

Dietary Nitrate Supplementation and Physical Performance in Chronic Respiratory Disease

**A thesis submitted for the degree of Doctor of
Philosophy (PhD)**

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Abdullah Saleh M Al Sulayyim

Airway Disease,
National Heart and Lung Institute,
Royal Brompton Hospital,
Imperial College London

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Declaration of originality

I declare that I presented my own original work in this thesis. Where appropriate, acknowledgement of other persons contributions has been acknowledged.

Statement of contribution

The studies presented in this thesis were conducted between 2019 and 2022 during my PhD candidature at the National Heart and Lung Institute, Imperial College London. This thesis was written entirely by myself under the supervision of Professor Nicholas Hopkinson and Professor Michael Polkey.

Professors Hopkinson and Polkey developed the original idea for the research studies included in this thesis. I designed and wrote the trial protocols with the assistance of Professor Hopkinson.

In chapter three, a systematic review was conducted in collaboration with my colleague Mr Ali Alasmari. For this study, I collected the specified data (including exercise capacity, blood pressure parameters, heart rate, oxygen saturation, VO_2 , FeNO, and FMD), and then ran the meta-analysis. For the studies presented in Chapters four, and five subject trial visits, data collection, outcome assessments including exercise parameters (including six-minute walk tests, incremental shuttle walk tests, and endurance shuttle walk tests), and analyses were undertaken by myself.

Venous blood sampling for plasma nitrate, nitrite, arginine, and ADMA was undertaken by myself, as was the initial sample processing. A member of Professor Jane Mitchell's lab assisted me in running plasma nitrate and nitrite assays and measuring them using a plate reader machine. Data obtained from the plate reader ran by myself. For the platelet

aggregation test, I received the test assays from Professor Tim Warner (Queen Mary University of London). For each assay, I draw the blood sample, process it in our research lab while Professor Warner's lab performs the flow cytometry measurements. Then I run statistical analyses myself on the raw data.

All sources of information have been referenced within the text.

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Dedications

I dedicate this thesis to my parents and lovely wife, as well as my sons. Hopefully, it will make you proud.

Publications arising from this thesis

- Alsulayyim AS, Alasmari AM, Alghamdi SM, et al "Impact of dietary nitrate supplementation on exercise capacity and cardiovascular parameters in chronic respiratory disease: a systematic review and meta-analysis" BMJ Open Respiratory Research 2021;8:e000948. doi: 10.1136/bmjresp-2021-000948.

Abstract

Exercise limitation is a cardinal feature in individuals with chronic respiratory disease (CRDs). This limitation occurs directly due to impairment and disability of one or more of the physiological systems (pulmonary system, cardiovascular system, and skeletal muscle), which contribute to breathlessness and reduced physical activity. Dietary nitrate supplementation can enhance NO availability via a nitrate-nitrite-NO pathway, and has been proposed as a potential complementary approach to improve exercise capacity in people with CRDs. However, studies to date have generally been short-term and existing studies on dietary nitrate supplementation in people with lung disease have mostly focused on COPD, typically in less severe disease. The objectives of this thesis were as follows: 1) to further extend the knowledge and synthesise the current evidence and evaluate the impact of dietary NO_3^- supplementation on exercise capacity and cardiovascular parameters in CRDs (**Chapter 3**), 2) to investigate the effects of acute NO_3^- administration on exercise performance, blood pressure, and endothelial function in hypoxic patients with pulmonary hypertension (**Chapter 4**), and 3) to investigate if there is a link between blood nitrate levels and the severity of lung disease (**Chapter 5**). Results from Chapter 3 indicated that dietary nitrate supplementation has the potential to reduce cardiovascular risk by lowering blood pressure in people with COPD as well as improving exercise capacity. In Chapter 4, following acute consumption of nitrate-rich beetroot juice (NR-BRJ), study participants walked for longer than following dosing with placebo. In Chapter 5, plasma nitrate levels negatively correlated with blood pressure parameters and positively correlated with the endothelial function and serum potassium level in stable COPD patients. However, no correlations were found between the plasma nitrate and breathlessness or any other marker of disease severity. The findings

suggest that dietary nitrate supplementation might be used as a non-pharmacological intervention to augment exercise in people with CRDs and protect against vascular events. Larger and longer trials are required to determine the optimal dose and to understand the possible mechanisms of dietary nitrate supplementation in pulmonary circulation.

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CHAPTER 1: Introduction

1.1 Overview of Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a heterogenous disease that occurs due to chronic inflammatory processes and pathological changes in the small airways (e.g., obstructive bronchitis) and destruction of lung parenchyma (emphysema) (Figure 1.1). It is characterised by persistent respiratory symptoms and airflow limitation caused by inhalation of noxious particles such as cigarette smoke or gases. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines COPD as the following:

“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”(Global Initiative for Chronic Obstructive Lung Disease, 2021).

Approximately 400 million individuals have been diagnosed with COPD worldwide (Adeloye et al., 2015). COPD is the third leading cause of mortality in the world (Lozano et al., 2012). The World Health Organization (WHO) estimates that more than three million people die each year from obstructive lung diseases, which is equivalent to 6% of all deaths worldwide (World Health Organization, 2017, December, 1). In the United Kingdom (UK), an estimated 1.2 million people are diagnosed with COPD, making COPD the second most common lung disease in the UK (British Lung Foundation, 2016).

The airflow limitation is a result of chronic inflammation including narrowing of the small airways (obliterative bronchiolitis) and parenchymal destruction (emphysema) which impairs the elastic lung recoil (O'Donnell and Laveneziana, 2006).

Developing COPD occurs due to a complex interaction between genetic and environmental factors. In reviewing the genetics of COPD, Michael Cho and colleagues state that the

heritability of COPD is estimated at 40–50% (Cho et al., 2022). Regarding the environmental factors, cigarette smoking is the most common contributing risk factor for COPD (Forey et al., 2011). Other environmental factors include outdoor air pollution, occupational exposure to fumes and dust, biomass smoke inhalation, exposure to second-hand smoke, low socioeconomic status, early life lung growth impairment, and previous tuberculosis (Eisner et al., 2010, Yang et al., 2022).

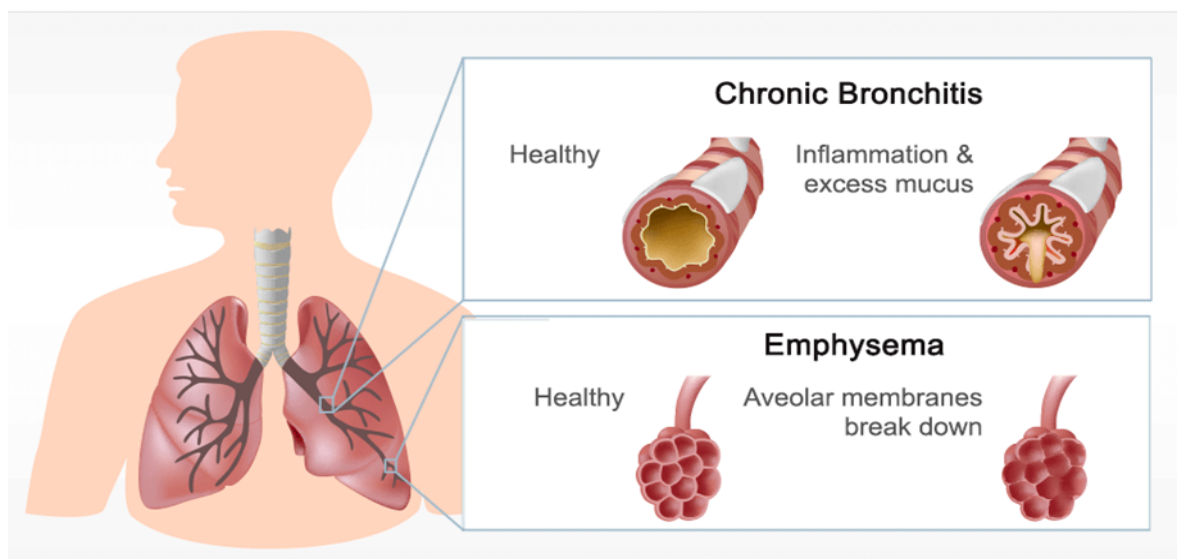


Figure 1.1: Airway with chronic bronchitis versus emphysema (Chronic Obstructive Pulmonary Disease (COPD), 2021, October 15).

A COPD diagnosis should be considered in individuals with persistent cough, sputum production, dyspnoea or a history of exposure to risk factors for the disease. For the diagnosis of COPD, spirometry with the presence of post-bronchodilator ratio of the forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC), less than 70% ($FEV_1/FVC < 0.7$) is necessary, which indicates the presence of airflow obstruction (Global Initiative for Chronic Obstructive Lung Disease, 2021). The severity of the airflow obstruction is determined by the reduction in percentage predicted of FEV_1 and is commonly stated as the GOLD stage (Table1.1).

Table 1.1 Global Initiative of Chronic Obstructive Lung Disease (GOLD) classification of airflow limitation severity.

Patients with $FEV_1/FVC < 0.70$		
GOLD 1	Mild	$FEV_1 \geq 80\%$ predicted
GOLD 2	Moderate	$50\% \leq FEV_1 < 80\%$ predicted
GOLD 3	Severe	$30\% \leq FEV_1 < 50\%$ predicted
GOLD 4	Very Severe	$FEV_1 < 30\%$ predicted

Abbreviations: GOLD: Global Initiative for Chronic Obstructive Lung Disease; FEV_1 : Forced Expiratory Volume in one second; FVC: Forced Vital Capacity.

Patients with COPD frequently present with other diseases (comorbidities) such as lung cancer, anxiety/depression, heart failure, ischemic heart disease, metabolic syndrome, diabetes, osteoporosis, gastroesophageal reflux disease, and pulmonary hypertension, which have a significant impact on disease prognosis and patient outcomes. However, cardiovascular disease is the most prevalent comorbidity and is considered the leading cause of mortality in individuals with COPD.

1.2 Pulmonary hypertension

1.2.1 Definition and classification of pulmonary hypertension

Pulmonary hypertension (PH) is defined as a mean pulmonary arterial pressure (mPAP) greater than 20 mmHg at rest that is measured during right heart catheterisation (Humbert et al., 2022). According to the guidelines for the European Society of Cardiology and the

European Respiratory Society (2022 ESC/ERS), the clinical classification of PH is categorised into five groups based on pathophysiological mechanisms, clinical presentation, haemodynamic characteristics and therapeutic management. These groups are group 1, pulmonary arterial hypertension (PAH); group 2, PH due to left heart disease; group 3, PH due to lung disease and/or hypoxia; group 4, PH due to pulmonary artery obstructions; and group 5, PH with unclear and/or multifactorial mechanisms (Table 1.2).

Table 1.2 Updated clinical classification of pulmonary hypertension.

GROUP 1 Pulmonary arterial hypertension (PAH)
1.1 Idiopathic
1.1.1 Non-responders at vasoreactivity testing
1.1.2 Acute responders at vasoreactivity testing
1.2 Heritable ^a
1.3 Associated with drugs and toxins ^a
1.4 Associated with:
1.4.1 Connective tissue disease
1.4.2 HIV infection
1.4.3 Portal hypertension
1.4.4 congenital heart disease
1.4.5 Schistosomiasis
1.5 PAH with features of venous/capillary (PVOD/PCH) involvement
1.6 Persistent PH of the newborn
GROUP 2 PH associated with left heart disease
2.1 Heart failure:
2.1.1 with preserved ejection fraction
2.1.2 with reduced or mildly reduced ejection fraction ^b
2.2 Valvular heart disease
2.3 Congenital/acquired cardiovascular conditions leading to post-capillary PH
GROUP 3 PH associated with lung diseases and/or hypoxia
3.1 Obstructive lung disease or emphysema
3.2 Restrictive lung disease
3.3 lung disease with mixed restrictive/obstructive pattern
3.4 Hypoventilation syndromes
3.5 Hypoxia without lung disease (e.g. high altitude)
3.6 Developmental lung disorders
GROUP 4 PH associated with pulmonary artery obstructions
4.1 Chronic thrombo-embolic PH
4.2 Other pulmonary artery obstructions ^c
GROUP 5 PH with unclear and/or multifactorial mechanisms
5.1 Haematological disorders ^d
5.2 Systemic disorders ^e
5.3 Metabolic disorders ^f
5.4 Chronic renal failure with or without haemodialysis
5.5 Pulmonary tumour thrombotic microangiopathy
5.6 Fibrosing mediastinitis
HF, heart failure; HIV, human immunodeficiency virus; PAH, pulmonary arterial hypertension; PCH, pulmonary capillary haemangiomatosis; PH, pulmonary hypertension; PVOD, pulmonary veno-occlusive disease. ^a Patients with heritable PAH or PAH associated with drugs and toxins might be acute responders. ^b Left ventricular ejection fraction for HF with reduced ejection fraction: ≤40%; for HF with mildly reduced ejection fraction: 41–49%. ^c Other causes of pulmonary artery obstructions include: sarcomas (high or intermediate grade or angiosarcoma), other malignant tumours (e.g. renal carcinoma, uterine carcinoma, germ-cell tumours of the testis), non-malignant tumours (e.g. uterine leiomyoma), arteritis without connective tissue disease, congenital pulmonary arterial stenoses, and hydatidosis. ^d Including inherited and acquired chronic haemolytic anaemia and chronic myeloproliferative disorders. ^e Including sarcoidosis, pulmonary Langerhans's cell histiocytosis, and neurofibromatosis type 1. ^f Including glycogen storage diseases and Gaucher disease (Humbert et al., 2022).

1.2.2 Epidemiology of PH in COPD

Individuals older than 65 years are more likely to develop PH because of the presence of underlying cardiac and pulmonary diseases (Hoeper et al., 2016). It is difficult to know the prevalence of PH in COPD patients due to the lack of valid data during the right heart catheterisation, variations in definition and diagnostic criteria, as well as the lack of large epidemiologic studies in this population. However, the reported prevalence of PH in moderate to severe COPD varies from 10.2% to 91% (Wrobel et al., 2012, Divo et al., 2012).

1.2.3 Pathology and pathophysiology

The development of PH in COPD is due to structural and/or functional factors (Table 1.3). Structural changes such as remodeling, decreased total number of pulmonary vessels, and pulmonary thrombosis cause a fixed rise of PAP while functional changes including acidemia, dynamic pulmonary hyperinflation, endothelial dysfunction, polycythemia, inflammation, and parenchymal destruction contribute to having more dynamic effects (Wrobel et al., 2012). The pathogenesis underlying this is associated with hypoxia, which causes pulmonary vasoconstriction and vascular remodelling, resulting in intimal thickening and muscularisation of arterioles, which also increases pulmonary vascular resistance (PVR), and resulting in exercise limitation (Chaouat et al., 2008).

Table 1.3 Structural and functional PH in COPD (Wrobel et al., 2012).

Structural/fixed PH	Functional/dynamic PH
Loss of pulmonary vascular bed	Hypoxia
Pulmonary vascular remodelling	Acidemia
Inflammation	Pulmonary hyperinflation
Thromboembolic disease	Airway resistance
	Endothelial dysfunction
	Polycythemia

1.3 Exercise limiting factors in COPD

Exercise limitation is a cardinal feature in individuals with COPD. This limitation occurs directly due to impairment and disability of one or more of the physiological systems (pulmonary system, cardiovascular system, and skeletal muscle), which contribute to the onset of dyspnoea. In COPD patients, dyspnoea is a primary cause that prevents these individuals from exercising and being physically inactive. This dyspnoea is due to a mismatch between central neural drive and the respiratory mechanical/muscular response (neuromechanical uncoupling). The mechanistic cause of this dyspnoea is multifactorial (Figure 1.2) including ventilatory constraints, an imbalance between oxygen supply and demand, and peripheral skeletal muscle dysfunction.

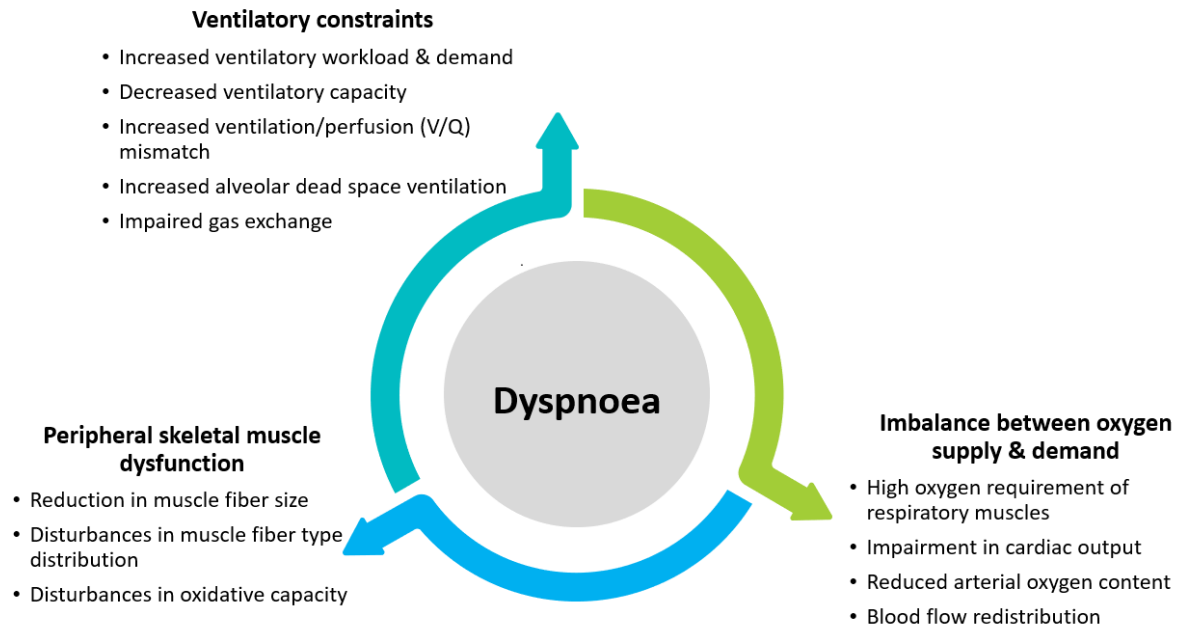


Figure 1.2: Multifactorial causes of dyspnoea and exercise limitation in COPD.

1.3.1 Ventilatory constraints

In individuals with COPD, structural changes in lung parenchyma cause changes in lung mechanics that contribute to reduced ventilatory capacity and increased ventilatory workload and demand. The presence of expiratory flow limitation, which is the hallmark and pathophysiological characteristic of COPD, results from the combination effects of decreased elastic lung recoil (emphysema) and increased airway narrowing (chronic bronchitis) causing an increase in both airway resistance and end-expiratory lung volume (EELV) during exercise, which leads to dynamic hyperinflation (DH) (O'donnell et al., 2001, O'Donnell et al., 2017). The development of DH has several consequences for the respiratory system, including a decrease in lung compliance, which increases the elastic loading of the inspiratory muscles, flattening and shortening the muscle fibers in the diaphragm and in inspiratory muscles, which affects the efficiency and ability to generate pressure during inspiration (O'Donnell and Laveneziana, 2006), and the development of intrinsic positive end expiratory pressure (PEEP), which must be overcome in order to initiate inspiration (Vogiatzis and Zakynthinos, 2012).

During exercise, individuals with COPD have an increased ventilatory demand and a significant loss in their ability to increase ventilation. As a result, those patients tend to have a rapid and shallow breathing pattern at rest and during exercise, which results in a reduction in expiratory time and the development of DH. This increase in ventilatory demand is mostly caused by increased ventilation/perfusion (V/Q) mismatch, which results in increased alveolar dead space ventilation, impaired gas exchange, and alveolar hypoxia and hypoxaemia (Panagiotou et al., 2013).

1.3.2 Imbalance between oxygen supply and demand

Due to the pathophysiological changes in COPD, the oxygen cost of breathing increases during exercise (9.3 mL O₂/L of ventilation) compared to healthy individuals (4.2 mL O₂/L of ventilation) (Levison and Cherniack, 1968, Aaron et al., 1992). To overcome the increased elastic and resistive loads, COPD patients develop not only DH but also a large expiratory intrathoracic pressure (ITP), which both contribute to several haemodynamic effects. A high ITP decreases systemic venous return and cardiac output, which results in a reduction in right ventricular (RV) preload. Moreover, ITP and high intrinsic positive airway pressure cause a compression of the vascular bed, resulting in an increase in pulmonary vascular resistance (PVR), which in turn increases RV afterload and subsequently decreases left ventricular (LV) preload (Pinsky, 2005). COPD is also associated with haemodynamic changes, including increased cardiac transmural pressure and left shift of the interventricular septum, leading to LV diastolic dysfunction (Vizza et al., 1998, Morrison et al., 1987).

Reduced cardiac output leads to reduced mixed venous oxygen saturation, which exacerbates the effect of V/Q mismatch. Commonly, individuals with COPD present with hypoxemia and the presence of this hypoxemia is due to a physiological shunt, which is defined as low V/Q ratios of lung areas, and high physiological dead space, defined as high V/Q ratios of lung areas (West and Luks, 2021). Despite the importance of this hypoxaemia as a factor limiting exercise tolerance, it has been exemplified by the fact that the administration of supplemental oxygen reduces exercise-induced arterial hypoxemia and improves exercise capacity (Suntharalingam et al., 2017, Jarosch et al., 2017).

1.3.3 Peripheral skeletal muscle dysfunction

Skeletal muscle dysfunction is commonly associated with COPD, and it's because of inactivity and disuse affecting the lower limbs (Man et al., 2005). Approximately 30% of COPD patients display quadriceps weakness and atrophy (Shrikrishna et al., 2012). Evidence shows that skeletal muscle weakness as well as a reduction in quadriceps muscle bulk can be identified in early disease (Shrikrishna et al., 2012). Additionally, quadriceps weakness has been confirmed to predict mortality and to be associated with reduced survival in COPD (Swallow et al., 2007). Also, several studies have confirmed a shift in skeletal muscle fibre type towards fast twitch fibres and a reduced number of oxidative type I muscle fibres in the quadriceps muscle (Swallow et al., 2007, Natanek et al., 2013b, Natanek et al., 2013a, Maddocks et al., 2014).

1.4 Nitric oxide (NO), overview

Nitric oxide (NO) is a vital physiological mediator in the body. It is the most critical endothelium-derived substance that works as a vasodilator on smooth muscles (Davis et al., 2001). NO is also recognised as a key regulator in the endothelium oxidative stress resulting from reactive oxygen species (ROS) and inflammation (Sandoo et al., 2010). As a result of its high reactivity, NO has a short half-life, ranging from 2 milliseconds (Liu et al., 1998) to seconds (Kelm, 1999, Crawford et al., 2006). In the human body, NO is produced in two different ways: by an endogenous pathway (oxygen-dependent) via the nitric oxide synthase (NOS) system, and by an exogenous pathway (oxygen-independent) NO_3^- - NO_2^- -NO pathway (Figure 1.3).

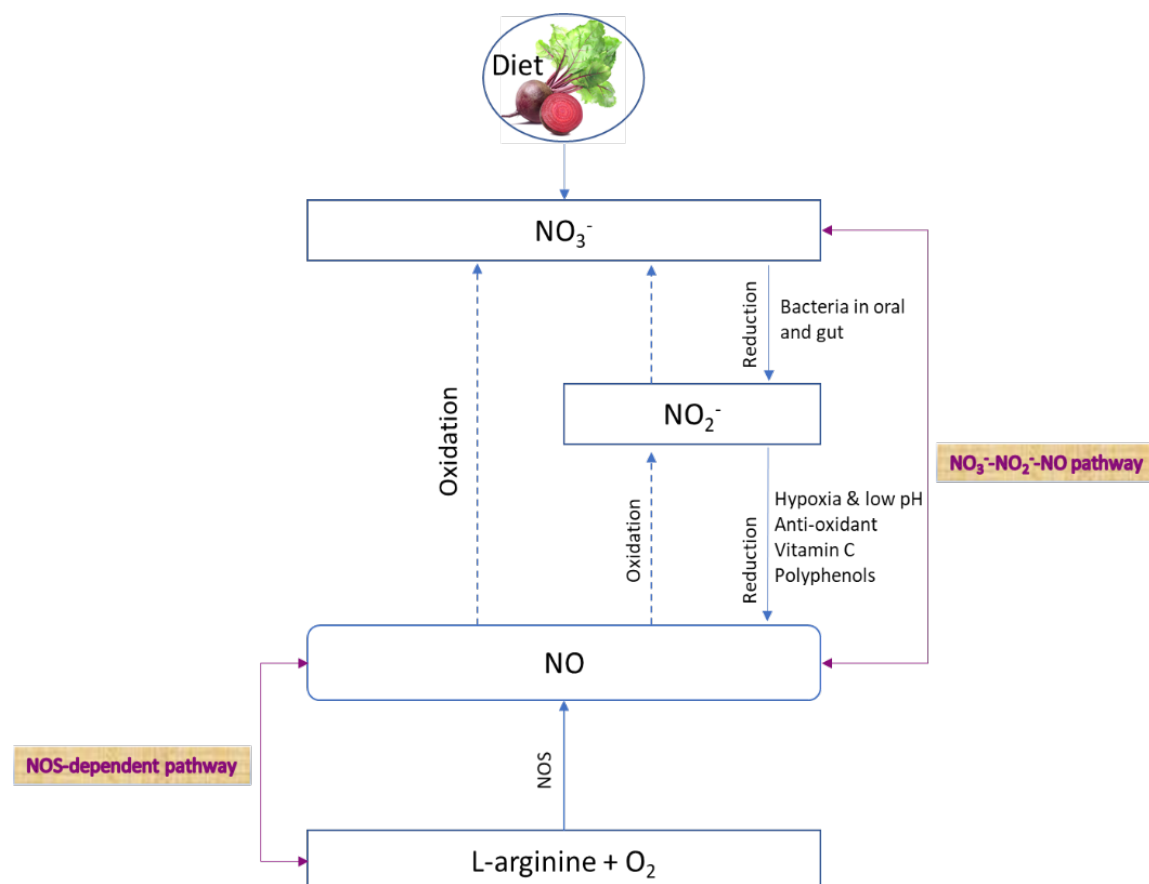


Figure 1.3: The pathways of nitric oxide (NO) production. Above: NO_3^- - NO_2^- -NO pathway. Down: The NOS-dependant pathway. Redrawn from Jones *et al.* 2014 (Jones, 2014).

Abbreviations: O₂: oxygen; NOS: Nitric oxide synthase; NO: Nitric oxide; NO_2^- : Nitrite; NO_3^- : Nitrate.

1.4.1 The NOS-dependent pathway

The enzyme NOS stimulates NO production by converting L-arginine to NO. This pathway was first discovered in the late 1980's by Moncada *et al* (Moncada et al., 1989). NOS exists in three distinct isoforms; the macrophage or inducible isoform (iNOS), the neuronal isoform (nNOS), and the endothelial isoform (eNOS), which produces nitric oxide in the vasculature (Moncada and Higgs, 1993, Alderton et al., 2001). The NOS-dependent reaction between L-arginine and O_2 is complex and dependent on a variety of cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4) haem, and calmodulin, and any limitations in the bioavailability of these cofactors would compromise NO production (Alderton et al., 2001). Immediately after being synthesised, NO is oxidised to NO_3^- via oxyhaemoglobin, and to NO_2^- in the plasma via a reaction catalysed by the plasma protein ceruloplasmin (Dejam et al., 2005, Shiva et al., 2006). The available literature has shown the impairment of NO production from NOS-dependent pathway is associated with various diseases such as COPD (Dinh-Xuan et al., 1991), cerebral vascular disease (Förstermann, 2010), sarcopenia (Hall et al., 2011) and type 2 diabetes mellitus (Lin et al., 2002) and this would imply the importance of NO production.

1.4.2 NO_3^- - NO_2^- - NO pathway

The inorganic anions (NO_3^- & NO_2^-) were previously considered as inert end-products of NO metabolism (Moncada and Higgs, 1993). However, in 1994, an alternative pathway was discovered involving the serial reduction of NO_3^- to NO_2^- and then to NO and other nitrogen oxides (Figure.1.3) (Benjamin et al., 1994, Lundberg et al., 1994). This alternative pathway might complement the NOS-dependent pathway specifically in hypoxic conditions in which

the NOS-dependent production of NO is reduced (Bryan, 2006, Lundberg and Govoni, 2004, Lundberg and Weitzberg, 2009, Van Faassen et al., 2009). Furthermore, this pathway not only uses endogenously produced NO_3^- , but can also be increased by exogenous inorganic NO_3^- from the diet, mainly through the consumption of NO_3^- rich foods such as green leafy vegetables and beetroot. In this pathway, oral commensal bacteria play a key role as it's considered essential for the first step in the nitrate-nitrite-NO pathway and is responsible for the enzymatic reduction of the higher nitrogen oxide nitrate to nitrite (Lundberg et al., 2004).

1.4.2.1 Entero-salivary circulation

Following ingestion, inorganic NO_3^- is completely absorbed from the upper gastrointestinal tract, which takes about 60 minutes to reach the systemic circulation, where it mixes with NO_3^- that derives from the oxidation of NOS-dependent pathway (Lundberg et al., 2008). NO_3^- anion is relatively stable with a half-life of 5-8 hours (Wagner et al., 1983). Approximately 60% of ingested inorganic NO_3^- is excreted in the urine (Lundberg and Govoni, 2004, Wagner et al., 1983), while the remainder (~25%) circulates in the plasma and is actively absorbed by the salivary glands via transporter protein (sialin), where it is concentrated up to 20 fold in saliva (Lundberg and Govoni, 2004, Petersson et al., 2015, Qin et al., 2012, Spiegelhalder et al., 1976).

In the oral cavity, commensal facultative anaerobic bacteria residing on the dorsal surface of the tongue utilise reductase enzymes to reduce ~20% of salivary NO_3^- to NO_2^- . These bacteria utilise nitrate as an alternative electron acceptor to produce adenosine triphosphate (ATP) in the absence of oxygen (Duncan et al., 1995). Human studies have demonstrated the importance of these bacteria by finding that commercial antibacterial mouthwash eliminated

the effect of dietary nitrate loading on blood pressure. In this way, the mouthwash killed the commensal facultative bacteria in the mouth, preventing the production of nitrites and NO (Bondonno et al., 2015, Duncan et al., 1995, Govoni et al., 2008). The plasma NO_2^- has approximately 1-5 minutes half-life (Lundberg et al., 2008). In response to dietary ingestion of NO_3^- , peak plasma concentrations of nitrate and nitrite are reached after 1-2 hours and 2-3 hours, respectively (Wylie et al., 2013). This delayed peak in plasma NO_2^- compared with plasma NO_3^- indicates the importance of the entero-salivary circulation, particularly the oral microbiome and its vital role in the reduction process of NO_3^- to NO_2^- .

1.4.2.2 NO synthesis from NO_2^-

Lastly, one-step reduction in the blood and tissues at the end of the NO_3^- - NO_2^- -NO pathway generates NO from NO_2^- . This reaction is catalysed by various NO_2^- reductases including aldehyde oxidase (Li et al., 2008), cytochrome P-450 (Kozlov et al., 2003), xanthine oxidase (Zhang et al., 1997), deoxyhaemoglobin (Cosby et al., 2003), deoxymyoglobin (Rassaf et al., 2007a, Shiva et al., 2007), and mitochondrial respiration complexes (Kozlov et al., 1999). A crucial point to consider is that oxygen availability differs for different tissues, and organs indicating regional differences in oxygen tension (Carreau et al., 2011, Hirai et al., 2018). As oxygen is limited, NO production from the NOS-dependent pathway may be hindered or impeded (Giraldez et al., 1997, Lundberg et al., 2008, Østergaard et al., 2007). Conversely, the NO_3^- - NO_2^- -NO pathway does not rely on the presence of oxygen and is enhanced by conditions of hypoxia (Castello et al., 2006, Millar et al., 1998) and low pH (Modin et al., 2001), both of which may be present in exercising skeletal muscle, together with an increased level of deoxymyoglobin.

1.4.2.3 The oral microbiome and NO bioavailability

As mentioned earlier, the oral microbiome plays a crucial role as a source of NO in the NO_3^- - NO_2^- -NO pathway (Hyde et al., 2014). In the oral cavity, the type and quantity of bacteria impact the initial reduction from NO_3^- to NO_2^- (Doel et al., 2005, Hyde et al., 2014, Velmurugan et al., 2016). Additionally, using antibacterial mouthwash (Govoni et al., 2008, McDonagh et al., 2015, Woessner et al., 2016) and spitting (Webb et al., 2008) lowers the increase in plasma NO_2^- following oral NO_3^- ingestion. Furthermore, foodstuffs containing competing components such as thiocyanate might impede the initial NO_3^- absorption in the gastrointestinal system (Olea and Parras, 1992).

1.5 Dietary nitrate sources, consumption, and health concerns of nitrate ingestion

Green leafy vegetables are the primary source of dietary NO_3^- in the human diet and account for approximately 85% of daily NO_3^- consumption (Gangolli et al., 1994). Other sources of NO_3^- include drinking water (15-20%) and animal products (10-15%), to which it is added as preservatives. Vegetables including rocket, spinach, kale, lettuce, and beetroot contain over 250 mg or 4 mmol of nitrate per 100 g of fresh weight (Gangolli et al., 1994, Hord et al., 2009, Lidder and Webb, 2013).

Personal nitrate and nitrite consumption varies greatly, and the mean intake estimates from population studies vary among nations and even regions. According to the estimates, the average daily intake of nitrate in the United States is 40–100 mg/day, while the average intake in Europe is 31–185 mg/day, with higher intakes in Mediterranean countries and lower

intakes in Northern Europe (Hord et al., 2009). This variation in NO_3^- intake is significantly dependent on several factors including growing conditions (geography and season), the types of vegetables consumed, fertilizer usage, the postharvest time, and storage and transportation conditions. (Kyriacou et al., 2019, Hord et al., 2009). Unlike NO_3^- , the concentration of NO_2^- in food is very low. The highest concentrations of NO_2^- are found in processed and cured meats (0-0.89 mg per 100 g) as a preservative, preventing *Clostridium botulinus* growth and improving taste and appearance (Hord et al., 2009). The NO_2^- content of vegetables is lower than NO_3^- , with an average concentration of less than 10 mg per kg and a maximal concentration of not exceeding 100 mg per kg (Hord et al., 2009, Pennington, 1998). Because NO_2^- is relatively low in foods, the average intake of NO_2^- is lower than that of NO_3^- (Pennington 1998), with intake estimates ranging from 0 to 20 mg (Pennington, 1998).

Health concerns have been raised several times throughout history regarding the intake of NO_3^- and NO_2^- due to carcinogenic potential (McKnight et al., 1999) and methaemoglobinemia (Comly, 1945). In 1945, Comly *et al.* first reported cases of infant methemoglobinemia ('blue baby syndrome') linked to well-water that had high nitrate (140 parts per million, equivalent to 0.619 gm of nitrate to the litre)(Comly, 1945) where methemoglobinemia is a condition resulting from the NO_2^- mediated oxidation to ferric iron (Fe^{3+}) in oxyhaemoglobin, which causes the haemoglobin molecule to be unable to bind oxygen, potentially causing hypoxia (Fan and Steinberg, 1996, McKnight et al., 1999). In the 1970s, more concern about dietary nitrate appeared when Spiegelhalter *et al.* (1976) in Germany and Tannenbaum *et al.* (1976) in the USA revealed that dietary nitrates could theoretically be converted into N-nitrosamines by the reaction of N_2O_3 (derived from the

acidification of salivary nitrites) with secondary amines in the diet (Spiegelhalder et al., 1976, Tannenbaum et al., 1976). However, in 1999, Avery *et al.* contended the early reports of methemoglobinemia following well-water ingestion in babies (Comly, 1945) were probably the result of bacteria contaminating the water, which reduces nitrate to nitrite (Avery, 1999). There was however a lack of epidemiological studies support for most of these studies and in 2003 the Joint FAO/WHO Expert Committee on Food Additives (JECFA) stated that epidemiological data indicated no consistently increased risk for cancer associated with increasing consumption of nitrates in humans (Speijers and Brandt, 2003).

1.6 Applications and forms of nitrate supplementation

Several approaches exist for NO_3^- supplementation. NO_3^- salts such as sodium NO_3^- (NaNO_3) and potassium NO_3^- (KNO_3), have been used in numerous research studies as solutions or capsules (Bescós et al., 2011, Kapil et al., 2010, Larsen et al., 2010). Researchers have also used BRJ, which is rich in NO_3^- and contains other bioactive compounds, which may contribute to beneficial effects in mammals. For example, it has been suggested that betaine can enhance muscular strength, power, and endurance (Hoffman et al., 2011), and is used for the treatment of cardiovascular disease (Borsook and Borsook, 1951, Van Zandt, 1951). However, a systematic review conducted in 2017 found no evidence for the impact of betaine supplementation on muscle strength and power performance (Ismaeel, 2017). Other bioactive components in BRJ include polyphenols, quercetin and resveratrol, all of which may contribute to mitochondrial biogenesis and increase aerobic capacity (Davis et al., 2009, Ganio et al., 2010, Lagouge et al., 2006). Because these bioactive compounds could interfere with interpretation of nitrate effects during clinical studies, a BRJ that is depleted of NO_3^- by

passage through an ion exchange resin column was developed to be identical in colour, taste, smell, and texture (Kelly et al., 2014, Lansley et al., 2011). In this way, the effects of nitrate itself can be analysed and identified from the potential effects of other bioactive ingredients in BRJ. Though this may in turn underestimate the beneficial effects of BRJ.

1.7 Therapeutic effects of dietary nitrate supplementation

1.7.1 Cardiovascular

Pharmaceutical drugs that act on NO signalling are well demonstrated, and they may either increase bioavailability or reduce NO breakdown in the body. For example, glyceryl trinitrate, which causes non-specific arterial and venous dilatation by free radical NO release, activates guanylate cyclase and increases production of guanosine 3' and 5'monophosphate, phosphorylating proteins in smooth muscle. Another example is cyclic guanosine monophosphate (cGMP) specific phosphodiesterase type 5 inhibitors (e.g., Sildenafil), which specifically inhibit the activity of the enzyme in the smooth muscle cells of vessels, preventing degradation of cGMP, leading to pulmonary artery vasodilation as well as inhibiting the remodeling of the pulmonary vasculature (Ahmed et al., 2021, Mónica and De Nucci, 2019).

In terms of dietary NO_3^- supplementation, a diet rich in fruits and green leafy vegetables has been shown to lower the risk of morbidity and mortality from cardiovascular disease (Joshipura et al., 2001), possibly due to the lower blood pressure associated with the high nitrate content in this type of diet (Appel et al., 1997). The existing research suggests that both acute (Kapil et al., 2010, Vanhatalo et al., 2010, Webb et al., 2008) and short term (less

than 3 months) (Bailey et al., 2010, Bailey et al., 2009a, Larsen et al., 2007, Larsen et al., 2010, Vanhatalo et al., 2010) dietary nitrate supplementations can effectively reduce resting BP. In 2008, Webb *et al.* reported that acute dose of BRJ (500 ml, 22.5 mmol of nitrate) significantly decreased systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) over 24 hrs with peak reductions of 10.4 ± 3.0 mmHg ($p < 0.01$) 2.5 hrs following consumption, 8.1 ± 2.1 mmHg ($p < 0.01$) 3 hrs following consumption, and 8.0 ± 2.1 mmHg ($p < 0.01$) 3 hrs following consumption, respectively (Webb et al., 2008). These effects were correlated with peak rises in plasma nitrite level and were abolished by interrupting the entero-salivary circulation by asking the participants to spit and not swallowing their saliva (Webb et al., 2008). In a subsequent trial, the same group used three different doses (4, 13, and 24 mmol) of potassium NO_3^- and found dose-dependent increases in plasma nitrite concentration and reductions in SBP and DBP by 9 mmHg and 6 mmHg, respectively (Kapil et al., 2010). The changes in BP returned to baseline after 24 hours (Kapil et al., 2010, Webb et al., 2008) and these changes in BP using different forms and doses of dietary nitrate supplementation demonstrate the importance of using NO_3^- as an effective strategy to improve vascular health and BP. These results have been replicated after short term supplementation. In 2006, Larsen *et al.* conducted a randomised, double-blind, crossover study with 17 healthy volunteers to investigate the impact of dietary nitrate supplementation on BP. They found that DBP following three days of sodium NO_3^- supplementation with a dose of ($0.1 \text{ mmol.kg}^{-1} \cdot \text{day}^{-1}$) was reduced by 3.7 mmHg ($p < 0.02$). In Kapil and colleagues' study, patients with hypertension were randomly allocated to receive 250 ml of BRJ or placebo (nitrate-depleted BRJ) per day for four weeks. They found that BP measured by three different methods was significantly reduced following dietary nitrate supplementation: mean clinic BP ($7.7/2.4$ mm Hg ($3.6-11.8/0.0-4.9$), $P < 0.001$, $P = 0.05$), mean 24-hour ambulatory BP ($7.7/5.2$

mm Hg (4.1-11.2/2.7-7.7), $P < 0.001$ for both, and mean home BP (8.1/3.8 mm Hg (3.8-12.4/0.7-6), $P < 0.001$, $P < 0.01$ (Kapil et al., 2015).

At this time, as far as I know, Pavitt *et al* (2020). conducted the longest intervention study, which was an eight-week intervention trial. In this study, 165 individuals with COPD were recruited to receive 140 ml of BRJ or nitrate-depleted BRJ for eight weeks during pulmonary rehabilitation (Pavitt et al., 2020). Results showed significant reduction in SBP 5mmHg; $P < 0.0005$, DBP 5 mmHg; $P < 0.0005$, and MAP 5mmHg; $P < 0.0005$ (Pavitt et al., 2020).

Importantly, these effects of dietary NO_3^- on lowering blood pressure appear to be connected to its conversion to NO_2 and ultimately to NO , which is considered a significant endothelium relaxing factor that causes smooth muscle relaxation and vasodilation (Gruetter et al., 1979).

1.7.2 Endothelial function

The endothelium is a vital component of the vasculature because it contributes to an atheroprotective environment through the action of endothelial cell-derived vasoactive factors. NO plays an important signalling role in vascular homeostasis (Furchgott and Zawadzki, 1980), and its deficiency results in endothelial dysfunction (ED). ED is a term that refers to functional alterations in vascular homeostasis (Lind et al., 2011). It develops when the endothelium is unable to function normally and commonly associated with atherosclerotic diseases, including coronary artery disease, occlusive stroke, and peripheral artery disease (Heiss et al., 2006). In the presence of ED, the vascular wall stimulates smooth muscle proliferation, inflammation, and the build-up of lipid-rich material (Münzel et al.,

2008). Additionally, it promotes platelet activation, coagulation, thrombus formation, and lipoprotein oxidation (Münzel et al., 2008). ED has a complex pathophysiology that includes multiple mechanisms. The common mechanisms mediating ED are summarised as follows: a) decreased NO bioavailability; b) increased reactive oxygen species (ROS) formation; c) endothelium failure to mediate vasodilation; c) excessive adhesion molecules, growth factors, inflammatory molecules (e.g., cytokines), ROS, and chemokines; d) altered (excessive or impaired) angiogenesis; e) enhanced thrombosis and disrupted fibrinolysis (Davignon and Ganz, 2004).

ED and low NO bioavailability are associated with the initiation and progression of heart failure (Marti et al., 2012), and this is due to increased oxidative stress, which in turn results in lower NO production (Hare and Stamler, 2005, Ontkian et al., 1991). The cytokines released as a result of ED inhibit eNOS expression (Smith et al., 1996, Agnoletti et al., 1999). Thus, these activities decrease NO bioavailability and impair endothelial function, accelerating the onset and progression of heart failure (Parodi et al., 2007). The existing evidence illustrates that both acute and short term of dietary NO_3^- supplementation significantly improve exercise capacity, blood pressure and quality of life in heart failure with preserved ejection (Eggebeen et al., 2016, Zamani et al., 2015, Zamani et al., 2017). In 2015, Zamani *et al.* and colleagues investigated the effect of acute dosage of dietary NO_3^- in the form of BRJ (12.9 mmol) on exercise capacity via supine cycle ergometer, among heart failure individuals with preserved ejection fraction (>50%) and found significant increase in exercise duration (mean duration 0.8 ± 1.3 minutes, $p = 0.02$) (Zamani et al., 2015). Likewise, Eggebeen *et al.* (2016) found that endurance time increased in patients with heart failure and preserved

ejection fraction following chronic dietary NO_3^- supplementation in the form of BRJ (6.1 mmol for one week) compared to placebo (BRJ: 449 ± 180 s versus PL: 363 ± 125 ; 24% increase, $p = 0.002$) (Eggebeen et al., 2016). In contrast, in a double-blind, randomised crossover study with ten individuals with heart failure and reduced ejection fraction, exercise tolerance, resting or exercising blood pressure, central haemodynamics, skeletal muscle oxygenation or the $\dot{V}\text{O}_2$ of exercise did not significantly change following nine consecutive days of dietary NO_3^- supplementation compared to placebo (Hirai et al., 2017). The results from studies indicate that heart failure with preserved ejection fraction and heart failure with reduced ejection fraction have different responses to dietary NO_3^- supplementation on exercise outcomes. This variation may be because heart failure with preserved ejection fraction has displayed abnormally low peripheral oxygen extraction in comparison with heart failure with reduced ejection fraction (Dhakal et al., 2015). The mechanism behind the improvement in exercise efficiency in heart failure following the dietary NO_3^- supplementation is that more effective delivery of NO results in a decrease in systemic vascular resistance and an increased O_2 delivery to exercising muscle (Zamani et al., 2017, Zamani et al., 2015, Hirai et al., 2017, Eggebeen et al., 2016).

1.7.3 Platelet function and aggregation

It is widely established that platelets have a role in atherosclerosis, thrombosis, and the development of coronary artery disease (Davì and Patrono, 2007). Platelet adhesion and aggregation are inhibited by NO, which is produced either by the vascular endothelium (Radomski et al., 1990, Radomski et al., 1987, Alheid et al., 1987). It has been shown that

dietary NO_3^- supplementation significantly inhibits platelet aggregation in humans (Webb et al., 2008, Richardson et al., 2002). The mechanism of this inhibition is likely to be linked to the NO activation of cyclic guanosine monophosphate (Moro et al., 1996) and the formation of nitrotyrosine, which inhibits platelet function (Richardson et al., 2002).

1.8 Proposed mechanisms of dietary nitrate on exercise efficiency

Dietary NO_3^- supplementation has been shown to reduce the oxygen cost of exercise (increased exercise efficiency). The mechanisms behind this are linked to being either independent or a combination of improvements in muscle contractile efficiency, mitochondrial function, and skeletal muscle blood flow.

1.8.1 Muscle contractile efficiency

Skeletal muscle requires a source of adenosine triphosphate (ATP) to function. ATP can be generated by glycolysis or oxidative phosphorylation, both of which involve substrates that are delivered to the skeletal muscle via blood flow. It can also be regenerated from adenosine diphosphate (ADP) using muscle stores of phosphocreatine (PCr). The mechanism underlying the improved exercise efficiency following NO_3^- supplementation is that NO_3^- reduces the ATP demand of skeletal muscle contraction. In 2010, Bailey *et al.* and colleagues were the first to demonstrate an increased muscle contractile efficiency during exercise following dietary NO_3^- supplementation. They conducted a double-blind, randomised, placebo-controlled crossover study of six days of NO_3^- supplementation in the form of BRJ (5.1 mmol) in seven healthy

young males. They found overall ATP turnover (using Phosphorus-31 magnetic resonance spectroscopy) was reduced, with reduced ADP and phosphate accumulation following dietary NO_3^- supplementation. The possible mechanism behind this is related to observations indicating that NO may modulate the ATP cost of force production by inhibiting calcium cycling (Hart and Dulhunty, 2000, Viner et al., 2000) and slowing cross-bridge cycling kinetics (Galler et al., 1997, Heunks et al., 2001). The ATP cost of force production in skeletal muscle is detected by actin and myosin interaction (actomyosin-ATPase) and calcium pumping within the sarcoplasmic reticulum (Calcium-ATPase). NO inhibits calcium release into the sarcoplasmic reticulum by reducing cross-bridge cycling, hence lowering ATP consumption.

1.8.2 Mitochondrial efficiency

During exercise, energy demands increase, so oxygen consumption increases to support ATP production. It has been demonstrated that following NO_3^- ingestion, human skeletal muscle mitochondria become more efficient (increased P/O ratio) (Larsen et al., 2011). The P/O ratio is a measure of mitochondrial oxidative phosphorylation efficiency. It is expressed as the amount of oxygen consumed per ADP phosphorylation to ATP, with a higher ratio indicating more efficient oxidative phosphorylation (Hinkle, 2005). In 2011, Larsen *et al.* performed a randomised, double-blind, crossover study in healthy subjects to determine the effect of three days of NaNO_3 ingestion (NO_3^- 0.1 mmol/kg/day) on mitochondrial efficiency (P/O ratio), which was taken from the vastus lateralis muscle. They found that the P/O ratio in skeletal muscle mitochondria increased by 19% following NaNO_3 ingestion compared to placebo. The change in P/O ratio was also correlated to the reduction in whole-body oxygen consumption during exercise. This is most likely due to down-regulation of two mitochondrial uncoupling

proteins: adenine nucleotide translocase (ANT) and uncoupling protein 3 (UCP3) (Larsen et al., 2011).

Unlike the findings of Larsen *et al.* (2011), a study indicates that administering dietary NO_3^- in the form BRJ (NO_3^- 26 mmol) for seven days significantly decreased the oxygen cost of exercise but did not show a change in markers of mitochondrial coupling such as the P/O ratio, and ANT and UCP3 expressions (Whitfield et al., 2016). This suggests that the observed reduction in oxygen consumption is due to a reduction in the ATP cost of force production, as Bailey *et al.* (2010) have reported previously (Bailey et al., 2010). Accordingly, these results suggest that improved exercise efficiency is not solely due to changes in mitochondrial efficiency following NO_3^- supplementation. The discrepancy in these results might very likely be due to the method of NO_3^- administration. For example, Larsen *et al.* (2011) administered NaNO_3 supplementation for three days (Larsen et al., 2011), whereas Whitfield *et al.* (2016) administered BRJ for seven days (Whitfield et al., 2016). As a result, it is plausible that these two supplements, administered at varying doses and durations, act through different mechanisms.

1.8.3 Skeletal muscle blood flow

NO is a prominent endothelial relaxing factor that acts via the secondary messenger cyclic guanosine monophosphate to cause smooth muscle relaxation and vasodilation (Gruetter et al., 1979). This is revealed by a decrease in blood pressure following NO_3^- administration (Larsen et al., 2006, Webb et al., 2008). NO is therefore thought to increase blood flow to metabolically active skeletal muscle, thereby enhancing oxygen delivery to local metabolic demands and allowing prolonged exercise. In addition, it has been shown that NO_3^-

supplementation increases blood flow to exercising skeletal muscles. Ferguson *et al.* (2013) demonstrated that supplementing rats' diets with NO_3^- (1 mmol/kg/d) for five days reduced exercising mean arterial blood pressure and increased hindlimb blood flow and vascular conductance during exercise, primarily in the fast twitch type IIb muscle fibres (Ferguson *et al.*, 2013b). It is evident from the improvement in skeletal muscle blood flow that NO plays a significant role in regulating perfusion and thereby increasing oxygen supply to exercised muscles. In this regard, it appears that NO_3^- has a selective effect on specific fibre types. This was also replicated in subsequent studies of microvascular oxygen delivery, which showed an improvement in oxygen delivery at the beginning of muscle contractions in rats during the electrically induced twitch spinotrapezius muscle contractions (Ferguson *et al.*, 2013a).

In humans, there is suggested evidence that NO_3^- supplementation has a preferential effect on type II muscle. According to Henneman *et al.* (1965), size principle and skeletal muscle fibres are recruited hierarchically according to their recruitment threshold. For example, smaller, slower type I (slow twitch) motor units are recruited first to meet low muscle force demands, whereas larger, faster type II (fast twitch) motor units are recruited as muscle force demands increase (Henneman *et al.*, 1965). In 2013, Breese *et al.* conducted a randomised, double-blind, crossover study in nine healthy subjects to investigate the effects of 4-6 days of NO_3^- supplementation in the form of BRJ (8 mmol of NO_3^-) on oxygen uptake ($\dot{V}\text{O}_2$) and muscle deoxyhaemoglobin kinetics during an initial increase in cycling work rate from a low-intensity (20 W) to a moderate-intensity work rate (where mostly type I muscle is recruited to produce force) and a subsequent increase to a severe-intensity work rate when additional force production is mainly achieved by more recruitment of type II muscle. They found that ($\dot{V}\text{O}_2$)

and muscle deoxyhaemoglobin kinetics were sped up in the moderate-severe exercise intensity but not in the low-moderate exercise intensity following the NO_3^- supplementation in compared to placebo (Breese et al., 2013). These findings suggest that NO_3^- supplementation exerts a predominant physiological effect on type II skeletal muscle than on type I skeletal muscle, and this may be related to the lower microvascular PO_2 found in type II muscle at rest and during contractions (McDonough et al., 2005).

1.9 Role and response of nitrate in hypoxia

Exercise under hypoxic conditions is associated with decreased muscle oxidative function and impaired exercise tolerance. In two landmark randomised controlled trials, the British Medical Research Council Long-Term Domiciliary Oxygen Trial (1981) and the Nocturnal Oxygen Therapy Trial (1980), long-term oxygen therapy which is defined as more than 15 hours of oxygen a day, provided an advantage for survival. These findings have been confirmed in further trials (Cooper et al., 1987, Gulbas et al., 2012, Strom, 1993). Similarly, it has been proven that ambulatory oxygen improves exercise performance in moderate to severe COPD patients (Bradley et al., 2007, Bradley and O'Neill, 2005, Nonoyama et al., 2007).

Previously, it was confirmed that NO production via the NOS-dependent pathway is dependent on the presence of molecular oxygen and that its production may be affected under hypoxic conditions. Therefore, the NO_3^- - NO_2^- -NO pathway is upregulated, which may restore systemic NO levels.

It has been demonstrated that NO_3^- supplementation increases exercise performance in hypoxic conditions (Masschelein et al., 2012, Pawlak-Chaouch et al., 2016, Shannon et al., 2016, Vanhatalo et al., 2011). In a recent study, Cocksedge *et al.* (2020) performed a randomised, double-blind, crossover study in ten healthy males to evaluate the effect of dietary NO_3^- supplementation in the form of BRJ (18.6 mmol of NO_3^-) on oxygen uptake and exercise tolerance in normoxia, hyperoxia, and hypoxia conditions. They found increases in peak oxygen uptake ($p < 0.05$) under hypoxia conditions but not in normoxia or hyperoxia ($p > 0.05$) following acute dietary NO_3^- supplementation (Cocksedge et al., 2020). Therefore, the ergogenic potential benefits of dietary NO_3^- supplementation may be enhanced in conditions where skeletal muscle is hypoxic.

The findings that dietary NO_3^- supplementation can improve exercise performance in healthy individuals have fueled interest in the possible benefits for individuals with COPD and other respiratory diseases where oxygen delivery to muscles is reduced and pulmonary vascular and systemic dysfunction may also be present.

At the onset of this thesis, eleven studies have investigated the effects of dietary NO_3^- supplementation on exercise capacity and cardiovascular parameters in chronic respiratory disease (see Chapter 3 for further details).

1.10 Aims, research questions, and hypothesis

The purpose of this thesis was to further extend the knowledge and synthesis the current evidence and evaluate the impact of dietary NO_3^- supplementation on exercise capacity and cardiovascular parameters in chronic respiratory disease. A secondary aim of this thesis was to investigate the effects of acute NO_3^- administration on exercise performance, blood pressure, and FMD in pulmonary hypertension patients who are using ambulatory oxygen or desaturate on exercise. The third aim of this thesis was to investigate if there was a link between blood nitrate levels and the severity of respiratory disease. The work presented in this thesis addresses the following research questions:

1. Does the published literature support the hypothesis that dietary nitrate supplementation improves cardiovascular parameters and exercise capacity in people with chronic respiratory disease?
2. Does acute dietary nitrate supplementation, in pulmonary hypertension patients who are using ambulatory oxygen or desaturate during exercise, improve exercise endurance?
3. Are blood nitrate and nitrite levels associated with breathlessness and other measures of disease severity in respiratory disease?
4. Does a low level of exhaled nitric oxide (NO) reliably predict low blood nitrate and nitrite levels?

CHAPTER 2: Description of methods

2.1 General methods

Three studies were conducted to fulfil this thesis. The first study was a systematic review and meta-analysis (Chapter 3), which synthesised the current evidence and evaluated the impact of dietary nitrate supplementation on exercise capacity and cardiovascular parameters in chronic respiratory disease. The other two clinical studies (Chapters 4, 5) were a double-blind, randomised, placebo-controlled trial (EDEN-OX) in patients with pulmonary hypertension (PH) and an observational study (EON-Lung) to investigate whether there is a link between blood levels of nitrates and the severity of the disease. In these two clinical studies, 85 participants were included, 20 with PH (EDEN-OX trial) and 65 with COPD (EON-Lung trial).

2.2 Ethical approval

The EDEN-OX study (Chapter 4), titled “Effect of dietary nitrate supplementation on exercise performance in pulmonary hypertension”, and the EON-Lung study (Chapter 5), titled “Effect of Nitrate levels in Lung Disease - an observational study”, were approved by the London Chelsea Research Ethics Committee (REC reference 15/LO/0975) and the Health Regulator Authority (HRA) and Health and Care Research Wales (HCRW) (REC reference 19/EE/0256), respectively (see Appendix 2). Written informed consent was taken from all participants included in these studies, and research was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Table 2.1 Outcome Measures for each study

Outcome	EDEN-OX	EON-Lung
Pulmonary Function Test	✓	✓
Anthropometry	✓	✓
MRC Dyspnoea Scale	✓	✓
CAT Score	✓	✓
Six-minute Walk Test		✓
Incremental Shuttle Walk Test	✓	
Endurance Shuttle Walk Test	✓	
Plasma Nitrate and Nitrite Sampling	✓	✓
Endothelial Function	✓	✓
Exhaled Nitric Oxide		✓
Blood Markers of Endothelial and Inflammatory Function		✓
Oral Microbiome Sampling		✓

Abbreviations: EDEN-OX– Effect of Dietary Nitrate Supplementation on Exercise Performance in Hypoxia; EON-Lung– Effect Of Nitrate levels in Lung Disease; MRC – Medical Research Council; CAT – COPD Assessment Test.

2.3 Study subjects

Participants were recruited between October 2020 and March 2022 from the outpatient clinics at the Royal Brompton and Harefield NHS Foundation Trust or through the department's research audit database. Participants recruited for the EDEN-OX study have a diagnosis of PHT (WHO group 3), which is defined as pulmonary hypertension resulting from lung disease and/or hypoxia. For the EON-Lung study, all participants were recruited with a diagnosis of COPD.

Exclusion criteria included clinically unstable patients (within one month of exacerbation), significant comorbidity limiting exercise tolerance, significant renal impairment (estimated glomerular filtration rate (eGFR) $<50 \text{ ml} \cdot \text{min}^{-1}$), hypotension (systolic blood pressure $<100 \text{ mmHg}$), and pregnancy. Subjects were withdrawn if they had a significant adverse event or if they declined to participate.

2.4 Anthropometric measurements

Height (cm) was measured without shoes using a wall mounted measure, and weight (kg) was also measured using standardised scales. The Body Mass Index (BMI, $\text{kg} \cdot \text{m}^{-2}$) was computed using this equation: $\text{BMI} = \text{weight (kg)} / \text{height (m)}^2$.

2.5 Blood pressure measurement

Blood pressure was measured using an automated blood pressure monitor (Omron M3 Comfort BP monitor (HEM-7134-E-Omron Health Care. Inc., Japan). In order to take measurements, subjects were instructed to rest for at least 10 minutes, seated upright in a chair, with their arms at the level of their hearts. Each participant was assigned the proper

cuff pressure size. A mean of two measurements (one minute apart) was recorded. The following formula was used to calculate mean arterial pressure:

$$(MAP): MAP = (SBP + (DBP \times 2))/3$$

2.6 Body composition

2.6.1 Fat free mass measurement

Fat free mass was measured by bioelectrical analysis (BIA) and this value is dependent on body water content. This technique measures the electrical resistance between the wrist and ankle. BIA was conducted in a semi supine position using a Bodystat 4000 device (Bodystat, Isle of Man, United Kingdom). Bodystat 4000 device was calibrated as per the manufacture specifications before measuring BIA. Two electrodes were placed on the right hand and right foot (Figure 3.1). Measurements were recorded before participants performing any exercise. Fat free mass index (FFMI) was measured by dividing FFM by height in metres squared ($\text{kg} \cdot \text{m}^{-2}$).

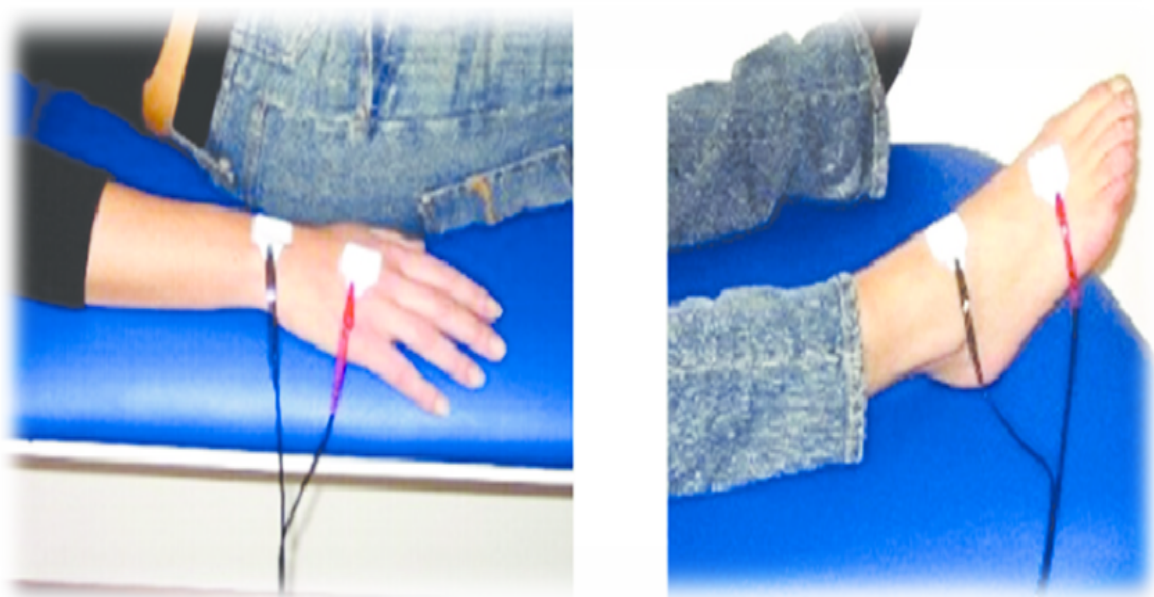


Figure 2.1: Electrode position for Bodystat 4000.

2.7 Exercise capacity

2.7.1 The incremental shuttle walk test (ISWT) and endurance shuttle walk test (ESWT)

The ISWT was developed by Professor Sally Singh and colleagues to assess exercise capacity in patients with chronic respiratory disease by using a field walking test. This test is incremental and progressive, which stimulates and stresses the individual to symptom-limited exercise performance (Singh et al., 1992). The ISWT is a valid tool for measurement of cardiopulmonary exercise capacity in chronic respiratory disease and provokes a similar physiological response to cardiopulmonary exercise testing (Singh et al., 2014). The minimum important difference (MID) of the ISWT is between 35.0 and 36.1 metres (Evans and Singh, 2019).

In this thesis, the ISWT was conducted based on the European Respiratory Society (ERS) and American Thoracic Society guidelines (ATS) (Holland et al., 2014).

The patient was instructed to walk between two cones, 10 metres apart. Cones were located nine metres apart to avoid abrupt changes in direction (Figure 2.2). The instructions were played to subjects from an audio recording (CD, Glenfield Hospital, Leicester, UK), with the following instructions:

“Walk as long as possible there and back along the 10 metre course keeping to the speed indicated by the bleeps on the audio recording. You will hear these bleeps 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 bleep and at the other end when you hear the next”(Holland et al., 2014, Holland et al., 2015).

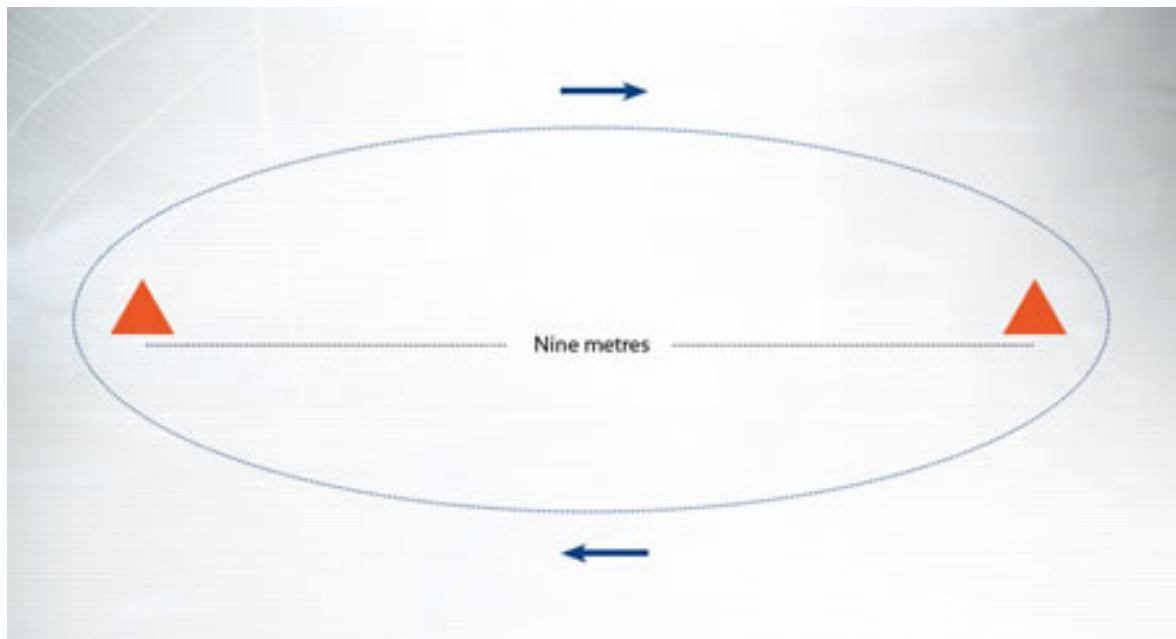


Figure 2.2: ISWT course layout.

The ISWT consists of 12 levels, and each level lasts for one minute. The walking speed for the first level was set at $0.50 \text{ m}\cdot\text{sec}^{-1}$ (20 seconds per shuttle), with an increment of $0.17 \text{ m}\cdot\text{sec}^{-1}$ per minute (Singh et al., 1992, Holland et al., 2014, Holland et al., 2015). At the end of each level, the subject was instructed to increase his or her speed and a warning was given if the subject did not walk within 0.5 metres of the cone before the beep; “you are not going fast enough; try to make up the speed this time”(Holland et al., 2014, Holland et al., 2015). Reasons for termination of the test are: 1) the subject indicates that they are unable to continue; 2) the operator determines that the subject is not fit to continue; or 3) the operator assesses that the subject was not able to maintain the speed of the test, which was defined as the subject being outside the 0.5 m marker away from the cone on the second following 10 metre length (Holland et al., 2014, Holland et al., 2015). Oxygen saturation, heart rate, blood pressure, dyspnoea, and fatigue Borg scale were recorded prior to the test, immediately afterwards, and after the recovery. The total duration of the ISWT is 20 minutes. Subjects were permitted to use their prescribed oxygen. The type of oxygen delivery device and the

methods of its use were recorded to maintain these factors constant for retesting. In order to account for a learning effect, two tests were conducted with 30 minutes rest between tests (Holland et al., 2014, Holland et al., 2015), and the best distance was selected for analysis.

The ESWT was first described in 1999. This test is used to measure exercise endurance. It is not incremental and is conducted at 85% of the maximal walking speed that can be attained on the ISWT (Revill et al., 1999). It has been stated that there is no significant difference between the test and retest for the ESWT (Revill et al., 2009, McKeough et al., 2011). However, for this thesis, the ESWT practice was conducted in purpose for familiarisation and to evaluate if the walking speed was correct. The MID of the ESWT following pulmonary rehabilitation is between 174 and 279 seconds (Zatloukal et al., 2019). Following bronchodilation intervention, a change of 65 seconds (95% CI 45-85 seconds) was detected to be perceivable by patients (Pepin et al., 2011).

The ESWT was performed along the same course layout as mentioned for the ISWT (Figure 2) using the European Respiratory Society (ERS) and American Thoracic Society (ATS) guidelines (Holland et al., 2014). Subjects were allowed to use their prescribed oxygen during the ESWT. Oxygen saturation, heart rate, blood pressure, dyspnoea, and fatigue Borg scale were recorded prior to the test. During the ESWT, heart rate and oxygen saturation were continuously monitored using a Pulseox-300i (Konica Minolta, Basildon, UK). The instructions were played to subjects from an audio recording (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 et al., 2015).

The ESWT consists of a warm-up period which lasts about one and a half minutes (Holland et al., 2014, Holland et al., 2015). Following this, a triple bleep is played, indicating the start of the 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 et al., 2014).* The reasons for termination of the test are the same reasons that were described for the ISWT. Peak heart rate, blood pressure, and Borg scales were recorded as well as the recovery time.

2.7.2 Six minute walk test (6MWT)

The six-minute walk test (6MWT) was performed according to the American Thoracic Society guidelines (Enright, 2003). Subjects walked as far as they could on a 30 metre course indoor with a flat corridor. Subjects were instructed to rest for 10 minutes before starting the walking test. Prior to the test, oxygen saturation, heart rate, blood pressure, and Borg scales were recorded. Throughout the 6MWT, the standardised encouragement phrases were given to subjects, and the test was terminated at the end of 6 minutes, at which point peak oxygen saturation, heart rate, blood pressure, and Borg scales were recorded, and the total distance walked was measured as well.

2.8 Pulmonary function testing

Pulmonary function test measurements were performed at the respiratory physiology department at the Royal Brompton Hospital according to ERS/ATS guidelines (Brusasco et al., 2005). The forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, residual volume (RV), total lung capacity (TLC), and diffusion capacity for carbon monoxide (DLco) were measured using the SpirostickTM PC spirometry system (Love Medical Ltd, Manchester, UK), Jaeger MasterScreenTM Pulmonary Function Laboratory (nSpire Health Ltd, Hertford, UK) with rigorous quality assurance and daily equipment calibration. All pulmonary function tests were performed with participants taking their regular inhaled therapies. These measurements were not repeated if subjects had been performed in the preceding 3 months. Standardised pulmonary function testing reference values were based on the European Coal and Steel Community reference values (Gibson, 1993).

2.9 Fractional exhaled nitric oxide (FeNO)

FeNO levels were measured according to the ATS clinical practice guidelines (Dweik et al., 2011) using the NIOX VERO[®] device (Circassia, London, UK). The MID of FeNO is the change of at least 20% increase or decrease following an intervention in asthmatic patients (Dweik et al., 2011). The measurement of FeNO requires the participant to exhale slowly into a mouthpiece for approximately 10 seconds, as instructed by a visual monitor on the device screen. The exhaled NO value is measured in parts per billion (ppb). According to the ATS clinical practice guidelines FeNO measurements were interpreted as low (<25 ppb),

intermediate (25-50 ppb), and high (>50 ppb) (Dweik et al., 2011). The average of three measures of FeNO was recorded.

2.10 Health-related quality of life assessments

2.10.1 The medical research council (MRC) dyspnoea scale

The MRC dyspnoea scale consists of five statements (Figure 2.3) that describe the degree of breathlessness related to daily activities. The MRC dyspnoea scale ranges from none (grade 1) to almost complete incapacity dyspnoea (grade 5). This assessment score has been shown to be well correlated with measurements of lung function as well as exercise capacity (Stenton, 2008, Kelly et al., 2012).

The MRC Breathlessness 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

Figure 2.3: Medical Research Council (MRC) dyspnoea scale.

2.10.2 COPD assessment test (CAT)

The COPD assessment test (CAT) is an eight-item symptom score where subjects rate their responses on a scale of 0-5, which gives a maximum total score of 40, and the higher score indicates poor health status (Figure 3.4). This assessment test has been shown to be responsive both to exacerbations and to pulmonary rehabilitation (Jones et al., 2009, Dodd et al., 2011). The CAT is validated in assessing health status in COPD (Jones et al., 2009), and the MID is a change of 2 points (Kon et al., 2014).

Your name:

Today's date:



How is your COPD? Take the COPD Assessment Test™ (CAT)

This questionnaire will help you and your healthcare professional measure the impact COPD (Chronic Obstructive Pulmonary Disease) is having on your wellbeing and daily life. Your answers, and test score, can be used by you and your healthcare professional to help improve the management of your COPD and get the greatest benefit from treatment.

For each item below, place a mark (X) in the box that best describes you currently. Be sure to only select one response for each question.

Example: I am very happy 0 ☒ 1 2 3 4 5 I am very sad

		SCORE
I never cough	0 1 2 3 4 5 I cough all the time	
I have no phlegm (mucus) in my chest at all	0 1 2 3 4 5 My chest is completely full of phlegm (mucus)	
My chest does not feel tight at all	0 1 2 3 4 5 My chest feels very tight	
When I walk up a hill or one flight of stairs I am not breathless	0 1 2 3 4 5 When I walk up a hill or one flight of stairs I am very breathless	
I am not limited doing any activities at home	0 1 2 3 4 5 I am very limited doing activities at home	
I am confident leaving my home despite my lung condition	0 1 2 3 4 5 I am not at all confident leaving my home because of my lung condition	
I sleep soundly	0 1 2 3 4 5 I don't sleep soundly because of my lung condition	
I have lots of energy	0 1 2 3 4 5 I have no energy at all	
		TOTAL SCORE

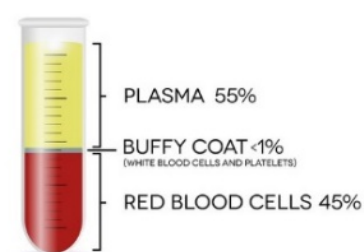
COPD Assessment Test and the CAT logo are trademarks of the GlaxoSmithKline group of companies.
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Figure 2.4: COPD Assessment Test (CAT) questionnaire.

2.11 Blood sampling and processes

2.11.1 Venous blood sampling

4mL of venous blood samples were collected into spray-coated EDTA and Lithium Heparin (LH) vials at baseline (0 hours) and at three hours. Upon collection the vials were gently inverted 8-10 times. Samples were processed as quickly as possible, and within 5 minutes. If there was a delay, the vials were placed at 2-8°C before processing. The samples were centrifuged at 860g for 10 minutes at 4°C to obtain sample separation as shown below:



Following centrifugation, plasma was removed carefully and transferred to a 1.5mL Falcon tube. The aliquoted plasma was transferred to dry ice and stored at -80°C prior further processing. Blood biochemistry tests (urea and electrolytes U&E), CRP and LFT tests, iron studies, and brain natriuretic peptide (BNP) were all analysed by the hospital's laboratory. In addition, in collaboration with experts in these areas of research, I performed the following blood analyses:

2.11.2 Plasma NOx measurement

NO has a relatively short half-life. One method to estimate total NO production is to calculate the sum of both (NO_3^- and NO_2^-) in the plasma, indicated as plasma NOx. In order to assess plasma NOx concentration, the Griess method was used due to its high reproducibility and minimal interference from plasma constituents (Figure 2.5) (Giustarini et al., 2008).

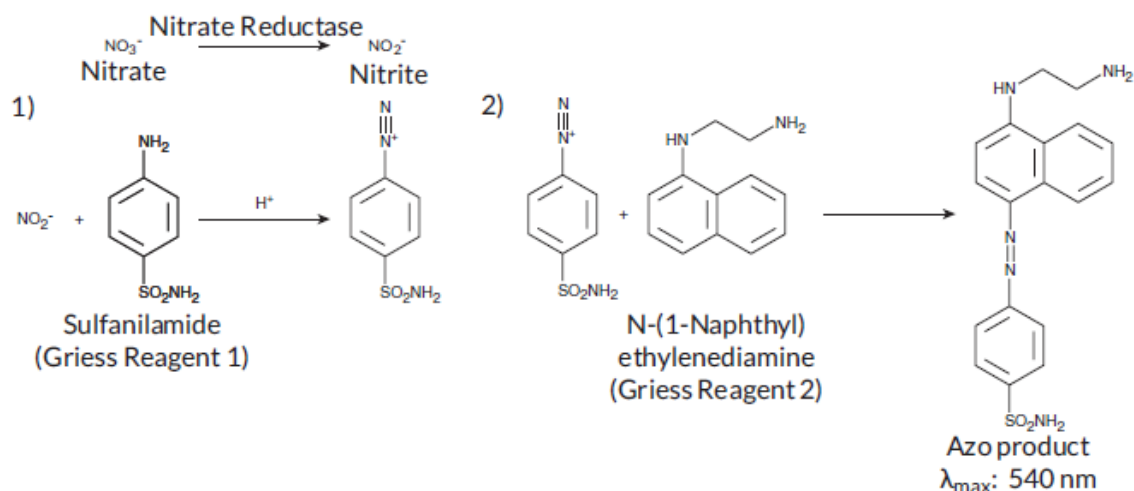


Figure 2.5: Griess reaction for measurement of plasma nitrate metabolites.

I used the nitrate/nitrite colorimetric assay kit from Cayman Chemical (Ann Arbor, MI, USA). Samples in LH vials were defrosted and ultrafiltered using a 30kDa filter. In two steps, total nitrate/nitrite were determined: (a) nitrate first converted to nitrite utilizing NADH-dependent nitrate reductase enzyme, and (b) the Griess reagents was added, which converted nitrite into a deep purple azo-compound. The level of azo-compound is measured by absorbance at 540 nm using a microplate reader (TECAN Infinite M200PRO, Grödig, Austria). A calculation of nitrate was done by subtracting nitrite from the value of NO_x . The standard curves were then plotted to represent all absorbance measurements (see Figures 2.6 & 2.7. Figure 2.8 shows photographs of the NO_x plate post-test.

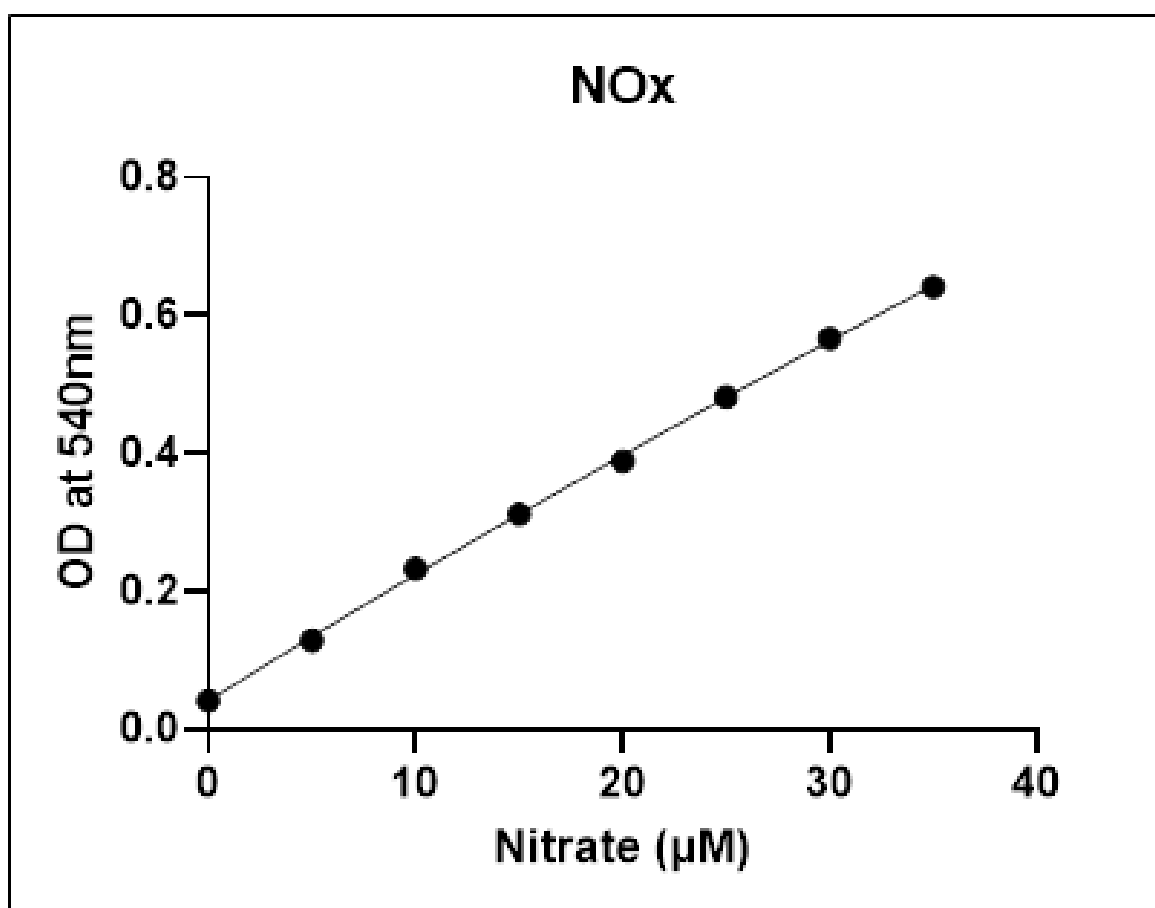


Figure 2.6: Standard curve for determination of total NO_x levels.

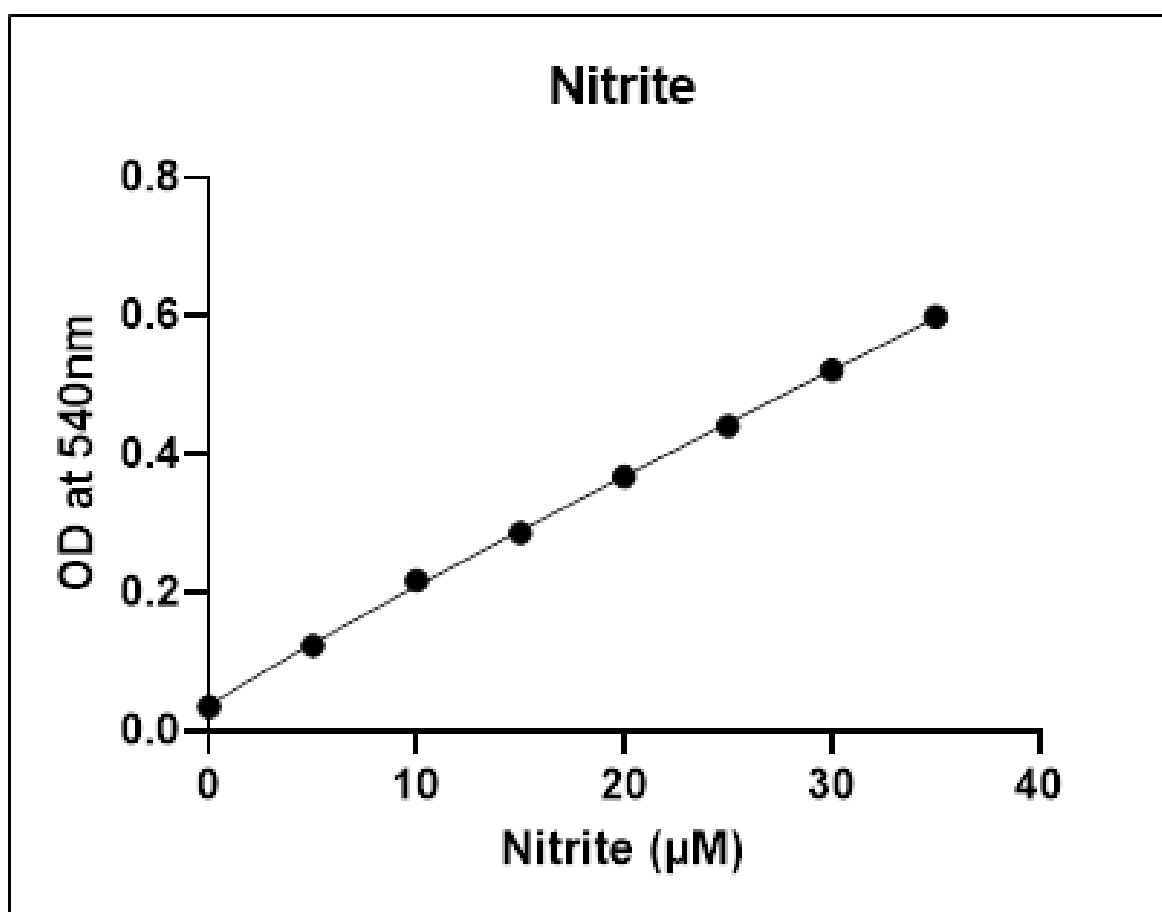


Figure 2.7: Standard curve for determination of nitrite levels.

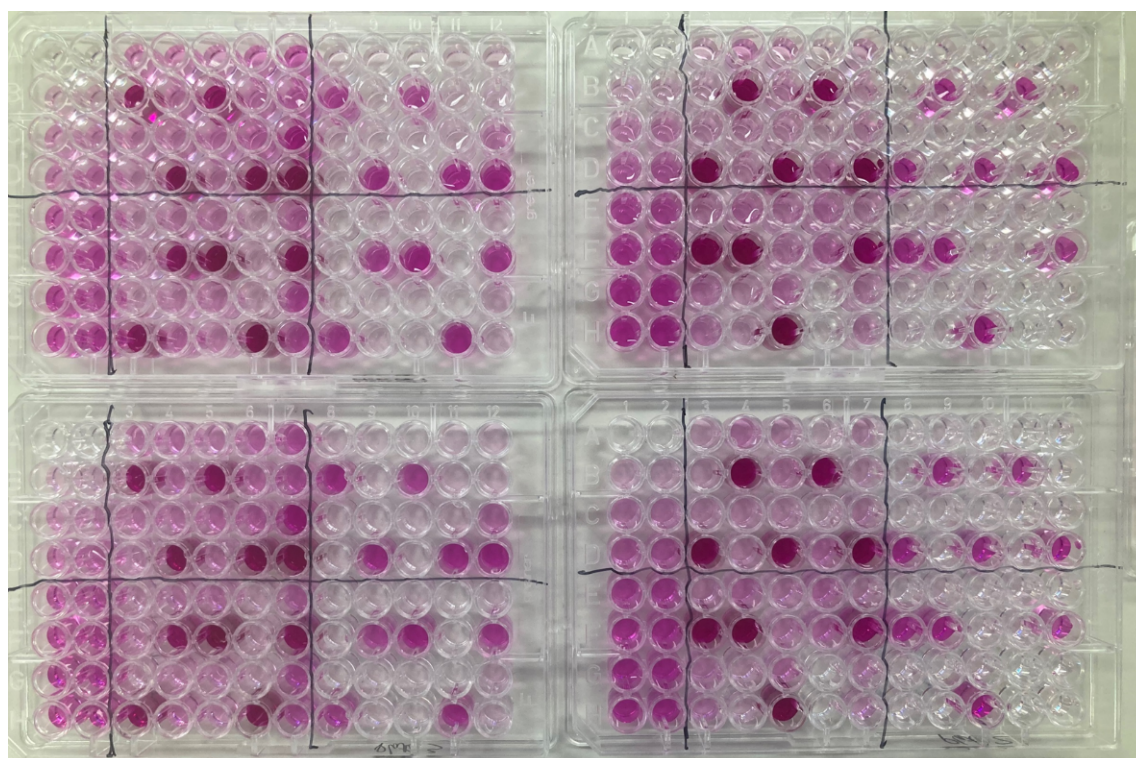


Figure 2.8: Photograph of plasma NOx plate.

2.11.3 Arginine and asymmetric dimethylarginine (ADMA) measurement

ADMA and L-arginine levels in plasma samples were quantified using a commercially available competitive ELISA kit (EA207/192, DLD Diagnostika), according to the manufacturer's instructions. Briefly, ADMA and L-arginine are pre-bound to the solid phase of ELISA microplate wells. Prior to the addition of samples into the ELISA microplate, plasma ADMA and L-arginine undergo acylation. Subsequently, plasma samples are dispensed into the ELISA microplates, followed by antisera, containing antibodies specific to ADMA/L-arginine (rabbit-anti-N-acyl-ADMA and rabbit-anti-N-acyl-Arginine). These antibodies bind ADMA/L-arginine associated with the solid phase or that of, contained in plasma samples (suspension). Thereafter, unbound antigen-antibody complexes are removed, and solid phase bound antigen-antibody complexes are detected via the reaction of tetramethylbenzidine and horseradish peroxidase conjugated to an anti-rabbit secondary antibody. Optical density is then determined (450nm) and the levels of ADMA/L-arginine are thus inversely proportional to the absorbance values.

Aliquoted 50 ml samples of the pre-treated standards, controls, and samples were pipetted into the wells of the microtiter plate, and 50 ml of antiserum solution was added to each well. In the next step, the plate was shaken on a horizontal shaker for 10 minutes. The plate was incubated at room temperature (20 - 25 °C) for 90 minutes. After incubation, each well's solution was removed, and the wells were washed four times with wash buffer (250 ml). After adding 100 µL of enzyme conjugate solution to each well, the plate was shaken horizontally for 30 minutes at room temperature. The wells were then washed again with the wash buffer four times. After washing, 100 µL of substrate solution was pipetted into the wells and the plate was incubated on a horizontal shaker for 20–30 minutes. We stopped the reaction with

a stopping solution and measured the optical density at 450 nm (reference wavelength 570–650 nm) with a microtiter plate reader (Sunrise, TECAN, Crailsheim, Germany). The detection limit was 0.05 $\mu\text{mol/L}$ with a standard range from 0.1 to 6.0 $\mu\text{mol/L}$. Intra-assay coefficient of variation (CV) of ADMA assay was 7.5% at 0.81 $\mu\text{mol/L}$ and 4.5% at 1.76 $\mu\text{mol/L}$, whereas inter-assay CV was ranged from 8.3% to 10.3% (Schnabel et al., 2005, Schulze et al., 2004). Figures 2.9 & 2.10 illustrate standard curves for the measurement of ADMA and arginine.

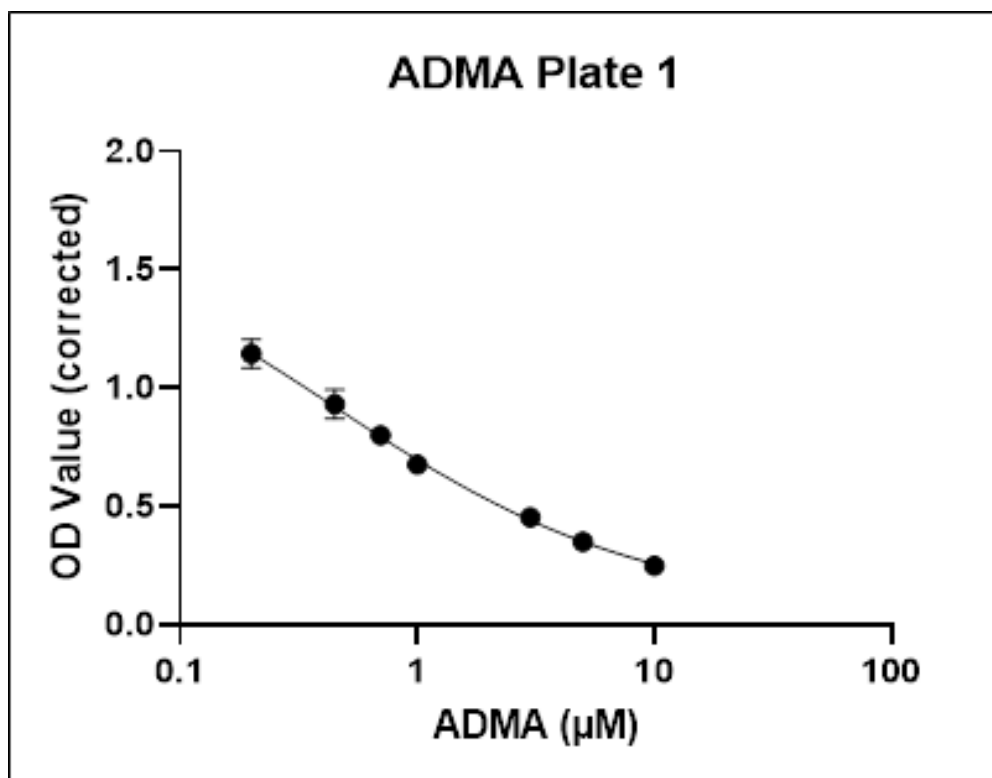


Figure 2.9: Standard curve for determination of plasma ADMA levels using ELISA (DLD Diagnostika).

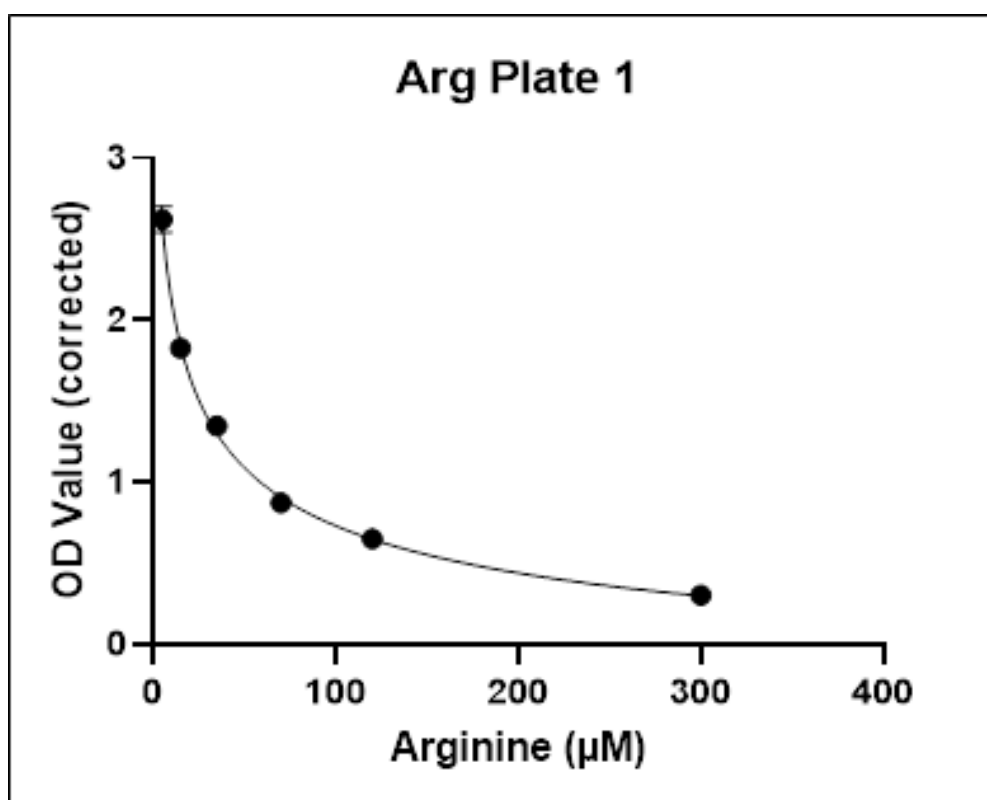


Figure 2.10: Standard curve for determination of plasma arginine levels using ELISA (DLD Diagnostika).

2.11.4 Platelet activation biomarkers measurement

The biomarkers for platelet aggregator expression and P-selectin expression % in whole blood with a 96-well plate were analysed using flow cytometry. I conducted these analyses under the supervision of Professor Tim Warner's vascular inflammation laboratory at Queen Mary University. This method includes a range of agonists with different concentrations, which were individually tested for multiple pathways of platelet activation (Figure 2.11). Plates were pre-prepared with a range of concentrations of freeze-dried agonists (including vehicle), packaged in a vacuum-sealed environment, and stored at room temperature until needed.

Blood samples were collected into 3.8 % trisodium citrate vials and pipetted into agonist-containing wells to initiate aggregations. This was performed within 5 minutes to maximise the influence of the vascular environment upon platelet responses. The following agonists

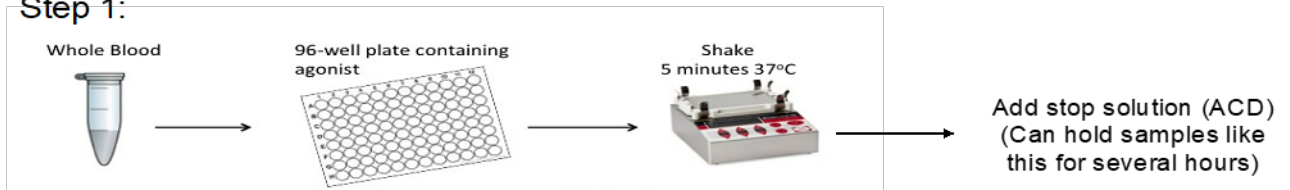
were used to stimulate the aggregation: arachidonic acid (0.03, 0.1, 0.3, 0.6 μ M), collagen (0.1, 0.3, 1, 3 μ g/ml), thrombin PAR1-receptor activating peptide (TRAP; 0.1, 0.3, 1, 3 μ M) and thromboxane A₂ mimetic U46619 (0.1, 0.3, 1, 3 μ M).

A 40 μ l of blood was peptide into 96-well plate-based aggregometry assay. The plate was then placed on the plate heater/shaker (BioShake iQ, Q Instruments) for 5 minutes at 1000rpm and 37°C and then fixed using 160 μ l of acid citrate dextrose (ACD), which was stored at 4°C. 10 μ l of anti-CD61 monoclonal antibody (1:25; clone VI-PL2, Biolegend) conjugated to allophycocyanin (APC) in PBS to label and identify the platelet. Whole blood samples were stained at room temperature for 30 minutes of staining before being fixed in 180 μ l of a bovine serum albumin (BSA)/dextrose saline solution containing 0.1% formalin (Sigma-Aldrich). We identified the platelets by forward and side scatter as well as CD61 positivity using a Novocyte 3000 flow cytometer (ACEA), and then counted the total number of platelets in each experimental well.

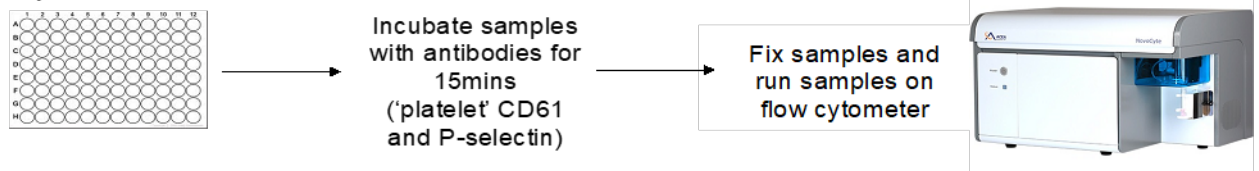
A strong correlation exists between P-selectin expression on the platelet surface and platelet aggregate size (Merten and Thiagarajan, 2000). The P-selectin expression was analysed as the same as the analysis method mentioned for platelet aggregation. In addition, 10 μ l of each experimental well was transferred into a separate well containing 10 μ l anti-CD61-APC (1:25; clone VI-PL2, Biolegend), and anti-CD62P-PE (1:25; clone AK-4, Biolegend) or isotype control (1:25, clone MOP-21, Biolegend) to identify platelets and quantify P-selectin expression. Samples were analysed using a Novocyte 3000 flow cytometer (ACEA) and with expression levels calculated from 10,000 acquired platelet events. Results were expressed as a percentage of positive platelets for P-selectin.

Overview of platelet function approach

Step 1:



Step 2:



Step 3:

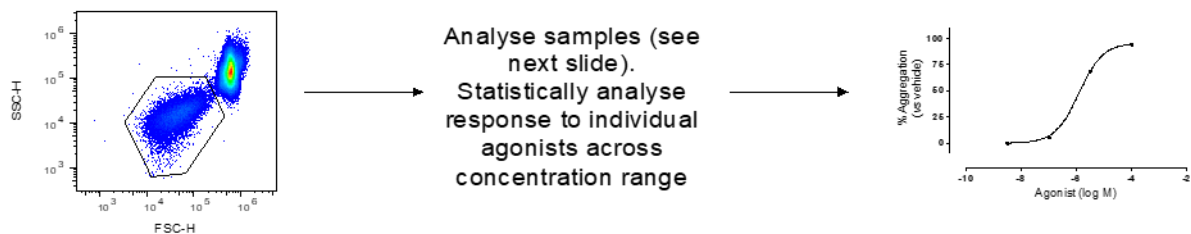


Figure 2.11: Process and analysis method of platelet activation assays. Image provided by the vascular inflammation laboratory at Queen Mary University.

2.12 Flow mediated dilatation (FMD)

Flow mediated dilatation (FMD) was evolved in 1992 and is a non-invasive technique to assess endothelial function (Celermajer et al., 1992). It has been shown that FMD is an endothelium-dependent process that is mediated by NO (Green, 2005). Large peripheral arteries (brachial, radial and femoral) are the sites for assessing the FMD. This test employed a temporary limb occlusion to cause an-ischaemia induced reactive hyperaemia, resulting in an increase in shear stress and dilation in the conduit artery (Pyke and Tschakovsky, 2005). FMD was measured as a percentage change of the baseline diameter. The following equation was used to calculate the FMD percentage change:

$$FMD \% = \frac{Peak\ Diameter - Baseline\ Diameter}{Baseline\ Diameter} \times 100$$

2.12.1 Participant preparation

Participants rested in a supine position for 10 minutes while the non-dominant arm was outstretched fully supported, resting on pillows in a quiet, temperature-controlled room. Participants were examined at the same time at each visit and were instructed to keep the same diet prior to each visit. Caffeine consumption was restricted on testing days, and all measurements were performed before the exercise tests.

2.12.2 Peripheral artery selection

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

2.12.3 Equipment

The FMD measurement is dependent on determining the arterial wall. A mobile application-based ultrasound solution with high resolution was used (Philips Lumify Ultrasound system). Image acquisition was performed using a Lumify L12-4 broadband linear array transducer with a frequency range (4.0-12 MHz). An acquiring video loop was used to allow for image acquisition to be taken at the end of the diastole. A modified arm supporter was used to support the arm and hold the ultrasound transducer (Figure 2.12).

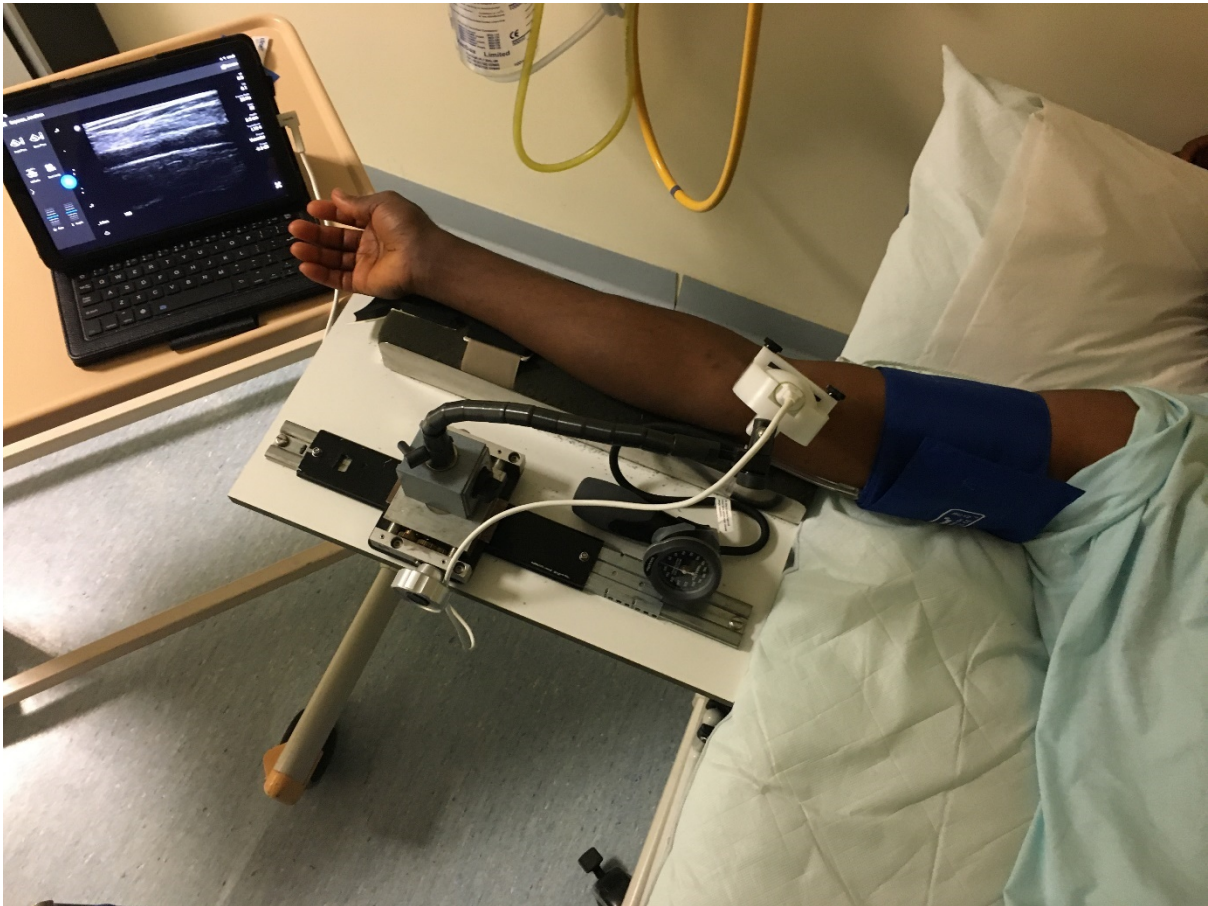


Figure 2.12: Positioning of broadband linear array transducer over brachial artery.

2.12.4 Vascular occlusion

A temporary vascular occlusion was required, generating reactive hyperaemia and shear stress. Vascular occlusion was conducted using a haemodynamometer and inflated cuff pressure around the upper arm for five minutes to supra-systolic pressure (200-250 mmHg), allowing for complete occlusion (Kooijman et al., 2008). After 5 minutes, the cuff was deflated and the dilation measurement was recorded.

2.12.5 Reactive hyperaemia measurements

Following cuff deflation, brachial artery dilation measurements were recorded at 30 second intervals (30 seconds, 90 seconds, and 120 seconds)(Black et al., 2008), and image acquisition was for 10 seconds.

2.12.6 Image analysis

The brachial artery diameter was measured from longitudinal images using ImageJ1.53e software (Fiji, National Institutes of Health and Laboratory for Optical Computational instrumentation, University of Wisconsin, USA. <https://fiji.sc>) where the lumen-intima interface is identified (Figure 2.13) (Schneider et al., 2012).

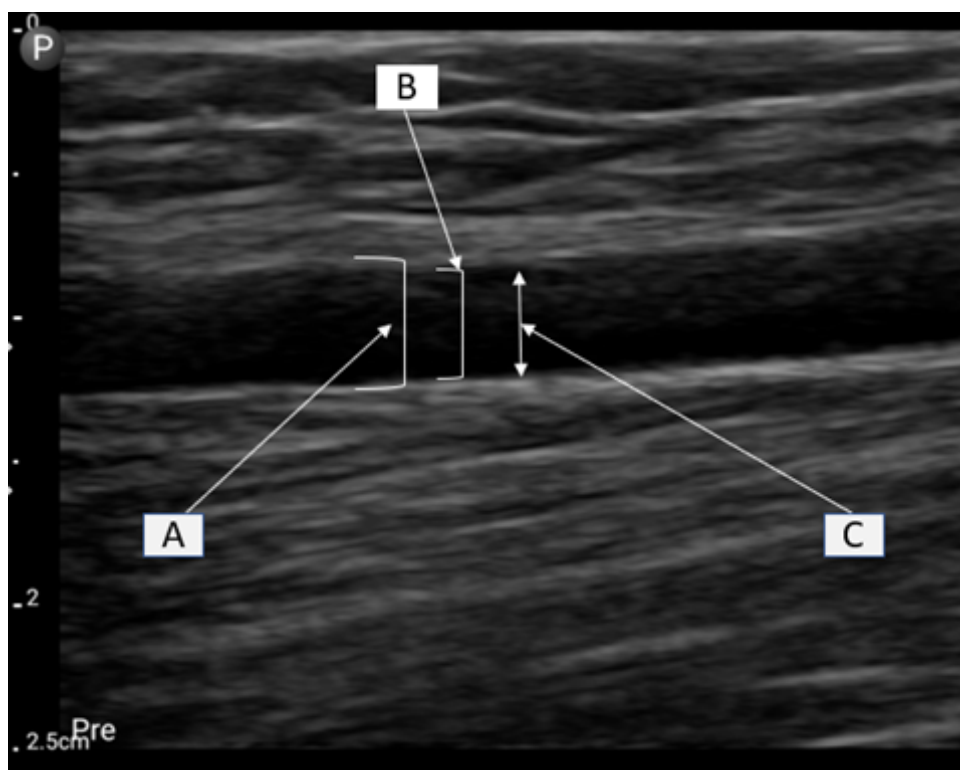


Figure 2.13: Example of imaging acquired of brachial artery using a Philips Lumify Ultrasound system (L12-4 broadband linear array transducer). A-media to media interface; B-Intima to intima interface; C-diameter identified.

2.12.7 FMD reproducibility

The evidence has shown that the FMD is a reproducible measure for assessing the endothelial function of the conduit artery. In a large multicentre (seven Italian centres), including 135 healthy subjects, Ghiadoni *et al.* (2012) showed the reproducibility of FMD measurements, and were not significantly different between the centres ($p= 0.91$). The FMD intra-session coefficient of variation was $9.9 \pm 8.4\%$ (from 7.6 to 11.9% across centres) and the FMD inter-session coefficient of variation was $12.9 \pm 11.6\%$ (from 11.6 to 16.1% across centres) (Ghiadoni *et al.*, 2012). Moreover, Charakida *et al.* (2013) evaluated the reproducibility of FMD in a multicentre trial including 375 participants across 19 vascular imaging centres in six European countries and showed that FMD was a reproducible measure and the range for absolute the technical error of measurement between sonographers was 0.5-1.1% with a good correlation between FMD percentage values with an intraclass correlation coefficient of 0.74 (Charakida *et al.*, 2013).

For this thesis, all FMD measurements were performed by myself according to a standardised protocol. I conducted a reliability/repeatability study to assess the operator repeatability for FMD measurements on ten healthy volunteers. FMD measurements were conducted at the same time at each visit. Participants were instructed to keep the same diet before each visit and avoid any strenuous exertion. A coefficient of variation and intraclass correlation coefficient (ICC) methods were used using the intrarater reliability test, which reflects the variation of data measured by one rater across two or more trials (Tables 2.2 & 2.3) (Koo and Li, 2016).

An ICC for the operator within the repeated measures of FMD was 0.78, which indicated "good reliability" between two visits (Table 2.3). The agreement between FMD measurements at different visits was also assessed by Bland-Altman analysis (Figure 2.14). This figure shows the Bland-Altman plot with mean difference and SD (0.2897 ± 0.94406) of absolute FMD (cm) for all participants, and the majority of values were within the 95% confidence interval (CI: -1.56065 to 2.14005).

In conclusion, the Philips Lumify Ultrasound system (Lumify L12-4 broadband linear array transducer) demonstrates a reliable, repeatable, and good agreement in FMD measurements in healthy volunteers. The Philips Lumify Ultrasound system will be used in the EDEN-OX study. Future significant changes will likely represent true changes in FMD and are not related to operator error.

Table 2.2 Mean, standard deviation and coefficient of variation of flow mediated dilatation (FMD)

Subjects	FMD at Visit 1	FMD at Visit 2
1	4.98	4.59
2	4.17	4.41
3	6.07	3.73
4	5.76	4.87
5	3.93	5.15
6	4.42	4.62
7	4.60	4.80
8	4.46	3.80
9	4.03	3.42
10	3.92	4.05
Mean $\pm$ SD	4.63 $\pm$ 0.75	4.34 $\pm$ 0.56
Coefficient of variation (CV)	0.16	0.13

Table 2.3 Intraclass Correlation Coefficient

Intraclass Correlation Coefficient							
	Intraclass Correlation ^b	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.633a	.023	.911	4.807	7	7	.028
Average Measures	.775	.044	.953	4.807	7	7	.028

Two-way random effects model where both people effects and measures effects are random.

- a. The estimator is the same, whether the interaction effect is present or not.
- b. Type A intraclass correlation coefficients using an absolute agreement definition.

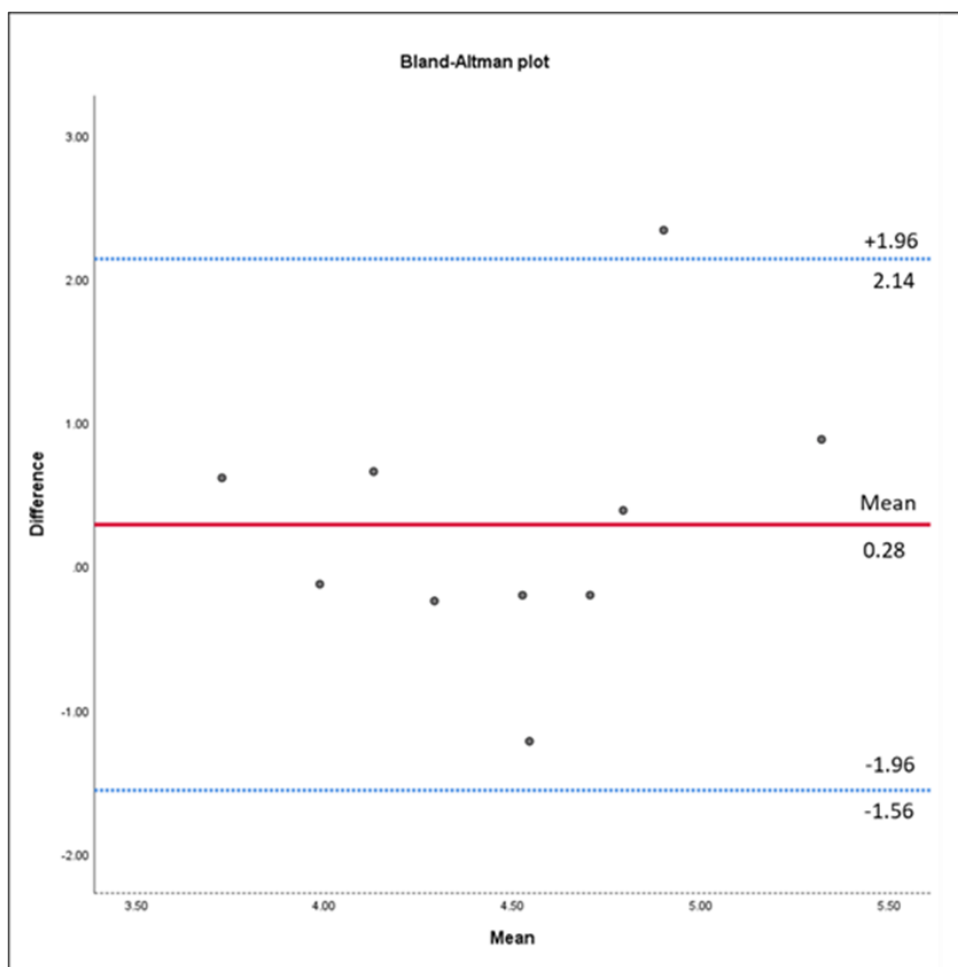


Figure 2.14: Bland Altman plot of brachial artery flow mediated dilatation (FMD) on two occasions.

2.13 Peripheral arterial tonometry (EndoPAT) test

The EndoPAT is a non-invasive technique that assesses vascular endothelial function by using finger plethysmography to detect the reactive hyperaemia-pulse wave amplitude (RH-PAT) at rest and shear stress (Yamaoka-Tojo, 2017). The RH-PAT index was calculated based on the ratio of pulse wave amplitude (PWA) signals after the cuff was released compared with the baseline using a computer algorithm. This change in PWA was recorded by pneumatic finger probes (Figure 2.15). These probes (PAT sensors) are a thimble-shaped, producing a uniform pressure around the distal phalanx of the finger, allowing pulsatile volume changes to be recorded.



Figure 2.15: Endothelial function assessed by EndoPAT™ 2000.

2.13.1 Participant preparation

Participants rested in a supine position for 15 minutes in a quiet, dimly lit temperature-controlled room (22–24°C) to reach a cardiovascular steady-state and reduce variations in vascular tone (Figure 2.16).



Figure 2.16: Patient position and EndoPAT device setup.

2.13.2 Equipment

Measurements were conducted using (Itamar Medical Ltd, Caesarea, Israel) according to the manufacture's protocol and instructions (Appendix 3).

2.13.3 Vascular occlusion

Probes were placed in the index fingers of both hands, and a cuff was placed on the non-dominant upper arm. The occlusion pressure was at least 60 mmHg above the systolic pressure (minimally 200 mmHg, and maximally 300 mmHg) and the digital pulse volume was measured at baseline, during the test and after five minutes of occlusion time.

2.13.4 Reactive hyperaemia measurements

Following five minutes of occlusion blood flow of the brachial artery using an inflated cuff pressure on one arm, the cuff was deflated allowing NO release from the endothelium which causes vasodilation, and this can be measured by the PAT sensors through beat to beat finger PWA.

2.13.5 EndoPAT reproducibility

Evidence has shown that the EndoPAT is a reproducible measure for assessing endothelial function. In a large cohort study involving 123 adults, Brant *et al.* (2013) showed the reproducibility of PAT measurements was not significantly different between the exams of each subject ($p = 0.48$) and there were no systemic errors between the tests (mean of differences = -0.03 ± 0.5). The measurement error was 0.35, with a coefficient variation of 18.0% and an ICC of 0.61 (Brant et al., 2013).

CHAPTER 3: Impact of dietary nitrate supplementation on exercise capacity and cardiovascular parameters in chronic respiratory disease: a systematic review and meta-analysis

3.1 Introduction

Exercise limitation is a common feature in individuals with chronic respiratory disease despite optimum medical treatment including pulmonary rehabilitation and pharmacotherapy (Elbehairy et al., 2019, Kelly et al., 2012, Philip et al., 2019). Factors contributing to breathlessness and reduced physical activity include altered pulmonary mechanics and cardiovascular function as well as skeletal muscle impairment (Maddocks et al., 2014, Shrikrishna et al., 2012, O'Donnell and Laveneziana, 2006, Jackson et al., 2013). Nitric oxide (NO) is a ubiquitous signalling molecule with a key role in endothelial function, and a relationship between plasma nitrite levels and exercise performance has been identified (Rassaf et al., 2007b, Dreißigacker et al., 2010). Dietary nitrate supplementation, which increases NO availability via a nitrate-nitrite-NO pathway has therefore been proposed as a potential complementary approach to improve exercise capacity in people with cardiorespiratory disease.

In healthy adults, endurance exercise capacity increases following dietary NO_3^- supplementation (McMahon et al., 2017, Van De Walle and Vukovich, 2018) and evidence suggests that NO_3^- supplementation with beetroot juice (BRJ) can reduce oxygen consumption (VO_2) during submaximal exercise and increase the time to reach exhaustion during high-intensity training (Bailey et al., 2009b, Lansley et al., 2011, Breese et al., 2013). Of note, dietary nitrate supplementation has been shown to improve exercise performance under hypoxic conditions (Masschelein et al., 2012) and there is evidence for an effect in some clinical conditions for example, chronic obstructive pulmonary disease (COPD) (Berry et al., 2015, Kerley et al., 2015, Kerley et al., 2019, Pavitt et al., 2020), peripheral vascular disease (PVD) (Kenjale et al., 2011) and heart failure (Eggebeen et al., 2016, Zamani et al., 2015).

In addition to exercise limitation, vascular comorbidities are common in people with lung disease and clinical guidelines highlight the need to identify and optimally treat them (Hopkinson et al., 2019). If dietary nitrate supplementation can be shown to have a beneficial effect on exercise capacity and / or vascular comorbidities, it has the potential to improve outcomes in this patient group.

We therefore aimed to evaluate the available evidence regarding the impact of nitrate supplementation on exercise capacity and cardiovascular parameters in individuals with respiratory disease.

3.2 Methods

The review was registered in the International Prospective Register of Systematic Reviews (PROSPERO) database (no. CRD42019130123). It was conducted based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

3.2.1 Search strategy

The following databases have been searched electronically from inception to March 2021: PubMed, CINHAL, MEDLINE (Ovid), Cochrane and Embase. The terms used in this search were ‘respiratory diseases’, ‘chronic obstructive pulmonary disease’, ‘COPD’, ‘chronic obstructive airways disease’, ‘emphysema’, ‘bronchitis’, ‘bronchiectasis’, ‘interstitial lung disease’, ‘ILD’, ‘cystic fibrosis’, ‘pulmonary hypertension’, ‘PHT’, ‘nitrate’, ‘beetroot’, ‘dietary nitrate’ and ‘nitrate supplementation’. A search filter was applied by using medical subject heading (MeSH) terms. This search strategy was conducted in collaboration with a librarian to ensure

this review contained the appropriate and necessary keywords. Full search strategy from all databases is presented in Appendix 1.

3.2.2 Inclusion criteria

I included both randomised clinical trials and crossover studies.

The PICO format was used in our search strategy:

1. P: population included adults diagnosed with CRD such as COPD, interstitial lung disease (ILD), bronchiectasis or pulmonary hypertension (PH) either undergoing usual care or taking part in pulmonary rehabilitation.
2. I: intervention was dietary NO_3^- supplementation delivered to patients with CRD.
3. C: comparator was a placebo group for interventional studies.
4. O: outcomes included both primary outcomes (exercise capacity and blood pressure) and secondary outcomes such as cardiovascular parameters: heart rate [HR], oxygen saturation [O_2 sat], plasma nitrate [NO_3^-] and nitrite [NO_2^-] levels, peak and iso-time VO_2 , endothelial function (flow-mediated dilatation [FMD]) and fractional exhaled nitric oxide [FeNO].

3.2.3 Exclusion criteria

1. Studies examining children under 18 years.
2. Any papers written in a language other than English.
3. Conference abstracts or unpublished data.

3.2.4 Data extraction

Data were extracted into a standardised Microsoft Excel spreadsheet form (Microsoft Corp., Redmond, WA, USA). We contacted the corresponding authors for missing data. Together with (AMA) I independently screened titles and abstracts of potential studies. A third reviewer (NSH) was available to resolve any disagreements. The form included data about change in exercise capacity following dietary NO_3^- supplementation. Other data such as cardiovascular parameters (HR, systolic blood pressure [SBP], diastolic blood pressure [DBP], mean arterial pressure [MAP] and O_2 sat), VO_2 , FMD, FeNO, intervention protocol (e.g. dose, duration, and delivery method), exercise protocol (type and duration) and placebo were extracted.

3.2.5 Data analysis

The synthesis of results described the outcomes of interest, such as exercise capacity, VO_2 , exercise endurance time, plasma NO_3^- level, plasma NO_2^- level, FeNO, SBP, DBP, MAP, HR, O_2 sat, and FMD. Where appropriate meta-analysis was conducted to estimate the pooled differences and 95% CIs in the outcomes of interests between nitrate and placebo conditions. For cross over studies, endpoint values were extracted from the end of the study after receiving the supplements (nitrate or placebo) and included in meta-analysis as if from a parallel designed trial (Elbourne et al., 2002, Higgins, 2011). The random effect model was applied because of the variety in several factors (e.g. exercise protocol, dose and duration of NO_3^- supplementation). Continuous data are reported as the mean difference (Δ). Standardised mean difference (SMD) was used when the same outcome was assessed with different measures (e.g. exercise capacity assessed using different walking tests). Heterogeneity among included studies was evaluating using the I^2 value. The statistical

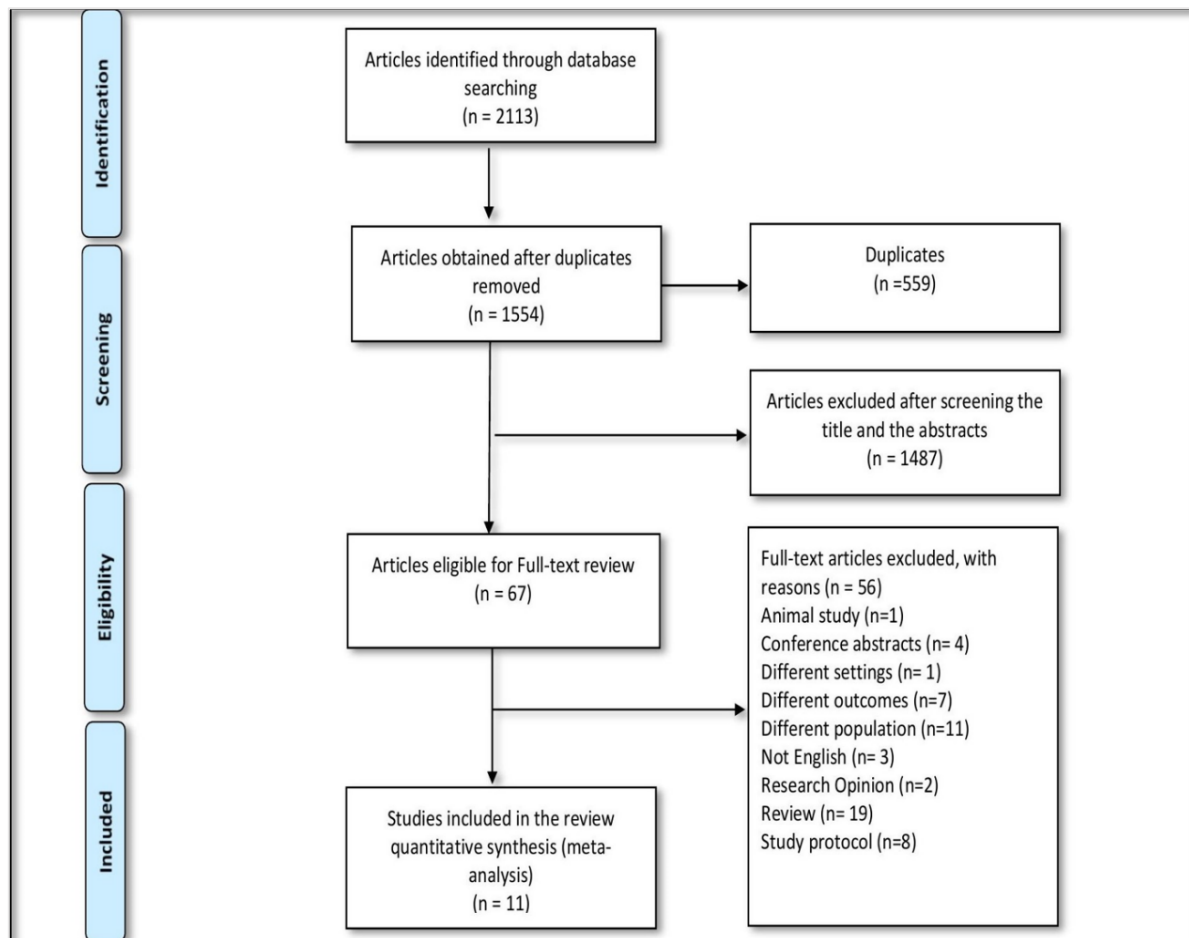
analyses were performed using the Cochrane Collaboration's Review Manager software (RevMan V. 5.4.0).

3.3 Results

3.3.1 Selection of studies

Initially, 2,113 articles were identified through the database searches, 1,554 after removing duplicates with 67 articles eligible for full-text review following title and abstract screening. Following full-text review, 11 studies met the inclusion criteria for the present systematic review (Figure 3.1).

Figure 3.1: Flowchart of study selection (Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow diagram)



3.3.2 Study characteristics

Among the 11 randomised controlled trials (RCT), two used a parallel design and nine used a crossover design. All 11 were published between 2015 and 2020. Eight studies were conducted in Europe, two in the United States of America, and one in Australia. Studies were categorised based on the reported duration of NO_3^- supplementation as “acute effect” (single dose of nitrate supplementation, 2.5 - 3 hrs before exercise session) (n=3), “short-term” (less than three months in usual care) (n=7) or “during pulmonary rehabilitation” (n=1). One study provided both acute effect and short-term data (Beijers et al., 2018). The total number of participants was 282, including 15 with pulmonary arterial hypertension (Henrohn et al., 2018) and 267 with COPD (Behnia et al., 2018, Beijers et al., 2018, Berry et al., 2015, Curtis et

al., 2015, Friis et al., 2017, Kerley et al., 2015, Kerley et al., 2019, Leong et al., 2015, Pavitt et al., 2020, Shepherd et al., 2015) with sample sizes ranging from 8 to 122. Age of participants (mean $\pm$ SD) was 66 ± 3 years, and the majority (57%) were male. Ten trials used beetroot juice (BRJ) as the source of nitrate (n=10), and one used sodium NO_3^- (NaNO_3) (Beijers et al., 2018). The dose of NO_3^- used ranged from 6.45 mmol (Behnia et al., 2018) to 16 mmol (Henrohn et al., 2018). A full description of the included studies is presented in (Table 3.1).

Table 3.1 Description of the included studies

Authors / Design	Study Sample	Nitrate dose	Placebo	Duration	Wash-out	Results (Following nitrate vs. Placebo)
Behnia et al, 2018/RCT	N = 25 GOLD stage 1-4 Age (y): 68±9 Sex (M/F): 13/12	70 ml BRJ+ 180 ml Black currant juice	70 ml water+ 180 ml black currant juice	8 d	No	- No effect on VO ₂ at peak (p>0.05)) - Significant change SBP: 11mmHg (p<0.05) at peak exercise in the nitrate group only compared to pre nitrate - Significant increase in FeNO (ppb) in nitrate group (p<0.05) - No effect on DBP (p>0.05) - No effect on heart rate (p>0.05)
Beijers et al, 2018/RXT	N = 18 GOLD stage 1-2 Age (y)= 67 ± 8 Sex (M/F) = 13/5 FEV ₁ % = 69.2	sodium nitrate (8mmol)	NaCl ingestion	Acute and 7 d	7 d	- No effect on endurance cycle time (p=0.08) - No effect on VO ₂ on Day 1 and Day 7 during submaximal cycling at 70% Wmax (p=0.56) - Significant increase in plasma NO ₃ ⁻ level on Day 1 and Day 7 (p<0.001) - Significant increase on NO ₂ ⁻ level on Day 1 (p<0.001) and Day 7 (p=0.003) - No effect on SBP on Day 1 and Day 7 at 150 min(p=0.66) - No effect on DBP on Day 1 and Day 7 at 150 min (p=0.35) - No effect on physical activity, counts/min (p=0.53) - No effect on heart rate on Day 1 and Day 7 at 150 min (p=0.76)
Berry et al, 2015/RXT	N = 15 GOLD stage 1-2 Age (y): 70±9 Sex (M/F): 12/3 FEV ₁ % =61.8	140 ml BRJ (7.58 mmol)	163 ml prune juice (0.01 mmol nitrate)	Acute	7 d	- 28.8 sec. longer in endurance cycle time (p=0.031) - No effect on VO ₂ at end exercise,(P=0.43) - Significant increase in plasma NO ₃ ⁻ level (p<0.001) - Significant increase in plasma NO ₂ ⁻ level (p=0.001) - Significant change in resting SBP: 8.2 mmHg, p=0.019 - Significant change iso-time DBP: 6.4 mmHg (p=0.001) - Significant change end exercise DBP: 5.6 mmHg (p=0.08) - No effect on HR, p=0.70 - No effect on O ₂ saturation, p=1.0
Curtis et al, 2015/RXT	N = 21 GOLD stage 2-4 Age (y): 68±7 Sex (M/F): 16/5 FEV ₁ % =50.1	140 ml BRJ(12.9 mmol)	140 ml ND-BRJ	Acute	7 d	- No effect on median endurance time (P=0.50) - Significantly lower iso-time VO ₂ (p=0.04) - Significant increase in plasma NO ₃ ⁻ level (p<0.0001) - No effect on SBP - Significant change DBP: 7mmHg (p=0.01) - No effect on MAP (p=0.07) - No effect on heart rate (p=0.06) - No effect on O ₂ saturation (p=0.26)
Friis et al, 2015/RXT	N = 15 GOLD stage 2-4 Age (y): 63±13 Sex (M/F): 9/6 FEV ₁ % =44.7	140 ml BRJ	140 ml ND-BRJ	7 d	7 d	- No effect on exercise capacity (p=0.46) - No effect on VO ₂ (p=0.31) - Significant increase in plasma NO ₂ ⁻ level (p<0.01) - No effect on SBP (p=0.80) - Significant reduction on DBP (p<0.05) - No effect on heart rate (p=0.86) - No effect on physical activity (p>0.05)

Henrohn et al, 2018/RXT	N = 15 Pulmonary hypertension, WHO group 1 Age (y): 60±15 Sex (M/F): 7/8	140 ml BRJ (16 mmol)	140 ml ND-BRJ (0.118 mmol nitrate)	7 d	4-9 d	- No effect on exercise capacity (P= 0.445) - No effect on VO₂ (p=1.00) - Significant higher in FeNO levels (p<0.001) - Significant increase in plasma NO₃⁻ level (p<0.001) - Significant increase in plasma NO₂⁻ level (p<0.001) - No effect on SBP (p=0.482) - No effect on DBP (p=1.000) - No effect on heart rate (P= 0.191)
Kerley et al, 2015/RXT	N = 11 GOLD stage 2-4 Age (y): 69±7 Sex (M/F): 5/6 FEV ₁ % =43.4	140 ml BRJ+ 200 ml blackcurrant cordial	140 ml water + 200 ml blackcurrant cordial	Acute	7 d	- ISWT distance increased 25 m (p=0.005) - Significant increase in plasma NO₃⁻ level (p=0.000005) - Significant increase in plasma NO₂⁻ level (p<0.01) - Significant change SBP: 12 mmHg (p=0.03) - Significant change DBP: 2 mmHg (p=0.045) - Significant change MAP: 5 mmHg (p=0.018) - No effect on heart rate (p=0.19) - No effect on O₂ saturation (p=0.71)
Kerley et al, 2019/RXT	N = 8 GOLD stage 1-3 Age (y): 63±7 Sex (M/F): 5/3 FEV ₁ % =55	140 ml BRJ (12.9 mmol)	140 ml ND-BRJ (0.5 mmol nitrate)	14d	NA	- ISWT distance increased 56 m (p=0.0004) - Significant increase in plasma NO₃⁻ level (p=0.015) - Significant increase in plasma NO₂⁻ level (p=0.02) - No effect on FeNO level (p= 0.095) - No effect on SBP (p= 0.14) - No effect on DBP (p= 0.35)
Leong et al, 2015/RXT	N = 19 GOLD stage 2 Age (y): 67±8 Sex (M/F): 5/14 FEV ₁ % =62	140 ml BRJ (9.6 mmol)	140 ml ND-BRJ (0.0056 – 0.020 mmol nitrate)	3d	4d	- Endurance distance increased 79 m (p=0.494) - Increase time to fatigue by 6% (p=0.693) - Significant change SBP in safety phase: 10 mmHg at 1hr standing (p=0.001) and 7.5 mmHg at 4hr standing (p=0.029) - No effect on DBP in safety phase: 0.1 mmHg at 1hr standing (p=0.966) and 2.7 mmHg at 4hr standing (p=0.352)
Pavitt et al, 2020/RCT During PR	N = 122 GOLD stage 2-4 Age (y): 68±10 Sex (M/F): 69/53 FEV ₁ % =49	140 ml BRJ (12.9 mmol)	140 ml ND-BRJ	56 d	No	- Significant increase ISWT distance by 60m (p=0.027) - Significant change SBP: 5 mmHg (p<0.0005) - Significant change DBP: 5 mm Hg (p<0.0005) - Significant change MAP: 5.0 mm Hg (p<0.0005) - Significant change in the FMD in BRJ group +6.6 % (p= 0.046) - Significant increase in physical activity by 348 step/day (p=0.02)
Shepherd et al, 2015/RXT	N = 13 GOLD stage 1-2 Age (y): 65±8 Sex (M/F): NR FEV ₁ % =57	140 ml BRJ (13.5 mmol)	140 ml ND-BRJ (0.004mmol nitrate)	2.5 d	7 d	- No effect on 6MWD (p=0.17) - No effect on baseline VO₂(p=0.56) and end exercise VO₂ (p=0.88) - Significant increase in plasma NO₃⁻ level (p=0.002) - No effect on SBP (p=0.91) - No effect on DBP (p=0.25)

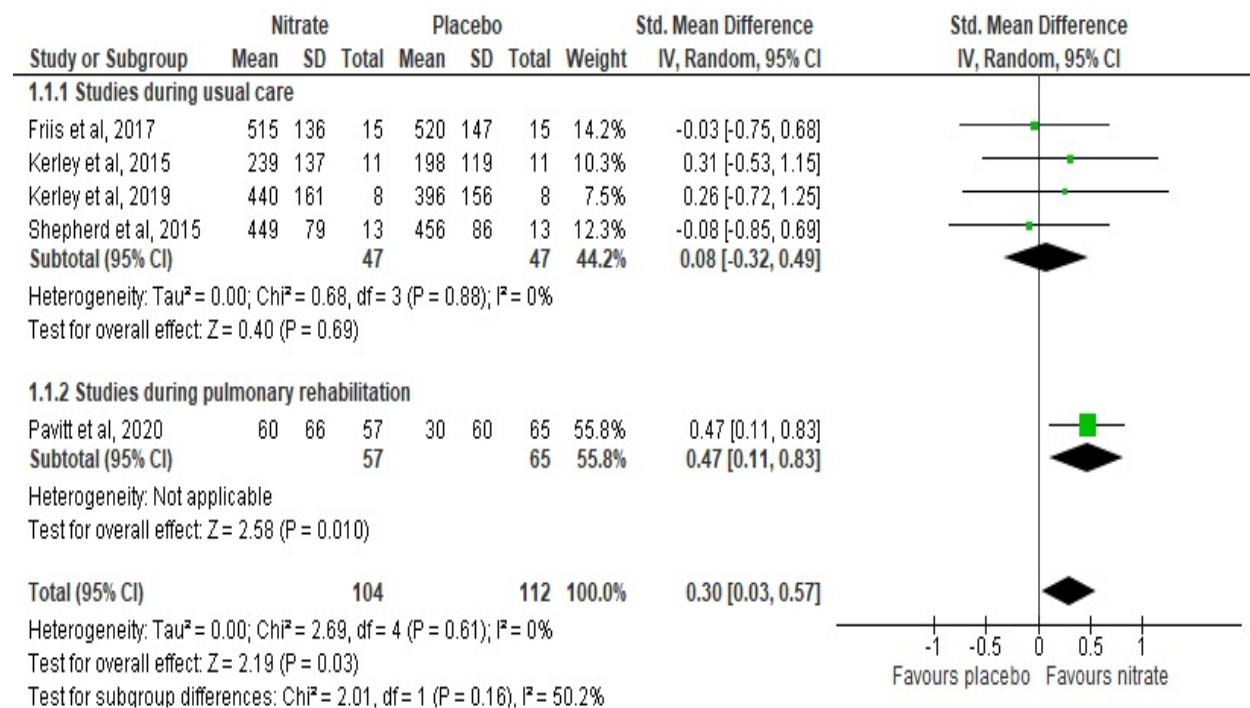
Abbreviations: RCT: Randomised control trial; RXT: Randomised crossover trial; N: number of participants who completed the trial; NA: Not available; COPD: Chronic obstructive pulmonary disease; PHT: Pulmonary arterial hypertension; BRJ: Beetroot juice; ND-BRJ: Nitrate-depleted beetroot juice; Y: Yes; NR: Not reported; D: days; VO₂: Oxygen consumption; FeNO: Fractional exhaled nitric oxide; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; MAP: Mean arterial pressure; ESWT: Endurance shuttle walk test; ISWT: Incremental shuttle walk test; 6MWD: 6 min walk distance; FMD: Flow mediated dilatation.

3.3.3 Exercise capacity

Data on exercise capacity or endurance were reported in ten studies using different tests including the incremental shuttle walk test (ISWT) (n=3), six- minute walk distance (6MWD) (n= 3), endurance time during cycle ergometry (n=3), and endurance shuttle walk test (ESWT) (n=1) in individuals with COPD (Beijers et al., 2018, Berry et al., 2015, Curtis et al., 2015, Friis et al., 2017, Kerley et al., 2015, Kerley et al., 2019, Leong et al., 2015, Pavitt et al., 2020, Shepherd et al., 2015) and pulmonary hypertension (Henrohn et al., 2018).

The impact of nitrate supplementation on peak exercise capacity measured using walking tests (ISWT and 6MWD) in people with COPD is shown in (Figure 3.2). Pooled analysis from five trials demonstrated an improvement in exercise capacity following nitrate supplementation compared to placebo, (SMD (95% CI), 0.30 (0.03 to 0.57), p=0.03) (Friis et al., 2017, Kerley et al., 2015, Kerley et al., 2019, Pavitt et al., 2020, Shepherd et al., 2015) although this effect was driven by the study in the context of PR. Thus supplementation was associated with increased walk distance in the context of pulmonary rehabilitation (SMD (95% CI), 0.47 (0.11 to 0.83), p=0.01), but no effect was identified in short term studies (0.08 (-0.32 to 0.49). A single trial in 15 patients with pulmonary hypertension patients taking BRJ for one week did not show a significant effect on 6MWD (Henrohn et al., 2018).

Figure 3.2: Forest plot for the effect of nitrate supplementation on peak exercise capacity measured with incremental shuttle walk test or 6-minute walk distance (in metres) in patients with chronic obstructive pulmonary disease



Berry et al. (2015) found an improvement in endurance exercise capacity during cycle ergometry at 75% maximal workload (Berry et al., 2015). In contrast, results from two trials providing nitrate supplementation (one using BRJ and one NaNO_3) did not demonstrate a significant improvement in endurance exercise time during cycle ergometry (Beijers et al., 2018, Curtis et al., 2015).

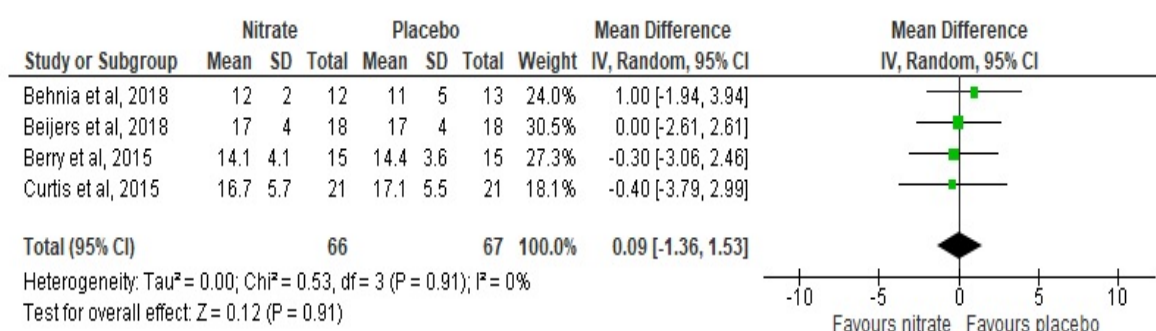
Similarly, Leong et al. (2015) investigated the effect of three days of BRJ on exercise endurance via ESWT at 85% VO_2 max, among stable moderate COPD patients and found no difference in exercise endurance following BRJ compared with placebo (Leong et al., 2015).

3.3.4 Effect of NO₃⁻ supplementation on physiological and cardiovascular parameters

3.3.4.1 Oxygen consumption

The impact of nitrate supplementation on peak VO₂ was reported in six studies (Figure 3.3) (Behnia et al., 2018, Beijers et al., 2018, Berry et al., 2015, Curtis et al., 2015, Friis et al., 2017, Henrohn et al., 2018). Pooled analysis from four trials demonstrated that peak VO₂ did not change following nitrate supplementation compared to placebo, (MD (95% CI), 0.09 ml/min/kg (-1.36 to 1.53), p=0.91 (Behnia et al., 2018, Beijers et al., 2018, Berry et al., 2015, Curtis et al., 2015).

Figure 3.3: Forest plot for the effect of nitrate supplementation on peak oxygen consumption (ml/min/kg) in patients with chronic obstructive pulmonary disease



However, VO₂ at isotime was reported in two studies; Curtis et al. (2015) report a significant decrease in isotime VO₂ after NO₃⁻ supplementation compared to a placebo (BRJ 16.6 ± 6.0 mL/min/kg, placebo 17.2 ± 6.0 mL/min/kg; p= 0.043) (Curtis et al., 2015). Differently, Berry et al. (2015) failed to find lower isotime oxygen uptake (median + IQR; BRJ 14.1+5.4, placebo 13.4+5.5; p=0.099) (Berry et al., 2015). I was unable to perform a meta-analysis of isotime oxygen uptake due to incomplete data. Isotime oxygen consumption and other cardiopulmonary exercise parameters are provided in (Table 3.2).

Table 3.2 Isotime oxygen saturation and other cardiopulmonary exercise parameters

Study	Parameter	Placebo	BRJ	P value
Berry et al, 2015	HR	112 [99, 124]	110 [97, 123]	0.300
	SBP	167.1 [151.7, 182.4]	160.1 [147.8, 172.5]	0.137
	DBP	86.3 [79.6, 92.9]	79.9 [72.8, 86.9]	0.001
	VO ₂ *	13.4 + 5.5	14.1 + 5.4	0.099
	O ₂ saturation	95.1[94.0, 96.2]	95.1 [93.9, 96.2]	0.895
Curtis et al, 2015	HR	122 (17)	121 (20)	0.30
	VO ₂	17.2 (6.0)	16.6 (6.0)	0.043
	O ₂ saturation	92 (4)	93 (4)	0.15

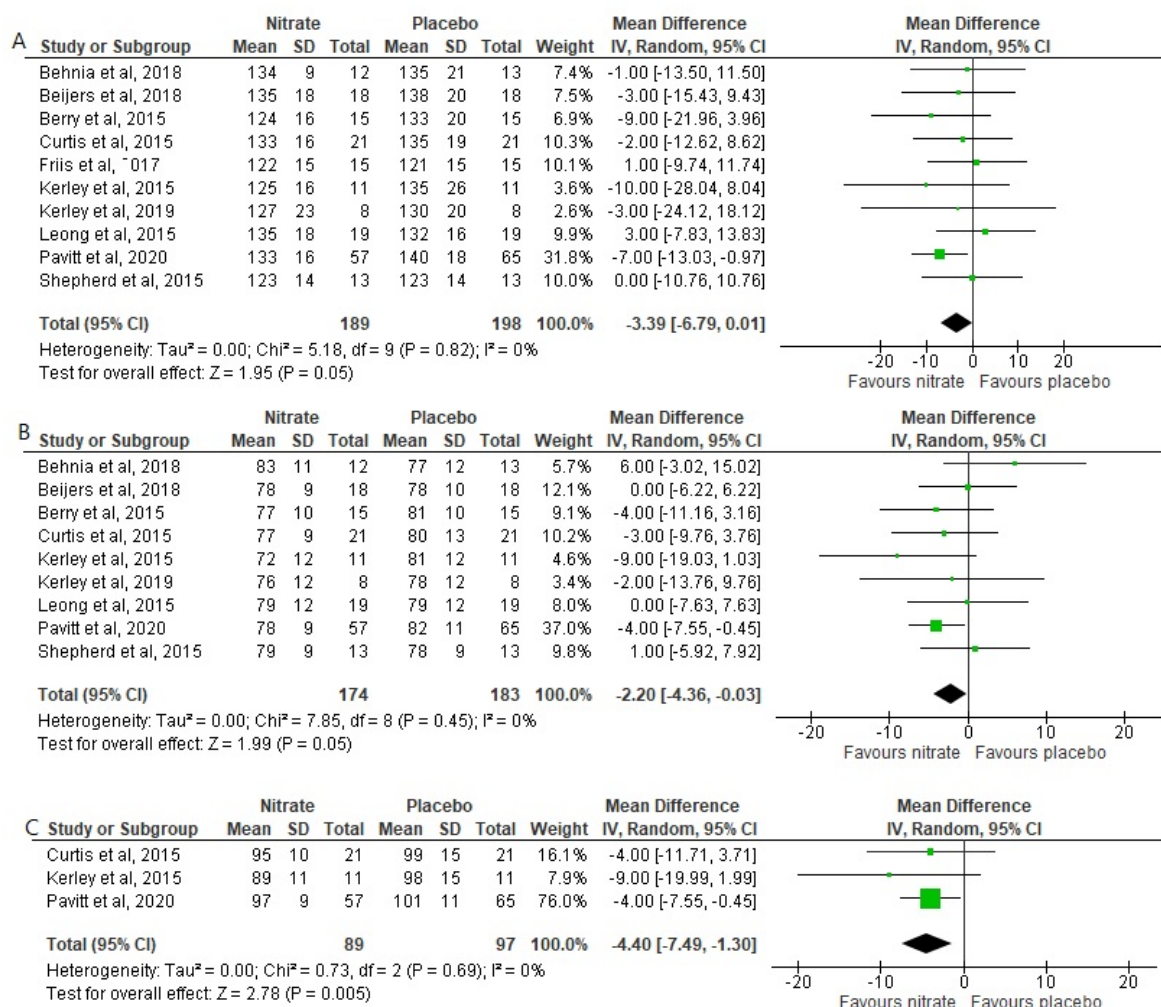
- *Berry et al, 2015: * Non-normally distributed variables are presented as medians and interquartile ranges. Normally distributed values are presented as means and [95% confidence intervals.*

- *Curtis et al, 2015: Data are presented as mean (SD).*

3.3.4.2 Blood pressure

Systolic and diastolic blood pressures were reported in all included studies while mean arterial pressure was reported in three studies (Curtis et al., 2015, Kerley et al., 2015, Pavitt et al., 2020). Meta-analysis for systolic, diastolic and mean arterial blood pressure in people with COPD found significant reductions compared to placebo (Figure 3.4); (MD (95% CI) was -3.39 mmHg (-6.79 to 0.01), $p=0.05$ for systolic blood pressure, -2.20 mmHg (-4.36 to -0.03), $p=0.05$ for diastolic blood pressure and -4.40 mmHg (-7.49 to -1.30), $p=0.005$ for mean arterial blood pressure (Behnia et al., 2018, Beijers et al., 2018, Berry et al., 2015, Curtis et al., 2015, Friis et al., 2017, Kerley et al., 2015, Kerley et al., 2019, Leong et al., 2015, Pavitt et al., 2020, Shepherd et al., 2015). However, in one study in individuals with pulmonary hypertension systolic and diastolic blood pressure did not significantly change following nitrate supplementation compared to placebo (Henrohn et al., 2018).

Figure 3.4: Forest plot for the effect of nitrate supplementation on (A) systolic blood pressure (mm Hg), (B) diastolic blood pressure (mm Hg) and (C) mean arterial blood pressure (mm Hg) in patients with chronic obstructive pulmonary disease

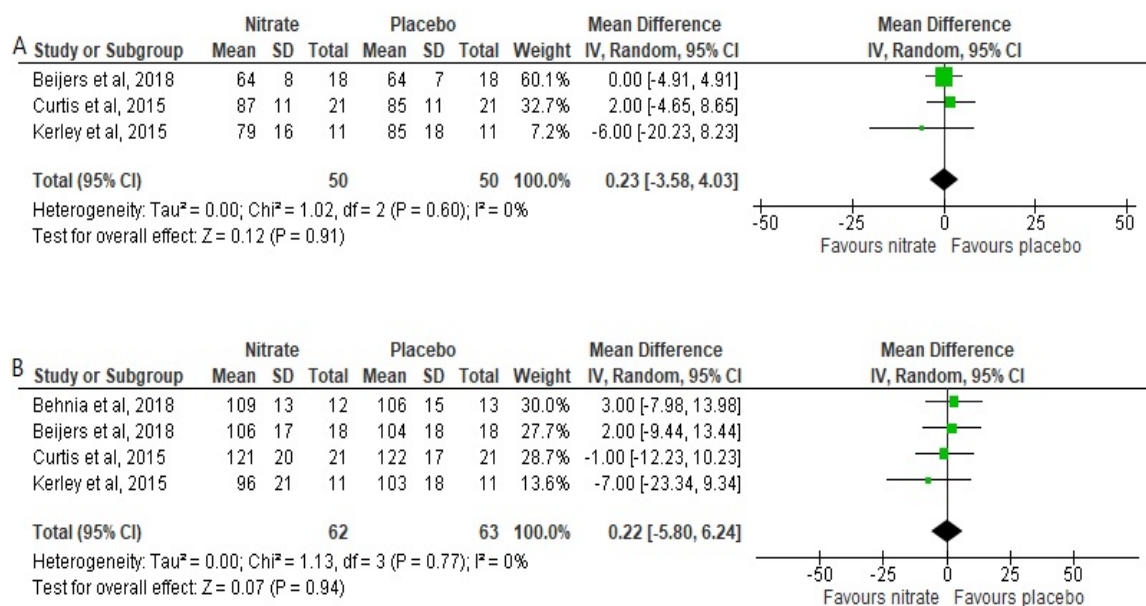


3.3.4.3 Heart rate

The impact of nitrate supplementation on HR was reported in seven studies (Behnia et al., 2018, Beijers et al., 2018, Berry et al., 2015, Curtis et al., 2015, Friis et al., 2017, Henrohn et al., 2018, Kerley et al., 2015). Pooled analysis from four trials of heart rate at rest and at peak of exercise in people with COPD is shown in (Figure 3.5). Following the meta-analysis, the (MD (95% CI) was 0.23 (-3.58 to 4.03), $p=0.91$ for heart rate at rest and 0.22 (-5.80 to 6.24), $p=0.94$ for heart rate at peak of exercise, showing no change in HR following nitrate supplementation

compared to placebo (Behnia et al., 2018, Beijers et al., 2018, Curtis et al., 2015, Kerley et al., 2015).

Figure 3.5: Forest plot for the effect of nitrate supplementation on (A) heart rate at rest (bpm) and (B) heart rate at peak (bpm) in patients with chronic obstructive pulmonary disease



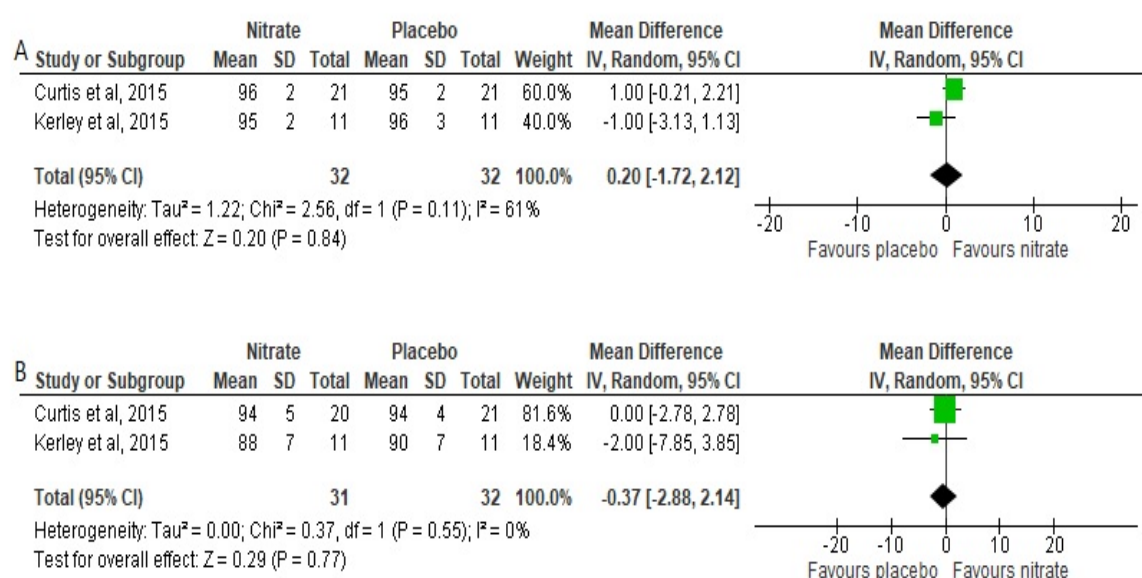
3.3.4.4 Endothelial function

Pavitt et al. (2020) assessed the impact of NO_3^- supplementation during pulmonary rehabilitation on endothelial function using brachial artery flow mediated dilatation (Pavitt et al., 2020), finding an improvement (increase) in FMD in the treatment group ($n=10$) compared to placebo ($n=10$), (median (IQR) percent change; +6.6% (0.6, 17.6), placebo -4.7% (-21.5,11.8), estimated treatment effect -20.3% (95% CI -33.8 to 3.4); $p=0.046$.

3.3.4.5 O₂ saturation

The impact of dietary NO₃⁻ supplementation on O₂ saturation was reported in three studies (Berry et al., 2015, Curtis et al., 2015, Kerley et al., 2015). Pooled analysis from two trials for oxygen saturation at rest and at peak exercise in COPD is shown in (Figure 3. 6). The (MD (95% CI) was 0.20 (-1.72 to 2.12) %, p=0.84 for oxygen saturation at rest and -0.37 (-2.88 to 2.14) %, p=0.77 for oxygen saturation at peak of exercise, showing no effect on oxygen saturation following nitrate supplementation compared to placebo (Curtis et al., 2015, Kerley et al., 2015). Curtis et al did demonstrate a reduction in area under the curve for oxygen saturation during exercise with nitrate supplementation compared to placebo (Curtis et al., 2015). Of note this study excluded individuals with resting hypoxia.

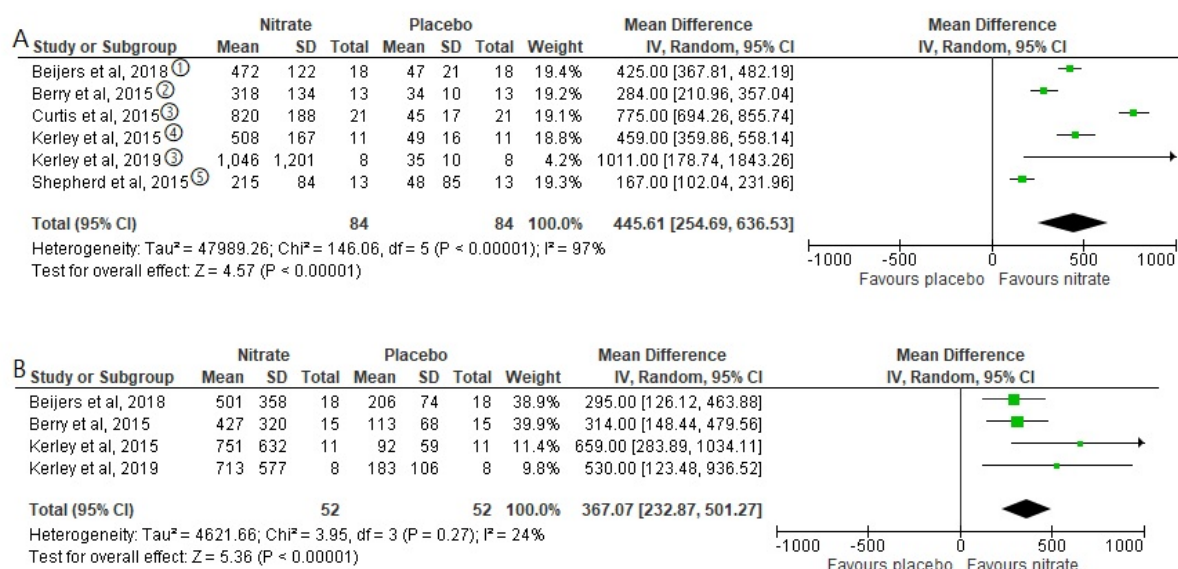
Figure 3.6: Forest plot for the effect of nitrate supplementation on (A) oxygen saturation at rest (%) and (B) oxygen saturation at peak (%) in patients with chronic obstructive pulmonary disease



3.3.5 Plasma NO_3^- and NO_2^- levels

Plasma NO_3^- and NO_2^- levels were measured in seven studies (Beijers et al., 2018, Berry et al., 2015, Curtis et al., 2015, Henrohn et al., 2018, Kerley et al., 2015, Kerley et al., 2019, Shepherd et al., 2015). Pooled analysis from six trials for plasma NO_3^- and NO_2^- levels in COPD individuals is shown in (Figure 3.7). Following the meta-analysis, the (MD (95% CI) was 445.61 (254.69 to 636.53), $p < 0.00001$ for plasma NO_3^- level and 367.07 (232.87 to 501.27), $p < 0.00001$ for plasma NO_2^- level, showing that levels of plasma NO_3^- and NO_2^- significantly increased following NO_3^- supplementation in compared to placebo (Beijers et al., 2018, Berry et al., 2015, Curtis et al., 2015, Kerley et al., 2015, Kerley et al., 2019, Shepherd et al., 2015).

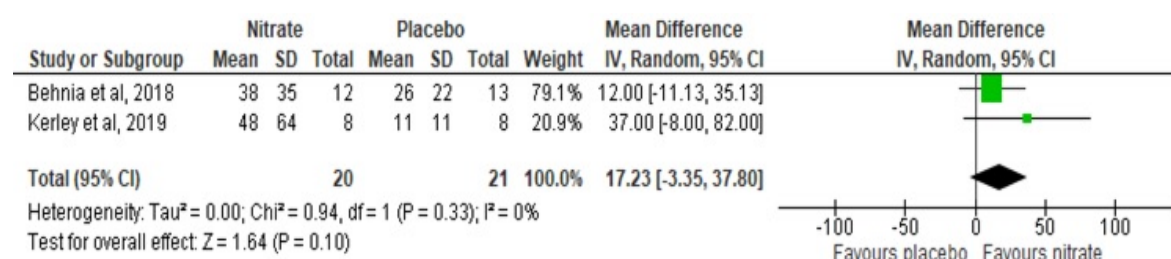
Figure 3.7: Forest plot for the effect of nitrate supplementation on (A) plasma nitrate (μM) and (B) plasma nitrite (nM) levels in patients with chronic obstructive pulmonary disease (1) sodium nitrate (8 mmol); (2) beetroot juice (BRJ) (7.58 mmol); (3) BRJ (12.9 mmol); (4) 140 mL of BRJ + 200 mL black currant cordial; (5) BRJ (6.75 mmol)



3.3.6 Fractional exhaled NO

The impact of nitrate supplementation on FeNO was measured in three trials, two conducted in individuals with COPD (Behnia et al., 2018, Kerley et al., 2019), and one in pulmonary hypertension (Henrohn et al., 2018). Pooled analysis from two trials for FeNO in COPD individuals is shown in (Figure 3.8). Following the meta-analysis, the (MD (95% CI) was 17.23 (-3.35 to 37.80)ppb, $p=0.10$ for FeNO, showing no consistent effect on FeNO following nitrate supplementation compared to placebo (Behnia et al., 2018, Kerley et al., 2019).

Figure 3.8: Forest plot for the effect of nitrate supplementation on fractional exhaled nitric oxide (ppb) in patients with chronic obstructive pulmonary disease



However, Henrohn et al. (2018) found that the level of FeNO in individuals with pulmonary hypertension increased at all flow rates (50 - 300mL/s) following nitrate supplementation compared to placebo; at a flow rate 50 ml/s (median of differences 18, 95% CI [11,26]), $P<0.001$ (Henrohn et al., 2018).

3.3.7 Risk of bias and evidence quality assessment

Using the Cochrane risk-of-bias assessment tool, (Higgins et al., 2011) the included studies showed considerable variation in the risk of bias, but most were limited by a lack of allocation concealment, blinding, and incomplete reporting of data (Figure 3.9).

The Grading of Recommendations, Assessment, Development and Evaluations (GRADE) (Cochrane Training, 2021) criteria were used to assess the overall evidence around specific outcomes (e.g. exercise capacity and blood pressure endpoints). For exercise capacity, the majority of studies were small and short term which limiting the precision of estimates. Studies did not focus clearly on disease severity, limiting the directness of the evidence to COPD phenotypes, particularly hypoxic patients. Further, studies used a variety of interventions (e.g. doses, duration, and delivery method), and outcome measures contributing to heterogeneity or inconsistency. Therefore, the quality of evidence by GRADE to support an effect of nitrate supplementation on exercise capacity in people with lung disease is low. In the specific context of pulmonary rehabilitation, only a single high quality randomised control trial, the largest and the longest of the included studies, was identified. Therefore, the total evidence by GRADE to support the impact of nitrate supplementation on exercise capacity in the context of pulmonary rehabilitation is moderate. Regarding blood pressure endpoints, studies were consistent, although the longest duration study is only eight weeks so taken together, the evidence to support an impact of nitrate supplementation on blood pressure is moderate.

Figure 3.9: Risk of bias summary: review authors' judgements about each risk-of-bias item for each included study

	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias
Behnia et al, 2018	?	-	?	-	?	+	?
Beijers et al, 2018	?	?	+	?	-	-	+
Berry et al, 2015	?	-	-	+	?	+	?
Curtis et al, 2015	+	+	+	+	?	+	+
Friis et al, 2017	+	?	?	-	+	+	?
Henrohn et al, 2018	?	?	+	-	?	+	?
Kerley et al, 2015	?	-	?	-	+	+	-
Kerley et al, 2019	+	?	?	-	?	-	-
Leong et al, 2015	+	?	?	-	-	+	?
Pavitt et al, 2020	+	+	+	+	+	+	?
Shepherd et al, 2015	+	?	?	-	?	?	?

 Low risk of bias
  Unclear risk of bias
  High risk of bias

3.4 Discussion

The main findings of this review into the effects of dietary nitrate supplementation in people with chronic respiratory disease are that, although it can augment the effects of pulmonary rehabilitation on exercise performance, a consistent short term effect on exercise capacity in the absence of exercise training has not so far been demonstrated. However, studies to date do suggest that dietary nitrate supplementation can lower blood pressure, perhaps by improving endothelial function, which is potentially important given the high prevalence of cardiovascular disease in people with COPD, especially if a single intervention could address both issues.

3.4.1 Significance of findings

NO is a vital physiological mediator in the body. It is produced in two different ways: by an endogenous pathway (oxygen-dependent) via the L-arginine NO synthase system, and by an exogenous pathway (oxygen-independent) via the reduction of dietary nitrate via nitrite to NO (Alderton et al., 2001, Lundberg et al., 2008). In the human diet, the main source of NO_3^- is green leafy vegetables, which have high concentrations of NO_3^- . NO_3^- reduction to NO is favoured by conditions found in exercising muscle, in particular hypoxia, acidosis and the presence of deoxyhaemoglobin and myoglobin. Effects on exercise in people with respiratory disease could be mediated through improved muscle mitochondrial efficiency or through effects on vascular endothelium either in the systemic or pulmonary circulation. Endothelial effects of NO are also likely to underpin the effects on blood pressure that were observed.

Regarding the impact of dietary nitrate supplementation on exercise capacity, the current meta-analysis includes studies in COPD which found an improvement in exercise capacity

(Berry et al., 2015, Kerley et al., 2015, Kerley et al., 2019, Pavitt et al., 2020) and others which did not (Behnia et al., 2018, Beijers et al., 2018, Curtis et al., 2015, Friis et al., 2017, Leong et al., 2015, Shepherd et al., 2015). Studies included heterogeneous COPD populations (e.g. COPD severity, age), and used different exercise protocols (e.g. ISWT, 6MWD, and endurance time during cycle ergometry). Furthermore, hypoxic patients who required oxygen supplementation, a patient phenotype that might be expected to benefit most the given that nitrite to NO conversion is enhanced in hypoxic conditions, were typically excluded. The duration of treatment and doses of nitrate used in trials also differed. The results from trials indicate that longer term studies in specific patient phenotypes are needed to see if nitrate supplementation can improve exercise capacity in the absence of a training stimulus. Likewise, although dietary nitrate supplementation was associated with a greater increase in walk distance during pulmonary rehabilitation, it is not clear how long this benefit might be sustained for - the 8-week ON-EPIC trial (Pavitt et al., 2020) is the longest study to date of this intervention in people with chronic respiratory disease.

Physiological parameters at peak exercise including VO_2 did not change significantly with nitrate supplementation compared to placebo (Behnia et al., 2018, Beijers et al., 2018, Berry et al., 2015, Curtis et al., 2015, Friis et al., 2017, Henrohn et al., 2018, Shepherd et al., 2015), although, one study found a significant reduction in VO_2 at iso-time during cycle exercise in COPD patients (Curtis et al., 2015). Again, these negative results could be due to an absence of effect or be a result of using insufficient dose or duration of supplementation. A dose response effect for reduction in oxygen consumption during exercise has previously been described in healthy individuals (Wylie et al., 2013).

Dietary nitrate supplementation has been shown to reduce blood pressure in individuals who are either normotensive (Hobbs et al., 2012) or hypertensive (Kapil et al., 2015). We found an overall effect to lower systolic, diastolic and mean arterial pressure in the studies reviewed here. People with lung disease are at high risk of cardiovascular disease, and this includes damage to the pulmonary vascular bed which can lead to pulmonary hypertension (Chaouat et al., 2005). There is also interesting data in individuals with idiopathic pulmonary hypertension which demonstrate that a low level of plasma nitrate is associated with increased mortality risk making it a potential prognostic indicator for pulmonary hypertension (Zhang et al., 2016). Although one study with pulmonary hypertension was identified by our search strategy it was small, we advise against overinterpreting it and further studies are needed.

Plasma NO_3^- and NO_2^- levels have potential to be used as a biomarker for NO availability (Kleinbongard et al., 2003). As expected, the available evidence showed that plasma NO_3^- and NO_2^- levels increased following dietary nitrate supplementation. The fractional exhaled nitric oxide has been used as a diagnostic test for asthma (Deykin et al., 2002, Smith et al., 2004). However, studies describing the FeNO level in people with COPD are inconclusive. In this review, two studies showed that FeNO level increased following nitrate supplementation (Behnia et al., 2018, Henrohn et al., 2018), while another study had a negative result (Kerley et al., 2019) so further work is needed to clarify this. Further work is needed to establish if FeNO can be used as a biomarker to monitor compliance in therapeutic trials of nitrate supplementation or even to adjust dose in individuals.

3.4.2 Strengths and limitations

A variety of lessons can be learned from this review. First, most of the trials covering the effect of dietary nitrate supplementation on exercise capacity in people with respiratory disease have focused on COPD, with only one on pulmonary hypertension. Most trials were short-term. Second, trials involved a variety of study designs, outcome measures, clinical phenotypes (severity of the disease, and of hypoxia in particular), exercise protocols, and dose and duration of nitrate supplementation. Third, it will be important to define whether there are different COPD phenotypes or subpopulations that can be categorised as nitrate responders or non-responders. Fourth, in most studies beetroot juice was used as the source of nitrate. It is possible that other bioactive compounds in the juice, that have antioxidant and anti-inflammatory properties including Vitamin C, carotenoids, phenolics and betalains, could contribute to beneficial effects. Many but not all studies have used nitrate depleted beetroot juice as a control which is ideal for identifying effects of nitrate itself, but runs the risk of underestimating the effect of beetroot juice itself if these other components have a role. Finally, none of the trials we identified for this review evaluate the effect of dietary nitrate supplementation on exercise capacity or cardiovascular parameters in people with rarer lung diseases such as ILD and cystic fibrosis.

3.5 Conclusion

Dietary nitrate supplementation has potential to reduce cardiovascular risk by lowering blood pressure in people with COPD as well as improving exercise capacity, though evidence for the latter is largely in the context of pulmonary rehabilitation. Importantly, further work is needed to understand whether it is the rehab setting that is giving the benefit (i.e. combining

supplementation with exercise etc) or whether it is purely due to the fact that participants were followed for a longer duration than in other non-PR studies. To date, no data exist which might support dietary nitrate supplementation for lung diseases other than COPD. The data support trials of dietary nitrate supplementation in COPD patients to address vascular endpoints and we would suggest that exercise capacity also be measured in such trials to see whether a 'dual benefit' can be elicited. Outside the context of pulmonary rehabilitation further trials are indicated to evaluate the value of dietary nitrate supplementation on exercise capacity in COPD, and specifically to identify the phenotypes most likely to profit from this intervention.

CHAPTER 4: Effect of dietary nitrate supplementation on exercise performance in pulmonary hypertension: The EDEN-OX trial

4.1 Introduction

Exercise limitation is a common feature in people with pulmonary hypertension (PH), caused by complex interactions between the pulmonary, cardiovascular, and musculoskeletal systems (Babu et al., 2017). Various factors can contribute to the development of PH. One of these is hypoxia, which causes pulmonary vasoconstriction and vascular remodelling resulting in thickening of the intima and neo-muscularisation of the arterioles, both of which increase pulmonary vascular resistance (PVR) (Stenmark et al., 2006).

It has been conclusively demonstrated that the endothelial dysfunction in the pulmonary vasculature is induced in part by decreased NO production from eNOS and/or decreased NO bioavailability (Demoncheaux et al., 2005, Giaid and Saleh, 1995).

The systematic review and meta-analysis presented in Chapter 3 of this thesis suggests that dietary nitrate supplementation has the potential to improve exercise performance as well as reducing cardiovascular risk in individuals with COPD by improving endothelial function and lowering blood pressure, although studies to date have generally been short term (Alsulayyim et al., 2021). Existing studies on dietary nitrate supplementation in people with lung disease have mostly focused on COPD, typically in less severe disease, but it is notable that a single dose of NR-BRJ consistently increased exercise capacity in people with COPD who needed to use home oxygen (Pavitt et al., 2021).

Therefore, in this chapter, I aimed to evaluate whether acute administration of dietary nitrate supplementation in the form of BRJ improves exercise performance in PH patients who are using ambulatory oxygen or desaturating on exercise. Furthermore, the effect of dietary nitrate supplementation on blood pressure, plasma nitrate levels, the area under oxygen

saturation curve to isotime, the area under heart rate curve to isotime, and endothelial function assessed using flow-mediated dilatation were also investigated.

4.2 Methods

4.2.1 Participant selection

Prior to enrolment in the study, all participants provided written informed consent. The study was approved by the London Chelsea Research and Ethics Committee (Ref: 15/LO/0975) and was carried out in accordance with the principles of the Helsinki Declaration. The study was registered in a publicly accessible database (<http://www.isrctn.com/ISRCTN14888729>) prospectively.

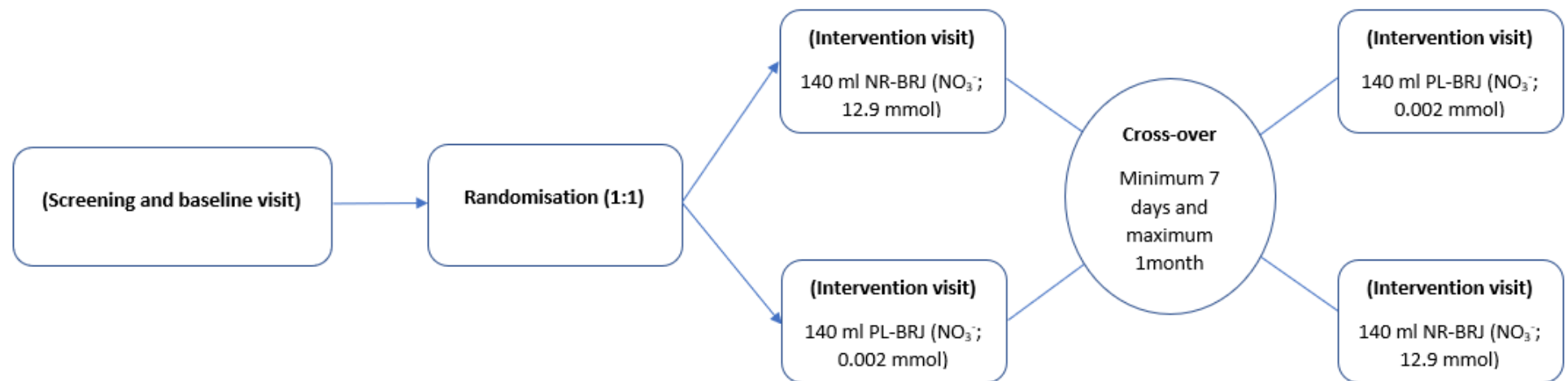
Participants were recruited if they had a diagnosis of PH (WHO group 3), which is defined as pulmonary hypertension caused by lung disease and/or hypoxia, requiring ambulatory oxygen or desaturating on exercise to <90%. Participants were recruited from outpatient pulmonary hypertension clinical services at the Royal Brompton Hospital between April 27th 2021 and March 4th 2022, with the last participant visit completed on March 25th 2022.

Exclusion criteria included clinically unstable patients (within one month of exacerbation), significant comorbidity limiting exercise tolerance, significant renal impairment (estimated glomerular filtration rate (eGFR) <50 ml.min⁻¹), hypotension (systolic blood pressure <100 mmHg), and pregnancy. Subjects were withdrawn if they had a significant adverse event or if they declined to participate.

4.2.2 Study design and randomisation

The study was a single centre, double blind, placebo-controlled, cross-over trial. Participants were randomised to receive 140 ml nitrate-rich beetroot juice (NR-BRJ) (NO₃⁻; 12.9mmol) or placebo of nitrate depleted beetroot juice (PL-BRJ) (NO₃⁻; 0.002mmol). Patients were

randomised 1:1 to the order in which they receive nitrate-rich beetroot juice versus placebo. Randomisation was performed using a computer-generated block randomisation list (block size 10) with consecutive numbers linked to identical preparations of placebo or active treatment. The list was generated by independent statistician. All through the trial and data processing, both the participants and the researcher were blind to treatment allocation. Figure 4.1 illustrates full details regarding study visits, interventions, and measurements.



Pre intervention visit guidance

- Avoid vigorous exercise in the 24 h before testing.
- Avoid a cooked breakfast & eat the same meal on the day of testing.
- Match caffeine consumption.
- Avoid the use of mouthwash or chewing gum.
- Intervention visits scheduled at the same time of day (+/- 2h).

Screening and baseline visit (Visit 1)

- Review of inclusion and exclusion criteria.
- Written informed consent obtained.
- Medical history review and clinical examination.
- Baseline ISWTx2, practice ESWT.
- Full pulmonary function tests (if not performed within 3 months).
- Resting BP, HR, and SaO₂.

Intervention visits (Visit 2 and Visit 3)

Pre-dosing (0 minute):

- Resting BP, HR, SaO₂
- FMD
- Plasma NO₃⁻/NO₂⁻ sampling.

Post-dosing (180 minutes):

- Resting BP, HR, SaO₂
- FMD
- Plasma NO₃⁻/NO₂⁻ sampling.
- ESWT

Figure 4.1: Study flow diagram.

Abbreviations: NO₃⁻ - Nitrate; NO₂⁻ - Nitrite; BRJ - Beetroot juice; PL - Placebo; ISWT - Incremental shuttle walk test; ESWT - Endurance shuttle walk test; BP - Blood pressure; HR - Heart rate; SaO₂ - Oxygen saturation; FMD - Flow mediated dilatation.

4.2.3 Intervention

The active intervention consisted of 140 mL of BRJ (NO_3^- ; 12.9 mmol) mixed with 2% organic lemon juice (140 mL BEET-IT® SPORT Shot, James White Drinks, Ipswich, UK). The placebo was a matched juice of 140 mL NO_3^- -deplete BRJ (NO_3^- ; 0.002 mmol), which was prepared by the same company using a standardised procedure of passing the juice through an ion exchange column using Purolite A520E ion exchange resin, which selectively eliminates NO_3^- (Lansley et al., 2011). The placebo was identical in taste and appearance, and both beverages caused beeturia. A dosage of 12.9 mmol of NO_3^- was chosen because plasma concentrations of NO_3^- and NO_2^- increase in a dose-dependent fashion up to 16.8 mmol of NO_3^- with a peak change at 2-3 h after administration (Wylie et al., 2013). In that trial, researcher reported no significant difference in VO_2 with doses of 4.2 mmol NO_3^- however a reduction in steady state exercise VO_2 was found with doses of 8.4 mmol and 16.8 mmol of NO_3^- (Wylie et al., 2013).

To make conditions as similar as possible, the participants were asked not to eat NO_3^- -rich foodstuffs for 48 hours before the intervention visits and to avoid cooked breakfasts on those days. Participants were also asked to match the amount of caffeine they consumed to reduce any variation in performance due to caffeine's ergogenic effects (Warren et al., 2010). Participants were also asked to avoid vigorous exercise for 24 hours before the intervention visits, as it is known to affect the endothelium's vasoconstrictor response (Elliott et al., 2018). Additionally, participants were instructed to refrain from using antimicrobial mouthwash and chewing gum on the morning of their visits, which has been found to reduce the activity of the oral bacteria flora that regulate the metabolism of the oral NO_3^- load (Govoni et al., 2008).

During the intervention visits, participants were observed consuming the active/placebo juices. Both the participant and the study researcher were blinded to the beverage selected.

Between intervention crossover visits, there was a minimum of seven days and a maximum of one month. The intervention visits were conducted at the same time of day (+/- 2 h). More details about study methods can be found in Chapter 2 (Description of Methods). The study protocol is summarised in Table 4.1.

Table 4.1 Summary protocol for a double-blind, placebo-controlled, randomised cross-over study of acute effect of dietary NO₃⁻ supplementation in individuals with pulmonary hypertension

	Screening and Baseline Visits	Intervention Visits
Inclusion/Exclusion Criteria	✓	
Informed Consent	✓	✓
Medical history, physical examination and review of exacerbations	✓	✓
Anthropometric measurements	✓	
Vital signs	✓	
Bioimpedance	✓	
Pulmonary Function Test	✓	
Venous blood for urea and electrolytes	✓	
Questionnaires	✓	
Incremental Shuttle Walk Test	✓	
Endurance Shuttle Walk Test	✓	✓
Randomisation	✓	
Beverage ingestion		✓

4.2.4 Study Protocol

Visit 1 (Screening and Baseline Visit)

- Review of inclusion and exclusion criteria
- Written informed consent obtained
- Medical history review and clinical examination including exacerbation history
- Anthropometric measurements
- Vital signs (including resting BP, HR and peripheral O₂ saturations (SpO₂))
- Bioelectrical impedance measure of body composition
- Full pulmonary function testing if not performed with three months
- Venous blood sample for renal function (if not tested in the previous 12 months)
- Quality of life assessments using the COPD Assessment Test (CAT) and assessment of breathlessness using the Medical Research Council (MRC) dyspnoea score
- Assessment of exercise capacity via two incremental shuttles walk test (ISWT)

followed by calculation of the endurance shuttle walk test (ESWT) speed

- Practice ESWT
- Randomisation

Visits 2 and 3 (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 SaO₂)

- Observation of dosing with either NO₃⁻-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 SaO₂)
- Endothelial function following a 10-minute in a supine position while the non-dominant arm was outstretched, fully supported, and rested on pillows in a quiet, temperature-controlled room
- ESWT with continuous monitoring of HR and SaO₂

4.2.5 Study Outcome measures

4.2.5.1 Primary outcome

1. Increase in exercise performance as measured by the ESWT time (seconds) according to ATS/ERS guidelines (Holland et al., 2014). The ESWT walking speed is estimated to be 85% of the peak O₂ uptake calculated from the best ISWT distance achieved (Singh et al., 1994).

4.2.5.2 Secondary outcomes

1. Area under the oxygen saturation curve to isotime during the ESWT.
2. Area under the heart rate curve to isotime during the ESWT.
3. Blood pressure parameters.
4. Nitrate levels.

5. Flow mediated dilatation.

4.2.6 Statistics and data analysis

The primary endpoint was the change in ESWT time. Given the cross-over design and considering 65 seconds to be a clinically significant difference in ESWT, and a within patient standard deviation of 60 seconds for repeated measures (Singh et al., 2014), the study required 16 subjects to reach 80% statistical power with a significance level of 0.05. A sample size of 20 subjects was required to allow for a 25% withdrawal rate. Intention-to-treat analysis was performed (i.e., per study arm allocation). Data are reported as mean and standard deviation (SD), or as median and interquartile range (IQR) if not normally distributed. Wilcoxon signed-rank tests or paired T-tests were used to assess differences between the two conditions. The analysis was conducted using SPSS version 26 for Windows (SPSS Software, Chicago, Illinois, USA) and GraphPad Prism version 9.3.1 for Windows (GraphPad Software, San Diego, California, USA). A rolling average of 15 seconds of individual ESWT data point was utilised as a method for comparing continuous oxygen saturation and heart rate between the two treatment conditions. The rolling average was calculated using Python programming language (3.8.12; <http://python.org>);Pandas package (1.3.5; <https://pandas.pydata.org/>) and then expressed as percentages of isotime (defined as the duration of the shortest of the two ESWT tests). Following that, individual responses were grouped for analysis of oxygen saturation and heart rate against percentage of isotime (as plotted at the midpoint of each 10th percentile of isotime). The area under the curve for each subject was measured, and a paired t-test was used to compare between the two treatment conditions. A p value of < 0.05 was considered to be statistically significant.

4.3 Results

4.3.1 Recruitment process

Participants were recruited between April 27th 2021 and March 4th 2022, with the last participant visit completed on March 25th 2022. 71 individuals were assessed for eligibility. 51 of them were deemed not suitable for the study; 36 declined to participate, two died, and 13 had other reasons. A total of twenty participants were randomly assigned to the study, ten to NO₃⁻rich BRJ first and ten to PL-BRJ first, with one subject withdrawing prior to study completion. The CONSORT diagram below outlines the recruitment process for the study (Figure 4.2).

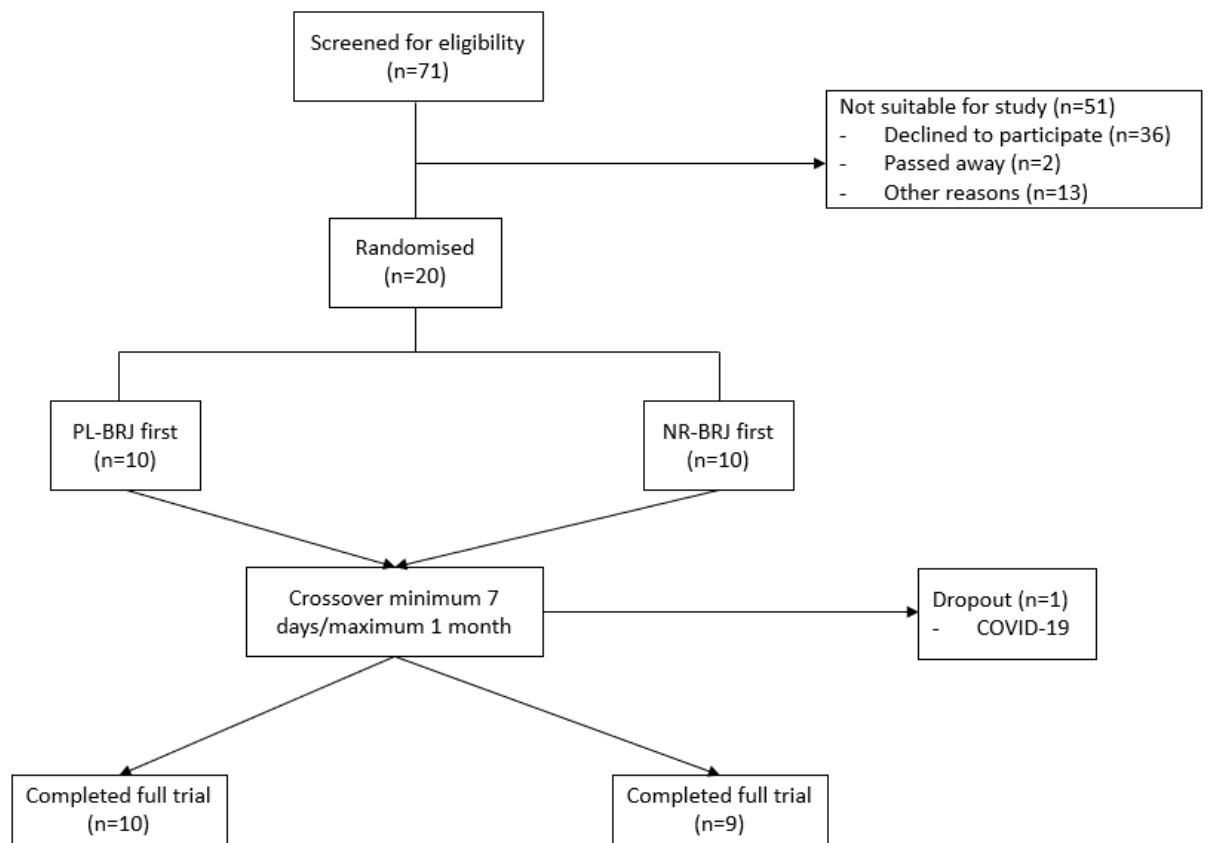


Figure 4.2: CONSORT recruitment diagram for enrolment and study completion in the EDEN-OX trial.

4.3.2 Baseline characteristics

Twelve men and seven women completed both dosing visits of the study. Table 4.2 displays the baseline characteristics of both subgroups in the sequence of intervention provided first and the whole group. Participants were fully compliant with the dosing protocol without any adverse effects, aside from beeturia as expected during the dosing visits.

Table 4.2 Baseline characteristics

Measurement	Whole group (n=20)	NR-BRJ first (n=10)	PL-BRJ first (n=10)	P
Age (years)	61.0 (14.9)	61.5 (13.8)	60.6 (16.7)	0.8
Gender, (% female: male)	40:60	20:80	60:40	0.06
BMI (kg/m ²)	25.2 (5.0)	26.8 (4.5)	23.7 (5.1)	0.1
FFMI (kg/m ²)	16.8 (3.4)	18.2 (2.7)	15.3 (3.4)	0.05
Smoking (pack years)	1.0 (1.0-2.0)	3.5 (0-30)	0 (0-31.7)	0.6
Diagnosis				
COPD n (%)	3 (15)	0 (0)	3 (30)	0.2
CTD n (%)	3 (15)	0 (0)	3 (30)	0.2
ILD n (%)	9 (45)	6 (60)	3 (30)	0.3
WHO Functional Class, n (%)				
II	4 (20)	3 (30)	1 (10)	0.2
III	16 (80)	7 (70)	9 (90)	0.2
Using of O ₂ , n (%)	16 (80)	8 (80)	8 (80)	1.0
PR attended, n (%)	6 (30)	3 (30)	3 (30)	1.0
MRC dyspnoea score	4 (2.2-4)	3.5 (2-4)	4 (2.7-4)	0.6
CAT score	20.8 (5.9)	22.1 (5.1)	19.6 (6.6)	0.3
Systolic BP (mm Hg)	121.4 (11.4)	123.3 (11.7)	119.5 (11.5)	0.4
Diastolic BP (mm Hg)	71.8 (10.1)	70 (10)	73.7 (10.4)	0.4
Lung function				
FEV ₁ (L)	1.8 (0.7)	1.9 (0.64)	1.7 (0.86)	0.5
FVC (L)	2.6 (1.0)	2.6 (0.72)	2.5 (1.2)	0.8
FEV ₁ : FVC (% predicted)	74.9 (18.4)	77.4 (19.9)	72.5 (17.8)	0.5
RV (% predicted)	77.5 (33.4)	67.4 (24.3)	87.6 (39.3)	0.1
TLC (% predicted)	73.0 (28.3)	66 (21.3)	80.1 (33.6)	0.2
RV/TLC	45.9 (16.8)	36.3 (7.4)	55.5 (18.4)	0.007
TLco (% predicted)	28.9 (14.0)	32.1 (15.0)	27.8 (13.3)	0.5
Kco (% predicted)	50.4 (21.2)	54.4 (17.1)	46.4 (24.8)	0.4
Best ISWT distance (m)	205.5 (133)	257 (158.1)	154 (80.7)	0.08
ESWT Time (Sec)	190 (117.2-306.5)	194.5 (125.7-469.5)	173 (87.2-263.5)	0.4

Data are presented as mean (SD), median (IQR) or percentage (%). P value is for independent t-test or Mann-Whitney test between groups. BMI, body mass index; FFMI, fat free mass index; COPD, Chronic obstructive pulmonary disease; CTD, connective tissue disease; ILD, Interstitial lung disease; WHO, World Health Organisation; PR, pulmonary rehabilitation; MRC, Medical Research Council; CAT, COPD Assessment Test; BP, blood pressure; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; TLco, transport factor for carbon monoxide; Kco, carbon monoxide transfer coefficient; ISWT, incremental shuttle walk test; ESWT, endurance shuttle walk test; NR-BRJ, nitrate-rich beetroot juice; PL-BRJ, placebo beetroot juice.

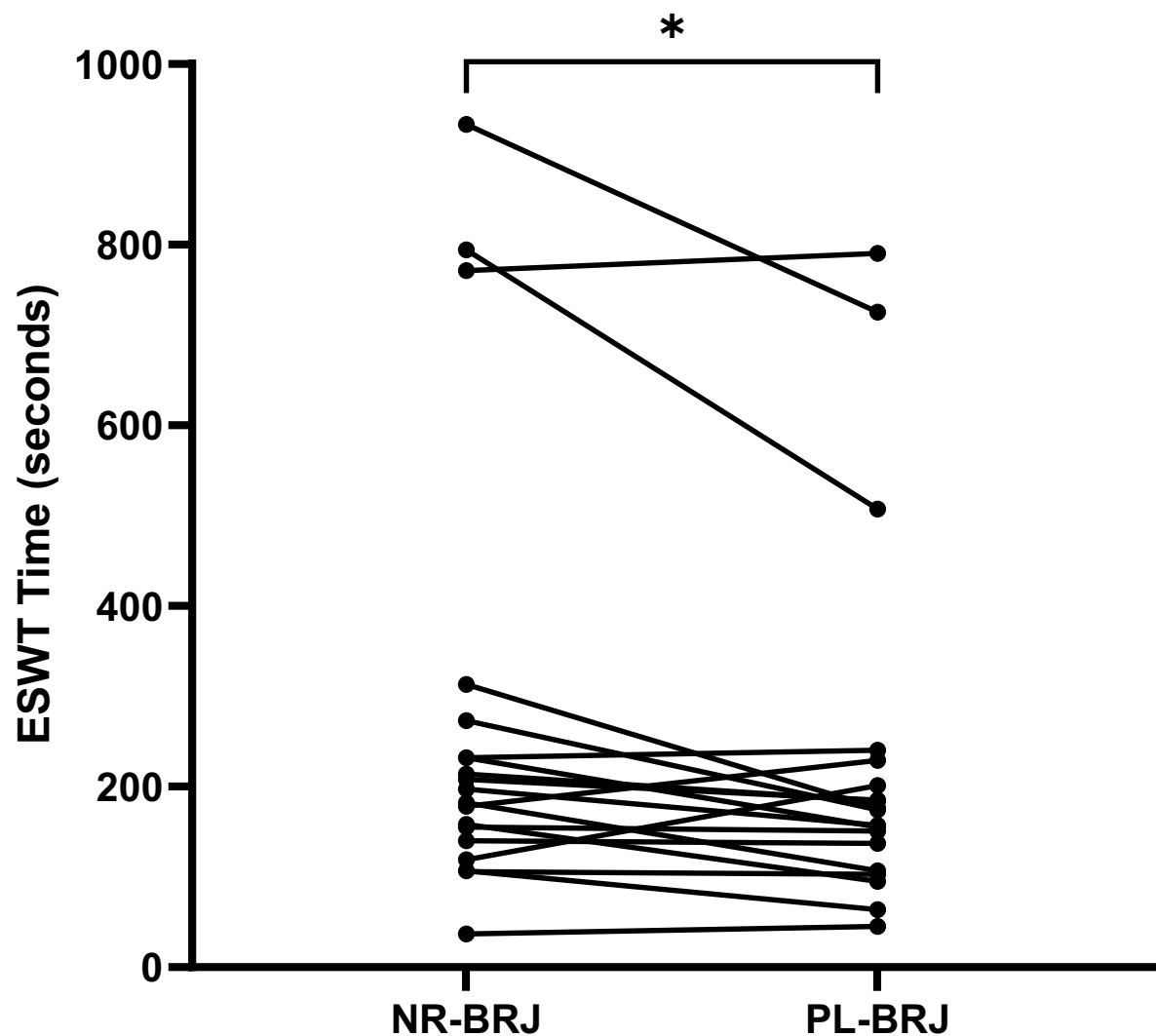
4.3.3 Effect of acute dietary nitrate supplementation on exercise capacity

Following dosing with NR-BRJ, study participants walked for longer than following dosing with PL-BRJ. The median (IQR) ESWT times were NR-BRJ 197 sec (140, 273) vs. PL-BRJ 174 sec (107, 229) with a median of differences (95% CI) 30 sec (6.19 -91.07); $p=0.02$ (Figure 4.3).

Three participants had substantially longer ESWT times than the rest of the cohort. As a sensitivity analysis I reviewed the results excluding these three from the dataset. The median (IQR) ESWT times were NR-BRJ 180 sec (124.3, 227.5) vs. PL-BRJ 174 sec (104, 184.8) with a median of differences (95% CI) 26.5 sec (-1.48 to 57.48); $p=0.0717$.

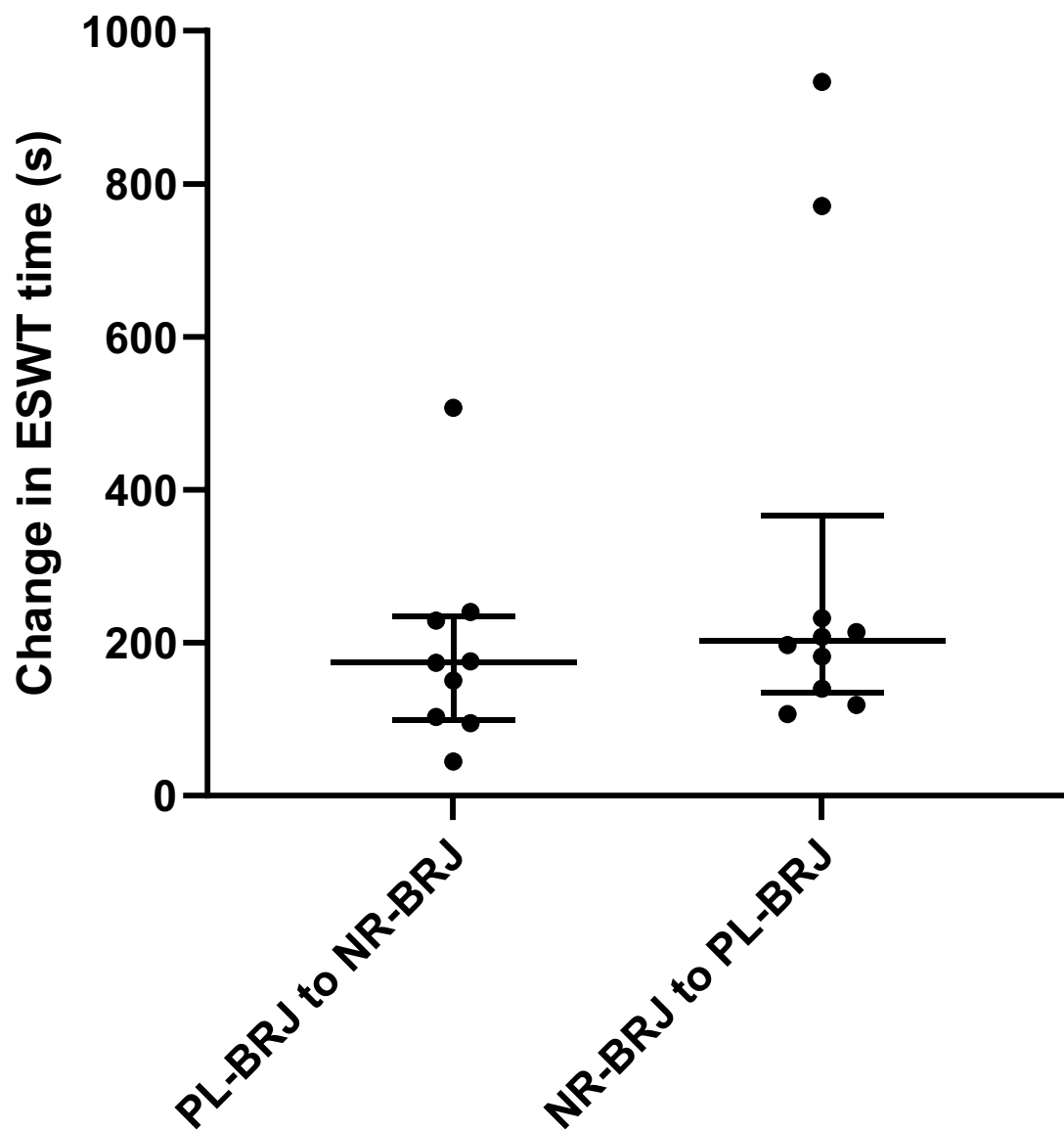
Recovery time (defined as return to baseline Borg dyspnoea scale prior to the ESWT) did not differ between intervention groups: NR-BRJ 282 sec (248.4, 329.4) vs. PL-BRJ 223 sec (214.8, 321.7); $p=0.111$. In terms of primary outcome order effect there was no evidence of intervention order effect on the median (IQR) change in ESWT time; if PL-BRJ was consumed first the ESWT time was 174.0 sec (99, 234.5) compared to 202.5 sec (134.8, 366.8) when NR-BRJ was consumed first; $p=0.3154$ (Figure 4.4).

Figure 4.3: Individual ESWT Time (seconds) measured in the NR-BRJ and PL-BRJ dosing conditions



Data are presented individual ESWT times (seconds) in both dosing conditions. Wilcoxon sign-rank test was used to compare the change in ESWT time between treatment conditions; NR-BRJ 197sec (140, 273) vs. PL-BRJ 174 sec (107, 229), with a median of differences (95% CI) 30 (6.19 -91.07); * $p < 0.05$.

Figure 4.4: Individual data points for the change in ESWT (seconds) were tested whether PL-BRJ or NR-BRJ was given initially



Data are shown as the median and interquartile range. Using the Mann-Whitney U test, the median (IQR) change in ESWT time was 174.0 (99, 234.5) seconds when PL-BRJ was consumed first, compared to 202.5 (134.8, 366.8) seconds when NR-BRJ was consumed first; $p=0.3154$.

4.3.4 Effect of acute dietary nitrate supplementation on physiological parameters during exercise

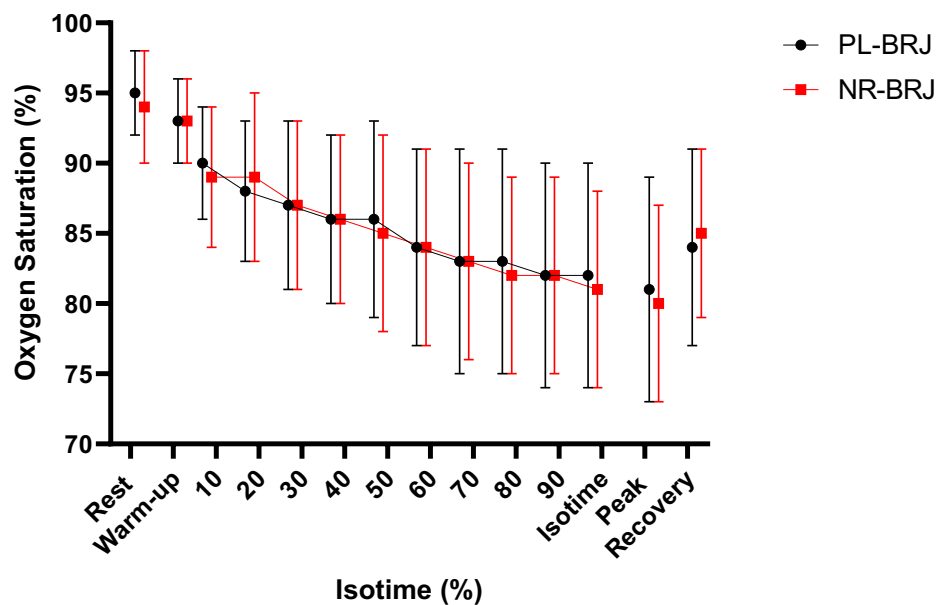
Due to the withdrawal of one participant from the study and failure of data recording for two participants, paired data for 17 individuals were available for analysis. There was no statistically significant change in O₂ saturations between the two treatment conditions during rest, warm-up, each 10th percentile of isotime, isotime, peak exercise, and recovery (Table 4.3 and Figure 4.5).

There was no statistically significant difference in heart rates between the two treatment conditions during rest, warm-up, each 10th percentile of isotime, isotime, peak exercise, and recovery (Table 4.3 and Figure 4.6).

Table 4.3 Effect of dietary nitrate supplementation on oxygen saturation and heart rate during exercise

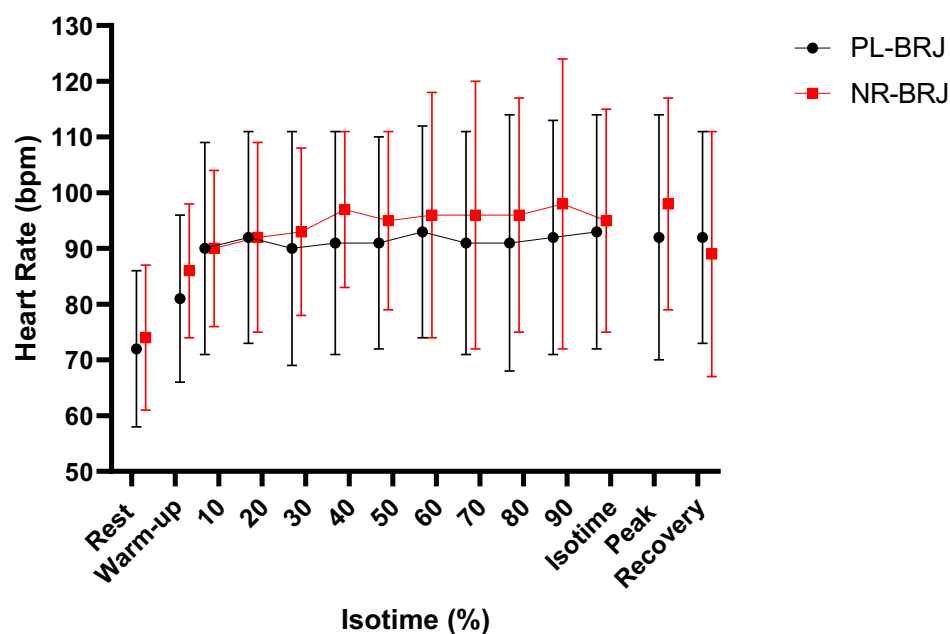
Period of ESWT	NR-BRJ O ₂ Saturation (%)	PL-BRJ O ₂ Saturation (%)	p value	NR-BRJ HR (bpm)	PL-BRJ HR (bpm)	p value
Rest	94.4 (3.6)	94.7 (2.9)	0.5315	73.7 (12.8)	71.7 (13.5)	0.2247
Warm-up	92.8 (3.6)	93.2 (2.9)	0.5645	85.7 (12.0)	81.0 (15.2)	0.0669
10th	89.2 (5.4)	89.8 (4.4)	0.7366	90.2 (13.5)	89.9 (19.1)	0.9213
20th	88.6 (5.8)	87.6 (5.1)	0.4872	92.1 (16.6)	91.9 (19.2)	0.9491
30th	87.3 (5.8)	87.0 (6.0)	0.8468	93.2 (14.7)	90.3 (21.3)	0.4455
40th	86.1 (6.2)	86.3 (5.9)	0.8055	97.2 (13.6)	90.9 (20.1)	0.1052
50th	85.0 (6.5)	85.6 (6.9)	0.7551	95.1 (15.9)	90.6 (15.5)	0.2947
60th	84.1 (6.6)	84.1 (7.4)	>0.9999	95.6 (21.9)	92.5 (19.0)	0.5437
70th	83.2 (7.0)	83.3 (7.9)	0.8513	96.0 (24.3)	90.5 (20.4)	0.3693
80th	82.2 (7.3)	83.1 (7.7)	0.4867	95.7 (21.4)	90.5 (23.3)	0.3258
90th	82.2 (7.0)	82.1 (8.1)	0.8740	97.8 (25.9)	91.7 (20.8)	0.3403
Isotime	80.5 (6.9)	81.5 (8.1)	0.2740	94.8 (20.1)	93.4 (20.7)	0.7179
Peak exercise	79.5 (7.1)	81.2 (8.3)	0.1825	98.3 (19.2)	92.4 (22.3)	0.3605
Recovery	85.1 (5.8)	84.0 (7.8)	0.2070	89.2 (21.7)	91.8 (18.5)	0.3138

Figure 4.5: Effect of dietary nitrate supplementation on O₂ saturation during exercise



Data presented as mean (SD). The graph represents the O₂ saturation at rest, warm-up, each 10th percentile of isotime, isotime, peak and recovery. A paired t-test was used to compare the area under the curves to isotime, and there was no significant separation of the curves representing the two treatment conditions ($p=0.906$).

Figure 4.6: Effect of dietary nitrate supplementation on heart rate during exercise



Data presented as mean (SD). The graph represents the heart rate at rest, warm-up, each 10th percentile of isotime, isotime, peak and recovery. A paired t-test was used to compare the area under the curves to isotime, and there was no significant separation of the curves representing the two treatment conditions ($p=0.226$).

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

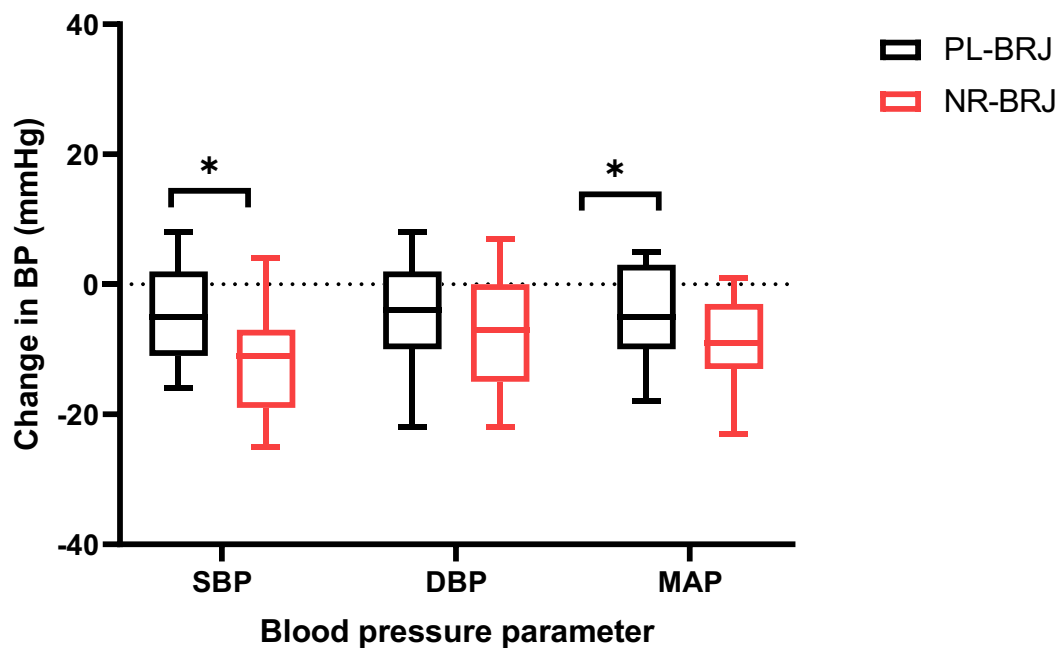
The changes in resting blood pressure parameters after the consumption of nitrate-rich beetroot juice (NR-BRJ) and placebo (PL-BRJ) are presented in Figure 4.7. There were significant differences in the reduction in systolic blood pressure (12 ± 8 mmHg NR-BRJ vs. 5 ± 8 mmHg PL-BRJ; $p=0.01$) and mean arterial pressure (9 ± 7 mmHg NR-BRJ vs. 5 ± 7 mmHg PL-BRJ; $p=0.02$) following dosing of NR-BRJ compared to PL-BRJ dosing (Table 4.4). The reduction in diastolic blood pressure was not statistically significant following dosing of NR-BRJ or PL-BRJ (7 ± 8 mmHg NR-BRJ vs. 5 ± 9 mmHg PL-BRJ; $p= 0.135$).

Table 4.4 Effect of dietary nitrate supplementation on blood pressure and endothelial function

	NR-BRJ			PL-BRJ			Between groups (95%CI)	p value
	Pre	Post	Change	Pre	Post	Change		
SBP mmHg	122.1 ± 10.8	110.6 ± 10.9	-11.5 ± 8.0	117.8 ± 10.6	112.8 ± 10.4	-5.0 ± 7.5	-6.5 (-11.7 to -1.2)	0.01
DBP mmHg	71.7 ± 10.3	64.5 ± 6.7	-7.2 ± 8.1	69.0 ± 8.3	64.4 ± 7.1	-4.6 ± 8.9	-2.5 (-6.0 to 0.8)	0.135
MAP mmHg	88.5 ± 9.2	79.8 ± 6.6	-8.6 ± 7.0	85.3 ± 7.2	80.5 ± 6.3	-4.7 ± 7.3	-3.9 (-7.4 to -0.4)	0.02
FMD %	6.8 ± 4.3	10.2 ± 5.2	3.4 ± 5.4	9.3 ± 4.3	8.0 ± 4.8	-1.3 ± 4.7	4.7 (1.4 to 8.0)	0.007

Data are presented as mean $\pm$ SD. Paired t-Test was used to compare blood pressure parameters and endothelial function between the different dosing conditions. Between-treatment conditions effect treatment estimates are presented as a mean of differences (95% CI). p-value <0.05 is considered significant. NR-BRJ, nitrate-rich beetroot juice; PL-BRJ, placebo beetroot juice.

Figure 4.7: Effect of dietary nitrate supplementation on blood pressure

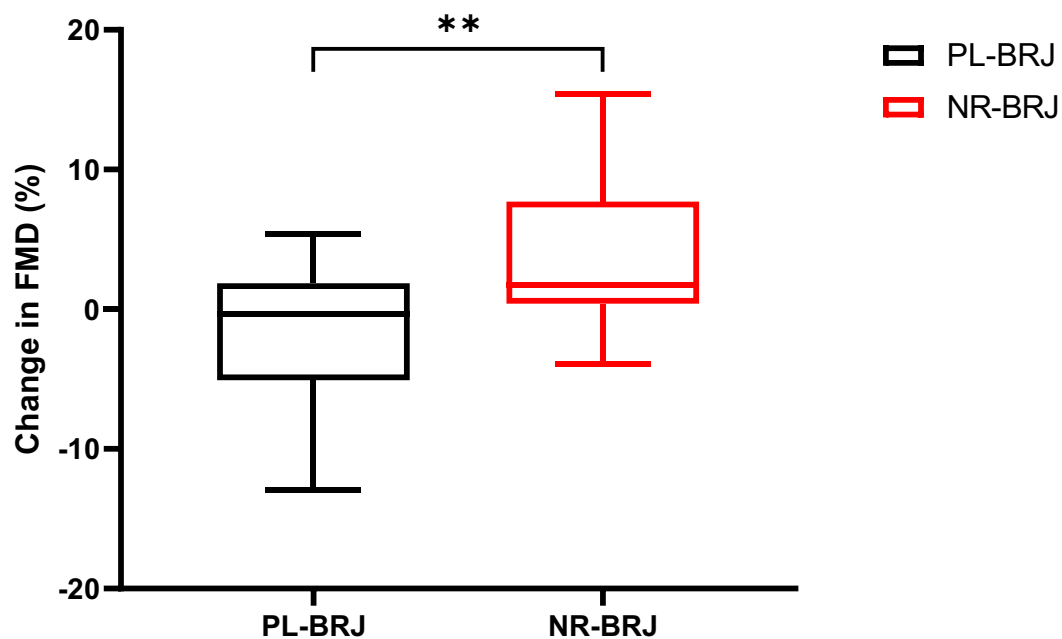


Data are presented as 25th -75th percentile with the solid line representing the median value, and the whiskers the minimum to maximum values. Paired t-Test was used to compare blood pressure parameters between the different dosing conditions; *significantly different from placebo, $p < 0.05$.

4.3.6 Effect of acute dietary nitrate supplementation on endothelial function

Figure 4.8 depicts the percentage changes in FMD after consuming of NR-BRJ and PL-BRJ. Data were only provided for 19 participants since one withdrew from the study. At 180 min, FMD following dosing with NR-BRJ increased $3.40 \pm 5.47\%$ compared with PL-BRJ -1.33 ± 4.78 , with a mean of differences 4.73 (95% CI 1.44 to 8.02); $p = 0.007$.

Figure 4.8: Effect of dietary nitrate supplementation on endothelial function



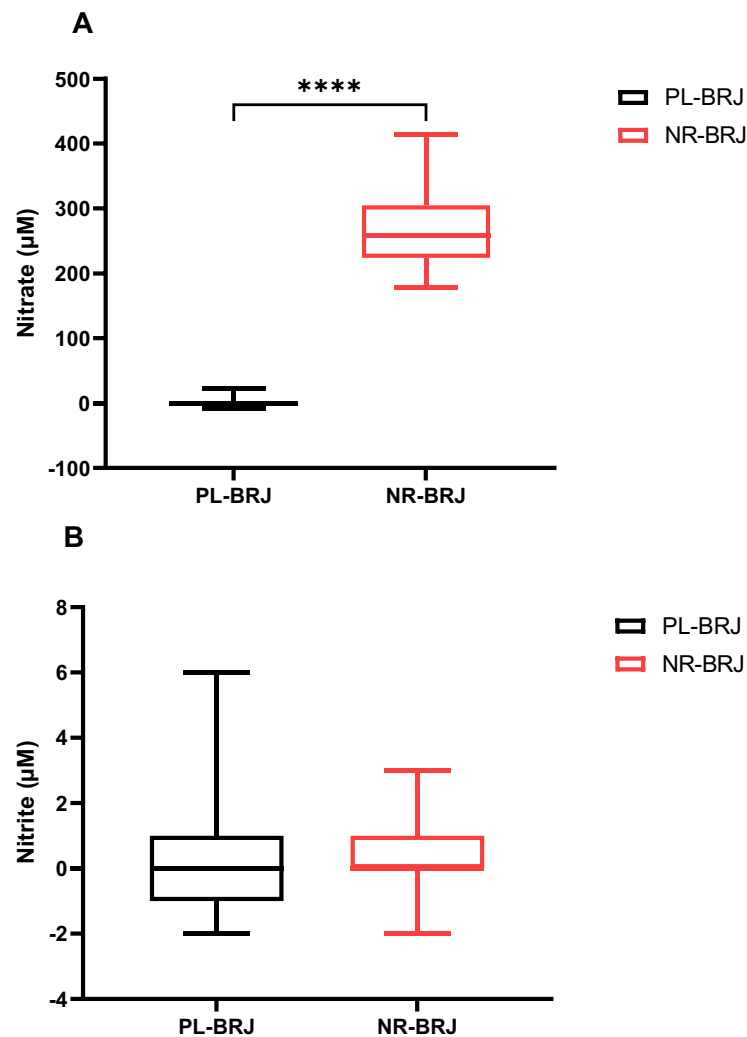
Data are presented as 25th -75th percentile with the solid line representing the median value, and the whiskers the minimum to maximum values. Paired t-Test was used to compare % changes in FMD between the different dosing conditions; **significantly different from placebo, $p < 0.05$.

4.3.7 Effect of dietary nitrate supplementation on nitrate and nitrite levels

Figure 4.9 illustrates the changes in plasma nitrate and nitrite concentrations 3 hours after consuming active or placebo juice. Following NR-BRJ, plasma nitrate concentrations significantly increased $258 \mu\text{M}$ (224, 305) compared to PL-BRJ $0.0 \mu\text{M}$ (-4.0, 3.0) with a median of differences (95% CI) $256 \mu\text{M}$ (237.9 to 304.9); $p < 0.0001$; Figure 4.9 A.

There were no significant changes in plasma nitrite concentrations following dosing with NR-BRJ $0.0 \mu\text{M}$ (0.0, 1.0) and PL-BRJ $0.0 \mu\text{M}$ (-1.0, 1.0) μM ; $p = 0.9086$; Figure 4.9 B.

Figure 4.9: (A) Change in plasma nitrate (μM) following active or placebo juice (B) Change in plasma nitrite (μM) following active or placebo juice



(A) Changes in plasma NO_3^- concentrations

(B) Changes in plasma NO_2^- concentrations

Data are presented as median (IQR) with whiskers representing minimum to maximum values. Wilcoxon sign-rank test was used to compare the change in plasma nitrate and nitrite concentrations between treatment conditions; ****significantly different from placebo, $p < 0.0001$.

4.4 Discussion

4.4.1 Main findings

The main findings of this study were that, in individuals with pulmonary hypertension requiring ambulatory oxygen or desaturating on exercise to <90%, acute dietary nitrate supplementation (12.9 mmol) in the form of NR-BRJ resulted in an increase in exercise performance assessed using the ESWT, an improvement in endothelial function measured using FMD, and a reduction in resting systolic and mean arterial pressure, accompanied by a significant rise in plasma nitrate level.

4.4.2 Significance of findings

The primary outcome of the EDEN-Ox study was to investigate the effect of dietary nitrate supplementation in the form of beetroot juice on exercise performance. The findings of the present study extend the evidence base that dietary nitrate supplementation have the potential to improve exercise performance and reduce cardiovascular risk by improving the endothelial function, lowering blood pressure in people with respiratory disease (Alsulayyim et al., 2021, Pavitt et al., 2021, Pavitt et al., 2020).

The effect on exercise capacity is consistent with a recent study that was conducted by our group using the same protocol that demonstrated a significant improvement in endurance time following an acute dose of (12.9 mmol) of NR-BRJ in hypoxic COPD (Pavitt et al., 2021). Furthermore, our findings correspond to a previous study in COPD that showed a significant increase in exercise performance during pulmonary rehabilitation for the 8 week ON-EPIC trial (Pavitt et al., 2020). However, our findings contrast with those of Henrohn et al. (2018), who

conducted a randomised, double-blind, placebo-controlled crossover trial to investigate the effect of dietary nitrate supplementation in the form of NR-BRJ in 8 women and 7 men with pulmonary hypertension (WHO group 1). In this study, participants received the NR-BRJ (16mmol) for one week and this failed to show an improvement in exercise capacity using the 6MWD test; $p=0.445$ (Henrohn et al., 2018). This may be because the ESWT is more sensitive test of exercise capacity.

There is a lack of clinical studies investigating the effect of nitrate supplementation on patients with pulmonary hypertension, although in 36 patients from different pulmonary hypertension groups (20 WHO group 1 PAH, 10 WHO group 2 (PH due to left heart disease), and 6 WHO group 3) inhaled nitrite at two doses (45 and 90 mg) had beneficial acute haemodynamic effects that resulted in lowering pulmonary capillary wedge pressure, PVR, and pulmonary arterial pressures (Simon et al., 2016).

The 30 seconds improvement in ESWT we observed with NR-BRJ was below the MCID of 65 seconds (95% CI 45-85) proposed based on response to bronchodilation in people with COPD (Pepin et al., 2011). The ESWT changes in that study represented 13-15% of baseline values, which is similar to the percentage difference that we observed in the present study. An MCID for the ESWT specifically in pulmonary hypertension has yet to be defined. It remains to be established whether the effect we observed will translate into improved experience of physical activity on a day to day basis. This was a single dose study, and further work will also need to be undertaken to see if the effect on exercise capacity persists or is enhanced by sustained treatment.

Studies in healthy participants have established that dietary nitrate supplementation increases exercise performance in hypoxic conditions (Kelly et al., 2014, Masschelein et al., 2012, Muggeridge et al., 2014, Shannon et al., 2016). As a result of exercising muscle in particular hypoxia, acidosis and the presence of deoxyhaemoglobin and myoglobin, the NOS-dependent production of NO is impaired and the alternative pathway ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO}$) is enhanced (Dauncey, 2012, Shannon et al., 2016, Vanhatalo et al., 2011). This would imply that dietary nitrate supplementation might have greater benefits under hypoxic conditions and that hypoxic patient phenotypes would benefit more. NO is the primary stimulus for cyclic guanosine monophosphate (cGMP) production in pulmonary vascular smooth muscle cells through activation of the soluble guanylate cyclase. In individuals with PH, cardiovascular comorbidity is common and endothelial dysfunction in the pulmonary vasculature is induced in part by decreased NO generation from eNOS and/or decreased NO bioavailability (Demoncheaux et al., 2005, Giaid and Saleh, 1995). In addition, NO synthesis is oxygen dependent, and a decrease in NO bioavailability under hypoxic conditions induces acute and chronic hypoxic pulmonary vasoconstriction.

The majority of study participants (16 out of 19) in this study were on treatment with phosphodiesterase-5 (PDE5) inhibitors. These suppress the activity of the enzyme in the smooth muscle cells of vessels, preventing degradation of cGMP leading to pulmonary artery vasodilation as well as inhibiting the remodeling of the pulmonary vasculature (Ahmed et al., 2021, Mónica and De Nucci, 2019). It is possible that dietary nitrate supplementation would have synergistic or additive effects, but to date this has not been investigated.

People with chronic lung disease have a high prevalence of cardiovascular disease. Dietary nitrate supplementation has been shown to lower blood pressure in healthy individuals (Wylie et al., 2013), older adults (Kelly et al., 2013), patients with peripheral artery disease (Kenjale et al., 2011), and COPD (Alsulayyim et al., 2021), though studies to date have been short term. Our findings confirm that this effect is seen in people with PH as well. Consistent with this, we also found a significant improvement in endothelial function using brachial artery flow-mediated dilatation. Improvement in endothelial function may have systemic as well as pulmonary vascular effects. The improvement in endothelial function we noticed is consistent with our previous studies demonstrating that dietary nitrate supplementation improved the %FMD by 20.3% in non-hypoxic COPD patients undergoing pulmonary rehabilitation (Pavitt et al., 2020) as well as by 11.9% in hypoxic COPD patients (Pavitt et al., 2021).

4.4.3 Strengths and limitations of the study

In this study, the study design allows for the removal of patient variation, thereby making crossover trials the preferred methodology. The study used a nitrate-depleted beetroot juice placebo beverage, allowing it to be conducted in a double-blind fashion, and combining both participant dependent and objective measures. There are some limitations to the study that must be addressed.

It is possible that other bioactive components in the juice, such as antioxidant and anti-inflammatory properties like vitamin C, carotenoids, phenolics, and betalains, may also contribute to positive effects. As such the present study addresses the effect of nitrate supplementation specifically and might underestimate the benefit of beetroot juice itself.

It should be highlighted that the majority of baseline samples had plasma nitrite levels that were below the limit of quantifiability. Possibly, this is linked to the absence of pretreatment of samples with a thiol blocking agent before centrifugation. As this treatment assures there is no flux of NO_2^- across the cell membrane, which could lower plasma concentrations following uptake by erythrocytes and white cells during processing.

As a single-dose study, although a biological effect has been demonstrated, there is still uncertainty as to whether the regular dosing and long-term treatment of dietary nitrate supplementation would have any meaningful effects or differences in clinical outcomes in pulmonary hypertension. The results of this study should be interpreted with caution, as all participants were classified with PH (WHO group 3). Therefore, further research is required to investigate the potential benefits of dietary nitrate supplementation in hypoxic conditions as well as in individuals with different pulmonary hypertension etiologies.

4.5 Conclusion

The acute administration of dietary nitrate supplementation in the form of NR-BRJ increased exercise performance and reduced blood pressure in individuals with pulmonary hypertension requiring ambulatory oxygen or desaturating on exercise to <90%. The findings suggest that dietary nitrate supplementation might be utilised as a non-pharmacological intervention to augment exercise in this population. To understand the possible mechanisms of dietary nitrate supplementation in pulmonary circulation and to prevent pulmonary vascular remodeling in pulmonary hypertension patients, larger and longer trials are required.

CHAPTER 5: Association of nitrate levels with disease severity in lung disease - an observational study: The EON-Lung trial

5.1 Introduction

In the preceding chapters of this thesis, we have shown that dietary nitrate supplementation in the form of beetroot juice has a variety of potentially beneficial effects in people with lung disease. These include improving exercise capacity and reducing blood pressure in people with COPD (Chapter 3); and enhancing exercise capacity, improving endothelial function, and reducing blood pressure in hypoxic PH patients (Chapter 4).

Although widespread dietary nitrate supplementation may be beneficial for patients with lung disease, trials to date have been relatively short-term and have studied a range of different endpoints, leaving it unclear what the optimum dose should be, how it should be monitored, and how it may vary between individuals. In most short-term studies evaluating exercise capacity, higher doses were used to enhance effectiveness.

Therefore, in this chapter, I aimed to investigate the association of nitrate levels with disease severity and symptoms in COPD. This would support the plausibility of this as a therapeutic direction, as well as determine if exhaled nitric oxide (FeNO) levels (outside of conditions like asthma, where lung inflammation increases FeNO) may be used as an indicator of a low blood nitrate level. This would be helpful in stratifying clinical trials and identifying the appropriate dose.

5.2 Methods

5.2.1 Participants

All participants provided written informed consent before enrolment in the trial. Participants were identified by clinical teams in the first instance via (i) outpatient attendance at the Royal

Brompton Hospital (ii) review of clinical databases and mailshot to potential participants. The study was carried out according to the principles of the Declaration of Helsinki.

Study inclusion criteria included adult (18 and over) with a clinical diagnosis of COPD. Exclusion criteria included clinically unstable patients within one month of pulmonary exacerbation or those who declined to consent.

5.2.2 Study design

This was a single centre, an observational study, requiring a single visit.

5.2.3 Study outcome measures

The primary outcome was the association between blood nitrate levels and MRC dyspnoea score. The secondary outcome was to establish whether a low of exhaled NO (FeNO) reliably predicts low blood nitrate and nitrite levels. In addition, I investigated whether blood levels of nitrate and nitrite levels are associated with CAT score, BP, lung function, walking distance, urea and electrolytes (U&Es), brain natriuretic peptide (BNP), resting O₂ saturation, blood markers of endothelial and inflammatory function, and endothelial function using Endo-PAT. Exploratory endpoints included vegetable and fruit consumption per week. Full explanation of methods and outcome measures can be found in Chapter 2 (Description of Methods).

5.2.4 Statistics and data analysis

Data was analysed using SPSS version 26.0 (IBM, SPSS, Illinois, United States) and Prism 9.2.0. (GraphPad Software Inc, California, USA). Baseline characteristics are presented as mean $\pm$ SD, median interquartile ranges (IQR), frequencies, and proportions. Normality tests were conducted on the data distribution. The correlation between variables was assessed using Pearson correlation coefficient for normally distributed variables, and Spearman's rho correlation coefficient for non-parametric variables. A formal sample size calculation was

not undertaken as we did not have data on the variability of these measures in these patient populations. A statistically significant difference was defined as a p-value of < 0.05 (there was no need to apply corrections for statistical multiplicity).

5.3 Results

5.3.1 Recruitment process

Participants were recruited between October 5th 2020 and August 8th 2021, with the last participant visit completed on August 10th 2021. 123 individuals were screened for eligibility. 58 of them were considered not suitable for the study; 21 declined to participate, and 37 had other reasons. A total of 65 participants were included in the analysis. The CONSORT diagram below describes the recruitment procedure for the study (Figure 5.1).

5.3.2 Baseline characteristics

The median (IQR) age of these 65 participants was 65 (54, 70) years, 72% were male, and the median (IQR) for FEV₁(% predicted), MRC dyspnoea score, plasma nitrate (μM), and FeNO (ppb) were 44 (29, 64), 3 (2, 4), 12 (9, 17), and 17 (12, 27); respectively (Table 5.1).

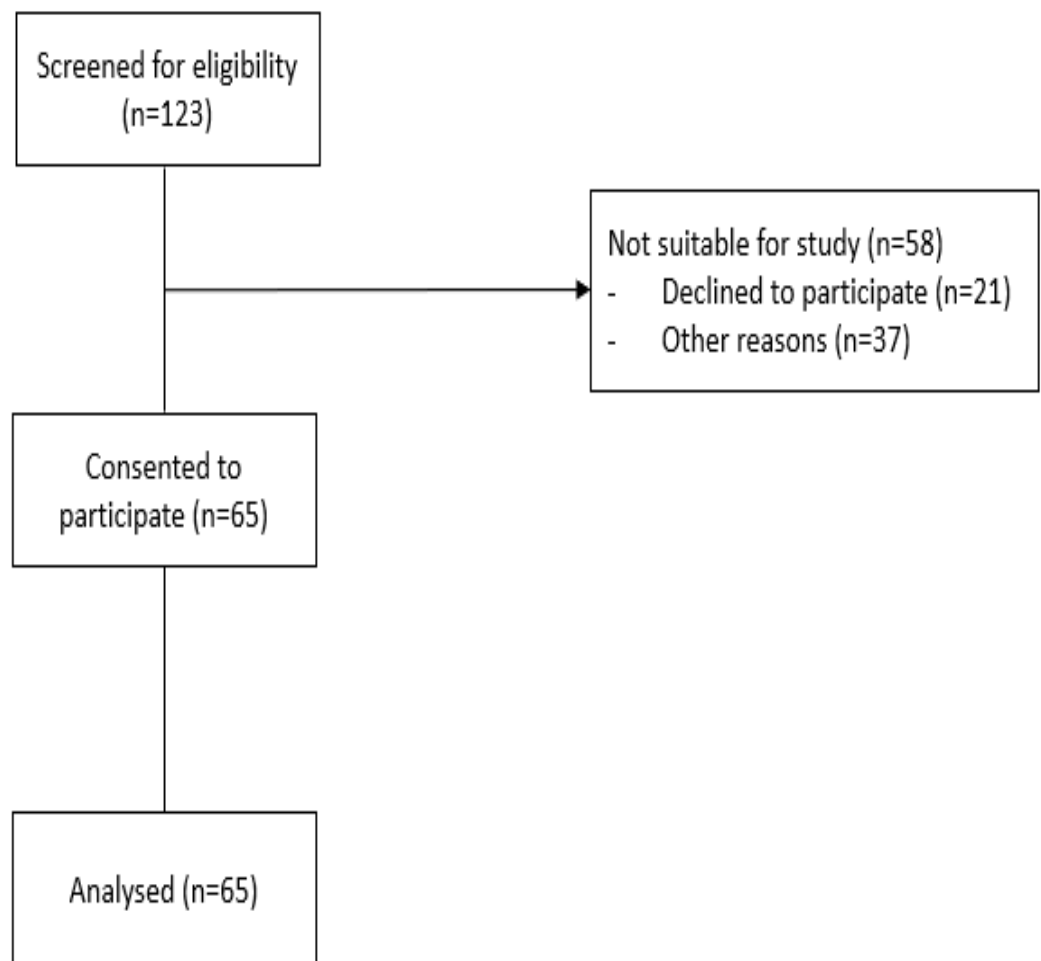


Figure 5.1: CONSORT recruitment diagram for enrolment and study completion in the EON-lung trial

Table 5.1 Baseline characteristics

Characteristics	Value
Age (years)	65 (54, 70)
Gender (Male), n (%)	47 (72.3)
Ethnicity, n (%)	
White British	51 (78.5)
White Irish	5 (7.7)
Black	6 (9.2)
Other	3 (4.6)
BMI (kg/m ²)	26.9 (22.2, 29.5)
Lung function	
FEV ₁ (% predicted)	43.7 (29.2, 64)
TLco	43.3 (32.6, 54.9)
Smoking history, n (%)	
Smoker	11 (16.9)
Ex-smoker	50 (76.9)
Never-smoke	4 (6.2)
Smoking pack years	33 (20, 40)
GOLD stage, n (%)	
I	6 (9.2)
II	20 (30.8)
III	23 (35.4)
IV	16 (24.6)
Inhalers, n (%)	
LABA	2 (3.1)
LAMA	26 (40)
LABA-ICS	33 (50.8)
LABA-LAMA	11 (16.9)
Number of exacerbations last year, n (%)	
No	28 (43.1)
1	22 (33.8)
2	9 (13.8)
> 3	6 (9.2)
Pulmonary Rehabilitation attended, n (%)	37 (56.9)
Using oxygen, n (%)	9 (13.8)
Comorbidities, n (%)	
Asthma	11 (16.9)
Coronary heart disease	6 (9.2)
DM	4 (6.2)
GERD	16 (24.6)
Hypertension	29 (44.6)
MRC dyspnoea score	3 (2, 4)
CAT score	20.9 ± 7.0
FeNO (ppb)	17 (12, 26.5)
Baseline HR	79.8 ± 13.7
Baseline systolic BP (mm Hg)	136.3 ± 19.3
Baseline diastolic BP (mm Hg)	74.3 ± 10.3
Baseline mean arterial pressure (mmHg)	94.9 ± 11.3
Baseline oxygen saturation (%)	95 (93, 97)

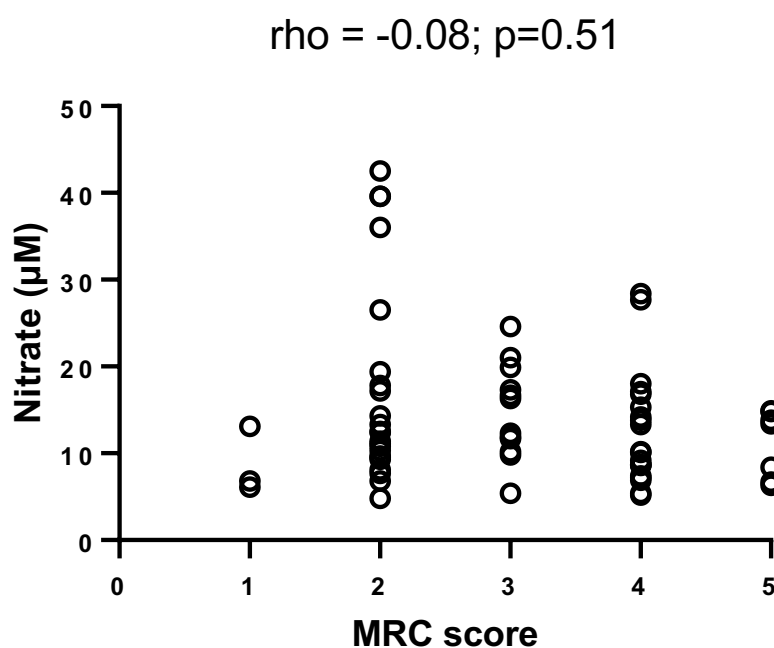
6MWT (m)	365 (280, 429)
Plasma NO ₃ ⁻ (μM)	12.2 (8.6, 16.9)
Plasma NO ₂ ⁻ (μM)	0.55 (0.44, 0.83)
Endothelial RHI score	1.9 ± 0.40
Aix@75 (%)	21.1 (13.2, 32.8)
Plasma L-arginine	81.3 ± 21.9
Plasma ADMA	0.50 (0.39, 0.63)
Arg./ADMA	157.8 (126.9, 194.6)
BNP	34 (22.5, 49)
eGFR	84 (75, 90)

Data are shown as mean ± standard deviation, median (IQR) or number (%). BMI, body mass index; FEV₁, forced expiratory volume in 1 second; TLco, transport factor for carbon monoxide; ICS, inhaled corticosteroid; LABA, long-acting beta agonist; LAMA, long-acting muscarinic agonist; DM, diabetic mellitus; GERD, gastroesophageal reflux disease; MRC, modified medical council; CAT, COPD Assessment Test; FeNO, fractional exhaled nitric oxide; BP, blood pressure; 6MWT, six-minute walk test RHI, reactive hyperemia index; Aix75, augmentation index corrected at 75 beat per minutes; ADMA, asymmetric dimethylarginine; eGFR, estimated glomerular filtration rate; BNP, brain natriuretic peptide.

5.3.3 Correlation between blood nitrate levels (NO₃⁻) and breathlessness (MRC score)

As shown in Figure 5.2, there was no correlation between plasma NO₃⁻ and MRC score (rho = -0.08, p=0.51).

Figure 5.2: Correlation between blood nitrate and MRC score

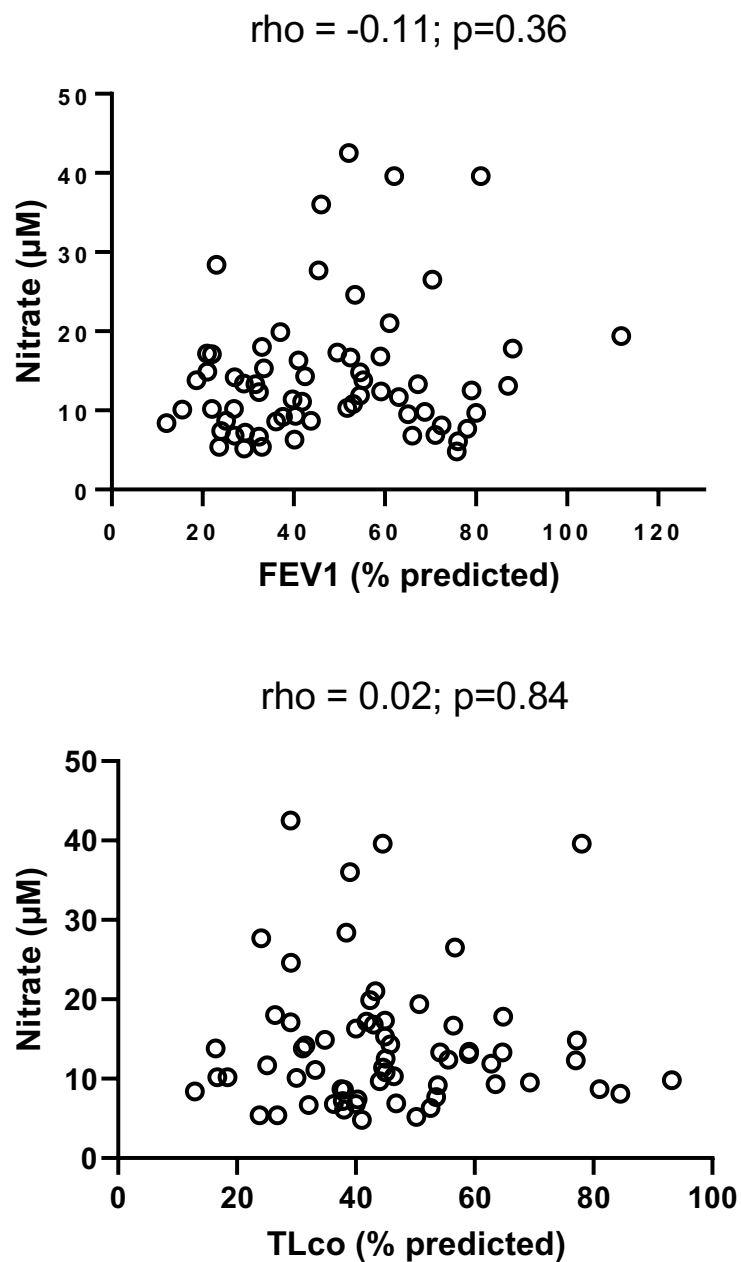


5.3.4 Correlation between blood nitrate levels (NO_3^-) and lung function

As shown in Figure 5.3, there was no correlation between plasma NO_3^- and $\text{FEV}_1\%$ (pred.)

($\rho = 0.11$, $p=0.36$ or $\text{TLco}\%$ (pred.) ($\rho = 0.02$, $p=0.84$).

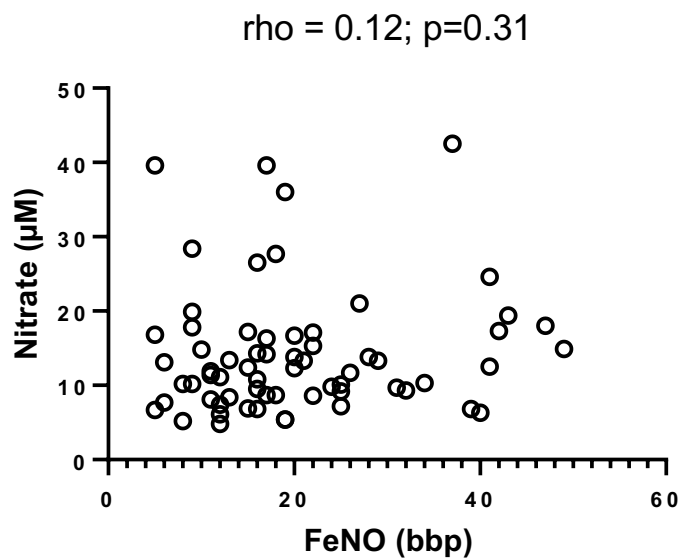
Figure 5.3: Correlation between blood nitrate and lung function



5.3.5 Correlation between blood nitrate levels (NO_3^-) and exhaled nitric oxide (FeNO)

Figure 5.4 showed no correlation between plasma NO_3^- and FeNO ($\rho = 0.12$, $p=0.31$).

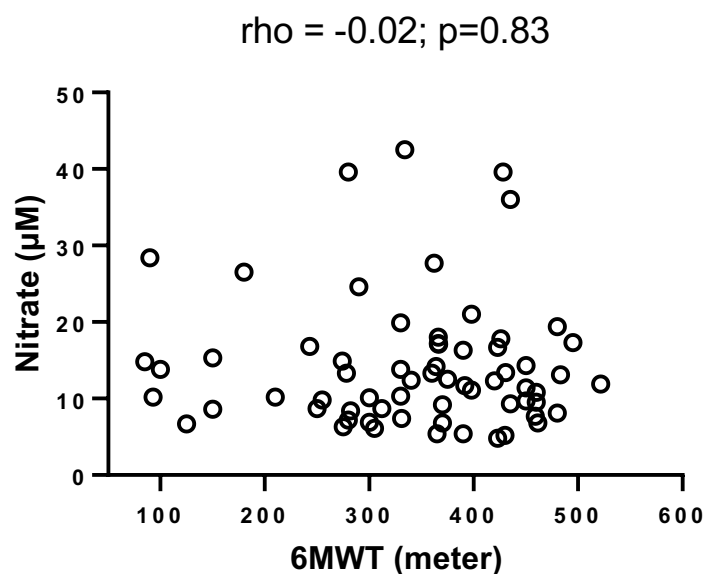
Figure 5.4: Correlation between blood nitrate and exhaled nitric oxide



5.3.6 Correlation between plasma NO_3^- and six minute walk test (6MWT)

Figure 5.5 showed no correlation between plasma NO_3^- and 6MWT ($\rho = -0.02$, $p=0.83$).

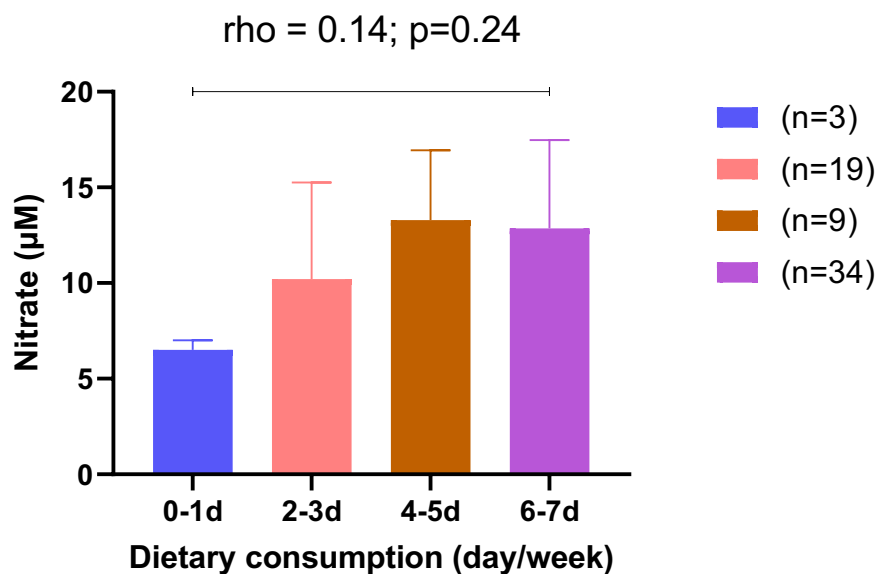
Figure 5.5: Correlation between blood nitrate and six minute walk test



5.3.7 Correlation between blood nitrate levels (NO_3^-) and dietary consumption (day/week)

As shown in Figure 5.6, there was no correlation between plasma NO_3^- and dietary consumption ($\rho = 0.14$, $p=0.24$). However, there was a significant difference between the low vegetable diet (3 or fewer days per week) and the high vegetable diet (4 or more days per week); $p=0.02$.

Figure 5.6: Correlation between blood nitrate and dietary consumption



5.3.8 Correlations between blood nitrate levels (NO_3^-) and other baseline variables

Table 5.2 presented the results of correlations of plasma NO_3^- and other variables. There were negative correlations between the plasma NO_3^- and DBP ($\rho = -0.24$, $p= 0.04$; Figure 5.7), and MAP ($\rho = -0.27$, $p= 0.02$; Figure 5.8). Plasma NO_3^- was positively correlated with the RHI score ($\rho = 0.38$, $p= 0.001$; Figure 5.9) and serum potassium level ($\rho = 0.26$, $p=0.03$; Figure 5.10).

Table 5.2 Correlations between plasma NO₃⁻ (μM) and baseline variables

Parameters	Correlation coefficient	p value
MRC score	-0.08	0.51
FeNO (ppb)	0.12	0.31
CAT score	0.15	0.20
SBP (mmHg)	-0.17	0.16
DBP (mmHg)	-0.24	0.04
MAP (mmHg)	-0.27	0.02
O ₂ saturation (%)	-0.18	0.14
FEV ₁ (% predicted)	0.11	0.36
TLco (% predicted)	0.02	0.84
6MWT (m)	-0.02	0.83
Urea (mmol/L)	0.22	0.07
Serum sodium (mmol/L)	-0.04	0.73
Serum potassium (mmol/L)	0.26	0.03
BNP (ng/L)	0.13	0.28
Creatinine (μmol/L)	-0.13	0.29
eGFR (ml/min)	-0.23	0.05
CRP (mg/L)	0.13	0.29
Endothelial RHI	0.38	0.001
Alx@75 (%)	-0.14	0.25
Arginine (μM)	-0.08	0.51
ADMA level (μM)	0.11	0.36
Arginine/ADMA ratio	-0.14	0.26
Platelet aggregation (%)	-0.06	0.60
P-selectin expression (%)	-0.22	0.08
Dietary consumption (day/week)	0.14	0.24

Figure 5.7: Correlation between blood nitrate and diastolic blood pressure

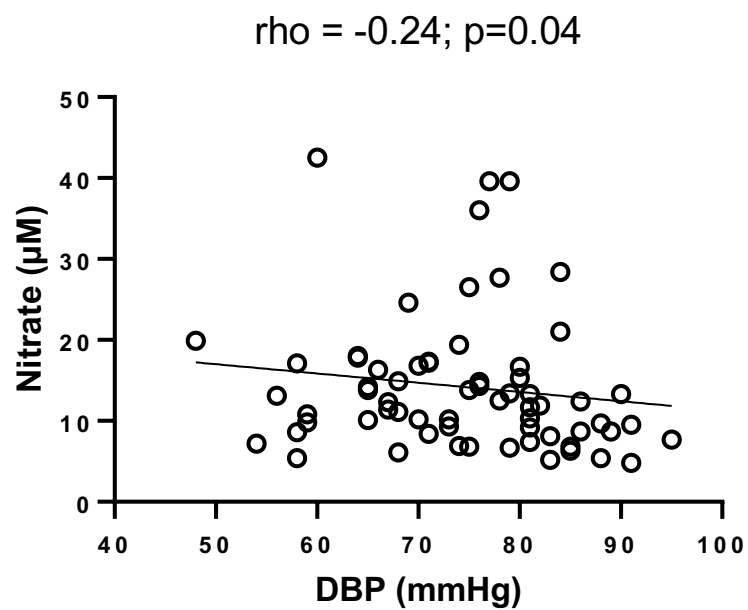


Figure 5.8: Correlation between blood nitrate and mean arterial blood pressure

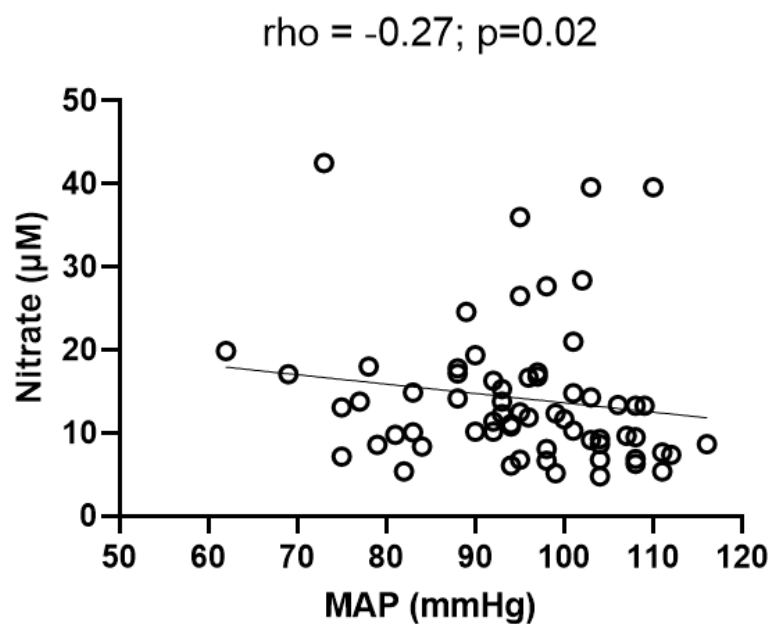


Figure 5.9: Correlation between blood nitrate and reactive hyperaemia index

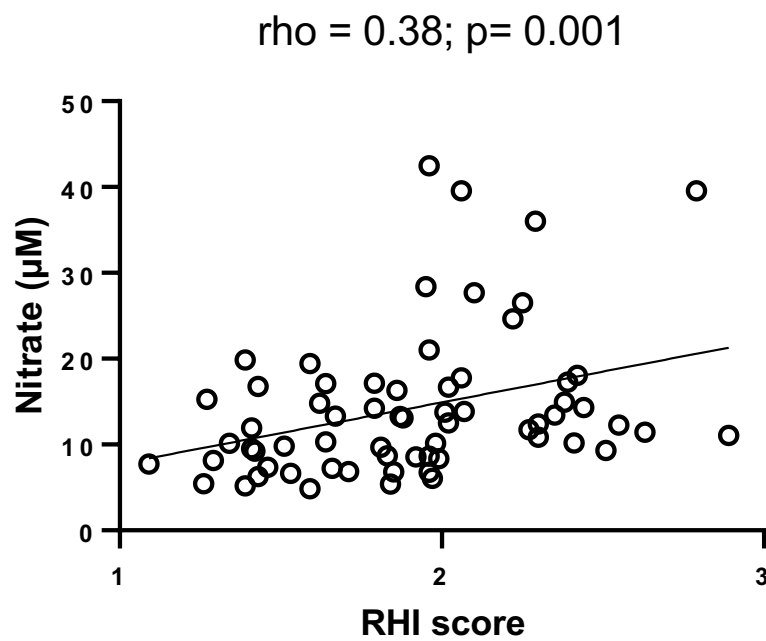
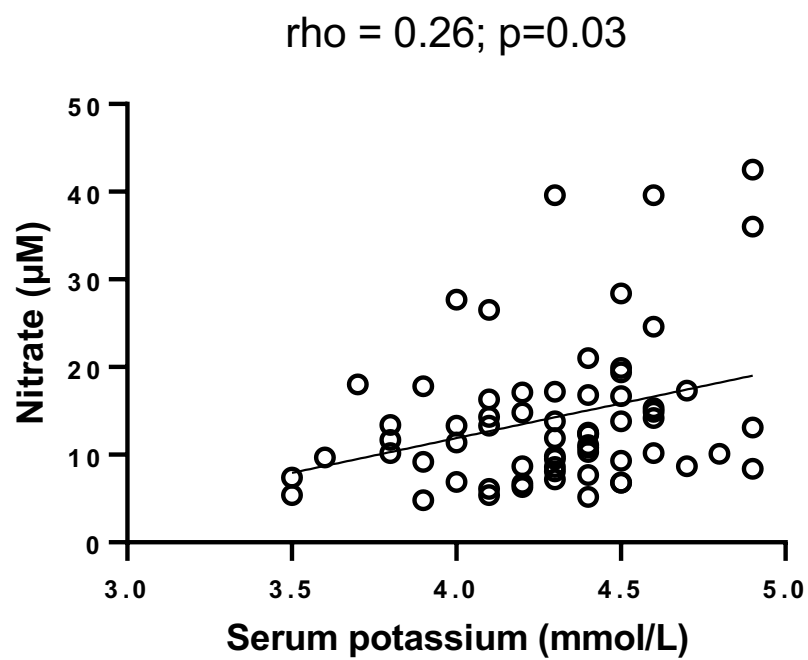


Figure 5.10: Correlation between blood nitrate and serum potassium



5.4 Discussion

The main findings of this study were that, at baseline, plasma nitrate levels negatively correlated with blood pressure parameters such as DBP and MAP in individuals with stable COPD. Furthermore, plasma nitrate levels were positively correlated with the endothelial RHI score and serum of potassium level. However, no correlations were found between the plasma nitrate and measures of disease severity including MRC score, exercise capacity and lung function parameters or any other variable. Nitrate levels were lower in those with a diet poor in vegetables.

The primary outcome of the EON-lung study was to investigate if there is a link between plasma nitrate levels and the MRC score in COPD. The results revealed that there was no association between plasma nitrate and MRC score. Only one study assessed the effect of dietary nitrate supplementation on the MRC score in people with COPD (Pavitt et al., 2020). In this study, Pavitt *et al* reported no significant differences in the change in MRC score between active and placebo treatment following a dose of (12.9 mmol) of NR-BRJ during pulmonary rehabilitation for the 8 week ON-EPIC trial; median (IQR) NR-BRJ -1 (-1.0, -0.0) versus placebo 0.0 (-1.0, 0.0), estimated treatment effect 0 (95% CI 0 to 0); $p=0.90$ (Pavitt et al., 2020).

FeNO is a well-established test for assessing asthmatic patients' airway inflammation and response to inhaled corticosteroids (Dweik et al., 2011). According to the guidelines for exhaled nitric oxide measurements (ATS/ERS 2005), nitrate-rich foods should be avoided as they can increase exhaled nitric oxide measurements (Olin et al., 2001, Zetterquist et al.,

1999). COPD patients have a low exhaled NO level (Kharitonov and Barnes, 2002). It has been concluded by previous studies that stable COPD patients have the same amount of exhaled NO as healthy individuals (Delen et al., 2000, Ichinose et al., 2000, Rutgers et al., 1999). This reduction in exhaled NO level is linked to the effect of smoking, which downregulates epithelial NOS (Su et al., 1998), and may imply increased oxidative stress that consumes NO in the formation of peroxynitrite (Eiserich et al., 1998). Furthermore, smoking has been shown to reduce plasma nitrate and nitrite levels in COPD population (Liu et al., 2007).

In this study, the findings showed no correlation between exhaled NO and plasma nitrate levels in individuals with stable COPD. This lack of correlation between plasma nitrate and exhaled NO indicates that the possibility of using the exhaled NO level to identify the appropriate nitrate dosing is challenging and cannot be used. However, it has been reported that there are no correlations between exhaled NO and plasma nitrate and nitrite levels in the asthma population (Nadif et al., 2014) and sputum nitrate and nitrite levels in patients with COPD (Silkoff et al., 2001, Ziora et al., 2007).

Fruits and vegetables contain nutrients such as potassium and non-nutrients like polyphenols, and the high potassium content of fruits and vegetables is associated with reduced cardiovascular disease (Blanch et al., 2015). Evidence suggests that consumption of inorganic nitrate has been shown to lower blood pressure, reduce arterial stiffness and platelet aggregation, and improve endothelial function in healthy individuals and in adults with cardiovascular risk (Jackson et al., 2018, Siervo et al., 2013). Similarly, in Chapter 3, our results of the systematic review and meta-analysis suggest that dietary nitrate supplementation has the potential to reduce cardiovascular risk by lowering blood pressure in people with COPD

(Alsulayyim et al., 2021). In line with this, we found that plasma nitrate was inversely related to blood pressure and positively related to endothelial function and potassium serum level. This may be because most of the participants (66%) in this study consumed high nitrate-rich foodstuffs (more than three days per week) although there was no observed correlation between dietary consumption per week and plasma nitrate level.

Plasma ADMA is produced endogenously and inhibits NOS production in endothelial cells, which reduces NO production (Cooke, 2004). It has been demonstrated that elevated ADMA concentrations are associated with COPD (Zinellu et al., 2018) and a variety of cardiovascular and metabolic diseases, including hypercholesterolemia (Böger et al., 1998), chronic renal failure (Jacobi and Tsao, 2008), chronic heart failure (Liu et al., 2016), and hypertension (Fujiwara et al., 2000). The results of our study revealed that plasma ADMA, arginine, and the arginine/ADMA ratio were not correlated with plasma nitrate. To the best of my knowledge, no published studies have investigated the effect of nitrate supplementation on plasma ADMA and arginine levels in COPD population. However, in individuals with pulmonary hypertension, Henrohn et al. (2018) found no significant change in arginine and ADMA plasma levels following NR-BRJ compared to placebo; plasma arginine (median of differences 0.15 μ M, 95% CI [-8.00 to 6.15,]), $P=1.00$; plasma ADMA (median of differences 0.05 μ M, 95% CI [-0.08 to 0.12,]), $P=0.23$ (Henrohn et al., 2018).

Finally, several limitations of this study must be acknowledged. The sample size was limited. This study was supposed to include 100 participants from three different populations: COPD, ILD, and PH. As a result, we did not conduct formal sample size calculations since we did not

have data on the variability of these measures in these patient groups. We also found it difficult to recruit for three groups of people with lung disease (COPD, PH, and ILD) due to the COVID-19 pandemic, which meant clinics were online, so we focused on the COPD group, which ended up with 65 participants.

5.5 Conclusion

Although plasma nitrate levels were not associated with the MRC score or FeNO, plasma nitrate levels were revealed to be negatively correlated with blood pressure and positively correlated with the endothelium RHI score and serum potassium level in individuals with stable COPD.

CHAPTER 6: Discussion and future work

6.1 Summary of thesis aims

This chapter summarises the main findings and clinical implications of this thesis. It also covers some future work, as well as some strengths and limitations.

Overall, the aims of this thesis were as follows: 1) to further extend the knowledge and synthesis the current evidence and evaluate the impact of dietary NO_3^- supplementation on exercise capacity and cardiovascular parameters in CRDs (Chapter 3); 2) to investigate the effects of acute NO_3^- administration on exercise performance, blood pressure, and FMD in pulmonary hypertension patients who are using ambulatory oxygen or desaturate on exercise (Chapter 4); and 3) to investigate if there is a link between blood nitrate levels and breathlessness and other measures of disease severity (Chapter 5).

6.2 Summary of main findings

6.2.1 Impact of dietary nitrate supplementation on exercise capacity and cardiovascular parameters in chronic respiratory disease: a systematic review and meta-analysis (Chapter 3)

The results of the systematic review and meta-analysis showed that dietary nitrate supplementation has the potential to improve exercise capacity as well as reduce cardiovascular risk by lowering blood pressure in people with COPD. The observed effects of dietary nitrate supplementation were moderate evidence to support the hypothesis that dietary nitrate supplementation lowers blood pressure. However, the GRADE evidence to support an improvement in exercise capacity following nitrate supplementation was low. This was based on a limited number of studies with considerable variations in study designs, outcome measures, clinical phenotypes, exercise protocols, and dose and duration of nitrate supplementation, and most trials focused on COPD and were short-term.

6.2.2 Effect of dietary nitrate supplementation on exercise performance in pulmonary hypertension: The EDEN-OX trial (Chapter 4)

As previously discussed in Chapter 3, existing studies on dietary nitrate supplementation in people with lung disease have mostly focused on COPD, typically in less severe disease, but it is notable that a single dose of NR-BRJ consistently increased exercise capacity in people with COPD who needed to use home oxygen (Pavitt et al., 2021). Therefore, Chapter 4 focused on the effect of acute dietary nitrate supplementation in the form of NR-BRJ on exercise performance in hypoxic PH patients who are using ambulatory oxygen or desaturating on exercise to <90%. Findings from the EDEN-OX trial demonstrated that a single dose of NR-BRJ (12.9 mmol) significantly increased the endurance time as well as significantly reduced systolic blood pressure and mean arterial pressure compared to a dose of PL-BRJ. In addition, we observed an improvement in endothelial function using brachial artery flow-mediated dilatation and a significant rise in plasma nitrate concentrations. The findings suggest that dietary nitrate supplementation might be used as a non-pharmacological intervention to augment exercise in this population.

6.2.3 Association of nitrate levels with disease severity in lung disease - an observational study: The EON-Lung trial (Chapter 5)

In spite of the fact that dietary nitrate supplementation may be beneficial to patients with lung disease, trials to date have been relatively short-term, and they have focused on a variety of endpoints, so it is unclear what the optimum dose should be, how it should be monitored, or how it varies from individual to individual. Therefore, we explored the relationship

between plasma nitrate level and disease severity and symptoms in COPD as well as whether FeNO levels could be used as an indicator of a low blood nitrate level.

Previous studies suggest that consumption of inorganic nitrate has been shown to lower blood pressure, reduce arterial stiffness and platelet aggregation, and improve endothelial function in healthy individuals and in adults with cardiovascular risk (Jackson et al., 2018, Siervo et al., 2013). In line with this, findings from the EON-lung trial demonstrated that at baseline, plasma nitrate level was negatively correlated with blood pressure parameters as well as positively correlated with the endothelial RHI score and serum potassium level in individuals with stable COPD. However, there was correlation between plasma nitrate level and MRC breathlessness score or FeNO level.

These findings suggest that the possibility of using the exhaled NO level to identify the appropriate nitrate dosing is challenging and cannot be used. In this population, no published studies have looked at the association and relationship between FeNO levels and plasma nitrate levels. However, it has been reported that there are no correlations between exhaled NO and plasma nitrate and nitrite levels in the asthma population (Nadif et al., 2014), as well as no correlation between exhaled NO and sputum nitrate and nitrite levels in patients with COPD (Silkoff et al., 2001, Ziora et al., 2007).

6.3 Limitations of the studies

In the meta-analysis, incomplete data posed a challenge to pooling results for some outcome measures such as isotime oxygen consumption. Moreover, there was a limited number of

studies evaluating the effect of dietary nitrate supplementation on exercise capacity or cardiovascular parameters in people with lung diseases other than COPD, such as PH and ILD.

In the EDEN-OX trial, this was a single-dose study, and it remains unclear if the regular dosing and long-term treatment of dietary nitrate supplementation would have any meaningful effects or differences in clinical outcomes in pulmonary hypertension. We selected the dose based on previous studies; however, future studies should examine the dose response and ceiling effects.

In the EON-lung trial, the sample size was small and the study was not powered. In addition, the COVID-19 outbreak shifted the target sample size from 100 individuals with COPD, PH, and ILD to only 65 participants with COPD.

6.4 Clinical implications

As demonstrated in this thesis, dietary nitrate supplementation (in the form of NR-BRJ) improves exercise capacity and endurance in patients with chronic lung disease and in hypoxic pulmonary hypertension. NR-BRJ was well tolerated and had no adverse effects. As a result, using NR-BRJ is readily available, safe, and inexpensive (70 mL of BEET-IT® SPORT Shot, James White Drinks, Ipswich, costs less than £1.93) to augment exercise and reduce cardiovascular risk by lowering blood pressure in people with lung diseases.

6.5 Future work

Our group has shown that NR-BRJ reduces the oxygen cost of exercise in COPD (Curtis et al., 2015). We also found that in hypoxic COPD patients, a single dose of NR-BRJ resulted in a clinically significant increase in endurance time (Pavitt et al., 2021). Therefore, it would be interesting to evaluate whether this improvement in physical performance translates into a meaningful improvement in physical activity in daily life in longer term study.

Furthermore, further research is required to investigate the potential benefits of dietary nitrate supplementation in hypoxic conditions with other lung diseases such as ILD as well as in individuals with different pulmonary hypertension etiologies.

Finally, there are several factors that may influence the oral microbiota and the metabolism of nitrate and nitrite in COPD including smoking history, poor dentition, inhaled medications, gastric acid suppressing medications, and gastro-oesophageal reflux. Understanding the oral microbiota could lead to novel probiotic approaches that allow identification of candidate organisms responsible for denitrification of nitrate to nitrite and NO and improve nitrate metabolism in patients with COPD.

6.6 Conclusion

The findings presented in this thesis suggest that dietary nitrate supplementation has the potential to improve exercise capacity and endurance as well as reduce cardiovascular risk by lowering blood pressure in people with COPD and in the specific phenotype of individuals with pulmonary hypertension who are hypoxic and require oxygen supplementation.

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Appendices

Appendix 1: Search strategies of specific database

Ovid MEDLINE(R) (1946 to March 2021)

#	Searches
1	respiratory diseases.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
2	chronic obstructive pulmonary disease.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
3	COPD.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
4	chronic obstructive airways disease.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
5	emphysema.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
6	bronchitis.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
7	bronchiectasis.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]

8	interstitial lung disease.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
9	ILD.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
10	cystic fibrosis.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
11	pulmonary hypertension.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
12	PHT.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
13	1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12
14	Respiratory Tract Infections/ or Humans/ or Respiratory Tract Diseases/ or Asthma/ or Lung/ or Pulmonary Disease, Chronic Obstructive/
15	Pulmonary Disease, Chronic Obstructive/
16	Pulmonary Disease, Chronic Obstructive/
17	Asthma/ or Pulmonary Disease, Chronic Obstructive/ or Humans/ or Lung Diseases, Obstructive/ or Adult/
18	Emphysema/ or Pulmonary Emphysema/
19	Bronchitis, Chronic/ or Bronchitis/
20	Bronchiectasis/

21	Lung Diseases, Interstitial/
22	Adult/ or Humans/ or Lung Diseases, Interstitial/ or Pulmonary Fibrosis/
23	Cystic Fibrosis/
24	Hypertension, Pulmonary/
25	Humans/ or Hypertension, Pulmonary/
26	14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 or 23 or 24 or 25
27	13 and 26
28	nitrate.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
29	beetroot.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
30	dietary nitrate.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
31	nitrate supplementation.mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]
32	28 or 29 or 30 or 31
33	Nitrates/
34	Vegetables/ or Nitrites/ or Nitrates/ or Dietary Supplements/ or Beverages/ or Humans/
35	Nitric Oxide/ or Nitrites/ or Diet/ or Nitrates/ or Blood Pressure/ or Humans/ or Dietary Supplements/

36	Oxygen Consumption/ or Exercise/ or Dietary Supplements/ or Nitrites/ or Nitrates/ or Nitric Oxide/ or Humans/
37	33 or 34 or 35 or 36
38	32 and 37
39	27 and 38

Embase (1947 to March 2021)

#	Searches
1	respiratory diseases.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
2	chronic obstructive pulmonary disease.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
3	COPD.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
4	chronic obstructive airways disease.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
5	emphysema.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
6	bronchitis.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
7	bronchiectasis.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]

8	interstitial lung disease.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
9	ILD.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
10	cystic fibrosis.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
11	pulmonary hypertension.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
12	PHT.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
13	1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12
14	Respiratory Tract Infections/ or Humans/ or Respiratory Tract Diseases/ or Asthma/ or Lung/ or Pulmonary Disease, Chronic Obstructive/
15	Pulmonary Disease, Chronic Obstructive/
16	Pulmonary Disease, Chronic Obstructive/
17	Asthma/ or Pulmonary Disease, Chronic Obstructive/ or Humans/ or Lung Diseases, Obstructive/ or Adult/
18	Emphysema/ or Pulmonary Emphysema/
19	Bronchitis, Chronic/ or Bronchitis/
20	Bronchiectasis/
21	Lung Diseases, Interstitial/
22	Adult/ or Humans/ or Lung Diseases, Interstitial/ or Pulmonary Fibrosis/
23	Cystic Fibrosis/
24	Hypertension, Pulmonary/

25	Humans/ or Hypertension, Pulmonary/
26	14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 or 23 or 24 or 25
27	13 and 26
28	nitrate.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
29	beetroot.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
30	dietary nitrate.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
31	nitrate supplementation.mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
32	28 or 29 or 30 or 31
33	Nitrates/
34	Vegetables/ or Nitrites/ or Nitrates/ or Dietary Supplements/ or Beverages/ or Humans/
35	Nitric Oxide/ or Nitrites/ or Diet/ or Nitrates/ or Blood Pressure/ or Humans/ or Dietary Supplements/
36	Oxygen Consumption/ or Exercise/ or Dietary Supplements/ or Nitrites/ or Nitrates/ or Nitric Oxide/ or Humans/
37	33 or 34 or 35 or 36
38	32 and 37
39	27 and 38

CINAHL (1960 to March 2021)

S1	respiratory diseases
S2	chronic obstructive pulmonary disease
S3	COPD
S4	chronic obstructive airways disease
S5	emphysema
S6	bronchitis
S7	bronchiectasis
S8	interstitial lung disease
S9	ILD
S10	cystic fibrosis
S11	pulmonary hypertension
S12	PHT
S13	S1 OR S2 OR S3 OR S4 OR S5 OR S6 OR S7 OR S8 OR S9 OR S10 OR S11 OR S12
S14	nitrate
S15	beetroot
S16	dietary nitrate
S17	nitrate supplementation
S18	S14 OR S15 OR S16 OR S17
S19	S13 AND S18

Cochrane database - up to March 2021:

ID	Search
#1	respiratory diseases
#2	chronic obstructive pulmonary disease
#3	COPD
#4	chronic obstructive airways disease
#5	emphysema
#6	bronchitis
#7	bronchiectasis
#8	interstitial lung disease
#9	ILD
#10	cystic fibrosis
#11	pulmonary hypertension
#12	PHT
#13	#1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12
#14	nitrate
#15	beetroot
#16	dietary nitrate
#17	nitrate supplementation
#18	#14 OR #15 OR #16 OR #17
#19	#13 AND #18

PubMed – up to March 2021

1. **respiratory diseases:** "respiration disorders"[MeSH Terms] OR ("respiration"[All Fields] AND "disorders"[All Fields]) OR "respiration disorders"[All Fields] OR ("respiratory"[All Fields] AND "diseases"[All Fields]) OR "respiratory diseases"[All Fields]
2. **chronic obstructive pulmonary disease:** "pulmonary disease, chronic obstructive"[MeSH Terms] OR ("pulmonary"[All Fields] AND "disease"[All Fields] AND "chronic"[All Fields] AND "obstructive"[All Fields]) OR "chronic obstructive

- pulmonary disease"[All Fields] OR ("chronic"[All Fields] AND "obstructive"[All Fields] AND "pulmonary"[All Fields] AND "disease"[All Fields])
3. **COPD:** "pulmonary disease, chronic obstructive"[MeSH Terms] OR ("pulmonary"[All Fields] AND "disease"[All Fields] AND "chronic"[All Fields] AND "obstructive"[All Fields]) OR "chronic obstructive pulmonary disease"[All Fields] OR "copd"[All Fields]
 4. **chronic obstructive airways disease:** "pulmonary disease, chronic obstructive"[MeSH Terms] OR ("pulmonary"[All Fields] AND "disease"[All Fields] AND "chronic"[All Fields] AND "obstructive"[All Fields]) OR "chronic obstructive pulmonary disease"[All Fields] OR ("chronic"[All Fields] AND "obstructive"[All Fields] AND "airways"[All Fields] AND "disease"[All Fields]) OR "chronic obstructive airways disease"[All Fields]
 5. **emphysema:** "emphysema"[MeSH Terms] OR "emphysema"[All Fields] OR "emphysemas"[All Fields] OR "pulmonary emphysema"[MeSH Terms] OR ("pulmonary"[All Fields] AND "emphysema"[All Fields]) OR "pulmonary emphysema"[All Fields]
 6. **bronchitis:** "bronchitis"[MeSH Terms] OR "bronchitis"[All Fields] OR "bronchitides"[All Fields]
 7. **bronchiectasis:** "bronchiectasies"[All Fields] OR "bronchiectasis"[MeSH Terms] OR "bronchiectasis"[All Fields] OR "bronchiectases"[All Fields]
 8. **interstitial lung disease:** "lung diseases, interstitial"[MeSH Terms] OR ("lung"[All Fields] AND "diseases"[All Fields] AND "interstitial"[All Fields]) OR "interstitial lung diseases"[All Fields] OR ("interstitial"[All Fields] AND "lung"[All Fields] AND "disease"[All Fields]) OR "interstitial lung disease"[All Fields]
 9. **cystic fibrosis:** "cystic fibrosis"[MeSH Terms] OR ("cystic"[All Fields] AND "fibrosis"[All Fields]) OR "cystic fibrosis"[All Fields]
 10. **pulmonary hypertension:** "hypertension, pulmonary"[MeSH Terms] OR ("hypertension"[All Fields] AND "pulmonary"[All Fields]) OR "pulmonary hypertension"[All Fields] OR ("pulmonary"[All Fields] AND "hypertension"[All Fields])
 11. **PHT:** "lung"[MeSH Terms] OR "lung"[All Fields] OR "pulmonary"[All Fields]) AND "hypertension" [All Fields]
 12. **nitrate:** "nitratation"[All Fields] OR "nitrates"[MeSH Terms] OR "nitrates"[All Fields] OR "nitrate"[All Fields]
 13. **beetroot:** "beetroot"[All Fields] OR "beetroots"[All Fields]
 14. **dietary:** "diet"[MeSH Terms] OR "diet"[All Fields] OR "dietary"[All Fields] OR "dietaries"[All Fields]
 15. **nitrate:** "nitratation"[All Fields] OR "nitrates"[MeSH Terms] OR "nitrates"[All Fields] OR "nitrate"[All Fields]
 16. **nitrate:** "nitratation"[All Fields] OR "nitrates"[MeSH Terms] OR "nitrates"[All Fields] OR "nitrate"[All Fields]
 17. **supplementation:** "supplemental"[All Fields] OR "supplementation"[All Fields] OR "supplementation"[All Fields] OR "supplementation's"[All Fields] OR "supplementations"[All Fields] OR "supplementation"[All Fields]

Appendix 2: Ethical approval letter



Dr Nicholas Hopkinson
Reader in Respiratory Medicine and Hon Consultant
Physician
NHLI
Respiratory Muscle Lab
Royal Brompton Hospital
Fulham Rd
SW3 6NP

Email: hra.approval@nhs.net
HCRW.approvals@wales.nhs.uk

04 February 2020

Dear Dr Hopkinson

**HRA and Health and Care
Research Wales (HCRW)
Approval Letter**

Study title:	EON-Lung: Effect Of Nitrate levels in Lung Disease - an observational study.
IRAS project ID:	266682
REC reference:	19/EE/0256
Sponsor	Imperial College London

I am pleased to confirm that [HRA and Health and Care Research Wales \(HCRW\) Approval](#) has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

Please now work with participating NHS organisations to confirm capacity and capability, in line with the instructions provided in the "Information to support study set up" section towards the end of this letter.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?

HRA and HCRW Approval does not apply to NHS/HSC organisations within Northern Ireland and Scotland.

If you indicated in your IRAS form that you do have participating organisations in either of these devolved administrations, the final document set and the study wide governance report (including this letter) have been sent to the coordinating centre of each participating nation. The relevant national coordinating function/s will contact you as appropriate.

Appendix 3: EndoPAT test protocol

1. Asked the participants for any deformities or injuries in their figure of non-dominant arm and I recommend them to abstain for at least 1 hour from caffeine and cigarettes smoking these could affect vascular tone.
2. Measured the blood pressure using the control arm (the arm that is not occluded during the EndPAT test).
3. Conducted the EndoPAT test in a quiet, dimly lit exam room with room temperature set at (22–24°C) to reduce fluctuations in vascular tone.
4. Asked the participants to switch-off their cell phones or silent them as well as remove watches, rings, or other jewelry on the hands or fingers.
5. Used the index finger as recommended; however, if this finger is unsuitable, use a different digit (except the thumb) as long as the same finger is used on both hands.
6. The patient should be supine and comfortable for 15 minutes to attain a cardiovascular steady state. Then I place the two arm supporters along each of the patient's sides.
7. Placed the occlusion cuff pressure on the arm to be occluded during the test. Applied it without excess pressure.
8. At this step, I clicked the "Patient Information" icon on the tool bar to create a new patient file. Then, entered the participant information including study ID, age, gender, height (cm), weight (kg), SBP/DBP.
9. Selected two new PAT probes and connect to the pneumo-electrical tubing.

10. Placed the connected probes into the sockets of the arm-supports and press the "Deflate" button on the top of the device and then asked the participants to insert index fingers completely into the probes, and pressed the "Inflate" button on the top of the Endo-PAT 2000 device.
11. Placed a foam anchor ring at the base of the adjacent middle finger to ensure the foam ring and the PAT sensor do not touch.
12. Final step before starting the test positioned the patient's arms so the forearms are supported on the arm supports and the fingers dangle freely off the edge of the support. Make sure the probes are not in contact with any object, including the arm support, foam ring, tubing, the mattress or another finger.
13. Explained to the patient that during the test you will inflate the arm cuff, and during that time they may feel some discomfort, numbness, or tingling and they should stay calm from moving the fingers throughout the test as this will create mechanical artifacts.
14. Clicked the "Standby" icon for 1 minute to inspect the tracings of the PAT signals from the two probes to confirm that they are free of artefactual signals.
15. Then, clicked the "Go" icon to start and at the same time starting the stopwatch, by clicking the "Start/Stop Timer" icon for five-minute for the baseline recording period.
16. When reaching the last few seconds of baseline recording, tell the patient that I am going to inflate the cuff for the occlusion phase and participant should stay relaxed and not move the fingers.

17. Rapidly inflate the cuff pressure to a supra-systolic pressure of 60mmHg above the patient's systolic pressure, whichever is higher, and start the stopwatch again for minutes. To confirm occlusion, increase the gain on the screen of the channel of the occluded side to 20,000 while keeping the gain of other side constant.
18. Toward the end of the occlusion period, deflate the cuff and tell the subject I am going to release the cuff and that they should continue to calm from moving their fingers and start/Stop Timer" icon again to initiate a five-minute post occlusion recording period.
19. After these five minutes, click the "Test Stop" icon to complete the study. The probes will automatically deflate.
20. The results of the test can be reached via the study file and run the automatic analysis. The occlusion period will be highlighted in blue, and the test result will be displayed, including the Reactive Hyperemia Index (RHI) and Heart Rate (HR), in the right-hand column of the screen.