . In both the intervention and placebo arm three individuals were unable to tolerate the beetroot juice due to gastrointestinal discomfort (nausea). .

3.5 Discussion

3.5.1 Key findings

The main finding of this study was that, in people with COPD undertaking an 8-week course of PR, supplementation with nitrate-rich BRJ augmented the improvement in exercise capacity, measured as ISWT compared to an otherwise identical but nitrate-depleted placebo. Active treatment was also associated with reductions in blood pressure, inferring improved endothelial function, and a greater daily step count compared to the control arm.

3.5.2 Significance of findings

Pulmonary rehabilitation is a high value and cost effective intervention (Zoumot, Jordan et al. 2014), associated with significant health benefits including an improvement in exercise capacity, a reduction in hospital admissions, a reduced number of in hospital bed days and reduced mortality (Steiner M 2017). The benefits of PR are thought to last approximately 12 months (Mereles, Ehlken et al. 2006). However, access to PR is limited (Philip, Gaduzo et al. 2019) by resource availability and it is important to ensure the benefit of programmes is maximised. The present results suggest that dietary nitrate supplementation, a relatively cheap intervention with few side effects, can augment the improvement in exercise capacity produced by PR. In those individuals able to tolerate BRJ, this study would suggest that it would be beneficial as part of a PR programme. Further trials are needed to assess if this outcome can be replicated and if daily dosing of dietary nitrate confers a greater benefit.

The estimated treatment effect of dietary nitrate supplementation on ISWT of 30m (10, 40) is close to but does not quite exceed the MCID for this measure, recently defined as between 35m and 36.1m (Evans and Singh 2019), but our findings are likely to be clinically relevant for several reasons. Firstly, whether the benefit of an intervention is meaningful depends on the nature of the intervention. The level of acceptable response needed for a complex intervention like PR is higher than the response needed to justify the small additional cost and burden of consuming a beetroot shot twice a week. Secondly, the improvement in health status associated with PR is partly driven by psychological factors and social interaction through participation in PR distinct from direct exercise training effects, so a direct comparison of the effects is inappropriate as the nitrate supplementation would not be expected to effect the former category. Thirdly, the finding that measures of physical activity improved with treatment compared to controls suggests an impact on daily activity as well as performance on exercise tests. The MCID for step count following PR is 600steps/day (Demeyer, Burtin et al. 2016), this study finding a median difference of 678steps/day. Fourthly, our data show improvements in blood pressure parameters, which are a surrogate of endothelial function in the treatment arm, suggesting beetroot juice impacts on the risk of vascular events in COPD which are known to be elevated (Bloom, Ricciardi et al. 2019). It has previously been shown that dietary nitrate supplementation can reduce both systolic and diastolic blood pressure (Larsen, Ekblom et al. 2006, Webb, Patel et al. 2008), the likely mechanism being that of NO acting as an important endothelial relaxing factor via the secondary messenger cyclic guanosine monophosphate resulting in smooth muscle relaxation and subsequent vasodilatation (Gruetter, Barry et al. 1979)We performed an additional responder analysis. Taking a threshold of 35m the number of responders in the active arm was 39 (68.4%) and in the placebo group 29 (44.6%) ($Chi^2 = 6.98$, $p = 0.008$).

Other supplements have been studied in COPD. A daily dose of Vitamin D (1,200 IU vitamin D3/day) for 6 months was found to confer no improvement in 6-minute walk test (Rafiq, Prins et al. 2017). A carbohydrate rich supplement containing 560 kcal of carbohydrate taken daily for the duration of a 7-week programme of pulmonary rehabilitation had no impact on either peak exercise capacity (ISWT) or exercise endurance (measured by the endurance shuttle walk test) (Steiner, Barton et al. 2003). A systematic review and meta-analysis found that creatine supplementation in COPD subjects does not improve exercise capacity in patients undergoing PR (Al-Ghimlas and Todd 2010). Angiotensin-converting enzyme inhibition reduced blood pressure but, actually attenuated the improvement in peak work rate after completion of PR (Curtis, Meyrick et al. 2016). Body composition has not been a focus of trials of dietary nitrate supplementation. It may be the case that those individuals with a reduced BMI or FFMI would achieve a greater response from this intervention. Future studies should assess the impact of BMI and or FFMI on dietary nitrate supplementation.

Cardiovascular comorbidity is common in COPD (Bloom, Ricciardi et al. 2019) and vascular responsiveness measured using FMD of the brachial artery is known to be significantly impaired in individuals with COPD compared to controls (Vaes, Spruit et al. 2017). It has been shown that dietary nitrate supplementation can reduce blood pressure in healthy individuals (Wylie, Kelly et al. 2013), older adults (Kelly, Fulford et al. 2013), peripheral arterial disease (Kenjale, Ham et al. 2011) and COPD (Berry, Justus et al. 2015, Curtis, O'Brien et al. 2015, Kerley, Cahill et al. 2015), though studies to date have been of short duration. Our finding that this effect on blood pressure is sustained over at least 8 weeks suggests that this intervention should be investigated further for possible impacts on cardiovascular events in COPD and other long-term conditions, over and above impacts

on exercise capacity and physical performance. The improvement in endothelial function we observed is consistent with another study showing that dietary nitrate supplementation improved it in people with hypercholesterolemia, with an increase in FMD (1.1 % (0.26), p = 0.003) (Velmurugan, Gan et al. 2016).

There are multiple potential NO-related mechanisms that may have contributed an increase in exercise capacity following dietary nitrate supplementation, which are reviewed in detail elsewhere (Jones, Thompson et al. 2018). These include increased skeletal muscle oxygen delivery through enhanced blood flow to muscle (Bailey, Winyard et al. 2009), reduced adenosine triphosphate (ATP) cost to generate a submaximal force, increased mitochondrial efficiency by reducing the oxygen cost of mitochondrial ATP resynthesis (Larsen, Schiffer et al. 2011, Jones, Thompson et al. 2018) and improved calcium handling in fast-twitch muscle fibres resulting in increased contractile force. We have shown previously that nitrate supplementation reduces the oxygen cost of exercise in people with COPD (Curtis, O'Brien et al. 2015) a condition known to be associated with skeletal muscle abnormalities including fibre type switch and mitochondrial dysfunction (Maddocks, Shrikrishna et al. 2014, Farre-Garros, Lee et al. 2019). In addition, effects on systemic and pulmonary circulation may improve cardiovascular function. In the present trial, nitrate supplementation may have enhanced the training effect of PR by increasing the level at which individuals were able to exercise, or by augmenting the training effect at an equivalent level of exertion. Alternatively, one or more effects of repeated dosing may have persisted beyond the end of the PR and dosing period. Importantly, since the outcome measures were made 7-14 days following the last dose of beetroot juice, our findings cannot be due to an acute effect of dietary nitrate supplementation as the half-life of nitrate in the body is less than 24 hours (Webb, Patel et al. 2008). By design this

study was not a mechanistic study. Mechanistic studies of dietary nitrate supplementation are confined to healthy individuals. Future work should assess the mechanism of dietary nitrate supplementation in individuals with specific disease states.

3.5.3 Impact of dietary nitrate supplementation

This study has found that dietary NO_3^- supplementation in association with PR improved exercise capacity, step count and METs. Whilst undertaking ADL individuals with COPD work close to their maximum oxygen capacity. The finding of an increased duration of time spent at greater than three METs in the dietary NO_3^-supplementation group suggests that the continued supplementation with dietary NO_3^- may allow for more efficient exercise and energy consumption.

Further trials need to be undertaken to assess the role of continued dietary NO_3^- supplementation after the completion of PR and outside of a PR programme to assess if there is an improvement of PAL, METs and step count.

3.5.4 Critique of Method

The study made use of standard measures used in PR programmes already running and 3 of the 4 sites were district general hospitals (DGH). By design, endothelial function outcomes were only measured at one site. An advantage of the use of standard PR programmes at DGH sites increases our confidence that the effects of nitrate supplementation are likely to be generalizable.

The completion rate for PR was 74%, similar to the 68% seen in the 2018 Royal College of Physicians PR audit (Steiner M 2018) and the dropout rates did not differ significantly between groups, suggesting that dietary nitrate supplementation is safe.

A strength of the study is the closely matched placebo, as the nitrate-deplete BRJ matched the nitrate-rich BRJ in every way except the nitrate content. The supplements were consumed without supervision. To check compliance empty bottles were collected and recorded prior to each PR session. Participants were also advised to avoid nitrate-rich foodstuffs 48hours prior to the study visits at the beginning and end of the trial and avoid antibacterial mouthwash and chewing gum. It is unlikely that this differed between groups in a way that might have influenced the results. The fall in blood pressure observed in the treatment group supports the notion that patients were adherent to treatment. We abandoned initial plans to measure blood nitrate levels because of logistical difficulties which could have provided further evidence about compliance. Measurement of salivary nitrate and nitrite content could also have been an option.

3.5.5 Conclusion

Dietary nitrate supplementation in combination with pulmonary rehabilitation increased exercise capacity, lowered blood pressure and improved endothelial function in people with COPD compared to placebo. Further longer-term trials are needed to see if these benefits are sustained over time.

CHAPTER 4: EFFECT OF DIETARY NITRATE SUPPLEMENTATION ON EXERCISE PERFORMANCE IN HYPOXIA

4.1 Background

The second aim of this thesis was to assess if an acute dose of dietary NO_3^- administered to individuals with Chronic Obstructive Pulmonary Disease (COPD), who experience hypoxaemia and require supplemental oxygen, would improve their exercise performance.

Chronic obstructive pulmonary disease is defined by progressive airflow limitation and is a highly heterogeneous condition with individuals presenting with cough, sputum production and dyspnoea (https://goldcopd.org/). As the airflow limitation progresses the risk of alveolar hypoxia and subsequent hypoxaemia increases. The mechanistic basis for the development of hypoxaemia in individuals with COPD is the presence of a ventilation/perfusion (V/Q) mismatch (Rodriguez-Roisin, Drakulovic et al. 2009). This V/Q mismatch is the result of destructive emphysema and small airways disease (Kent, Mitchell et al. 2011). In the emphysematous phenotype there is increased ventilation of poorly perfused lung units (or a high V/Q ratio) resulting in an increased physiological dead space (Wagner, Dantzker et al. 1977) and in the airways disease phenotype there is increased perfusion to poorly ventilated regions of lung as a result of bronchial obstruction (or a low V/Q ratio) resulting in a physiological shunt (Wagner, Dantzker et al. 1977). The impact of VQ mismatch is multiplied in conditions of reduced cardiac output

because this requires greater oxygen extraction relative to delivery by peripheral tissue which leads to lower mixed venous saturation. Hyperinflation in COPD impairs cardiac output as can pulmonary vascular disease and co-morbid cardiac disease. The reported prevalence of hypoxaemia in COPD is between 1.4% (Sundh and Ekstrom 2017) and 7% (Wells, Estepar et al. 2016). Hypoxaemia is associated with an increase in mortality and a reduction in exercise tolerance, quality of life and skeletal muscle function (Kim, Benditt et al. 2008). Forty years ago, two landmark randomised controlled trials, the British Medical Research Council Long-Term Domiciliary Oxygen Trial (1981) and the Nocturnal Oxygen Therapy Trial (1980) found a survival benefit of the use of long-term oxygen therapy, defined as more than 15 $h.day^{-1}$, a finding confirmed in further studies (Cooper, Waterhouse et al. 1987, Strom 1993, Gulbas, Gunen et al. 2012). There is also evidence that ambulatory oxygen improves exercise performance in individuals with moderate to severe COPD (Bradley and O'Neill 2005, Bradley, Lasserson et al. 2007, Nonoyama, Brooks et al. 2007). It is unclear, however, if the improvement in exercise performance is of clinical significance. The use of ambulatory oxygen, despite improving exercise capacity, does not appear to be associated with an improvement in survival (Sadaka, Montgomery et al. 2018). The modulation of the oxygen cost of exercise in individuals in COPD would therefore be of therapeutic benefit.

Nitric oxide (NO) has gained traction as a modulator of exercise performance. NO is a ubiquitous signalling molecule involved in a number of process at a tissue and cellular level including; mitochondrial and cellular respiration (Brown and Cooper 1994, Shiva, Huang et al. 2007), glucose uptake in skeletal muscle (Merry, Lynch et al. 2010), skeletal muscle contraction (Reid 1998, Viner, Williams et al. 2000, Stamler and Meissner 2001), regulation of vasodilatation (Moncada and Higgs 1993, Joyner and Tschakovsky 2003),

neurotransmission (Garthwaite 2008) and fatigue development (Percival, Anderson et al. 2010). NO is continually produced, with increased production under conditions of exercise (Balon and Nadler 1994), by two pathways: the oxygen (O_2)-dependent nitric oxide synthase (NOS) pathway and the O_2-independent Nitrate (NO_3^-)-Nitrite (NO_2^-)-NO pathway. It is possible to augment this NO_3^--NO_2^--NO pathway through the supplementation of exogenous NO_3^-. The NO_3^--NO_2^--NO pathway is also upregulated under conditions of hypoxia and low pH (Lundberg and Weitzberg 2009). In 2007, the landmark study by Larsen *et al* (Larsen, Weitzberg et al. 2007) found that following the administration of sodium nitrate ($NaNO_3$; 0.1 $mmol.kg^{-1}.day^{-1}$) to healthy individuals the was a significant reduction in the submaximal cost of O_2 cost ($\dot{V}O_2$) of exercise. This finding was unexpected and went against the dogma of exercise physiology: that there is a tight coupling between the $\dot{V}O_2$ and workload. This has led to a great deal of research into the role of dietary NO_3^- supplementation as a way to improve exercise efficiency and performance.

The upregulation of the NO_3^--NO_2^--NO pathway under conditions of hypoxia (Lundberg and Weitzberg 2009) is suggestive there may be increased gains in exercise efficiency and performance under this condition. Studies in healthy individuals have suggested that dietary NO_3^- supplementation improves the $\dot{V}O_2$ of submaximal exercise under conditions of hypoxia (Vanhatalo, Fulford et al. 2011, Masschelein, Van Thienen et al. 2012, Kelly, Vanhatalo et al. 2014, Muggeridge, Howe et al. 2014, Vanhatalo, Jones et al. 2014, Bakker, Engan et al. 2015, Carriker, Mermier et al. 2016, Shannon, Duckworth et al. 2016). These finding have not been overlooked by researchers interested in improving exercise in individuals with COPD. Dietary NO_3^- supplementation trials in individuals with COPD have suggested an improvement in exercise endurance and the $\dot{V}O_2$ of submaximal exercise

(Berry, Justus et al. 2015, Curtis, O'Brien et al. 2015, Kerley, Cahill et al. 2015, Kerley, James et al. 2019). Notably, work from our lab (Curtis, O'Brien et al. 2015) showed that in non-hypoxaemic COPD patients nitrate supplementation reduced the oxygen cost of exercise but did not improve performance. The failure to show an improvement in exercise endurance time, despite showing a reduction in the oxygen cost of exercise, may be related to the day to day variability in the symptoms of individuals with COPD and the small sample size (n=20). However, in a more hypoxic context where oxygen delivery to muscles is more restricted and where there may also be pulmonary vascular involvement there is a greater chance that nitrate supplementation might have a clinically useful impact.

4.2 Hypothesis

The hypothesis of this study was to investigate the effect of an acute dose of dietary NO_3^-, in the form of concentrated beetroot juice (BRJ), given to individuals with COPD who are hypoxaemic and require supplemental oxygen would improve their exercise performance compared to a robust placebo (PL).

4.3 Methods

4.3.1 Participant selection

All participants provided written informed consent prior to enrolment in the study, which was approved by the London Chelsea Research and Ethics Committee (Ref: 15/LO/0975) and was conducted in line with the principles of the Declaration of Helsinki. The study was registered prospectively on a publicly accessible database (http://www.isrctn.com/ISRCTN14888729).

Study inclusion criteria included individuals with COPD GOLD grade II-IV, based on the NICE (www.nice.org.uk) and GOLD (https://goldcopd.org/) criteria, who had already been prescribed by their physician supplemental oxygen in the form of long-term oxygen therapy and ambulatory oxygen therapy during exercise, based on the British Thoracic Society guidelines (Hardinge, Annandale et al. 2015) (Table 4.1). Arterial blood gas sampling was not performed as part of this study. Historic arterial blood gases, taken prior to oxygen prescription, were if available analysed. Participants were recruited for the outpatient clinical services at the Royal Brompton Hospital, between the 4th November 2016 and the 8th August 2017, with the last participant last visit completed on the 15th January 2018.

Table 4.1. British Thoracic Society guidance for the use of long-term oxygen therapy in individuals with COPD

Guidance for the use of long-term oxygen therapy in individuals with COPD
Stable COPD and a resting $P_aO_2 \leq 7.3$ kPa
Stable COPD and a resting $P_aO_2 \leq 8$ kPa with evidence of peripheral oedema, polycythaemia (haematocrit ≥ 55%) or pulmonary hypertension
Individuals with resting hypercapnia if they fulfil all other criteria for long-term oxygen therapy

Abbreviations: COPD – Chronic Obstructive Pulmonary Disease; P_aO_2 – Partial pressure of oxygen

Exclusion criteria included clinical instability (i.e. less than one month after an exacerbation of COPD), significant other comorbidity limiting exercise tolerance, significant renal impairment (defined as an estimated glomerular filtration rate <50

mL.min^{-1}), hypotension, (systolic blood pressure < 100 mmHg), pregnancy, the use of nitrate based medications or phosphodiesterase V inhibitor or other reasons to benefit from NO_3^- supplementation (i.e. ischaemic heart disease and peripheral arterial disease). Subjects were withdrawn if they had a significant adverse event or if they withdrew their consent for participation.

4.3.2 Study design and randomisation

This was a single centre, double-blind, placebo-controlled, randomised crossover study comparing dietary NO_3^- supplementation in the form of concentrated Beetroot Juice (NO_3^- ; 12.9 mmol) to a matched placebo (PL) of NO_3^--deplete BRJ (NO_3^-; 0.002 mmol). Participants were randomised 1:1 using a computer-generated block randomisation list with consecutive numbers linked to identical preparations of either BRJ or PL, block size 10, produced by an independent statistician. Both the participants and the researcher were blinded throughout the study and during data analysis to treatment allocation. Figure 4.1 shows the study flow diagram.

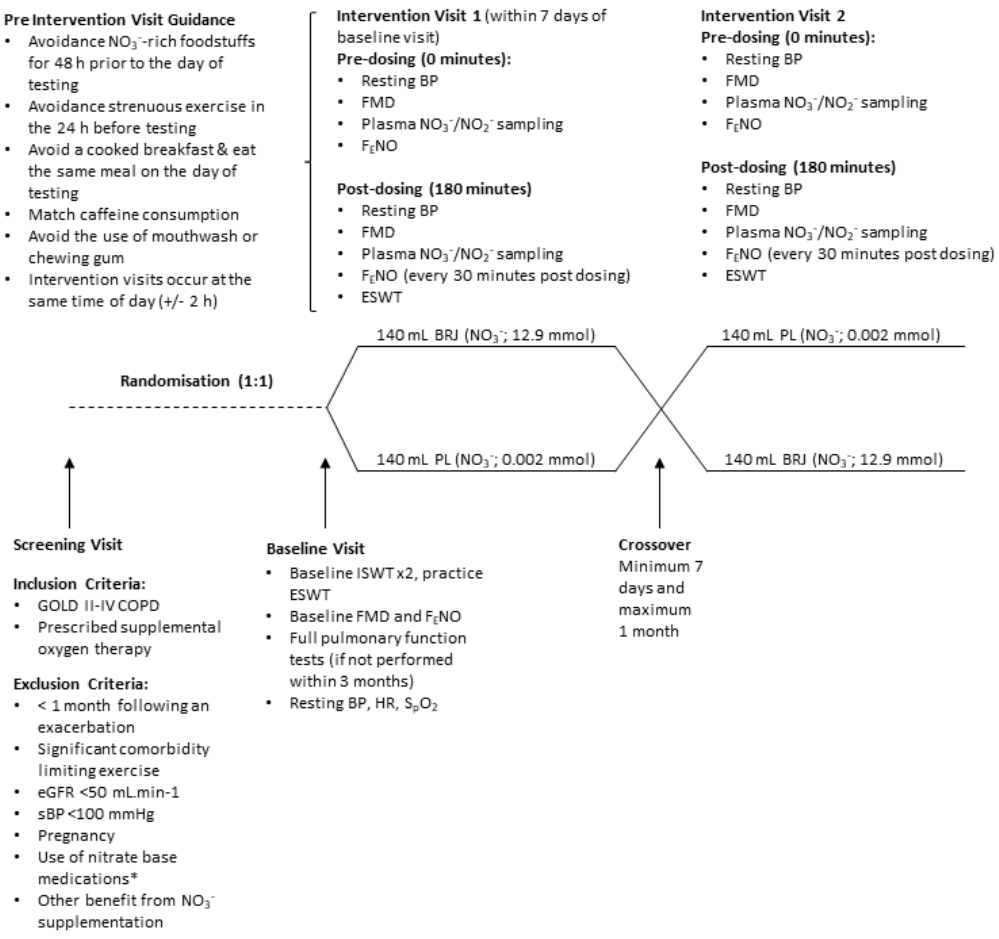


Figure 4.1 Trial Flow Diagram

Abbreviations: GOLD – Global Initiative for Chronic Obstructive Lung Disease; COPD – Chronic Obstructive Pulmonary Disease; eGFR – Estimated Glomerular Filtration Rate; sBP – Systolic Blood Pressure; BP – Blood Pressure; FMD – Flow Mediated Dilatation; NO_3^- - Nitrate; NO_2^- - Nitrite; F_ENO – Fractional Exhaled Nitric Oxide; ISWT – Incremental Shuttle Walk Test; ESWT – Endurance Shuttle Walk Test; HR – Heart Rate; S_pO_2 – Oxygen Saturations; BRJ – Beetroot Juice; PL – Placebo

4.3.3 Intervention

The active intervention was 140 mL concentrated BRJ (NO_3^-; 12.9 mmol) cut with organic lemon juice (2%) (140 mL BEET-IT® SPORT Shot, James White Drinks, Ipswich, UK). The PL was a matched beverage of 140 mL NO_3^--deplete BRJ (NO_3^-; 0.002 mmol) (produced by James White Drinks, Ipswich, UK) which was generated by the standardised method of passing the juice, prior to pasteurisation, through an ion exchange column, containing Purolite A520E ion exchange resin, which selectively removes NO_3^- (Lansley, Winyard et al. 2011) (See Chapter 5 where the quantity of NO_3^- and NO_2^- have been assessed in each beverage). The PL beverage was identical in taste and appearance with both beverages causing beeturia. A dose of 12.9 mmol of NO_3^- was selected as the plasma concentrations of NO_3^- and NO_2^- increase in a dose-dependent fashion up to 16.8 mmol of NO_3^- with peak change at 2-3 h post dosing (Wylie, Kelly et al. 2013). In that study, no difference was found at doses of 4.2 mmol NO_3^- but a reduction in the $\dot{V}O_2$ of steady state exercise was found at doses of 8.4 mmol and 16.8 mmol of NO_3^- (Wylie, Kelly et al. 2013). The manufacturer reports that BEET-IT® SPORT Shot (James White Drinks, Ipswich, UK) contains 12.9 mmol NO_3^- and in a previous study (Curtis, O'Brien et al. 2015) 12.9 mmol of NO_3^- in this preparation was shown to reduce the $\dot{V}O_2$ of submaximal exercise in individuals with COPD whilst performing a cardiopulmonary exercise test.

Prior to attendance for the intervention visits participants were requested to avoid NO_3^--rich foodstuffs for 48 h and to avoid a cooked breakfast on these days to create as standardised condition as possible, reducing differing levels of dietary NO_3^- consumption as a source of variation in study measurements. Participants were also requested to match their caffeine consumption to avoid the known ergogenic effect of caffeine (Warren, Park et al. 2010). In the 24 h period prior to the intervention visits participants

were requested to avoid strenuous exertion, given the known effect on the vasoconstrictor response of the endothelium (Elliott, Alsalahi et al. 2018).

Participants were also asked to avoid the use of antimicrobial mouthwash or chewing gum on the days of these visits, which has been shown to reduce the oral facultative bacteria whose NO_3^- reductase activity is essential for the metabolism of oral NO_3^- load (Webb, Patel et al. 2008). On the days of the intervention visits, following a 10-minute rest period and undertaking baseline assessments participants were observed consuming the randomised juices, empty bottles were collected and batch numbers recorded, with the participant and the study researcher being blinded to which beverage was being consumed. The intervention visits were undertaken at the same time of day (+/- 2 h) and there was a minimum of seven-days to one-month washout period between intervention visits.

4.3.4 Study Protocol

Visit 1 (Screening Visit)

- Review of inclusion and exclusion criteria
- Written informed consent obtained
- Spirometry (FEV1, FVC and FEV1/FVC ratio) reviewed
- Venous blood sample for renal function (if not tested in the previous 12 months)

Visit 2 (Baseline Visit)

- Review of written consent and answering of any questions participants may have
- Medical history review and clinical examination including exacerbation history

- Anthropometric measurements
- Vital signs (including resting BP, HR and peripheral O_2 saturations (S_pO_2)
- Bioelectrical impedance measure of body composition
- Full pulmonary function testing if not performed with three months
- Quality of life assessments using the COPD Assessment Test (CAT) and the Hospital Anxiety and Depression (HAD) scale and assessment of breathlessness using the Medical Research Council (MRC) dyspnoea score
- Assessment of exercise capacity via two incremental shuttle walk test (ISWT) followed by calculation of the endurance shuttle walk test (ESWT) speed
- Practice ESWT
- Randomisation

Visits 3 and 4 (Intervention Visits)

- Review of written consent and answering of any questions participants may have
- Medical history review and clinical examination including exacerbation history
- Following a 10-minute rest period assessment of vital signs (including resting BP, HR and S_pO_2)
- Observation of dosing with either NO_3^--rich BRJ or PL, with empty bottle collection and recording of batch numbers
- 180 minutes following beverage ingestion assessment of resting vitals (including resting BP HR and S_pO_2)
- ESWT with continuous monitoring of HR and S_pO_2

There was a minimum seven days to maximum one month between intervention crossover visits. Intervention visits were undertaken at the same time of day (+/- 2

h). Further details on study methods can be found in Chapter 2: Description of Methods. Table 4.2 is a summary of the study protocol.

Table 4.2. Summary protocol for single-centre, double-blind, placebo-controlled, randomised cross-over study of dietary NO_3^- supplementation to a matched PL in individuals with COPD who require supplemental oxygen

	Screening Visit	Baseline Visit	Intervention Visits
Inclusion/Exclusion Criteria	☒		
Informed Consent	☒	☒	☒
Spirometry	☒		
Venous blood for urea and electrolytes		☒	
Medical history, physical examination and review of exacerbations		☒	☒
Anthropometric measurements		☒	
Vital signs		☒	
Measurement of body composition		☒	
Full pulmonary function testing		☒	
Questionnaires		☒	
ISWT		☒	
ESWT		☒	☒
Randomisation		☒	
Consumption of beverage			☒

Abbreviations: ISWT – Incremental Shuttle Walk Test; ESWT – Endurance Shuttle Walk Test

4.3.5 Primary and Secondary Outcome Measures

1. Change in exercise performance, assessed using the ESWT time (seconds) according to the ATS/ERS guidelines (Holland, Spruit et al. 2014, Rochester, Vogiatzis et al. 2015). The

walking speed at which the ESWT is set is 85% of the peak O_2 uptake calculated from the best ISWT distance achieved (Singh, Morgan et al. 1994). **Primary Outcome Measure.**

2. Area under the oxygen saturation curve to isotime during the ESWT. **Secondary Outcome Measure.**
3. Area under the heart rate curve to isotime during the ESWT. **Secondary Outcome Measure.**
4. Blood pressure parameters. **Secondary Outcome Measure.**

4.3.6 Sample size, data analysis and statistics

This was a superiority trial, with the NO_3^--rich BRJ as the intervention and the NO_3^--deplete BRJ as the control intervention, to assess the change in the ESWT time. Given the cross-over design and taking 65 sec to be the minimal clinically significant difference in the ESWT, and a within individual standard deviation of 60 sec for repeat testing (Singh, Puhan et al. 2014). To have an 80% statistical power, with a significance level of 0.05, 16 subjects would be required to complete the study. To allow for a 25% withdrawal rate a sample size of 20 subjects was anticipated (www.sealedenvelope.com). Data analysis was conducted on an intention to treat basis (i.e. per study arm allocation). Data are presented as mean and standard deviation (SD) or if not normally distributed as median and interquartile range (IQR). Differences in response between groups were assessed using paired T-tests or a Wilcoxon signed-rank test as appropriate. Treatment effect was estimated using the Hodges-Lehman estimates of shift parameters. Analysis was performed using SPSS version 24 for Windows (SPSS Software, Chicago, Illinois, USA) and Stata version 16.1 for Windows (StataCorp 2019, Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC). To perform the comparison of continuous S_pO_2 and heart rate between the two treatment conditions, individual ESWT data periods was

subjected to a 30 sec rolling average using MATLAB (MATLAB and Statistics Toolbox Release 2017a, The MathWorks, Inc., Natick, Massachusetts, USA) and then expressed as percentages of isotime (defined as the duration of the shortest of the two ESWT). These individual responses were then grouped to allow analysis of heart rate and S_pO_2 against percentage of isotime (plotted at the midpoint of each 10th percentile of isotime). The area under the curve was assessed for each individual subject and the two treatment conditions compared using a Wilcoxon signed-rank test. Figures were prepared using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, California, USA). A p value of < 0.05 was considered to be statistically significant.

4.4 Results

4.4.1 Recruitment process

Participants were recruited between 4th November 2016 and the 8th August 2017, with the last participant last visit completed on the 15th January 2018. 67 individuals were screened for eligibility (Figure 4.2 shows the study CONSORT diagram). Of these, 31 declined to participate, seven had a comorbidity precluding participation and nine were not using supplementary oxygen. Twenty participants were randomised to take part in the study, ten randomised to NO_3^--rich BRJ first and ten randomised to PL-BRJ first. No subjects withdrew during the study.

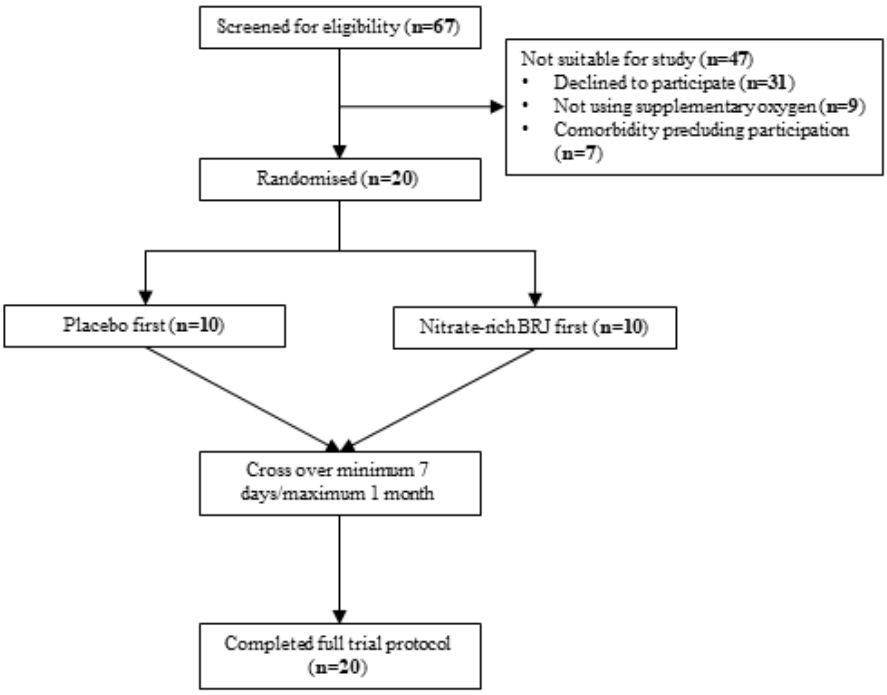


Figure 4.2. CONSORT diagram of study enrolment and completion into effect of dietary nitrate supplementation on exercise performance in hypoxia (EDEN-Ox) study

4.4.2 Baseline characteristics

Twenty participants completed the full study protocol, ten randomised first to receive NO_3^--rich BRJ and ten randomised first to PL. Table 4.3 shows the baseline characteristics for both subgroups in order of intervention given first and the whole group. The participants taking part in this study had severe or very severe COPD (GOLD grade III or IV), this was matched regardless of the intervention taken first. Both groups were well matched, particularly for exercise endurance – median (IQR) baseline ESWT for NO_3^--rich BRJ first was 172 sec (137, 267) vs PL first was 181 sec (158, 193); p = 0.6. Baseline O_2 prescription was also well matched – median (IQR) O_2 prescription for NO_3^--rich BRJ first

was 4 L/min (2, 6) vs PL first was 2 L/min (2, 4); p = 0.2. Subsequent to randomisation all individuals completed the cross-over to the alternative intervention, with no withdrawals. All participants were 100% compliant with the dosing protocol, as observed by direct observation by the study researchers. There were no serious adverse events reported. Beeturia was reported by all participants.

Table 4.2. Baseline characteristics

Measurement		Nitrate-rich BRJ First (n=10)	Placebo First (n=10)	p-value	Whole Group (n=20)
Sex (%Female)		30	50	1.0	40
Age (years)		68 (62, 73)	67 (64, 76)	0.7	67.6 (8.5)
Caucasian (%)		100	100	1	100
Smoking (Pack Years)		28 (14, 50)	64 (57, 96)	0.006	52 (21.6)
BMI ($kg.m^{-2}$)		24.3 (20.9, 29.0)	26.2 (21.4, 30.1)	0.5	25.2 (4.7)
FFMI ($kg.m^{-2}$)		18.4 (15.8, 20.1)	18.0 (14.0, 20.2)	0.8	18.1 (15.8, 19.9)
Inhaled Medications					
	SABA (%)	91	100	0.4	95
	LABA-ICS (%)	91	78	0.4	95
	LAMA (%)	91	100	0.4	85
Antibiotic Rescue Pack (%)		36	22	0.5	30
Prophylactic Antibiotic (%)		9	44	0.08	25
Baseline Resting O_2 Saturations F_iO_2 0.21 (%)		92 (89, 94)	92 (89, 93)	0.8	92
Pre-LTOT Prescription Baseline P_aO_2 (kPa)		6.9 (6.0, 7.3)	6.6 (6.4, 7.2)	0.6	6.8
Long Term Oxygen Prescription (%)		100	100	1.0	100
Ambulatory Oxygen Prescription ($L.min^{-1}$)		4 (2, 6)	2 (2, 4)	0.2	3.0 (2.0, 6.0)
CAT Score		20 (18, 29)	19 (15, 28)	0.6	21 (8.0)
MRC Dyspnoea Score		4 (4, 4)	4 (4, 4)	1.0	4 (4, 4)
HAD Score A		4 (3, 7)	7 (2, 10)	0.3	4.0 (2.3, 8.8)
HAD Score D		4 (4, 5)	5 (4, 7)	0.7	4.5 (4.0, 5.6)
Systolic BP (mmHg)		139 (123, 149)	135 (115, 139)	0.1	137 (121, 143)
Diastolic BP (mmHg)		76 (66, 83)	70 (65, 80)	0.4	73 (65, 82)
MAP (mmHg)		94 (91, 104)	91 (85, 94)	0.2	92 (80, 100)
Lung Function					
	FEV1 (L)	0.7 (0.6, 1.0)	0.7 (0.3, 1.0)	0.3	0.7 (0.6, 1.0)
	FVC (L)	2.7 (1.9, 3.1)	1.6 (1.4, 3.2)	0.3	2.7 (1.6, 3.1)
	FEV1/FVC Ratio	0.3 (0.3, 0.3)	0.3 (0.2, 0.4)	1.0	0.3 (0.3, 0.3)
	RV %Predicted	211 (181, 235)	212 (188, 233)	0.8	212 (186, 233)
	TLco %predicted	33 (19, 45)	36 (28, 44)	0.9	32 (19, 44)
GOLD Stage					
	3 (%)	22	45	1.0	35
	4 (%)	78	55	1.0	65
ISWT Distance (meters)		300 (280, 360)	370 (220, 280)	0.04	279 (70)
ESWT Time (secs)		172 (137, 267)	181 (158, 193)	0.6	179 (152, 193)

Data are presented in order of intervention taken first. Data shown are median (IQR) or number (percentage). P values are for Mann-Whitney U test between groups analysis.

4.4.3 Effect of acute dietary nitrate supplementation on the endurance shuttle walk test time

The primary outcome measure was available for all 20 individuals. The individual ESWT times for both treatment conditions are shown in Figure 4.3. All individuals walked for longer after consuming the NO_3^--rich BRJ than after the placebo beverage and the median (IQR) ESWT time was statistically significantly different between the groups: NO_3^--rich BRJ 194.6 sec (147.5, 411.7) vs PL 159 sec (121.9, 298.5), estimated treatment effect 62 sec (95%CI: 33, 106); p 0.00089.

There was no statistical difference in recovery time between the intervention groups (recovery time defined by each individual returning to their baseline Borg dyspnoea scale which was recorded prior to the ESWT): NO_3^--rich BRJ 155.3 sec (144.8, 195.5) vs PL 148.8 sec (117.9, 208.5); p= 0.9. There was no evidence of intervention order effect on the median (IQR) change in ESWT time; if PL was consumed first the ESWT time was 60.0 sec (21.8, 88.4) vs 43.1 sec (14.03, 155.3) is the NO_3^--rich BRJ was consumed first; p = 0.82.

There was no difference in breathlessness or fatigue between interventions at peak exercise, as assessed by the modified Borg dyspnoea and fatigue scale. Peak median (IQR) modified Borg dyspnoea scale: NO_3^--rich BRJ 5.0 (4.0, 5.0 vs PL 4.0 (4.0, 6.8); p = 0.5. Peak median (IQR) modified Borg fatigue scale: NO_3^--rich BRJ 5.0 (3.0, 5.0) vs PL 4.0 (3.0, 6.5); p = 0.9.

There is a clear outlier in this dataset (Figure 4.3). When this individual's data was removed from analysis, all individuals still walked further following consumption the NO_3^- -rich BRJ. There was a statistically significant difference between the median (IQR) ESWT time with the outlier removed; NO_3^--rich BRJ 193.8 (145.5, 389.6) seconds vs PL 158.2 (121.6, 236.6) seconds; p = 0.0001. Regarding this specific individual at baseline assessment their best ISWT distance was 370 meters, using the ESWT conversion table the ESWT speed was calculated as 4.65 km/h which equates to ESWT level 11. All individuals undertook a practice ESWT, this individual's practice ESWT time was 599 seconds. The ESWT time that this individual achieved following consumption of the placebo beverage was 785 seconds. For both ESWT this individual reported peak Borg Dyspnoea scale of 8. This individual's data was included in the full analysis as it is felt to be a true representation of this individuals exercise endurance.

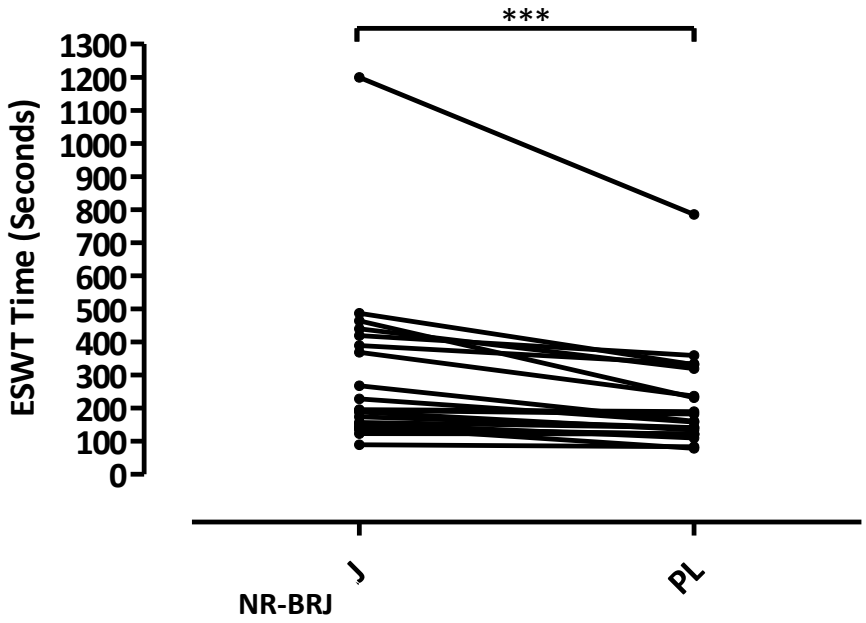


Figure 4.3. Individual ESWT Time (seconds) measured in the NO_3^--rich BRJ and PL dosing conditions

Data presented are individual ESWT times (seconds) in both dosing conditions. Wilcoxon sign-rank test was used to compare the change in ESWT time between treatment conditions; NO_3^--rich BRJ 194.6 sec (147.5, 411.7) vs PL 159 sec (121.9, 298.5), estimated treatment effect 62 sec (95%CI: 33, 106); *** p 0.00089.

Abbreviations: ESWT – Endurance Shuttle Walk Test; BRJ – Beetroot Juice; PL – Placebo

4.4.4 Effect of acute dietary nitrate supplementation on oxygen saturation during the ESWT

Data were only available for 18 individuals due to failure of data recording for two participants. O_2 saturations were recorded whilst all individuals were using their prescribed oxygen therapy. At rest and during the warm up there was no statistical significant difference in O_2 saturations between the two interventions. At all 10^{th} percentiles of isotime, isotime and peak exercise there was a statistically significant difference between the O_2 saturations (Figure 4.4 and Table 4.3). The area under the curve (AUC) to isotime for O_2 saturations shows a statistically significant separation between treatment conditions, area (SE), was 0.9 (0.07) (95%CI 0.8, 1.0); p = 0.002.

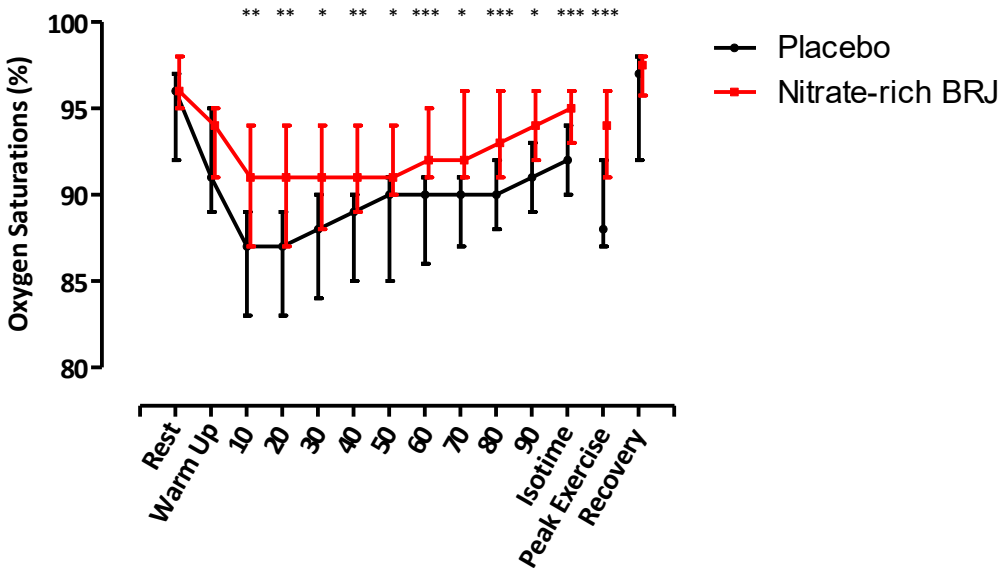


Figure 4.4. Isotime O_2 saturation analysis in the NO_3^--rich BRJ and PL dosing conditions

Data presented are median (IQR) of O_2 saturations in the two dosing conditions NO_3^--rich BRJ (red) and PL (black) at rest, warm up, each 10th percentile, isotime, peak exercise and recovery. Wilcoxon sign-rank test was used to compare the O_2 saturations between dosing conditions. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Abbreviations: % - percentage; BRJ – Beetroot Juice

Table 4.3. Isotime O_2 saturation analysis in the NO_3^--rich BRJ and PL dosing conditions

Period of ESWT	NO_3^--rich BRJ O_2 Saturations (%)	PL O_2 Saturations (%)	p-value
Rest	96 (93.8, 98)	96.0 (92.0, 97.3)	0.607
Warm up	93.5 (90, 95.3)	91.0 (88.5, 95.3)	0.118
10th	90.5 (85.8, 95.3)	86.5 (82.8, 89.5)	0.001
20th	90.5 (85.8, 94.0)	86.5 (82.8, 90.3)	0.001
30th	91.0 (87.8, 94.0)	88.5 (83.8, 90.3)	0.002
40th	91.0 (88.5, 94.5)	89.0 (84.8, 90.5)	0.001
50th	91.5 (88.8, 95.3)	89.5 (85.0, 91.3)	0.000275
60th	91.5 (89.8, 95.3)	89.50 (86.0, 91.3)	0.001
70th	92.5 (91.0, 96.5)	89.5 987.0, 92.0)	0.000061
80th	93.0 (91.0, 96.5)	90.0 (87.0, 92.0)	0.001
90th	94.0 (92.0, 96.5)	90.0 (87.0, 92.0)	0.000275
Isotime	95.5 (93.0, 96.5)	91.5 (89.0, 94.3)	0.000275
Peak Exercise	93.5 (91.0, 96.0)	88.0 (86.25, 92.0)	0.049
Recovery	97.5 (95.8, 98.0)	97.0 (92.0, 98.0)	1.0

Data shown are median (IQR) of O_2 saturations in the two dosing conditions NO_3^--rich BRJ and PL at rest, warm up, 10th percentiles of isotime, isotime, peak exercise and recovery. Wilcoxon sign-rank test was used to compare the O_2 saturations between dosing

conditions associated p values are presented in the table. There was a statistically significant difference at all 10th percentiles of isotime, isotime and peak exercise.

Abbreviations: NO_3^-- Nitrate; BRJ – Beetroot Juice; PL – Placebo; O_2 – Oxygen

4.4.5 Effect of acute dietary nitrate supplementation on heart rate during the ESWT

Data were only available for 18 individuals due to failure of data recording. The AUC to isotime for heart rate shows a statistically significant separation between treatment conditions, area (SE), was 0.86 (0.09) (95%CI 0.7, 1.0); p = 0.007. There was no significant difference in heart rate response at individual time points during the ESWT (Figure 4.5 and Table 4.4).

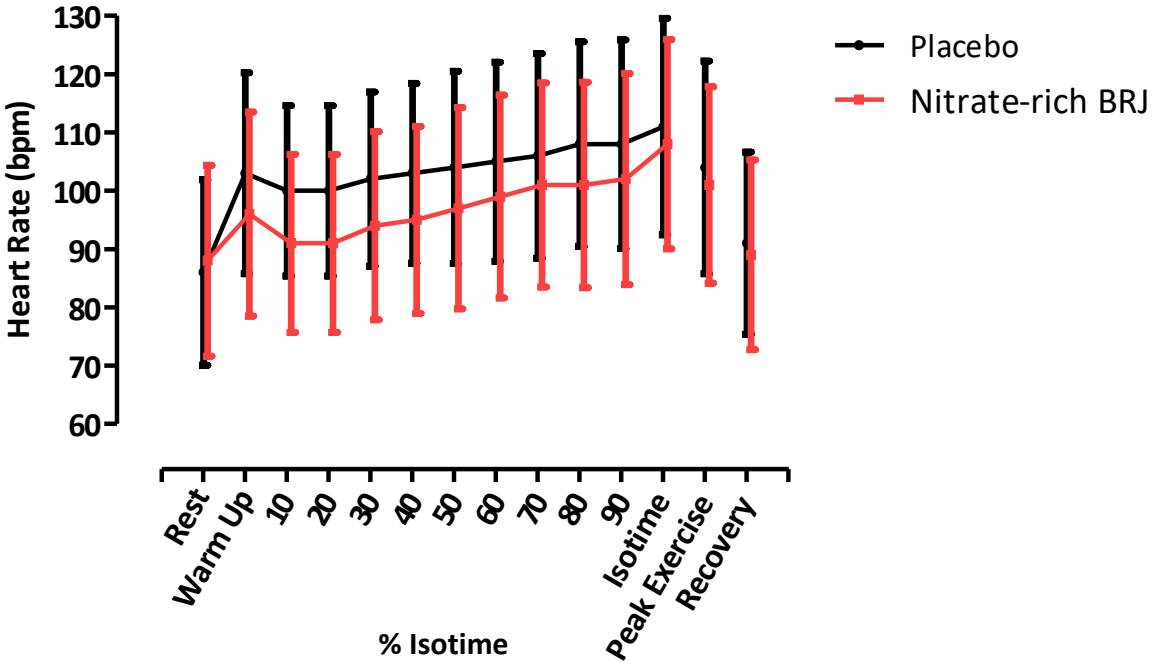


Figure 4.5. Isotime heart rate analysis in the NO_3^--rich BRJ and PL dosing conditions

Data presented are median (IQR) of heart rate in the two dosing conditions NO_3^--rich BRJ (red) and PL (black) at rest, warm up, each 10th percentile, isotime, peak exercise and recovery. Wilcoxon sign-rank test was used to compare the heart rate between dosing conditions. There was no statistically significant difference.

Abbreviations: % - percentage; BRJ – Beetroot Juice

Table 4.4. Isotime heart rate analysis in the NO_3^--rich BRJ and PL dosing conditions

Period of ESWT	NO_3^--rich BRJ Heart Rate	PL Heart Rate	p-value
Rest	87.5 (77.8, 90.8)	85.5 (73.5, 88.3)	0.204
Warm up	96.0 (88.3, 102.3)	103.0 (88.3, 108.0)	0.478
10th	91.5 (75.0, 106.0)	100.5 (72.8, 110.0)	0.276
20th	91.5 (75.0, 106.0)	100.5 (72.3, 110.0)	0.276
30th	94.0 (81.3, 108.3)	102.0 (76.3, 111.8)	0.331
40th	95.5 (82.5, 110.0)	103.0 (80.0, 111.8)	0.244
50th	97.5 (89.0, 110.3)	103.5 (86.0, 112.8)	0.246
60th	99.0 (90.3, 111.3)	104.5 (90.0, 114.5)	0.169
70th	101.0 (91.8, 112.0)	105.5 (92.5, 115.5)	0.162
80th	101.5 (93.5, 112.3)	107.5 (95.0, 118.5)	0.102
90th	102.0 (95.8, 113.3)	108.0 (97.5, 121.0)	0.064
Isotime	108.5 (95.8, 115.8)	111.0 (103.3, 123.0)	0.102
Peak Exercise	101.0 (87.8, 112.3)	103.5 (96.3, 111.0)	0.136
Recovery	88.5 (80.8, 98.0)	91.0 (78.5, 101.0)	1.0

Data shown are median (IQR) of heart rate in the two dosing conditions NO_3^--rich BRJ and PL at rest, warm up, 10th percentiles of isotime, isotime, peak exercise and recovery. Wilcoxon sign-rank test was used to compare the heart rate between dosing conditions

associated p values are presented in the table. There was no statistically significant differences in heart rate at any points during the ESWT.

Abbreviations: NO_3^-- Nitrate; BRJ – Beetroot Juice; PL – Placebo; O_2 – Oxygen

4.4.6 Effect of acute dietary nitrate supplementation on resting blood pressure parameters

Change in blood pressure parameters from baseline following the acute ingestion of NO_3^--rich BRJ and PL are shown in Figure 4.6. There was no statistically significant difference in the median (IQR) change in sBP following dosing with NO_3^--rich BRJ -1.5 (-15.0, 10.8) mmHg vs PL -0.5 (-10.5, 6.8) mmHg; p = 1.0. There was no statistically significant difference in the median (IQR) change in dBP following dosing with NO_3^--rich BRJ -4.0 (-14.0, 7.0) mmHg vs PL -1.0 (-9.3, 5.0) mmHg; p = 0.481. There was no statistically significant difference in the median (IQR) change in MAP following dosing with NO_3^--rich BRJ -5.0 (-15.3, 6 mmHg) vs PL -2.5 (-13.5, 7) mmHg; p = 0.359.

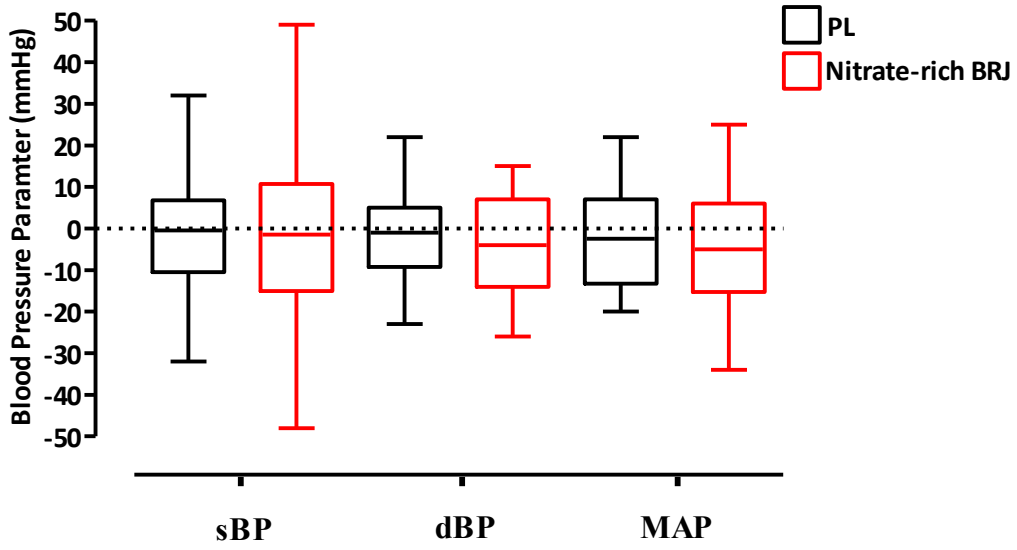


Figure 4.6 Change in blood pressure parameters in the NO_3^--rich BRJ and PL dosing conditions

Data are presented as 25th-75th percentile with the solid line representing the median value, and the whiskers the minimum to maximum values. Wilcoxon sign-rank test was used to compare change in blood pressure parameters relative to baseline blood pressure three hours prior to dosing with either intervention.

Abbreviations: Pl – Placebo; BRJ – Beetroot Juice; sBP – Systolic Blood Pressure; dBP – Diastolic Blood Pressure; MAP – Mean Arterial Pressure

There was no statistically significant change between any blood pressure parameters. sBP: NO_3^--rich BRJ -1.5 (-15.0, 10.8) mmHg vs PL -0.5 (-10.5, 6.8) mmhg; p = 1.0. dBP: NO_3^--rich BRJ -4.0 (-14.0, 7.0) mmHg vs PL -1.0 (-9.3, 5.0) mmHg; p = 0.481. MAP: NO_3^--rich BRJ -5.0 (-15.3, 6) mmHg vs PL -2.5 (-13.5, 7) mmHg; p = 0.359.

4.4.7 Adverse and serious adverse events

There were no adverse or serious adverse events during this study. Both interventions were well tolerated. Beeturia was universally reported for both interventions.

4.5 Discussion

4.5.1 Main findings

The major finding of this study was that an acute dose of dietary NO_3^- at a volume of 140 mL (NO_3^-: 12.9 mmol), in the form of a widely available BRJ (BEET-IT® SPORT Shot, James White Drinks, Ipswich, UK) resulted in a statistically significant increase in exercise performance – an estimated treatment effect of 62 sec (95%CI: 33, 106); p 0.00089) in individuals with COPD who require supplemental oxygen compared to a well matched placebo. This was accompanied by lower heart rate and higher oxygen saturations during the period to isotime. No difference in blood pressure parameters was noted.

4.5.2 Significance of findings

Of the studies investigating dietary NO_3^- supplementation in COPD this is the first focusing on individuals with COPD with hypoxaemia requiring oxygen supplementation. The prevalence of hypoxaemia in COPD is uncertain, being between 1.4% (Sundh and Ekstrom 2017) and 7% (Wells, Estepar et al. 2016). Hypoxaemia in COPD contributes to reduced quality of life, reduced exercise tolerance, reduced skeletal muscle function and increased mortality (Kim, Benditt et al. 2008). There is potential that an intervention that could improve whole-body oxygen efficiency would improve exercise endurance in this

subgroup of individuals with COPD. The present results suggest that dietary NO_3^- supplementation can improve exercise endurance in the subgroup of COPD who have exercise induced desaturation and require oxygen supplementation.

In conditions of hypoxia, the $_L$-arginine-NOS pathway is impaired and the NO_3^--NO_2^--NO pathway is potentiated by deoxyhaemoglobin, -myoglobin and hydrogen ions (Vanhatalo, Fulford et al. 2011, Dauncey 2012, Jones 2014, Shannon, Duckworth et al. 2016). This would suggest that nitrate supplementation would have more impact in hypoxia than normoxia. Studies in healthy individuals in hypoxic conditions have found that NO_3^- supplementation improves exercise efficiency (Masschelein, Van Thienen et al. 2012, Kelly, Vanhatalo et al. 2014, Muggeridge, Howe et al. 2014, Shannon, Duckworth et al. 2016), muscle oxygenation (Masschelein, Van Thienen et al. 2012)(17) and increases oxygen saturations (Masschelein, Van Thienen et al. 2012, Kelly, Vanhatalo et al. 2014, Muggeridge, Howe et al. 2014, Shannon, Duckworth et al. 2016). The finding in this hypoxaemic COPD cohort of increased oxygen saturations at isotime are consistent with the findings in healthy individuals.

This study only included individuals with COPD who had been prescribed long-term oxygen therapy in line with the BTS Guidelines for Home Oxygen Use in Adults (Hardinge, Annandale et al. 2015). There will be a cohort of individuals with COPD which are not sufficiently hypoxaemic to qualify for LTOT but desaturate on exercise, these individuals were not included in this study. Supplemental oxygen has been shown to improve outcomes of exercise endurance (Bradley, Lasserson et al. 2007) and in those individuals who desaturate on exercise to tolerate a higher level oof activity and therefore gain more

from training (Nonoyama, Brooks et al. 2007). Currently supplemental oxygen for exercise induced desaturation is only prescribed if the BTS criteria for LTOT are met (Hardinge, Annandale et al. 2015). It is in these individuals where supplementation with dietary NO_3^- may be of benefit. Despite being in a state of normoxia at rest, during exercise a state of hypoxaemia is likely to develop. It would be reasonable to conclude that there would be potentiation of the NO_3^--NO_2^--NO pathway during exercise (Vanhatalo, Fulford et al. 2011, Dauncey 2012, Jones 2014, Shannon, Duckworth et al. 2016) and therefore supplementation with dietary NO_3^- would result in the known improvements in exercise efficiency, muscle oxygenation and increased oxygen saturation.

The underlying mechanism is unclear, however the finding of relatively preserved oxygen saturation and lower heart rate during exercise in the nitrate supplemented condition are likely to reflect a combination of more efficient oxygen utilisation peripherally and some impact on central haemodynamics potentially related to reduced pulmonary hypoxic vasoconstriction. The effects are almost certainly not COPD-specific and work is needed to investigate possible benefits in other long term lung conditions associated with hypoxia including interstitial lung diseases and the various categories of pulmonary hypertension.

The minimal important difference (MID) of the ESWT in pharmacotherapy trials is 65 s (95% CI 45-85) (Pepin, Laviolette et al. 2011). The estimated treatment effect of dietary NO_3^- supplementation found in this study was 62.5 seconds, which falls slightly short of the MID of the ESWT. These findings are likely to be clinically significant for several reasons. In pharmacological trials where the ESWT time is the outcome, the interventions are likely to be given over a period of weeks or months. In this study the dietary NO_3^-

supplement was given in an acute dosing regimen. Despite this single acute dose protocol, all participants in this trial improved their exercise performance, suggesting a robust underlying physiological mechanism of benefit.

4.5.3 Methodological issues

The use of a robust placebo strengthens the reliability of the findings, as does the fact that the improvement in walking time was accompanied by an appropriate physiological response (lower heart rate and higher oxygen saturations). An additional strength was the use of a walking test which is of clinical relevance to patients as it reflects most individuals' main form of exercise and daily physical activity.

This was a single dose study and therefore questions remain as to the impact that regular dosing might have and whether this would translate into meaningful clinical effect. The dose used was selected based on previous studies but future work should investigate whether there is a dose response or ceiling effect.

For longer term treatment, as well as working out whether the treatment is effective will also need to consider dosing strategies – should these be the same for all individual? Should dosing be daily or intermittent?

Beetroot juice is cheap and readily accessible so has the potential to be used widely if effective. Further mechanistic work is needed to work out the relative impact of the

possible mechanisms, in particular the impact of muscle vs pulmonary or systemic circulation effects.

4.5.4 Conclusion

The acute dosing of dietary NO_3^- in the form of BRJ increased exercise performance and resulted in relatively preserved oxygen saturations and a reduction in heart rate at isotime in individuals with COPD who require supplemental oxygen. Providing a possible new intervention that could be used to augment exercise in this subgroup of individuals with COPD. This did not, however, translate into a reduction in blood pressure parameters. Given the inexpensive nature of BRJ and its safety profile this intervention warrants further investigation in individuals with different chronic lung diseases who are hypoxaemic.

CHAPTER 5: DIETARY NITRATE SUPPLEMENTAION – ASSESSMENT OF BIOAVAILABILITY IN COPD

5.1 Introduction

The results of the previous two Chapters have shown that (i) in the ON-EPIC trial dietary nitrate (NO_3^-) supplementation in the form of NO_3^--rich beetroot juice (BRJ) augmented the improvement in exercise capacity seen in people with COPD following an eight week programme of pulmonary rehabilitation (PR) and (ii) in the EDEN-OX trial a single dose of NO_3^- supplementation increased exercise performance in COPD patients who require who require supplemental oxygen.

A third aim of this thesis was to investigate the potential effects of NO3- supplementation on endothelial function, plasma NO_3^- and nitrite (NO_2^-) and exhaled nitric oxide (FeNO). These measures were undertaken as part of the studies in Chapter 3 and Chapter 4.

5.2 Background

The production of nitric oxide (NO) occurs via the oxygen (O2)-dependent nitric oxide synthase (NOS) pathway and the O_2-independent NO_3^--NO_2^--NO pathway (Lundberg and Govoni 2004). It has been shown that by exogenous supplementation of NO_3^- the O_2-independent formation of NO can be augmented.

Methods that could be used to assess the increased bioavailability of NO following supplementation include: increased levels of plasma NO_3^- and NO_2^- (Dreissigacker, Wendt et al. 2010, Wilkerson, Hayward et al. 2012, Porcelli, Ramaglia et al. 2015), or more indirectly via reduction in blood pressure parameters (Appel, Moore et al. 1997, Webb, Patel et al. 2008, Hord, Tang et al. 2009, Vanhatalo, Bailey et al. 2010) and via effects on endothelial function assessed as an increase in flow mediated dilatation (FMD) (Velmurugan, Gan et al. 2016, Walker, Bailey et al. 2019).

It has been well established that NO is a ubiquitous signalling molecule involved in a variety of processes: as a potent vasodilator (Furchgott and Zawadzki 1980), in platelet aggregation (Bredt 1999), inhibition of vascular smooth muscle cell proliferation (Forstermann 2010) and the prevention of atherosclerotic plaque formation (Alderton, Cooper et al. 2001). A reduction in NO is characteristic of impaired endothelial function (EF) (Sverdlov, Ngo et al. 2014).

The endothelial production of NO takes place via the O_2-dependent reduction of L-arginine via endothelial NOS (eNOS) (Forstermann, Pollock et al. 1991) with eNOS located in the vascular endothelium (Kobzik, Reid et al. 1994). Exogenous supplementation of NO_3^- augments the O_2-independent NO_3^--NO_2^--NO pathway independently of the endothelium (Benjamin, O'Driscoll et al. 1994) and has been found to improve EF in cardiovascular disease (Jackson, Patterson et al. 2018). FMD allows for an assessment of EF, with FMD being a measure of endothelium-dependent dilatory response to reactive hyperaemia (Jones, Dunn et al. 2019).

5.2.1 Flow mediated dilatation

FMD is a non-invasive bioassay of peripheral EF involving the temporary occlusion of the arm vasculature inducing an acute increase in shear stress (Celermajer, Sorensen et al. 1992) and is an established method used to assess EF (Thijssen, Black et al. 2011). FMD allows for the assessment of EF through the non-invasive measurement of the ability of an artery to respond to endothelial NO release during reactive hyperaemia produced by sheer stress.

Large peripheral arteries including the brachial, femoral and radial arteries are typically used for FMD assessment. The generation of shear stress is via the application temporary limb ischaemia, which generates an ischaemia induced reactive hyperaemia (Pyke and Tschakovsky 2005) and as a result arterial dilatation occurs. Studies have found that following supplementation of dietary NO_3^- a resultant increase in FMD occurs, with a percentage increase between 0.5-4% (Bondonno, Yang et al. 2012, Heiss, Meyer et al. 2012, Bakker, Engan et al. 2015, Lee, Stebbins et al. 2015, Velmurugan, Gan et al. 2016, Walker, Bailey et al. 2019).

5.2.2 Exhaled nitric oxide

In most research studies into the supplementation of NO_3^- the bioavailability of NO is typically assessed by measures of plasma NO_3^- and NO_2^- concentrations. Following a bolus of oral NO_3^-, plasma NO_3- concentration peaks at approximately 1-2 h and plasma NO_2^- concentration peaks at approximately 2-3 h, with both concentrations falling to the baseline values at around 24 h (Webb, Patel et al. 2008). It is difficult to measure the plasma NO_3^- and NO_2^- concentrations without direct access to processing facilities. It may

be possible to assess response to dietary NO_3^- ingestion through measurements of exhaled nitric oxide (FeNO).

F_ENO is an established measure to assess airways inflammation and response to inhaled corticosteroids in asthmatic individuals (ATS/ERS 2005). Airways NO is produced by inducible NOS (iNOS) located in the airway and circulatory endothelial cells and inflammatory cells located in the alveoli and large airways (Barnes, Dweik et al. 2010). ENO is a measure of the fraction of NO in exhaled breath at a flow rate of 50 mL.sec-1 (F_ENO_{50}) (Lane, Knight et al. 2004, Dahgam, Modig et al. 2014). The guidelines for the measurement of exhaled nitric oxide recommend the avoidance of NO_3^--rich foodstuffs (ATS/ERS 2005) as it has been established that NO_3^--rich food can increase the measures exhaled nitric oxide (Zetterquist, Pedroletti et al. 1999, Olin, Aldenbratt et al. 2001, Kroll, Werchan et al. 2018). The measurement of exhaled nitric oxide in individuals with COPD is of less clinical use. In this group exhaled nitric oxide is influenced by disease severity and smoking status (Malerba, Radaeli et al. 2014). Levels of measured exhaled nitric oxide in current smokers is usually reduced and conversely in non-smoking individuals with established COPD exhaled nitric oxide is higher than in healthy individuals (Maziak, Loukides et al. 1998, Clini, Cremona et al. 2000, Lim, Rani et al. 2018). At the time of writing this thesis only one study has assessed the response to dietary NO_3^- supplementation in individuals with COPD (Kerley, James et al. 2019). In this study Kerley *et al* undertook a 14-day double-blind, randomised, placebo-controlled, cross-over trial of NO_3^--rich BRJ (NO_3^-: 12.9 mmol) against a matched NO_3^--deplete (NO_3^-: 0.5 mmol) in eight individuals with COPD (Kerley, James et al. 2019). The baseline F_ENO_{50} was 10 +/- 8.6 ppb and following NO_3^- supplementation increased to 48 +/- 63.7 ppb vs placebo 11 +/- 10.9 ppb; p = 0.095 (Kerley, James et al. 2019).

5.3 Hypothesis

The hypotheses for this Chapter are three-fold:

(i) that dietary NO_3^- supplementation consumed in combination with a programme of pulmonary rehabilitation or consumed acutely would improve endothelial function

(ii) that acute supplementation of dietary NO_3^- would increase plasma NO_3^- and NO_2^- levels

(iii) that acute supplementation of dietary NO_3^- would increase ENO levels

5.4 Methods

5.4.1 Participant selection

All participants in this Chapter were drawn from the ON-EPIC and EDEN-OX studies described in Chapters 3 and 4 and had provided written, informed consent prior to the enrolment. Both studies were approved by research and ethic committees and registered prospectively on a publicly accessible database; the London Bridge Health Research and Ethics Committee (REC reference 14/LO/1474), (www.isrctn.com/ISRCTN27860457) (Chapter 3 - ON-EPIC) and the London Chelsea Research and Ethics Committee (REC Reference 15/LO/0975), (http://www.isrctn.com/ISRCTN14888729) (Chapter 4 EDEN-OX).

Inclusion criteria for these studies included individuals with COPD GOLD grade II-IV, based on the NICE (www.nice.org.uk) and GOLD (https://goldcopd.org/) criteria. Participants were recruited from the outpatient clinical services at the Royal Brompton Hospital. Recruitment took place between the 26th January 2015 and the 15th January 2018.

Exclusion criteria included clinical instability (i.e. less than one month after an exacerbation of COPD), significant other comorbidity limiting exercise tolerance, significant renal impairment (defined as an estimated glomerular filtration rate <50 $mL.min^{-1}$), hypotension, (systolic blood pressure < 100 mmHg), pregnancy, the use of nitrate based medications or phosphodiesterase V inhibitor or other reasons to benefit from NO3- supplementation (i.e. ischaemic heart disease and peripheral arterial disease). Participants were withdrawn if they had a significant adverse event or if they withdrew their consent for participation.

Within this Chapter data are analysed from 20 consecutive participants from the ON-EPIC study and all participants were analysed from the EDEN-OX study.

5.4.2 Study design and randomisation

ON-EPIC was a multi-centre, randomised, double-blind, placebo-controlled, parallel group study and EDEN-OX was a single centre, randomised, double-blind, placebo-controlled cross-over study. Participants were randomised 1:1 using a computer-generated block randomisation list, block size 10, produced by an independent statistician with consecutive numbers linked to identical preparations of either BRJ or Placebo. Both the participants and the researcher were blinded to treatment allocation throughout the study and during data analysis.

5.4.3 Intervention

Participants were randomly assigned (1:1) in a double-blind fashion to receive either the intervention beverage or placebo (PL) beverage. The intervention beverage was 140 mL of concentrated BRJ (NO_3^-; 12.9 mmol) cut with organic lemon juice (2%) (140 mL BEET-

IT® SPORT Shot, James White Drinks, Ipswich, UK). The PL was a matched beverage of 140 mL NO_3^--deplete BRJ (NO_3^-; 0.002 mmol) (produced by James White Drinks, Ipswich, UK) which was generated by the standardised method of passing the juice, prior to pasteurisation, through an ion exchange column, containing Purolite A520E ion exchange resin, which selectively removes NO_3^- (Lansley 2011). The PL beverage was identical in taste and appearance with both beverages causing beeturia.

5.4.4 Assessment of flow mediated dilatation

Within the ON-EPIC study protocol described in Chapter 3, 20 consecutive participants (10 in each intervention arm) who were able to attend the Royal Brompton Hospital underwent assessment at baseline for FMD and the same individuals were reassessed at completion of the study. The end of study assessment was undertaken at a minimum of seven days following the last consumption of study product at the end of pulmonary rehabilitation.

All participants taking part in the EDEN-OX study described in Chapter 4 underwent assessment for FMD. At both intervention visits baseline assessment was performed at time point zero immediately prior to consuming the intervention beverage (active or placebo) and 180 minutes after.

5.4.5 Assessment of plasma nitrate and nitrite

All participants taking part in EDEN-OX underwent assessment for plasma NO_3^- and NO_2^-. At each intervention visit a baseline plasma sample was drawn prior to consumption of

the intervention beverage. A second plasma sample was drawn for plasma NO_3^- and NO_2^- at time point 180 minutes after consumption of the intervention beverages.

5.4.6 Assessment of exhaled nitric oxide

All participants taking part in EDEN-OX underwent assessment for F_ENO_{50}. A baseline F_ENO_{50} was measured at the baseline assessment visit. During both intervention visits a baseline F_ENO_{50} was measured at time point zero minutes. Following this F_ENO_{50} was measured at 30 minute time intervals until 180 minutes. In total seven measures seven measures of F_ENO_{50} were taken at each intervention visit per participant.

5.4.7 Outcome measures

1. Change in endothelial function, assessed by FMD, following dietary NO_3^- supplementation in combination with PR (ON-EPIC)**.**

2. Change in endothelial function, assessed by FMD, following the acute supplementation with dietary NO_3^- (EDEN-OX).

3. Change in plasma NO_3^- and NO_2^- following the acute supplementation of dietary NO_3^-. (EDEN-OX)

4. Change in FeNO following acute supplementation of dietary NO_3^-. (EDEN-OX)**.**

5.4.8 Data analysis and statistics

Data are presented as mean and standard deviation (SD) or if not normally distributed data as median and interquartile range (IQR). For the experimental study described in

Chapter 3, the data is unpaired, differences in response between groups were assessed using an unpaired T-test or a Mann-Whitney U test as appropriate. For the experimental study described in Chapter 4, the data is paired, thus differences in response between groups were assessed using a paired T-test or a Wilcoxon sign-rank test as appropriate. To assess the effect of the different interventions on ENO a one-way ANOVA or a Kruskal-Wallis *H* test will be used as appropriate. Analysis was performed using SPSS version 24 for Windows (SPSS Software, Chicago, Illinois, USA). Figures were prepared using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, California, USA). A p value of < 0.05 was considered to be statistically significant. Analysis of FMD images was performed using ImageJ2 Image Analysis Software (Fiji, National Institutes of Health and Laboratory for Optical Computational instrumentation, University of Wisconsin, USA. https://fiji.sc).

5.5 Results

5.5.1 Nitrate and nitrite content of the nitrate-rich beetroot juice and placebo supplements

The manufacturer of the both the NO_3^--rich BRJ and NO_3^--deplete PL, James White Drinks (Ipswich, UK), report that 140mls NO_3^--rich BRJ contains 12.9 mmol of NO_3^- and that the NO_3^--deplete placebo contains 0.002 mmol NO_3^-. The NO_3^- and NO_2^- content of both interventions, the cryotubes used for testing and the N-Ethylmalemiide (NEM) stock solution was assessed (Table 5.1). The mean ± SD NO_3^- content of 140mls NO_3^--rich BRJ was 120,411.03 ± 5267.1 µM compared to the NO_3^--deplete PL with a NO_3^- content of 55.05 ± 0.68 µM.

Table 5.1. NO_3^- and NO_2^- content of the NO_3^--rich BRJ and placebo and constituents used for plasma concentration analysis

	NO_2^- (μM)				NO_3^- (μM)			
	Mean	**SD**	**SEM**	**%CV**	**Mean**	**SD**	**SEM**	**%CV**
NEM Stock Solution	0.07	0.03	0.02	45.41	22.37	2.33	1.34	10.40
Cryotube	0.09	0.02	0.009	17.15	2.44	0.53	0.31	22.33
PL	10.75	0.20	0.11	1.08	55.05	0.68	0.39	1.24
NO_3^- -rich BRJ	195.86	2.12	1.22	1.08	120411.03	5267.10	3040.96	4.37

Data displayed are mean NO_3^- and NO_2^- concentrations, standard deviation, standard error of the mean and percentage coefficient variation in the NEM stock solution, the cyrotubes and the intervention beverages.

Abbreviations: NO_2^- - Nitrite; NO_3^- - Nitrate; SD – Standard Deviation; SEM – Standard Error of the Mean; CV – Coefficient Variation; NEM – N-Ethylmaleimide; PL – Placebo; BRJ – Beetroot juice; SD – Standard Deviation; SEM – Standard Error of the Mean; %CV - Coefficient of Variation

5.5.2 Effect of dietary nitrate supplementation on plasma nitrite and nitrate concentration (EDEN-OX study)

Data on plasma NO_2^- and NO_3^- concentrations were available in 19 subjects (one subject declining venous sampling). Following supplementation with NO_3^--rich BRJ in individuals with COPD who require supplemental oxygen there was a statistically significant increase in plasma NO_2^- and NO_3^- concentrations compared to PL (Figure 5.2). The plasma NO_3^-

concentration 180 minutes following dosing with NO_3^--rich BRJ plasma increased significantly from baseline (baseline plasma NO_3^- concentration 62.59 µM (41.68, 77.29) vs 180 minutes post dosing plasma NO_3^- concentration 617.71 µM (556.25, 725.88); p = 0.00004.

The plasma NO_2^- concentration 180 minutes following dosing with NO_3^--rich BRJ plasma increased significantly from baseline (baseline plasma NO_2^- concentration 0.337 µM (0.222, 0.397) vs 180 minutes post dosing plasma NO_2^- concentration 0.620 µM (0.488, 0.673); p = 0.0003.

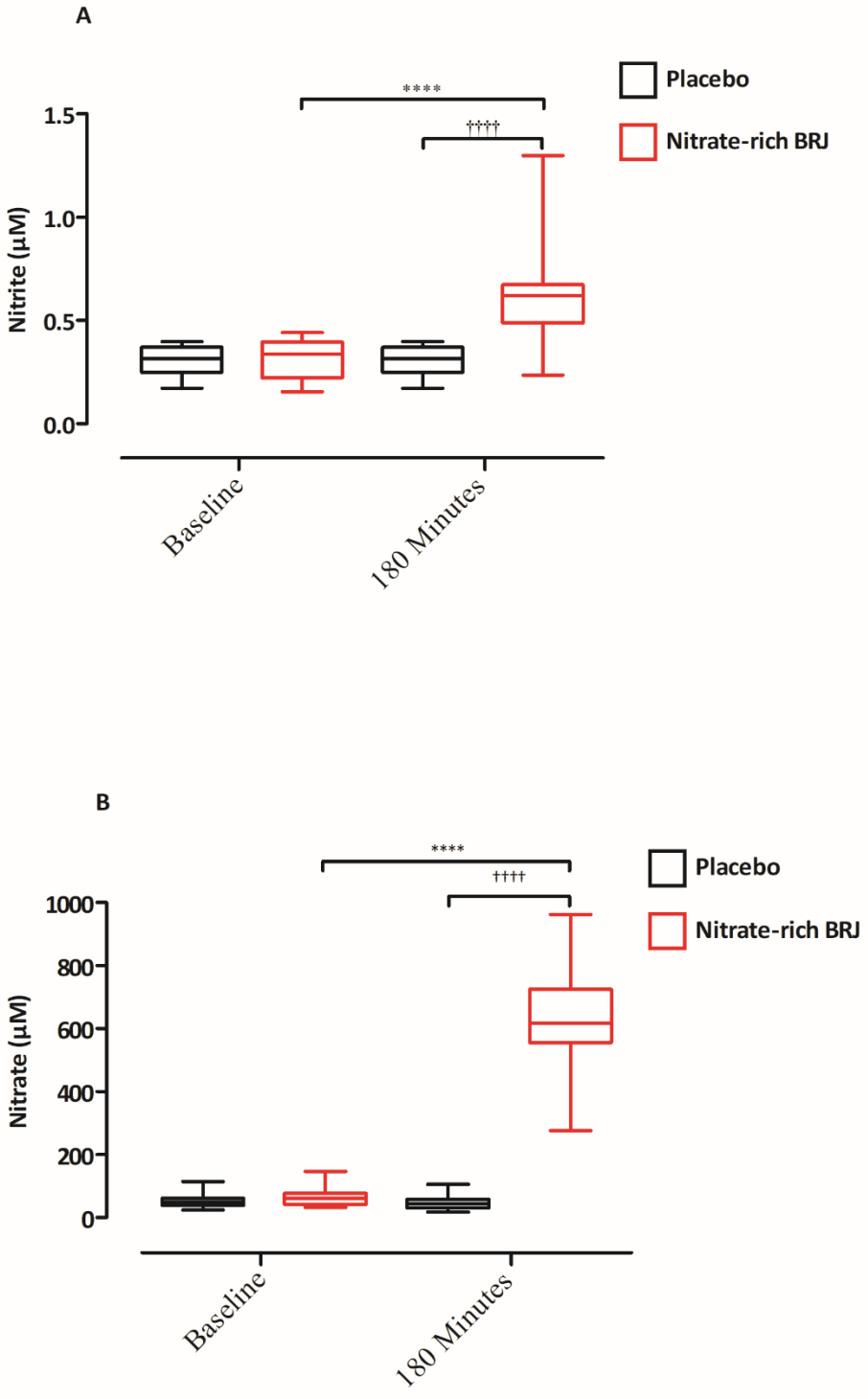

A
Placebo
Nitrate-rich BRJ
****
††††
Nitrite (µM)
1.5
1.0
0.5
0.0
Baseline
180 Minutes
B
Placebo
Nitrate-rich BRJ
****
††††
Nitrate (µM)
1000
800
600
400
200
0
Baseline
180 Minutes

Figure 5.1. The change in NO_3^- and NO_2^- concentrations following supplementation with NO_3^--rich BRJ and PL in individuals with COPD requiring supplemental oxygen

Data presented are median (IQR) with whiskers representing minimum to maximum values. Plasma NO_3^- and NO_2^- concentrations were measured at baseline (zero minutes) and 180 minutes after dosing with intervention beverage. Wilcoxon sign-rank test was used to compare change in plasma NO_3^- and NO_2^- concentrations between intervention groups. Mann-Whitney U test was used to compare change in plasma NO_3^- and NO_2^- concentrations between treatment conditions

Panel A. Change in plasma NO_2^- concentrations

There was a statistically significant difference between baseline plasma NO_2^- concentration and post dosing with NO_3^--rich BRJ plasma NO_2^- concentration (pre dosing plasma NO_2^- concentration in NO_3^--rich BRJ group 0.306 μM (0.227, 0.402) vs post dosing 0.620 μM (0.488, 0.673); **** p = 0.000076) (Wilcoxon sign-rank test). There was also a statistically significant difference between post dosing plasma NO_2^- concentrations in the different dosing conditions (PL post dosing plasma NO_2^- concentration 0.306 μM (0.227, 0.402) vs NO_3^--rich BRJ post dosing plasma NO_2^- concentration 0.620 μM (0.488, 0.673); †††† p = 0.000009) (Mann-Whitney u test).

Panel B. Change in plasma NO_3^- concentrations

There was a statistically significant difference between baseline plasma NO_3^- concentration and post dosing with NO_3^--rich BRJ plasma NO_3^- concentration (pre dosing

plasma NO_2^- concentration in NO_3^--rich BRJ group 62.59 µM (41.68, 77.29) vs post dosing 617 µM (556.25, 725.88); **** p = 0.00004) (Wilcoxon sign-rank test). There was also a statistically significant difference between post dosing plasma NO_3^- concentrations in the different dosing conditions (PL post dosing plasma NO_3^- concentration 45.31 µM (31.39, 58.84) vs NO_3^--rich BRJ post dosing plasma NO_3^- concentration 617.71 µM (556.25, 725.88); †††† p = 5.66 x10-11) (Mann-Whitney U test)

Abbreviations: BRJ – Beetroot Juice

5.5.3 Effect of dietary nitrate supplementation on exhaled nitric oxide (EDEN-OX)

Paired FeNO levels were available at all seven time points (zero minutes, 30 minutes, 60 minutes, 90 minutes, 120 minutes, 150 minutes and 180 minutes) for 16 participants. For four participants there was a device failure with no data being recorded.. There was a statistically significant difference in ENO following consumption of NO_3^--rich BRJ vs PL: 31 ppb (17,60); X2 (1) = 9.215, p = 0.0002 (Figure 5.2) (Table 5.3).

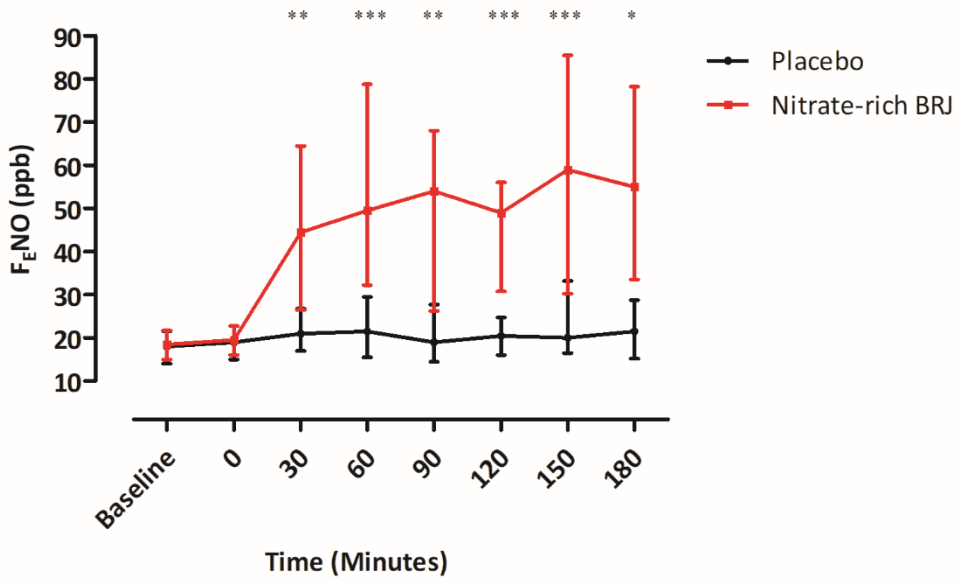


Figure 5.2. F_ENO levels at 30-minute time point following dosing with NO_3^--rich BRJ and PL

Data presented as median (dot or square) with whiskers representing the interquartile range. PL represented by black line and dot and NO3$^-$-rich BRJ represented by red square and line. Kruskal-Wallis H test was used to assess the effect of either dosing condition on ENO. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Abbreviations: F_ENO – Fractional Exhaled Nitric Oxide; BRJ – Beetroot Juice.

Table 5.3. F_ENO levels at 30-minute time points following dosing with NO_3^--rich BRJ and PL

	Median ENO (ppb)	IQR	X^2 (1)	p Value	PL Mean Rank ENO (ppb)	NO_3^--rich BRJ Mean Rank ENO (ppb)
Baseline	18	14, 22	0.018	0.8944	16	17
0 Minutes	19	15, 23	0.018	0.8944	16	17
30 Minutes	27	18, 47	10.650	0.0011	11	22
60 Minutes	31	20, 54	14.811	0.0001	10	23
90 Minutes	27	19, 57	11.692	0.0006	11	22
120 Minutes	27	19, 50	14.102	0.0002	10	23
150 Minutes	33	20, 60	14.365	0.0002	10	23
180 Minutes	31	17, 60	9.215	0.0024	11	22

Data presented are median (IQR). Kruskal-Wallis H test was used to assess the effect of either PL or NO_3^--rich BRJ on ENO levels. ENO levels measured at baseline visit (visit 1) and the subsequently at the intervention visits (visit 3 and 4). On intervention visit days ENO

was measured at seven time points. Zero minutes prior to dosing with intervention beverages followed by 30 minutes thereafter until 180 minutes.

Abbreviations: ENO – Exhaled Nitric oxide; IQR – Interquartile Range; X^2 – Chi-squared; PL – Placebo; NO_3^- - Nitrate; BRJ – Beetroot Juice

5.5.4 Correlation between plasma nitrate concentration and exhaled nitric oxide

There was no significant correlation between either baseline FeNO and plasma NO_3^- concentration (r = 0.322, p = 0.24, Figure 5.3) or 180-minute FeNO and plasma NO_3^- concentration (r = -0.02, p = 0.95, Figure 5.4).

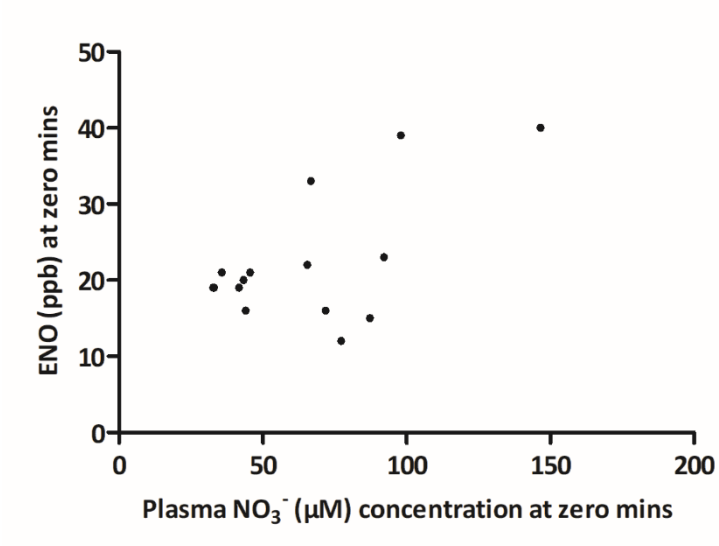


Figure 5.3. Correlation of F_ENO and Plasma NO_3^- concentration at baseline

Abbreviations: ENO – Exhaled Nitric Oxide; NO_3^- - Nitrate; µM - Micromole

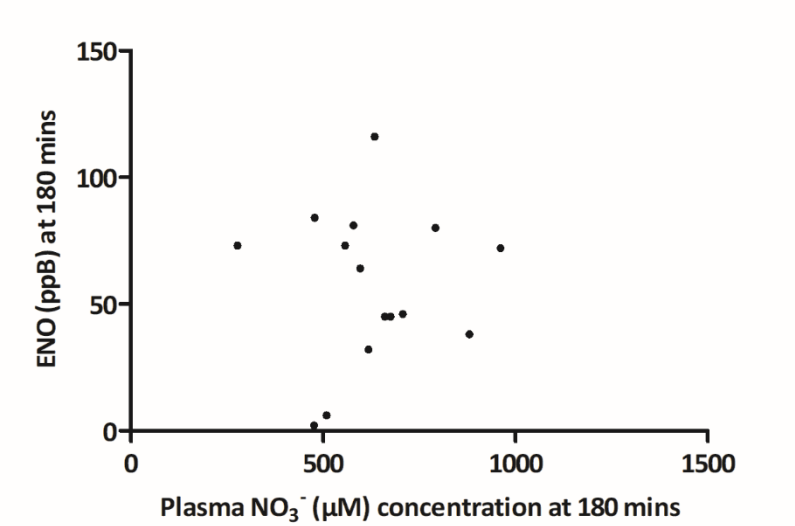


Figure 5.4. Correlation of F_ENO and Plasma NO_3^- concentration 180 minutes post dosing

Abbreviations: ENO – Exhaled Nitric Oxide; NO_3^- - Nitrate; µM - Micromole

5.5.5 Effect of dietary nitrate supplementation on flow mediated dilatation in combination with pulmonary rehabilitation (ON-EPIC)

In a planned sub study, FMD was assessed in 20 consecutive participants (NO_3^--rich BRJ n = 10, PL n = 10) at baseline and following an eight week, twice weekly programme PR (Figure 5.5 is an example of the images acquired). There was a statistically significant difference in the median (IQR) percentage change in the FMD in the $NO3^-$-rich BRJ group with an increase of 6.6 % (0.6, 17.6) vs PL with a reduction -4.7% (-21.5, 11.8); p = 0.046 (Figure 5.6).

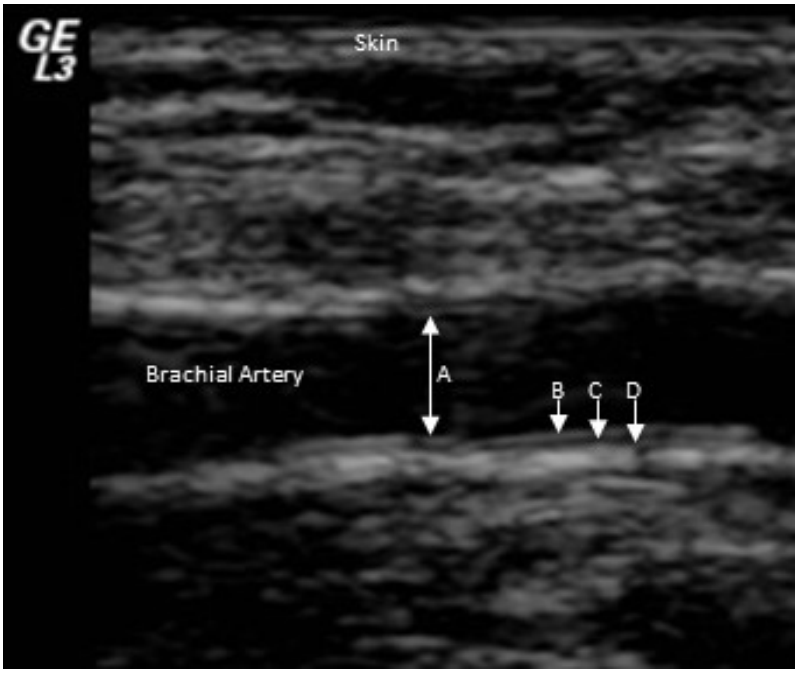


Figure 5.5. Ultrasound image of the brachial artery (longitudinal view)

Image acquisition using high-resolution doppler ultrasound (GE Logiq 3, GE Medical Systems, Milwaukee, Winsconsin USA) with a 10 MHz multi-frequency linear array probe in B-mode.

Annotation: A – brachial artery lumen; B – Intima; C – Myointimal Structures; D Adventitial Tunica

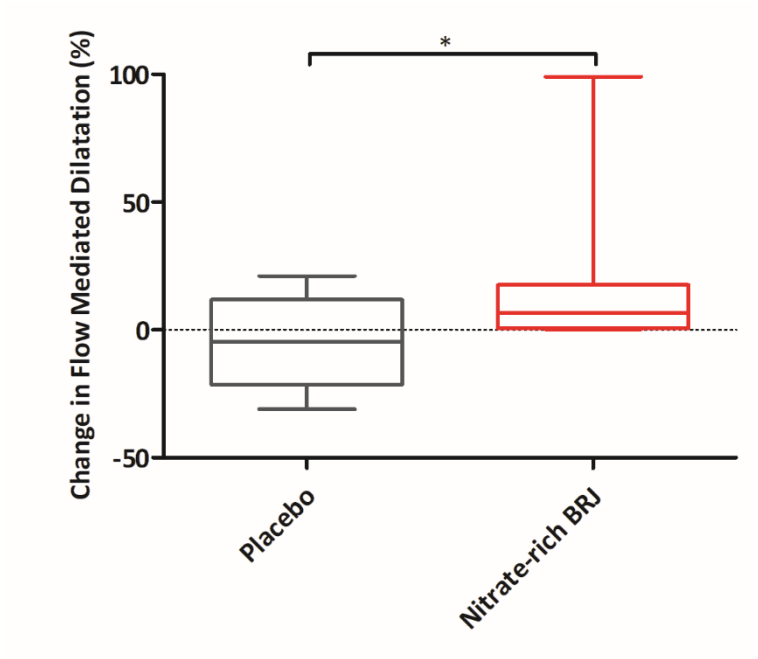


Figure 5.6. Percentage change in FMD following NO_3^--rich BRJ and PL dosing conditions and programme of PR

Data presented are IQR with the solid line representing the median value, and the whiskers the minimum to maximum values. Mann-Whitney U test was used to compare the percentage change in FMD in the NO_3^--rich BRJ group (red) and the PL (black) dosing conditions. There was a statistically significant difference in FMD percentage change in the NO_3^--rich BRJ group with an increase of 6.6% (0.6, 17.6) vs a reduction in the PL group of -4.7% (-21.5, 11.8), * p = 0.046.

Abbreviations: BRJ – Beetroot Juice; NO_3^- - Nitrate

5.5.6 Effect of dietary nitrate supplementation on flow mediated dilatation in individuals with COPD requiring supplemental oxygen (EDEN-OX)

Of 20 participants in EDEN-OX, paired data were only available for 18 (two declined FMD assessment). FMD was assessed at baseline (zero minutes and at 180 minutes following intervention drink consumption. There was a statistically significant difference in the percentage change in FMD in the NO_3^--rich BRJ group 4.1% (-1.1, 14.8) vs a reduction in the PL group -5.0% (-10.6, -0.6); p = 0.0003 (Figure 5.7).

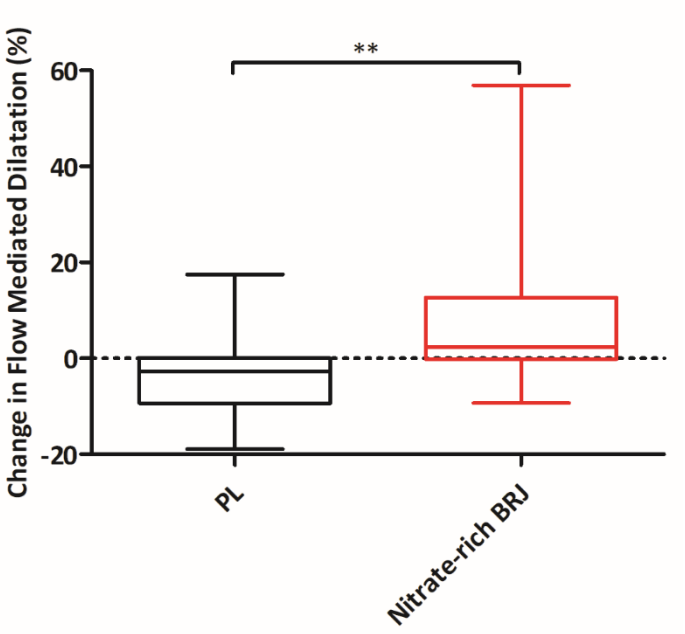


Figure 5.7. Percentage change in FMD following acute NO_3^--rich BRJ and PL dosing conditions in individuals with COPD requiring supplemental oxygen

Data presented are IQR boxes with the solid line representing the median value, and the whiskers the minimum to maximum values. Wilcoxon sign-rank test was used to compare the percentage change in FMD in the NO_3^--rich BRJ group (red) and the PL (black) dosing conditions. There was a statistically significant difference in FMD percentage change in the 4.1% (-1.1, 14.8) vs a reduction in the PL group -5.0% (-10.6, -0.6); ** p = 0.0003

Abbreviations: BRJ – Beetroot Juice; FMD – Flow Mediated Dilatation; NO_3^- - Nitrate

5.6 Discussion

The data in this Chapter confirm the bioavailability of NO following NO_3^- supplementation in the form of NO_3^--rich BRJ. This is evidenced first by confirmation that the "active" juice does indeed contain a high quantity of nitrate and that nitrate is present only at very low levels in the placebo juice. Secondly, plasma levels of nitrate and nitrate rose substantially following supplementation. Finally, NO_3^- supplementation was associated with improvements in endothelial function assessed using flow-mediated dilatation.

Acute dosing with NO_3^- in the EDEN-OX study improved endothelial function as expected. However, in ON-EPIC, supplementation of dietary NO_3^- in the form of NO_3^- -rich BRJ over a period of eight weeks in association with a programme of PR resulted in a persistent increase in FMD seven days after dosing suggests that serial dosing with NO_3^--rich BRJ confers a benefit sustained beyond the effects of acute dosing. The mechanisms for this are unclear and could represent vascular remodelling (increased NO bioavailability following NO_3^- supplementation) due to repeated nitrate dosing, or conceivably a change to the oral microbiome with increased populations of bacteria that reduce nitrate to nitrite synthesis.

The method of dietary NO_3^- supplementation in these studies was matched to a robust placebo which strengthens the findings described here. The FMD data provide mechanistic evidence that dietary NO_3^- leads to improved endothelial function and

peripheral arterial dilatation which would improve blood flow and thus oxygen delivery to skeletal muscle.

In those individuals with COPD who require supplemental oxygen we have also seen that following an acute dose if 12.9 mmol of NO_3^- there is a biological effect manifest as an increase in exercise capacity. This is likely to be due to action of the NO_3^--NO_2^--NO pathway which is enhanced in conditions of hypoxia and low pH (Lundberg and Weitzberg 2009) such as are present in the skeletal muscle of exercising individuals.

An increase in F_ENO was observed following NO_3^- dosing, suggesting that this could be used as a marker of treatment compliance. The increase in F_ENO maybe due to increased action of inducible NOS located in the airways following a NO_3^- bolus linked to the recycling of NO via the synergistic O_2-dependent and O_2-independent pathways. However, the poor association of F_ENO levels with plasma nitrate and nitrite levels suggests that this cannot be used to guide nitrate "dosing". The lack of correlation between plasma NO_3^- and F_ENO suggests that F_ENO is not a good biomarker of dietary NO_3^- dose. Despite F_ENO rising in all individuals following consumption of dietary NO_3^- there is individual variation in this increase. In the context of dietary NO_3^- supplementation F_ENO may be better used as a tool for intervention compliance as opposed to empty bottle checks and plasma NO_3^- and NO_2^- levels.

CHAPTER 6: GENERAL DISCUSSION AND FUTURE DIRECTIONS FOR RESEARCH

6.1 General discussion

NO is an essential free radical produced continuously at rest and during exercise (Balon and Nadler 1994). NO is involved in a variety of processes and functions at a tissue and cellular level in mitochondrial and cellular respiration (Brown and Cooper 1994, Shiva, Huang et al. 2007), glucose uptake in skeletal muscle (Merry, Lynch et al. 2010), skeletal muscle contraction (Reid 1998, Viner, Williams et al. 2000, Stamler and Meissner 2001, Merry, Lynch et al. 2010), glucose uptake in skeletal muscle (Merry, Lynch et al. 2010), regulation of vasodilatation (Moncada and Higgs 1993, Joyner and Tschakovsky 2003), neurotransmission (Garthwaite 2008) and fatigue development (Percival, Anderson et al. 2010). Recent research has focused on the modulation of NO bioavailability with particular attention paid to dietary NO_3^- supplements and their role in physiological responses to exercise (Jones, Thompson et al. 2018). NO_3^- supplements can be orally administered in the form of NO_3^--rich beetroot juice as a method to increase NO production. This increase in NO is reflected by the surrogate marker or elevated plasma NO_2^- and NO_3^- (Larsen, Weitzberg et al. 2007, Webb, Patel et al. 2008, Wylie, Kelly et al. 2013). Dietary NO_3^- supplementation, in healthy individuals, has been shown to improve muscle efficiency as seen in a reduction in the oxygen cost of exercise and induce an ergogenic effect. In individuals with COPD the role of dietary NO_3- supplementation is less clear cut but it has been shown to improve exercise capacity and reduce the oxygen cost of exercise (Berry, Justus et al. 2015, Curtis, O'Brien et al. 2015, Kerley, Cahill et al. 2015,

Kerley, James et al. 2019). The role for dietary NO_3^- supplementation in COPD is yet to be fully understood.

This thesis contributes to novel findings that address: 1) if dietary NO_3^- supplementation in combination with a programme of pulmonary rehabilitation improves exercise capacity; and 2) if dietary NO_3^- supplementation in hypoxic individuals with COPD improves exercise endurance.

6.1.1 Research questions addressed

The aim of this thesis was to investigate the potential for dietary NO_3^- supplementation to enhance exercise capacity and exercise endurance in individuals with COPD. We sought to answer the following questions:

1. Does dietary NO_3^- supplementation consumed prior to each exercise session as part of a programme of pulmonary rehabilitation enhance the exercise capacity gains in individuals with COPD?
2. Does dietary NO_3^- supplementation administered acutely in individuals with COPD who are hypoxic and require supplemental oxygen enhance exercise endurance?

6.1.2 The implications of dietary nitrate supplementation for exercise and training

Dietary NO_3^- supplementation appears to enhance aspects of the physiological response to exercise. Dietary NO_3^- appears to reduce blood pressure, lower the oxygen cost of exercise and in certain circumstances enhance exercise capacity. It has been shown that

up to 15 days supplementation can increase exercise performance during high-intensity constant work-rate exercise and maximal incremental exercise (Bailey, Fulford et al. 2010, Bailey, Fulford et al. 2010, Vanhatalo, Bailey et al. 2010, Lansley, Winyard et al. 2011). These outcomes are less consistent when supplementation is acute with some studies showing a positive effect (Kenjale, Ham et al. 2011, Lansley, Winyard et al. 2011) and some showing no effect (Cermak, Gibala et al. 2012, Peacock, Tjonna et al. 2012, Wilkerson, Hayward et al. 2012). The efficacy of dietary NO_3^- supplementation may well be affected by several individual factors: age, health, diet and baseline fitness or training status and factors related to the exercise bout: duration, intensity and nature of task performed. Dietary NO_3^- supplementation appears to have an ergogenic effect in exercise bouts of 5 to 30 minutes (Bailey, Winyard et al. 2009, Bailey, Fulford et al. 2010, Lansley, Winyard et al. 2011, Lansley, Winyard et al. 2011) with less evidence if the exercise bout is more than 40 minutes

6.1.3 Could dietary nitrate supplementation affect clinical practice?

The studies within this thesis have shown that in combination with PR and in hypoxic conditions dietary NO_3^- supplementation enhances exercise capacity and endurance, respectively. Dietary NO_3^- supplementation in the form of NO_3^--rich beetroot juice was well tolerated and did not result in increased adverse events. The NO_3^--rich beetroot juice used in these trials can be purchased readily in health food shops, supermarkets and online at low cost. For an eight-week programme of PR the cost of nitrate supplementation is less than £30. It is therefore not without reason that dietary NO_3^- supplementation could be used alongside exercise programmes at home or in combination with PR.

6.1.4 What long term issues need to be considered with dietary nitrate supplementation?

Dietary NO_3^- supplementation in the form of NO_3^--rich beetroot juice is well tolerated. Within the studies that comprise this thesis there were no significant adverse effects and there was no statistically significantly increase exacerbations of COPD. Universally NO_3^--rich beetroot juice resulted in beeturia. Most commonly NO_3^--rich beetroot juice caused gastrointestinal disturbance in the form of nausea.

The oral microbiome plays a pivotal role in the NO_3^--NO_2^--NO pathway (Hyde, Andrade et al. 2014). The bacteria which contribute to the oral microbiome can affect the initial reduction of NO_3^- to NO_2^- (Doel, Benjamin et al. 2005, Hyde, Andrade et al. 2014, Velmurugan, Gan et al. 2016). Burleigh *et al* assessed the influence seven days of dietary NO_3^- supplementation in the form of NO_3^--rich beetroot juice on the oral microbiome in healthy individuals and found that sustained dietary NO_3^- supplementation alters the oral environment in favour of commensal bacteria which are involved in the stepwise reduction of NO_3^- to NO_2^- (Burleigh, Liddle et al. 2019). No data is available on the long term sequalae of this altered oral microbiome nor how long it would take to revert once dietary NO_3^- supplementation is ceased.

There have previously been concerns regarding health risks associated with the ingestion of NO_3^- and NO_2^- in relation to carcinogenic potential (McKnight, Duncan et al. 1999) and methaemoglobinaemia (Comly 1987). These concerns appear to be unfounded and the evidence in humans lacking. The European Food Safety Authority recommend a single daily dose of 3.7 mg of NO_3^- .kg body weight^{-1}.day^{-1}, equivalent to 4.2 to 4.7 mmol of NO_3^-

for a 70 to 80 kg individual. This target is easily exceeded by a single portion of spinach (Hord, Tang et al. 2009). There are no long term follow up of dietary NO_3^- supplementation studies in health or disease on adverse events, so any true long term adverse events are not currently known.

6.1.5 Could the same outcomes be delivered by dietary manipulation?

The NO_3^- content provided by the 140 mL of NO_3^--rich beetroot juice (BEET IT ® SPORT shot, James White Drinks, Ipswich, UK) is the equivalent of 0.8 g or 12.9 mmol of NO_3^-. It would be feasible to attain the same NO_3^- load through consumption of approximately 400 g of dry weight green leafy vegetables. The average European diet contains approximately 4 mmol of NO_3^- (Gangolli, van den Brandt et al. 1994, Hord, Tang et al. 2009, Lidder and Webb 2013). Of ingested NO_3^- foodstuffs only 40% is absorbed and the remaining 60% being excreted (Wagner, Schultz et al. 1983, Lundberg and Govoni 2004) Dietary NO_3^- supplementation has been shown to produce its peak benefit in exercise if consumed two to three hours prior to undertaking a bout of exercise (Wylie, Ortiz de Zevallos et al. 2016). The benefit of dietary NO_3^- supplementation in the form of NO_3^--rich beetroot juice over dietary manipulation is that it is readily absorbed from the gastrointestinal tract (within one hour) and does not require any further digestion that would be required by dietary manipulation alone. When consumed with concomitant foodstuffs containing competing compounds such as thiocyanate (found in cruciferous vegetables) NO_3^- absorption can be hindered (Olea and Parras 1992). Individuals with COPD can experience dyspnoea in association with eating and therefore a 140 mL of NO_3^--rich beetroot juice may well be preferential to a 400 g dry weight of green leafy vegetables. NO_3^--rich beetroot juice is readily available to the public and is low in cost (at

the time of writing 140 mL of BEET IT ® SPORT shot costs less than £2.50), especially if purchased in bulk, which may also increase its acceptability over dietary manipulation.

6.2 Experience of research

Prior to my undertaking of this thesis I had limited experience of clinical research. The undertaking of the research studies that comprise this thesis has allowed me to gain experience and understanding of the processes of generating a research idea and subsequently generating a research protocol and undertaking the research study.

There were several aspects of my research studies that went well. The multi-centre nature of the study discussed in Chapter 3 (ON-EPIC) was a particular success. This allowed for a real-world assessment of the intervention, as opposed to a single centre assessment. The Royal Brompton and Harefield NHS Foundation Trusts' pulmonary rehabilitation programme was the first programme within the UK to be awarded accreditation by the Pulmonary Rehabilitation Services Accreditation Scheme. This accreditation is aspirational for all services and the outcomes achieved by this PR group are truly excellent. A multi-centre approach to this trial allowed for the recruitment of individuals attending different PR services, which whilst working to the ATS/ERS Pulmonary Rehabilitation guidelines are different in resources and attendee characteristics. This allowed for interpretation of the intervention in a real-world setting.

Whilst conducting this research I also became aware of the boarder issues around COPD and pulmonary rehabilitation. COPD is a highly heterogenous population and a one size all approach therefore is not beneficial. Phenotyping individuals (for example hypoxic

individuals with COPD requiring supplemental oxygen) allows for the identification of which factors are limiting exercise and thus allowing a personalised approach to both training and the use of adjunct agents like dietary NO_3^- supplementation.

6.3 Future research

The work described in this thesis thus provides clear evidence to support the idea that dietary nitrate supplementation can be beneficial for people with COPD, but a number of questions remain to be answered.

6.3.1 Questions arising from ON-EPIC

ON-EPIC: The main finding of this study was that, in people with COPD undertaking an 8-week course of PR, supplementation with nitrate-rich BRJ augmented the improvement in exercise capacity, measured as ISWT compared to an otherwise identical but nitrate-depleted placebo. Active treatment was also associated with reductions in blood pressure, greater daily step count and improved endothelial function compared to the control arm.

The main questions for further studies include:

1) Is this benefit sustained over time with a longer duration of treatment? – trials should concentrate on daily dosing outside of a programme of exercise over a longer period of time to assess response.

2) Will dietary nitrate supplementation improve these measures in the absence of the training stimulus provided by pulmonary rehabilitation? – trials should assess response outside of an exercise programme and assess response in physical activity levels

3) What is the optimum dosing strategy – intermittent, as used in this study or daily – trials should assess varied dosing regimes with a focus on daily dosing.

4) Are there specific patient phenotypes in which this approach is most helpful linked for example to disease severity or the presence of comorbidities, especially vascular ones – is there an optimal BMI where benefit may be greater.

5) Are there any biomarkers of response to supplementation such as FeNO?

6.3.2 Questions arising from EDEN-OX

EDEN-OX: The main finding of this study was that, in individuals with COPD who require ambulatory oxygen when undertaking exercise, acute supplementation with NR-BRJ increased their endurance capacity measured as ESWT compared to an otherwise identical but nitrate-deplete PL. Acute treatment was also associated with an increase in oxygen saturations and decrease in heart rate to isotime during exercise, and improved endothelial function.

This work was based on specific patient phenotype – exercise capacity improved whereas it had not in group's previous work with acute dosing in non-hypoxic COPD where the oxygen cost of exercise decreased but performance did not. This difference could have been due to a difference in physiological response or to the different exercise modality (walking vs cycling).

Questions for future research are:

1) Does the improvement in exercise capacity seen in hypoxic patients translate into improved daily physical activity (either more activity or activity accomplished with a lesser symptom burden).

2) Does the benefit seen here translate into other lung diseases associated with hypoxia such as interstitial lung disease and pulmonary hypertension.

Given the high prevalence of vascular co-morbidity in COPD the findings of blood pressure reduction and improvements in endothelial function are highly significant and warrant further investigation independent of the impact of beetroot juice on symptoms and exercise capacity.

Dietary nitrate supplementation in the form of beetroot juice is readily available and relatively cheap with the potential to scale up use quickly if benefits to patients are confirmed.

6.4 Conclusions

In summary, the information from this thesis indicates that dietary NO_3^- supplementation may be beneficial for individuals with COPD in combination with a programme of pulmonary rehabilitation and in the specific phenotype of individuals with COPD who are hypoxic and require supplemental oxygen enhancing exercise capacity and endurance.

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APPENDICIES

Appendix 1. Dietary Advice sheet

Royal Brompton & Harefield NHS
NHS Foundation Trust

Imperial College London

Dietary Advice for ON-EPIC

Dear [NAME],

Thank you for agreeing to take part in:

ON-EPIC Oral nitrate supplementation to enhance pulmonary rehabilitation in chronic obstructive pulmonary disease

You have been asked to consume **TWO bottles** of Beet It© Sport Shot (140 ml) **THREE hours** prior to attending pulmonary rehabilitation. Prior to attending for trial visits and pulmonary rehabilitation we kindly ask you adhere to the following advice:

- Please avoid nitrate-rich foods 48 hours prior to attending study visits and pulmonary rehabilitation. These include:
  - Green leafy vegetables – spinach, kale, lettuce and cabbage
  - Broccoli
  - Potato
  - Beetroot
  - Garlic
  - Dark chocolate
  - Nuts and seeds

- Avoid cooked breakfasts
- Please match your caffeine intake
- Please avoid the use of alcohol-based mouth washes
- Please avoid the chewing of chewing gum

For the study visits the researcher will advise you to avoid strenuous exercise 24 hours prior to the visit.

Yours sincerely

Dr Matt Pavitt

Clinical Research Fellow

Address: Muscle Laboratory

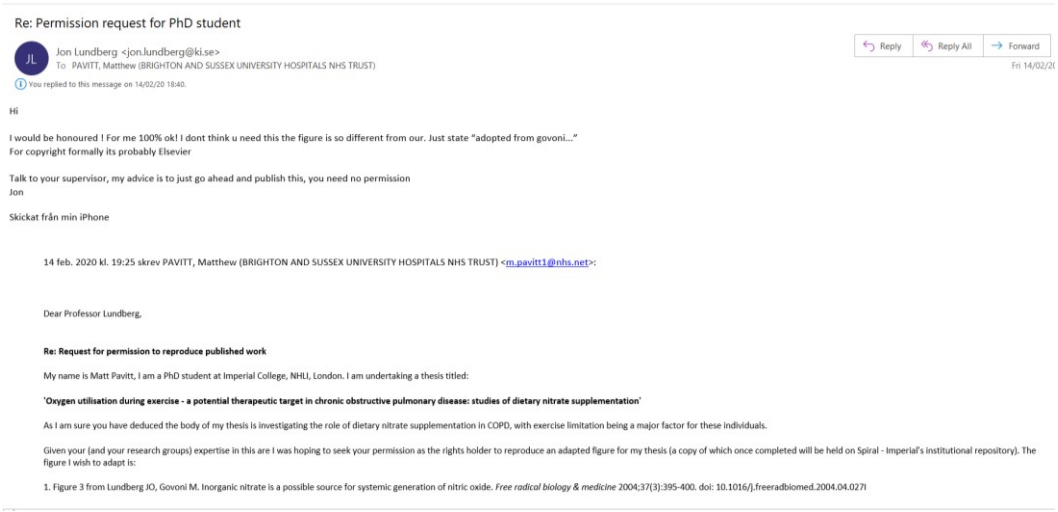
Royal Brompton Hospital

Fulham Road, London

SW3 6HP

Tel: 0207 351 8029

Appendix 2. Permissions



Re: Permission request for PhD student

Jon Lundberg <jon.lundberg@ki.se>
To PAVITT, Matthew (BRIGHTON AND SUSSEX UNIVERSITY HOSPITALS NHS TRUST)

Reply | Reply All | Forward

You replied to this message on 14/02/20 18:40.

Fri 14/02/2(

Hi

I would be honoured ! For me 100% ok! I dont think u need this the figure is so different from our. Just state "adopted from govoni..."
For copyright formally its probably Elsevier

Talk to your supervisor, my advice is to just go ahead and publish this, you need no permission
Jon

Skickat från min iPhone

14 feb. 2020 kl. 19:25 skrev PAVITT, Matthew (BRIGHTON AND SUSSEX UNIVERSITY HOSPITALS NHS TRUST) <m.pavitt1@nhs.net>:

Dear Professor Lundberg,

Re: Request for permission to reproduce published work

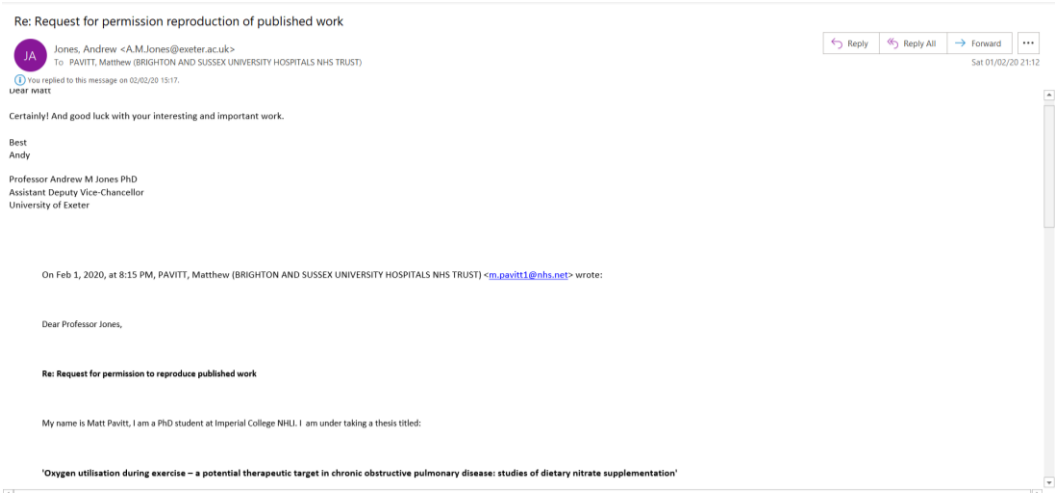
My name is Matt Pavitt, I am a PhD student at Imperial College, NHLI, London. I am undertaking a thesis titled:

'Oxygen utilisation during exercise - a potential therapeutic target in chronic obstructive pulmonary disease: studies of dietary nitrate supplementation'

As I am sure you have deduced the body of my thesis is investigating the role of dietary nitrate supplementation in COPD, with exercise limitation being a major factor for these individuals.

Given your (and your research groups) expertise in this are I was hoping to seek your permission as the rights holder to reproduce an adapted figure for my thesis (a copy of which once completed will be held on Spiral - Imperial's institutional repository). The figure I wish to adapt is:

1. Figure 3 from Lundberg JO, Govoni M. Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free radical biology & medicine* 2004;37(3):395-400. doi: 10.1016/j.freeradbiomed.2004.04.027I



Re: Request for permission reproduction of published work

Jones, Andrew <A.M.Jones@exeter.ac.uk>
To PAVITT, Matthew (BRIGHTON AND SUSSEX UNIVERSITY HOSPITALS NHS TRUST)

Reply | Reply All | Forward | ...

You replied to this message on 02/02/20 15:17.

Sat 01/02/20 21:12

Dear Matt

Certainly! And good luck with your interesting and important work.

Best
Andy

Professor Andrew M Jones PhD
Assistant Deputy Vice-Chancellor
University of Exeter

On Feb 1, 2020, at 8:15 PM, PAVITT, Matthew (BRIGHTON AND SUSSEX UNIVERSITY HOSPITALS NHS TRUST) <m.pavitt1@nhs.net> wrote:

Dear Professor Jones,

Re: Request for permission to reproduce published work

My name is Matt Pavitt, I am a PhD student at Imperial College NHLI. I am under taking a thesis titled:

'Oxygen utilisation during exercise – a potential therapeutic target in chronic obstructive pulmonary disease: studies of dietary nitrate supplementation'

