Dietary Nitrate Supplementation and Cardiovascular Risk Outcomes in Chronic Obstructive Pulmonary Disease

by

Ali Mubarak Alasmari

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Airways Disease,

National Heart and Lung Institute

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DECLARATION OF ORIGINALITY

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

STATEMENT OF CONTRIBUTION

The studies described in this thesis were performed between 2019 and 2021 during my PhD candidature at the National Heart and Lung Institute, Imperial College London. This thesis was written wholly by myself with the guidance of my supervisors Professor Nicholas Hopkinson and Professor Michael Polkey.

Professor Hopkinson and Professor Polkey developed the original idea for the research studies encompassed within this thesis. With the assistance of Professor Hopkinson, I designed and wrote the trial protocols.

The systematic review in chapter three was a collaborative work with my colleague Mr Abdullah Alsulayyim. In this work prespecified data collection was undertaken by myself (including cardiovascular related outcomes of BP parameters, HR, and FMD), then I ran the meta-analysis for these outcomes. For studies presented in Chapters four, five, and six subject trial visits, outcome assessments, collection and analyses were undertaken by myself including collecting and analyses home BP measurements from patients' BP log sheet. I collected and processed blood sampling for plasma nitrate, nitrite, arginine, ADMA at our research facility. A member from Professor Jane Mitchell lab helped me run the above-mentioned plasma on prespecified assays and measure them by plate reader machine. Data obtained from the plate reader ran by myself. I conducted the exhaled nitric oxide on the patients and analysed the data. For the endothelial function assessment by the EndoPAT®

Device, I did all the tests and analysed the outcomes data. The exercise capacity test (6 MWT) all assessed and analysed by myself as well. For the platelet aggregation test, I received the test assays from Professor Tim Warner (Queen Mary University of London), I draw the participants blood in each assay, process it in our research lab, while the measurements on the flow cytometry done by Professor Warner lab, and provide me with the raw data which I ran the statistical analyses by myself.

Venous blood sampling was undertaken by me as was the initial sample processing. When this was not possible assistance was provided by Dr Keir Philip (research fellow). I conducted all statistical data analysis presented in this thesis.

All sources of information have been referenced within the text.

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ABSTRACT

It has been shown that acute/short-term dietary nitrate supplementation improves cardiovascular (CV) risk outcomes. However, it is not clear if any long-term beneficial effect is present. So, the overall aim of this thesis was to investigate the effect of prolonged consumption of daily nitrate rich beetroot juice (NR-BRJ) on CV risks in patients with COPD over three months.

A pooled result from the meta-analysis I conducted at the start of my PhD has shown a reduction in BP due to short term consumption of nitrate supplement. However, the longest and largest study combined the intervention with pulmonary rehabilitation leading to inconclusive results.

Therefore, I undertook a 3-month randomised controlled trial (the ON-BC study) recruiting COPD patients with systolic blood pressure (SBP) ≥130 mmHg. We observed for the first time a sustained reduction in SBP in the (NR-BRJ) intervention group as compared to placebo (Pl-BRJ) median (IQR): Δ -4 mm Hg, (-6, -2), vs. Δ -1 mmHg (-2, 1); an estimated treatment effect -3 mm Hg, (-6, 1), $P<0.001$), respectively. This was associated with a clinically significant improvement in exercise capacity (6-minute walk distance).

To explore the potential mechanisms associated with the observed change in SBP, I studied several vascular markers. Novel findings in this section include a fall in plasma asymmetric dimethylarginine (ADMA), and an increase in the plasma L-arginine/ADMA ratio. Endothelial function assessed using peripheral arterial tonometry (PAT) improved following NR-BRJ supplement, but there was no change in platelet aggregation.

In summary, this thesis has shown that dietary nitrate supplementation in the form of NR-BRJ exerts a sustained (12 weeks) effect on blood pressure in people with COPD, accompanied by improvements in other markers of endothelial function and vascular risk. Further longer-term trials are needed to examine the effects of BRJ at CVD event rates and establish whether the effect on exercise capacity translates into a meaningful improvement in daily life physical activity.

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I consider myself fortunate to have worked as part of the respiratory muscle lab's unique team. I owe gratitude to the support of my colleagues and friends for cheering me along. Thank you to Mr. Saeed Alghamdi, and Mr. Abdullah alsulayyim for your support and friendship. Sara and Dr. Keir thank you for sharing this journey with me, your valuable advice, and comments were very helpful.

A massive thank you must go to all the participants who took the time to make such a valuable contribution to this thesis. I can't thank you enough for sharing your experience which taught me more about COPD.

Thanks a lot, to my friends (Dr. Ziyad Alshehri, Dr. Fahad Alahmadi, and Dr. Hamad Almohammadi) for their unlimited care, love, and encouragement.

I dedicate this thesis to:

My parents, thank you for showing the love and support during this Ph.D., I hope that I have made you proud. I also want to thank my brothers and sisters Saeed, Ruquaiah, Mudhi, Sharifah, Saud, and Abdullah, thank you for everything.

Suhilah nothing can happen without you. My gorgeous children (Abdullah, Dana, Jawana, and Lana), thank you for your love, patience, and being at my side on this journey. I hope as your dad I can make you proud.

GLOSSARY OF ABBREVIATIONS

ABPM	Ambulatory blood pressure monitoring
ACE	Angiotensin-converting enzyme
ACEi	Angiotensin-converting enzyme inhibitors
ARBs	Angiotensin receptor blockers
ADMA	Asymmetric dimethylarginine
ADP	Adenosine diphosphate
AECOPD	Acute exacerbation COPD
BMI	Body mass index
BNP	Brain Natriuretic Peptide
BP	Blood pressure
BRJ	Beetroot juice
cAMP	Cyclic adenosine monophosphate
CAT	COPD assessment test
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive proteins
CVD	Cardiovascular disease
cGC	Cyclic guanylate cyclase
cGMP	Cyclic guanosine monophosphate
CS	Cigarette smoking
DBP	Diastolic blood pressure
DDAH	Dimethylarginine dimethylaminohydrolase
EC	Endothelial cell
EF	Endothelial function

ED	Endothelial dysfunction
eGFR	Estimated Glomerular filtration rate
eNOS	Endothelial nitric oxide synthase
FeNO	Fractional exhaled nitric oxide
FEV_1	Forced expiratory volume in one second
FMD	Flow Mediated Dilatation
FVC	Forced vital capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GTP	Guanosine triphosphate
QoL	Quality of life
HBPM	Home Blood Pressure monitor
HF	Heart failure
HR	Heart rate
LTA	Light transmission aggregometry
MAP	Mean arterial pressure
MRC	Medical Research Council
NADPH	Nicotinamide adenine dinucleotide phosphate
NICE	National Institute for Health and Care Excellence
NO	Nitric oxide
NOS	Nitric oxide synthase
OS	Oxidative stress
PAT	Peripheral arterial tone
Pl	Placebo
PKA	Cyclic AMP-dependent protein kinase

PKG	cyclic GMP-dependent protein kinase
PPB	Parts per billion
PRP	Platelet-rich plasma
ROS	Reactive oxygen species
6MWD	six minute-walk test distance
SBP	Systolic blood pressure diastolic
WHO	World Health Organization
XOR	Xanthine oxidoreductase

Publications and abstracts arising from this thesis

- Alsulayyim, A. S., Alasmari, A. M., Alghamdi, S. M., Polkey, M. I., & Hopkinson, N. S. (2021). 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*, *8*(1), e000948.
- The Effect of Dietary Nitrate Supplementation on Blood Pressure and Exercise Capacity in COPD: A 3-month Randomised, Double-Blind, Placebo-Controlled trial. Ali M Alasmari, Abdullah S Alsulayyim, Saeed M Alghamdi, Michael I Polkey, JA Mitchell, Nicholas S Hopkinson. NHLI Research Away Day 2021, Imperial College London.

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Chapter 1: Introduction

1.1 Chapter overview

This introductory chapter provides a background of chronic obstructive pulmonary disease (COPD) definition, prevalence, disease burden, aetiology, pathophysiology, and outlines the comorbidities associated with COPD in particular cardiovascular disease (CVD) (Section 1.2.1). In addition, I briefly discussed common risk factors contributing to CVD in COPD (Section 1.2.8). I further provided in depth details about hypertension in COPD population, and the recommended guidelines approach of treatment for hypertension (pharmacological and nonpharmacological) (Section 1.2.10). Section 1.2.12 discusses the current evidence supporting a role for dietary nitrate supplementation on hypertension and other CV risk markers from animals and human trials. This leads to the rationale statement for conducting this thesis research topic (Section 1.3). Section 1.4 provides an overview of the research aims, and hypotheses.

1.2 Background

1.2.1 COPD definitions and epidemiology

Chronic obstructive pulmonary disease (COPD) is the umbrella term for progressive heterogeneous arrays of chronic bronchitis and chronic pulmonary emphysema in the respiratory system (Figure 1.1). The most widely recognised definition of COPD is defined by the **G**lobal initiative for chronic **O**bstructive **L**ung **D**isease (GOLD):

> *"a common, preventable, and treatable disease characterised by recurrent respiratory signs and airflow restriction due to airway and/or alveolar abnormalities typically caused by substantial exposure to noxious particles or gases. The chronic airflow limitation characteristic of COPD is caused by a mixture of small airway disease (e.g., obstructive bronchiolitis) and parenchymal damage (emphysema), the relative contribution of which varies from person to person, and with some*

significant extrapulmonary effects that may contribute to the severity in individual patients."

Global Initiative for Chronic Obstructive Lung Disease (GOLD) (Vestbo, 2013).

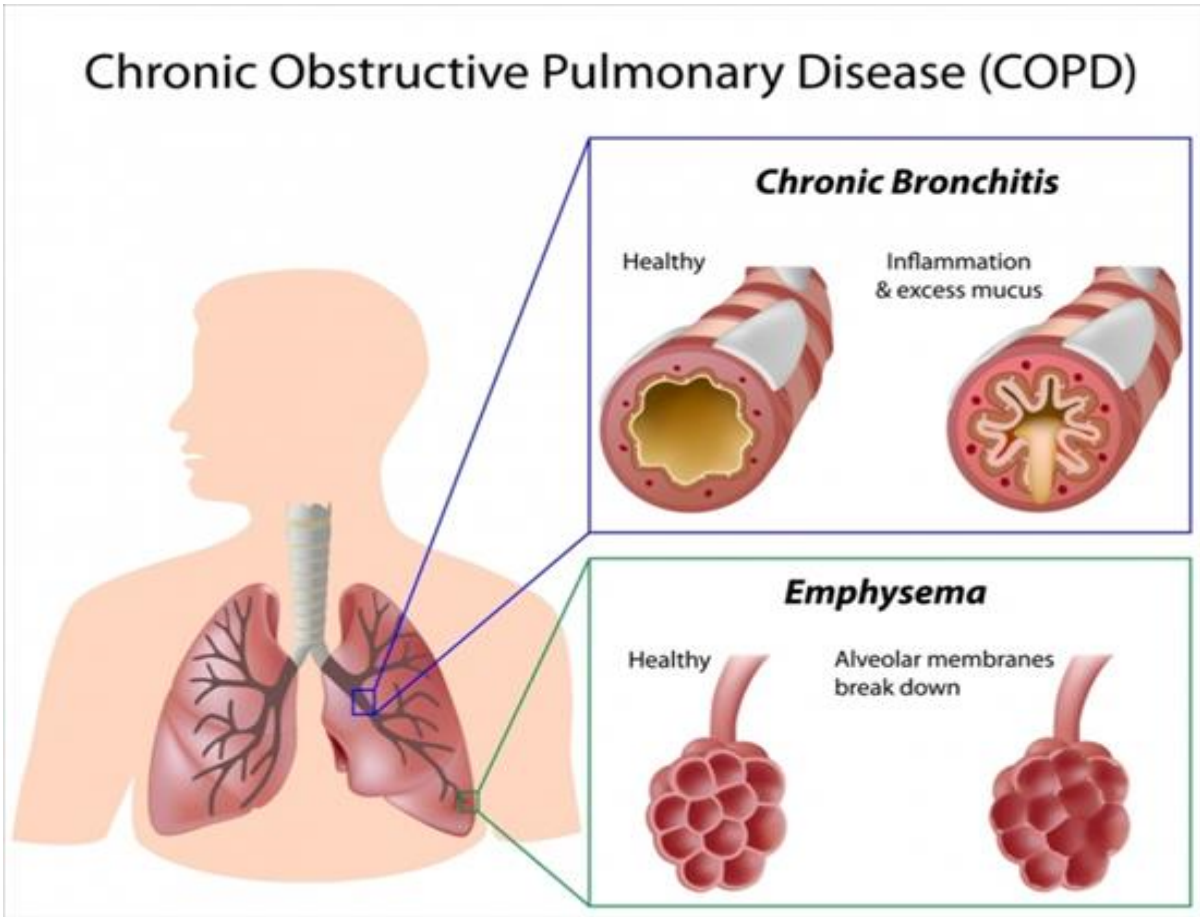


Figure 1-1 chronic bronchitis and emphysema make up COPD.Adapted from Applied Chest Imaging Laboratory, Feb 2021 "https://acil.med.harvard.edu/copd".

COPD is a prevalent chronic disease and a leading cause of mortality, bringing significant socio-economic strain to the UK and across the world (Chen & Mannino, 1999). A range of worldwide studies has examined the prevalence of COPD both in the developed and developing world and concurs on the severity and implications of this condition (Buist, 2007; Halbert, 2006; D. M. Mannino & Buist, 2007; Menezes, 2005; Tan, 2015). Published data from the **B**urden of **O**bstructive **L**ung **D**isease (BOLD) and the study of five Latin American countries (PLATINO) estimated that the prevalence of COPD ranges from 9% to 10%, which

affects 9.8% of men and 8.6% of women in the general population aged 40 years or older (Mathers & Loncar, 2006; Seemungal, Hurst, & Wedzicha, 2009; World Health Organization, 2017). The WHO 2016 report on COPD revealed there were 251 million cases of COPD reported globally (World Health Organization, 2017). Evidence from epidemiological studies showed that women are more susceptible than men to the effects of cigarette smoking, and therefore the overall trend for COPD prevalence in women is expected to rise worldwide (Foreman, 2011; Lopez Varela, 2010; D. M. Mannino, 2007; Silverman, 2000). In the UK, the prevalence of COPD is estimated to be 1.7% and 1.4% among males and females, respectively, accounting for approximately 1.2 million individuals diagnosed with COPD (British Lung Foundation, 2016).

Mortality statistics data released in 2015 by the WHO illustrates that COPD claimed the lives of 3.2 million people worldwide (World Health Organization., 2017). Currently, COPD is considered the third leading cause of death in the world, and it is predicted to rise (see Figure 1.2) (Mathers, 2006).

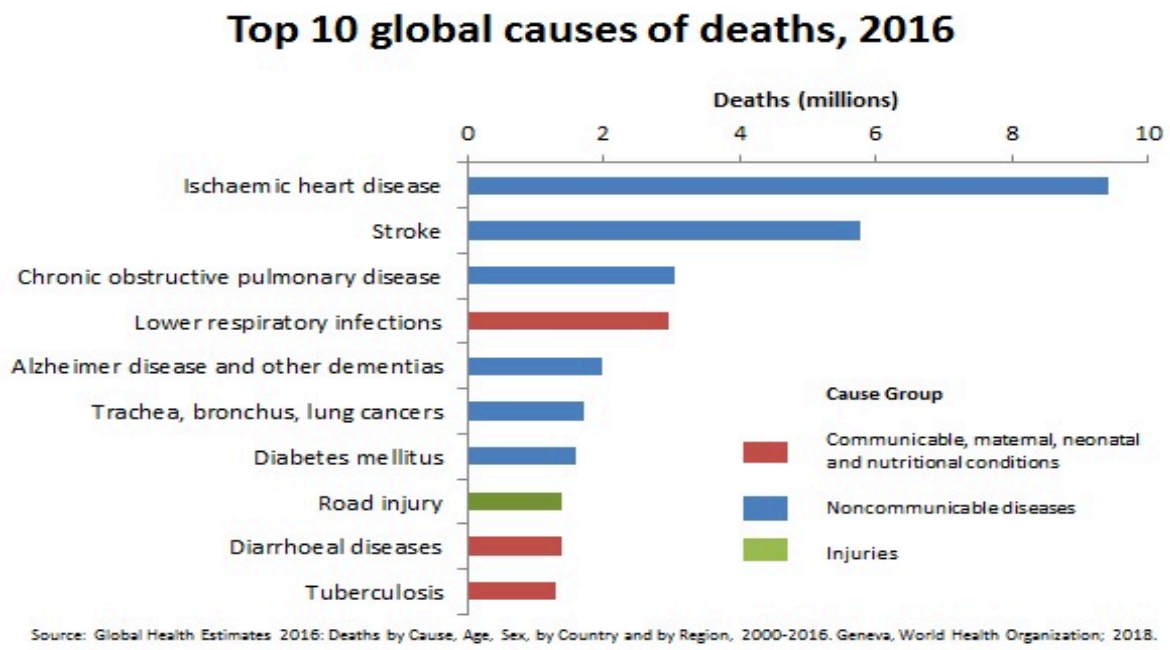


Figure 1-2: The ten leading causes of death in the world Adapted from (http://www.who.int/mediacentre/factsheets/fs310/en/).

In 2012, death statistics reported in the UK showed 26.1% of deaths due to lung diseases. 29,776 deaths were reported from COPD, which accounts for 5.3% of the total number of UK deaths (Snell, 2016).

In addition, COPD is a costly disease with a considerable socio-economic burden on the healthcare system. COPD acute exacerbation-related hospitalisations account for between 50% and 70% of COPD management costs (S. D. Aaron, 2014). In the United Kingdom, 140,000 hospital admissions were recorded in the emergency room for patients diagnosed with COPD, and 97% were admitted as an acute exacerbation of COPD (AECOPD) (British Lung Foundation, 2016). Approximately 20% of hospitalised patients with AECOPD were discharged; however, they had AECOPD flare-ups within 30 days (Jencks, Williams, & Coleman, 2009). In 2013, the UK healthcare system estimated the direct and indirect costs of patients with COPD at around £ 1.9 billion a year (Guarascio, Ray, Finch, & Self, 2013). This

cost is projected to rise to £2.32 billion in 2030, given that the COPD burden is expected to rise in the coming years as the British population ages, and exposure to risk factors increases (British Lung Foundation, 2016; G. B. D. C. R. D. Collaborators, 2017). As a result, the use and quality of healthcare resources are significantly affected.

1.2.2 Aetiology

Typically, COPD is a silent and progressive disease that gradually develops over many years. Both smokers and non-smokers are vulnerable to specific risk factors that are related to the cause of COPD (D. M. Mannino, 2007).

Cigarette smoking

Cigarette smoking (CS) is the predominant environmental risk factor for developing COPD in 80% to 90% of cases (Vestbo, 2013). Tobacco smokers have a higher prevalence of respiratory symptoms, airflow obstruction, lung structural changes, and an increased annual decline in FEV1% compared to non-smokers (D. M. Mannino, 2002). The risk of COPD development and disease severity are directly related to the total exposure (number of packs/years smoked) and predicted mortality (D. M. Mannino, 2007). In a large population study conducted from 1951 to 1991, British doctors mailed questionnaires to more than 34,000 patients to assess risks associated with long-term tobacco use (Doll, Peto, Boreham, & Sutherland, 2004). A series of observational studies revealed direct associations observed between smoking habit and high mortality, with approximately 50% of regular tobacco smokers reporting mortality associated with stroke and heart disease (Doll, Peto, Wheatley, Gray, & Sutherland, 1994) The study results also showed those who stopped smoking earlier

in their life (before middle-age) at a lower risk and increased subsequent expectation of life compared to those who continued smoking (Doll, 1994). However, this conclusion and many other conclusions from epidemiological (cross-sectional) studies assessing tobacco association with mortality are based on correlations, not cause-and-effect relationships (Yang, 2019).

Other environmental risk factors

COPD is also developed from environmental risk factors include occupational exposure to dust or fumes (Rennard & Vestbo, 2006). According to the NHANESIII study, 19.2% of COPD individuals and 31.1% of non-smokers were found to be exposed to occupational agents (Hnizdo, Sullivan, Bang, & Wagner, 2002). In developing countries, fractions of COPD are also seen in non-smokers due to exposure to wood smoke (biomass fuel) used for cooking with poor ventilation (K. R. Smith & Mehta, 2003). Other factors, such as outdoor air pollution and malnutrition, which may be related to lower socio-economic status, are all risks associated with developing COPD (Gershon, Warner, Cascagnette, Victor, & To, 2011).

Genetic/host risk factors

Alpha-1 proteinase inhibitor deficiency (AATD), also known as Alpha-1 antitrypsin deficiency, is an autosomal co-dominant genetic condition that is caused by mutations in the SERPINA1 gene (chromosome 14) and has been identified as a genetic risk factor for emphysema (Fregonese, Stolk, Frants, & Veldhuisen, 2008). Additionally, individuals with a history of early life infections and asthma/bronchial hyperreactivity also have a higher risk of acquiring COPD .

1.2.3 Symptoms, diagnosis, classifications, and prognosis

COPD is a silent disease that progresses over many years until symptoms impact the person to such a degree that they seek medical attention (GOLD, 2017). Many of these symptoms (see Table 1.1) often develop independently and with variable intensity in individuals aged 40 years or older (GOLD, 2017).

Table 1-1 The main clinical features of COPD

- Chronic cough, which may be daily and productive, but can also be intermittent and unproductive
- Breathlessness on exertion, initially intermittent and becoming persistent
- Sputum production: any pattern of sputum production may indicate COPD
- Frequent exacerbations of bronchitis
- A history of exposure to risk factors, especially tobacco smoke, occupational dust, home cooking, and biomass fuels.

Dyspnoea is the cardinal symptom of COPD and develops during physical activity at an early stage. Its impact is associated with a reduction in exercise performance and quality of life (Donaldson, Wilkinson, Hurst, Perera, & Wedzicha, 2005); and it is most commonly quantified using the Medical Research Council (MRC) dyspnoea scale (Figure 1.3) (Stenton, 2008).

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 1-3: MRC breathlessness scale From Stenton *et al.* (2008) with permission.

At later stages, it is also present at rest and limits the functional status of a person caused by hyperinflation (O'Donnell, Revill, & Webb, 2001). Reducing the symptoms of dyspnoea is thus recognised as a key goal in the treatment of COPD (O'Reilly, 2010).

Diagnosis and classification

If an individual above the age of 40 exhibits COPD symptoms (Figure 3) and has a history of exposure to risk factors, especially cigarette smoking, then a clinical diagnosis for COPD and a spirometry test should be considered, according to COPD guidelines (O'Reilly, 2010; Vestbo, 2013) The prominent COPD manifestation is airflow limitation, tested by spirometry and indicated by the reduced maximum forced expiratory flow in one second (FEV_1) to forced vital capacity (FVC) of less than 70% (FEV1/FVC < 0.7) which is not fully reversible with a post-bronchodilator (GOLD, 2017). The severity of airflow obstruction and how it is

classified is based on the percentage predicted value of FEV1% (Table 1.2). The GOLD guidelines suggest considering patient symptoms such as the MRC dyspnoea scale during the assessment of airflow limitation severity, as they are good indicators for COPD prognosis and management.

Table 1-2: GOLD and NICE classification for severity scoring of COPD

FEV1/FVC	**FEV1 % (Predicted)**	**GOLD**	**NICE (Updated 2010)**	**Symptoms**
<0.7	$FEV_1 \geq 80\%$	GOLD 1: Mild	STAGE 1: Mild	Shortness of breath when walking up a slight hill (MRC 2)*
<0.7	$50\% \leq FEV_1 < 80\%$	GOLD 2: Moderate	STAGE 2: Moderate	Shortness of breath when walking approximately 100 m (MRC 3 to 4)
<0.7	$30\% \leq FEV_1 < 50\%$	GOLD 3: Severe	STAGE 3: Severe	Shortness of breath when breathless when dressing or undressing (MRC 5)**
<0.7	$FEV_1 < 30\%$	GOLD 4: Very Severe	STAGE 4: Very Severe	

* Dyspnoea and disability assessed by the Medical Research Council dyspnoea scale
** or FEV1 < 50% with respiratory failure

In addition to the diagnosis of COPD via spirometry, there are different assessment tools suggested by GOLD to support the management approach in COPD. The updated GOLD report recommends including the ABE assessment tool in addition to spirometry stage results to enhance progression and prognosis (GOLD, 2017). The ABE tool (Figure 1.4) consists of three different categories where the assessment of COPD patients is based on the following

criteria: patients' grade of airflow obstruction; severity level of health-related quality of life using the dyspnoea (MRC scale), and COPD assessment test (CAT) questionnaire; and exacerbation history.

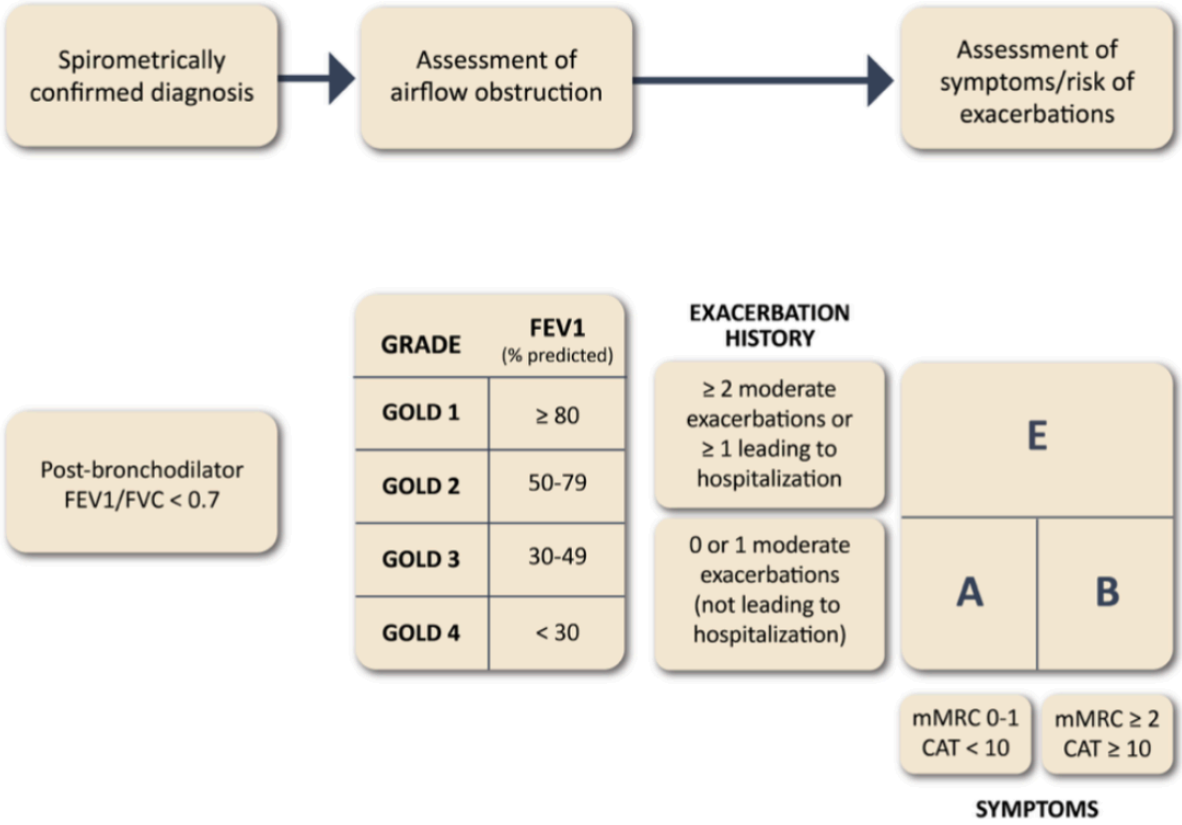


Figure 1-4: Gold COPD ABE assessment tool.Association between symptoms, classification of airflow limitation severity, and future risk of exacerbations. Adapt from the Global Strategy for Diagnosis, Management and Prevention of COPD 2023, on Nov 2022 http://www.goldcopd.

Prognosis

Prognosis in COPD is influenced by acute exacerbations (AECOPD) episodes of the condition, which are sustained worsening of symptoms (B. R. Celli & Barnes, 2007). Initially, AECOPD was defined in various ways in the past either as symptom-based or event-based (J. A. Wedzicha, Singh, & Mackay, 2014). The consensus definition stated by GOLD includes both an increase in symptoms and healthcare resource utilisation:

"*An event in the natural course of the disease characterised by a baseline change in the patient's dyspnoea, cough, and/or sputum that is beyond the normal day-to-day variations, is acute in onset, and may warrant a change in regular medication in a patient with underlying COPD*"

Global Initiative for Chronic Obstructive Lung Disease (GOLD) (Vestbo, 2013).

If these exacerbation symptoms are not well treated, AECOPD will develop (S. D. Aaron, 2014). AECOPD is associated with accelerated decline in lung function and requires a longer recovery period (J. A. Wedzicha & Seemungal, 2007). Typically, exacerbations show two patterns: sudden onset and gradual onset. Sudden onset is correlated with shorter recovery times, while gradual onset is associated with a longer recovery duration. The estimated average recovery time of AECOPD is between seven and ten days (Seemungal, Donaldson, Bhowmik, Jeffries, & Wedzicha, 2000). The exacerbation of chronic pulmonary disease tool (EXACT) is a validated patient-reported outcome tool to facilitate the assessment of symptomatic manifestations of COPD exacerbations in practice (P. W. Jones, 2011). Although GOLD classifications are based on spirometric cut-off levels, the risk of future exacerbations will increase with a higher GOLD rating, i.e., more severe (Vestbo, 2013). GOLD has reported classification criteria for exacerbation severity based on medication change and healthcare utilisation (Table 1.3).

Table 1-3: Severity of COPD exacerbations as defined by GOLD

SEVERITY	Level of Health Care Utilisation
MILD	Treated with SABDs only
MODERATE	Treated with SABDs plus antibiotics and/or oral corticosteroids

SEVER	Patients require to visit ER and/or hospitalisation

Note: SABD: Short-acting bronchodilators

Therefore, well management of COPD exacerbations is essential to improving symptoms and reducing AECOPD episodes requires sufficient healthcare utilisation (S. D. Aaron, 2014; Hillas, Perlikos, Tsiligianni, & Tzanakis, 2015).

1.2.4 Pathology, pathogenesis, and pathophysiology of COPD

Previous studies exploring the pathological process of COPD suggested that exposure of the body to the above-mentioned risk factors stimulates innate and adaptive inflammatory immune responses in the host (MacNee, 2005; MacNee & Tuder, 2009; Tuder & Petrache, 2012). Adaptive immunity is antigen-specific and is activated when it is exposed to pathogens, causing it to react more slowly than innate immunity. Innate immunity, on the other hand, is nonspecific and quickly reacts to general threats in the body, producing rapid responses with no memory (Bhat, Panzica, Kalathil, & Thanavala, 2015; Moldoveanu, 2009). Innate immunity is considered the first line of defensive mechanism in the lung, including removal systems such as mucociliary, epithelial barriers, and inflammatory cells. Several studies have reported the effects of cigarette smoking on cilia structures and function as a part of the innate immunity among COPD patients (Hogg & Timens, 2009; Sethi, 2000). Smoking shortens cilia length and reduces its function in the small airways, which impacts mucus removal (Chung & Adcock, 2008). Additionally, chronic exposure to inhaled noxious particles and gases has been revealed to activate epithelial cells and alveolar macrophages, which are the most abundant innate immune cells in the respiratory tract. Activation of these cells triggers the release of different inflammatory mediators in the lung, which attracts various inflammatory cells, including monocytes and neutrophils (Shapiro, 2001; Shapiro &

Ingenito, 2005). Recruiting these inflammatory cells results in stimulating the release of different proteases, which contributes to structural changes and obstruction in the airways, leading to emphysema, tissue destruction, and mucus hypersecretion (Stockley, Mannino, & Barnes, 2009).

Prolonged CS leads to the recruitment of inflammatory cells, such as neutrophils and macrophages in the airways, which stimulates damage to alveolar epithelial cells and produces reactive oxygen species (ROS) (Kirkham & Barnes, 2013). ROS molecules demonstrate a critical feature in the pathogenesis of COPD, resulting in an imbalance between the pro-oxidant system and the antioxidant system known as oxidative stress (OS). Stimulation of ROS production in epithelial cells in the lungs leads to an increase in OS biomarkers in sputum and systemic circulation. The formation of OS biomarkers in COPD suggests different pathways (Antus & Kardos, 2015). Superoxide radical anions O_2^- forms through nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH) oxidase, which is an important mediator involved in the endothelial dysfunction process (Church & Pryor, 1985). Additionally, it reacts with the nitrogen oxide radical NO·, produced from the L-arginine-nitric oxide synthase (NOS)-nitric oxide (NO) pathway, to produce peroxynitrite ($ONOO^-$) and hydroxyl radical HO· (S Moncada & Higgs, 2006). The reaction of O_2^- with perhydroxyl radicals leads to H_2O_2 production and then HO· production (Yin, Xu, & Porter, 2011). Therefore, OS exerts negative functional effects, including damage to lipids, proteins, and DNA, and facilitates initiation of pulmonary and systemic vascular endothelial apoptosis as well as the breakdown of lung elastin and bronchoconstriction (Domej, Oettl, & Renner, 2014; Rahman, 2005; Schulz, Gori, & Münzel, 2011; Song, Chen, & Liu, 2021).

Hogg *et al*. (2004) demonstrated the direct correlation between the progression of COPD and the increase in the percentage of neutrophils, macrophages, and other inflammatory immune cells (Hogg, Chu, , 2004). Subsequent responses of both types were identified on two predominant mechanisms. First, destruction of lung parenchyma and loss of elasticity leads to the collapse of peripheral airways (subtype emphysema), and second, small airway lumen narrowing develops due to remodelling of the damage process and is filled with mucous exudates (subtype chronic bronchitis) resulting from chronic inflammation (Barnes, 2000; GOLD, 2017; Hogg, 2004; Hogg, Chu, , 2004; Shapiro, 2001).

Emphysema is considered a disease that constitutes COPD. It is pathologically defined as an abnormal permanent enlargement and destruction of airspaces distal to the terminal bronchiole, accompanied by destruction of capillaries and loss of lung elasticity, leading to a decline in the alveolar surface area available for gas exchange (Barnes, 2000; GOLD, 2017). Classification of emphysema can be grouped into three main types (Finkelstein, 1995), though all may occur within an individual: 1) centriacinar (centrilobular): the most common type of emphysema, localised in the upper lung zones with dilatation and destruction of respiratory bronchioles; 2) panlobular emphysema: predominant in lung bases with the destruction of the entire alveolus and observed in AATD patients; and 3) paraseptal: known as distal acinar emphysema, which involves the distal airway structures, alveolar ducts, and alveolar sacs (Figure 1.5).

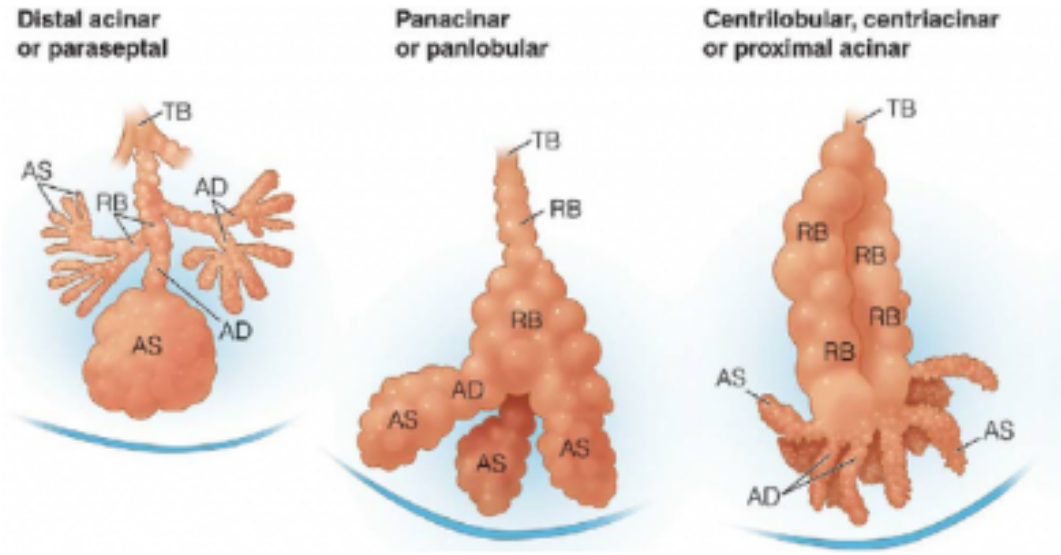


Figure 1-5: Lung emphysema subtypes: centriacinar, panacinar, and paraseptal Adapted from www.physio-pedia.com on Feb 2021 https://www.physio-pedia.com/Emphysema.

Meanwhile, chronic bronchitis is a clinical diagnosis recognised by the presence of a productive cough with sputum on most days during at least three consecutive months for more than two successive years (Elbehairy, Raghavan, , 2015; GOLD, 2017). Pathologically, it is described by inflammation and excessive mucus build-up in the bronchi or small airways (Figure 1.6).

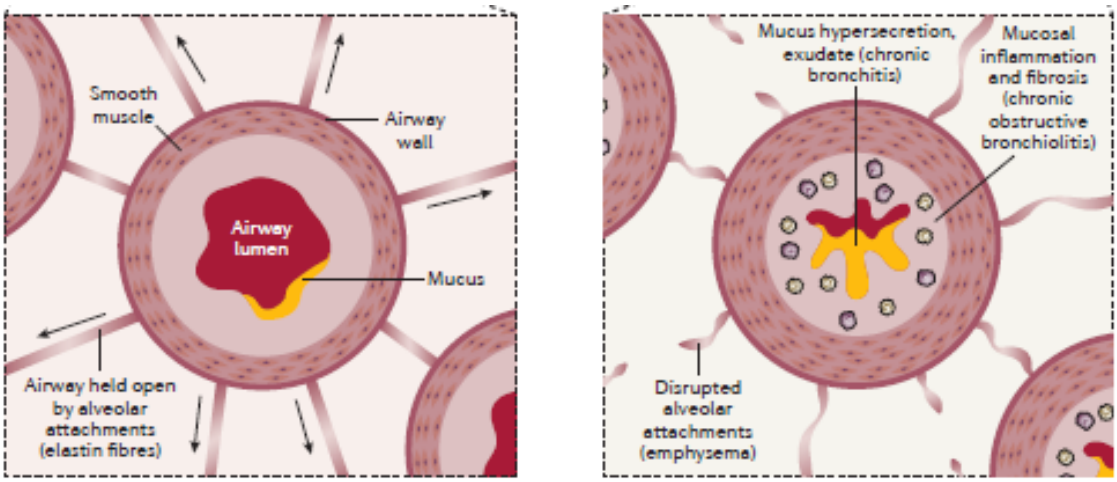


Figure 1-6: Normal airways vs. airways with chronic bronchitis Adapted from Barnes *et al.* (2015).

Furthermore, individuals with COPD might have both aspects of the disease. Long-term exposure to cigarette smoke or other noxious particles stimulates the host defences and releases an adaptive inflammatory immune response as a normal response (Hogg, Chu, , 2004). This inflammatory immune response becomes persistent inflammation in small and large airways as well as the lung parenchyma (Gooptu, Ekeowa, & Lomas, 2009). This results in destroyed parenchymal tissue (emphysema) and prevents the normal repair process of airway remodelling, causing the small airway to narrow, and leading to more diverse pathophysiological manifestations (Hogg, 2009) as outlined below:

1.2.4.1 **Expiratory flow limitation and gas trapping** are the most prominent pathophysiologies of COPD. The extent of intrinsic airway factors (such as mucosal inflammation, airway fibrosis, and secretions) and extrinsic airway factors (reduction of airway thickening from emphysema) increases the resistance and declines in small airway functions (FEV_1% and FEV1/FVC), resulting in airflow limitation during human exhalation and leading to air trapping and lung hyperinflation, which contributes to dyspnoea (Elbehairy, Ciavaglia, , 2015; Hogg, Chu, , 2004).

1.2.4.2 **Gas exchange abnormalities** are characterised by the abnormality of ventilation mechanics (hyperventilation) and reduced cardiac output, which results in ventilation/perfusion (V/Q) mismatch (O'Donnell, Laveneziana, Webb, & Neder, 2014). It is proposed that these physiological anomalies are due to structural anomalies observed in subtype emphysema, which are characterised by alveolar ventilation dysfunction and pulmonary vascular bed damage, resulting in a decreased surface area available for gas exchange and the ability to oxygenate blood (GOLD, 2017).

1.2.4.3 **Pulmonary arterial hypertension (PAH)** may develop at a late stage of COPD, inducing pulmonary hypoxia due to decreased partial oxygen pressure. Subsequently, hypoxic pulmonary vasoconstriction is initiated (Chaouat, Naeije, & Weitzenblum, 2008). This homeostatic mechanism in the pulmonary vasculature stimulates the narrowing of small pulmonary arteries, which reduces the flow of blood and, in severe stages, the destruction of the pulmonary vascular bed. As a result, persistent PHT induces right ventricular hypertrophy and leads to right heart failure (cor pulmonale) (MacNee, 1994). Endothelial dysfunction, a pathologic feature of PAH, is attributed to an impaired endogenous nitric oxide (NO) signalling pathway (Morrell, 2009).

1.2.4.4 **Mucus hypersecretion** is a feature of chronic bronchitis patients characterised by persistent active cough due to an increased number of goblet cells and enlarged submucosal glands caused by chronic airway inflammation related to smoking (GOLD, 2017).

1.2.4.5 **Exacerbations and comorbidities** episodes of recurrent pulmonary infections often interrupt patients' general health and respiratory symptoms. About 80% of exacerbations are mainly caused by respiratory viral infections (human rhinovirus) or bacterial infections; however, the underlying causes of one-third of serious exacerbations cannot be identified (B. R. Celli, 2007). Exacerbations lead to an increase in inflammatory mediators within the airways. These include IL-8 and TNF-a, which enhance the release of proteases and reactive oxygen species (ROS) and damage the epithelium lining of the airways, resulting in increased aggravation of systemic inflammation and airflow limitation (O'Donnell & Parker, 2006). Hence, to improve the patients' health and reduce symptoms during COPD exacerbations, close monitoring of comorbidities is vital (Hillas, 2015).

1.2.5 Systemic inflammation in COPD

Agustí and several authors use the term "systemic inflammation" to refer to increased levels of inflammatory markers of chemokines and cytokines in the circulatory blood of COPD individuals (A. G. Agusti, 2005; Choudhury, Rabinovich, & MacNee, 2014; Watz, 2008). These circulating inflammatory markers include neutrophils, leukocytes (white blood cells), C-reactive proteins (CRP), interleukins (IL-6 & 8), fibrinogen, monocyte chemoattractant proteins (T lymphocytes-CD8), and tumour necrosis factor-alpha (TNF-α); all of which are associated with poor clinical outcomes (Gan, Man, Senthilselvan, & Sin, 2004; S. F. Van Eeden & Sin, 2008). In addition, it was reported by Agustí that systemic inflammation was not found to be a persistent symptom as only 16% of COPD patients had systemic inflammation detected in their blood serum (Agustí, 2012).

CRP, fibrinogen, and leukocytes were the most frequently investigated markers in the studies due to their availability in clinical practice (Thomsen, 2013). The Copenhagen City Heart Study and clinical researcher traced higher rates of CRP, leukocyte, and fibrinogen markers in individuals with COPD and concluded that these markers correlate with a double to fourfold risk of cardiovascular disease and cancer, and subsequent risk of hospitalisation due to the comorbidities; Figure 1.7 (Rennard, 2005; Thomsen, Dahl, Lange, Vestbo, & Nordestgaard, 2012). This is consistent with findings from the ECLIPSE and two large Danish population studies, along with a rise in these markers also predicting all-cause mortality reported by the Lung Health Study and ECLIPSE (Dahl, 2007; Man, 2006; J. Miller, 2013).

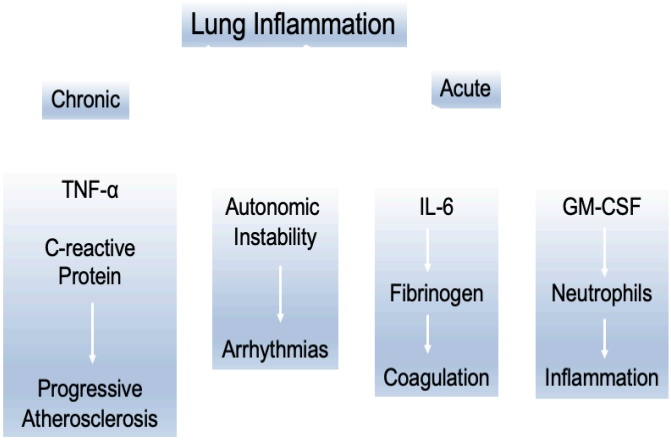


Figure 1-7: Circulatory systemic inflammatory markers in COPD that may contribute to CVD GM-CSF: granulocyte-macrophage colony-stimulating factor (GM-CSF); IL: interleukin; TNFα: tumour necrosis factor-alpha. Adapted from (Rennard, 2005).

However, the origin of the circulating systemic inflammation in COPD remains unclear and poorly understood. The source of systemic inflammation in COPD has been controversial for decades. Subsequently, various pathogenic mechanisms for systemic inflammation in COPD have been proposed.

A plethora of molecular mechanism studies in COPD suggests that systemic inflammation is initiated by three different pathogenic mechanisms. Some preliminary works in this area have proposed that the lungs' inflammatory response in COPD may be "spilt-over" inflammatory cytokines into the systemic circulation (Barnes & Celli, 2009; Sinden & Stockley, 2010). Although this hypothesis has been controversial for decades, a recent review has reported that studies in animals and humans show that protein movement can occur from the lung surface to the systemic circulation (Sinden, 2010). Recurrent episodes of AECOPD stimulate circulating inflammatory biomarkers such as neutrophils, CRP, IL-6, IL-8, TNFα, fibrinogen, and monocytes significantly (S. D. Aaron, 2014; Barnes, 2009).

Another possible mechanism suggests that the pulmonary inflammatory response is regulated differently. Vernooy and colleagues (2002) assessed the relationship between pulmonary and systemic inflammation in 35 participants (18 subjects with COPD vs 17 healthy smokers) (Vernooy, 2002). They measured components of soluble TNF receptors, TNF-α, and IL-8 in induced sputum and in plasma and concluded there were no direct correlations between the same markers in sputum and plasma (Vernooy, 2002). Similarly, Michel *et al.* examined the clinical responses of inhaled lipopolysaccharide with the inflammatory process in the airways and systemic circulation in 15 healthy subjects. The study results showed inflammatory processes in the airways and the systemic circulation as independent underlying mechanisms (Michel, Dentener, Corazza, Buurman, & Rylander, 2001).

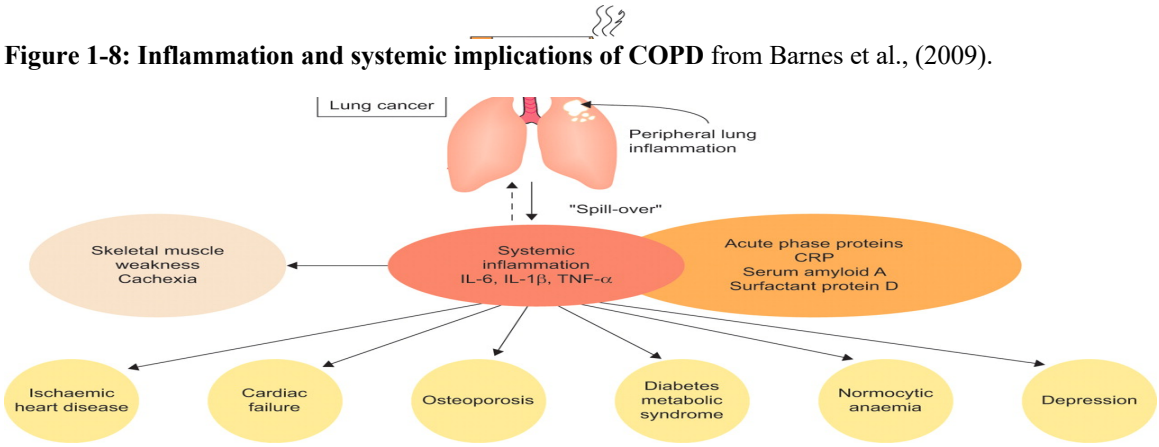
A third mechanism argues that tissue hypoxia may play a role in the process of systemic inflammation by enhancing cytokine production. Takabatake *et al.* (2000) studied the relationship between hypoxemia and activation of the TNF-α system i.e., TNF-α and soluble TNF receptors (sTNF-R; sTNF-R55 and -R75) levels, in COPD patients and 15 age-matched healthy controls (Takabatake, 2000). They concluded that PaO_2 and circulating TNF-α system marker levels were inversely correlated in patients with COPD compared to healthy subjects (Takabatake, 2000). Subsequently, the above-mentioned pathological process of systemic inflammation suggests a link between COPD, the cardiovascular disease process, and other extrapulmonary manifestations (S. Van Eeden, Leipsic, Paul Man, & Sin, 2012).

1.2.6 Comorbidities in COPD

Although for many years the definition of COPD emphasises the impact of risk factors such as cigarettes smoking in the respiratory tract, the updated GOLD report now includes "significant extrapulmonary effects that may contribute to the severity in COPD patients"

(GOLD, 2017). Recent evidence has reported that the pathogenesis and pathological effect of cigarettes smoking are beyond lung boundaries (Barnes, 2016). Its effects induce systemic cellular inflammation and systemic oxidative stress, subsequently releasing circulatory pro-inflammatory and procoagulant factors as mechanisms that trigger changes associated with a plethora of vascular and non-vascular comorbidities (A. G. Agusti, 2005). Vascular diseases include cardiovascular diseases (CVD), such as coronary heart disease (CHD), hypertension, atherosclerosis, pulmonary arterial hypertension (PAH), venous thromboembolism, whereas non-vascular diseases consist of comorbidities, such as dysfunction of skeletal muscles, osteoporosis, anaemia, diabetes, and metabolic syndrome Figure 1.8 (A. Agusti, 2007; Barnes, 2009; Fan & Meek, 2014; J. Miller, 2013).

Figure 1-8: Inflammation and systemic implications of COPD from Barnes et al., (2009).



Consequently, the GOLD report expressed COPD definition evolved beyond lung boundaries, stating that "airflow limitation is associated with an abnormal chronic inflammatory response and significant extrapulmonary effects may contribute to the severity in individual patients" (GOLD, 2017). Large longitudinal population-based cohorts (ECLIPSE, SPIROMICS, CanCOLD, and COPDGene) reported that approximately one-third (30%) of COPD participants had another medical morbidity, and 40% of those had two or more comorbidities (A. Agusti, 2010; Bourbeau, 2014; Couper, 2014; Wan, 2011). Comorbidities in COPD contributed to reduced health status, worsening prognosis, increased healthcare utilisations (cost), and an increased risk of death (Antonelli Incalzi, 1997). Accordingly, international COPD statements and treatment guidelines stress the benefits of early risk assessment and intervention for common comorbidities, such as CVD, which may improve COPD patient care pathways (Fabbri, 2012).

1.2.7 Cardiovascular comorbidities in COPD

The cardiovascular disease CVD or "cardiovascular disease continuum" hypothesis was proposed by cardiologists as a domino effect, initiated by risk factors that progress through numerous physiological pathways to the end-stage of heart disease (Dzau & Braunwald, 1991). CVD is known to be caused by blood clots (thrombosis) or atherosclerosis. The NICE guidance defined CVD as an umbrella disease, describing a range of conditions that affect the heart and/or the blood vessels (Duerden, O'Flynn, & Qureshi, 2015). According to the British Heart Foundation, coronary heart disease (CHD) is a very prevalent morbidity in people under the age of 75 years and the most common cause of hospitalisation and death. Additionally, CVD adds approximately £9 billion in direct costs to the UK healthcare system,

while the indirect costs to the UK economy are estimated to be approximately £19 billion (Factsheet, 2018). The WHO global statistical report demonstrated CVDs as the first cause of mortality globally, accounting for 17.9 million deaths in 2013 (World Health Organization., 2017). In 2016, cardiovascular disease (CVD) was the second main cause of death after cancer in the UK (Bhatnagar, Wickramasinghe, Williams, Rayner, & Townsend, 2015). The recognised risk of CVD is classified into two categories: modifiable and non-modifiable risk factors (Table 1.4).

Table 1-4: CVD Risks can be considered as modifiable or non-modifiable

Modifiable	Non-modifiable
Smoking	Ageing
Diabetes mellitus type 2 (T2DM)	Gender (male sex)
Physical inactivity	Race and ethnicity
Obesity	Genetic factors
Hypertension	
High cholesterol	
Poverty	

Although these risk factors increase the likelihood of developing atherogenesis and the rate of its development, CVD is considered a preventable disease. Large population-based studies demonstrate that CV risk factors and CVDs together are high multimorbidity seen within COPD individuals (Figure 9), with smoking as a common shared biological risk factor in COPD and CVDs (Lopez, Mathers, Ezzati, Jamison, & Murray, 2006; Sin, Wu, & Man, 2005).

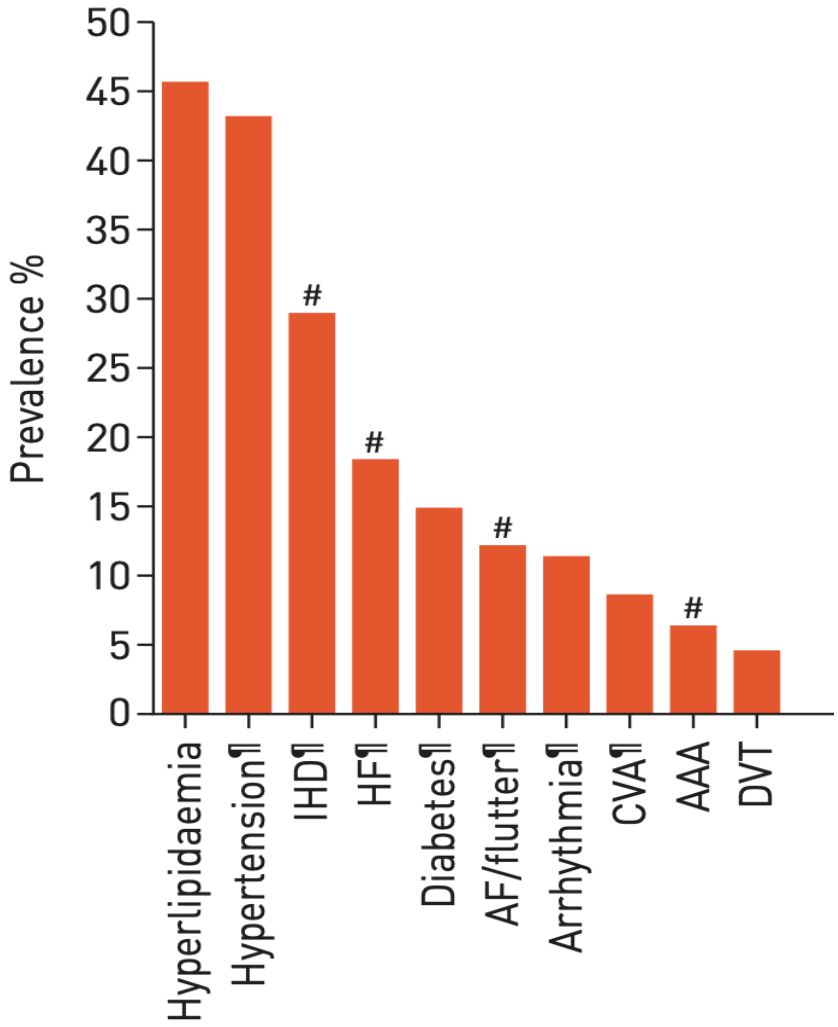


Figure 1-9: Prevalence of CV risk factors/CVDs in COPD Data are pooled from numerous studies and comorbidities with a prevalence (calculated as a weighted average based on study sample size) >5% are shown. IHD: ischaemic heart disease; HF: heart failure; AF: atrial fibrillation; CVA: cerebrovascular accident; AAA: abdominal aortic aneurysm; DVT: deep vein thrombosis; ¶: comorbidities with a significantly increased prevalence in patients with COPD compared with the general population. Adapted from Rabe *et al.*, with permission (M. C. Smith & Wrobel, 2014).

Epidemiological data show that at least 40% of COPD patients have concomitant CVDs (Barnett, 2012; Rabe, Hurst, & Suissa, 2018). Recently, more attention has been given to ischaemic heart disease (IHD), heart failure (HF), and cardiac arrhythmias due to their increasing prevalence and life-threatening risk in COPD patients. Saskatchewan longitudinal retrospective cohort data found that COPD patients present with a higher prevalence of IHD, especially coronary artery disease (CAD), as it correlated with a higher risk of hospitalisations among them (Curkendall, 2006). Another interesting findings by Soriano and

colleagues indicated airway limitation was present in 60% of hospitalised CVD patients, with CAD accounting for 87% of these patients (Soriano, 2010). These findings are in line with data from the PLATINO and Keio COPD studies, which reported CVD coexisted in 41% and 32% of the COPD patients, respectively (Menezes, 2005; Takahashi & Betsuyaku, 2015).

Having conducted an observational study, Reed *et al.* (2012) revealed high occult CAD present in approximately 53% of COPD patients compared to the matched control group. However, it was noted that COPD patients were sub-optimally utilising recommended treatments for CAD (Reed, 2012). The CONSISTE study, a case-control study, points out that the COPD group showed a significantly higher prevalence of IHD (12.5% versus 4.7%) compared to the matched control group. The CONSISTE univariate risk analysis concludes that COPD and hypertension are independent risk factors for IHD (de Lucas-Ramos, 2012). Another larger cohort study conducted in COPD by the Kaiser health care system in the U.S found that COPD individuals had a higher risk of hospitalisations for IHD, such as angina and myocardial infarction, compared to age- and gender-matched control subjects. Their data has also shown that arrhythmias and HF were common in the COPD group, which are both risk factors for stroke at 10% and 20%, respectively (Sidney, 2005). In a cross-sectional study, Boschetto *et al.* (2015) studied concurrent COPD in elderly patients with HF with respect to the occurrence, prognosis and therapeutic implications, and found that 30% of those hospitalised had coexisting COPD (Boschetto, 2013).

Mortality numbers reported by high-quality reviews in COPD suggest CVDs and lung cancer are the common causes of death in COPD patients (Sin, Anthonisen, Soriano, & Agusti, 2006). On the other hand, certain studies found COPD to be an important risk factor for sudden cardiac death and an independent risk factor for the development of IHD (Seemungal,

2009). Reported data from the US Lung Health Study revealed that 50% of mild-to-moderate COPD patients were hospitalised for CVD-related causes, and those patients were at high risk of both fatal and non-fatal cardiovascular events. Additionally, reported hospitalisations were increased by two- to-three-fold in patients in the highest quintile of CRP during the five-year period compared with those in the lowest quintile (Anthonisen, Connett, Enright, & Manfreda, 2002). These results are in line with data retrieved from observing in-hospital mortality in patients with STEMI who were treated by percutaneous coronary intervention. Compared to COPD, patients' data showed mortality was four times higher from coronary artery disease. They also concluded that COPD patients were less likely to be treated with beta-blockers. (Șerban, 2017).

Sympathetic overactivity commonly found in COPD is demonstrated as a risk factor for developing arterial stiffness and hypertension. The Copenhagen City Heart Study reported data showing resting heart rate, a marker of sympathetic activation, increased in severity from 73 beats/min in the mild group to 85 beats/min in the very severe group of COPD patients. After studying the patients, they found resting heart rate to be a predictor of the risk of developing CVD and all-cause mortality in COPD patients (Jensen, Marott, Allin, Nordestgaard, & Jensen, 2012).

More recent evidence highlights the profound impact of CVDs in COPD that may provoke dyspnoea, exercise intolerance, exacerbation frequency, and contribute to high utilisation of healthcare resources (J. Miller, 2013; Westerik, 2017). Hence, CV risk assessment should be established in the early stages of COPD development to minimise the potential for poor outcomes, as epidemiological data suggests CVDs play a crucial part in COPD outcomes (Vestbo, 2013). Nevertheless, the role of common cardiovascular risks and COPD

pathophysiological mechanisms need to be understood thoroughly to better prevent and hinder the progression of CVD risks.

1.2.8 Pathogenic mechanisms of cardiovascular risk factors in COPD

According to pathophysiological research, the CVD continuum is triggered by risk factors such as smoking, oxidative stress, inflammatory processes, and vascular remodelling, all of which initiate atherosclerosis and CVDs (Dzau, 1991). Due to the complexity of pathophysiological mechanisms, the link between CVD and COPD has not been fully elucidated despite having common risk factors. Nevertheless, several mechanisms have been proposed that underpin the association between COPD and CVD, including smoking, inflammation (Libby, Ridker, & Maseri, 2002), ageing (Benetos, 2001), oxidative stress (Lum & Roebuck, 2001), and hypoxia (Wright, Petty, & Thurlbeck, 1992).

CVD, particularly IHD, induces inflammatory responses that contribute to atherosclerosis (on Smoking, Control, & Prevention, 2010). Both IHD and COPD have a common aetiology and pathophysiology, which is reflected by the pathogenic mechanism of chronic systemic inflammation associated with the two diseases (Patel, Donaldson, Mackay, Wedzicha, & Hurst, 2012). Atherosclerosis is the principal cause of IHD, stroke, and peripheral vascular disease (Faxon, 2004). In atherosclerosis, arteries become narrowed because of lipid accumulation in the arterial wall and continuing inflammatory response (Libby, 2002). The cellular pathogenesis mechanism of atherosclerosis begins when oxidative stress markers injure endothelial cells, leading to an increase in the adhesion molecules of E-selectin, vascular cell adhesion molecule-1 (V-CAM-1), and intercellular adhesion molecule-1 (I-CAM-1), which then binds to blood leukocytes in the endothelium. The leukocytes penetrate the intima and activate inflammatory cells and fibrinogenic mediators, subsequently building

up atheromatous plaque and contributing to the development of thrombotic risk (Faxon, 2004). Smoking may influence the initiation of vascular atherosclerosis in COPD (Iwamoto, 2009). Furthermore, persistent systemic inflammation and oxidative stress through endothelial dysfunction and arterial stiffness may play roles in the pathogenesis of CVDs in COPD (Figure 1.10) (Rabe, 2018; S. Van Eeden, 2012).

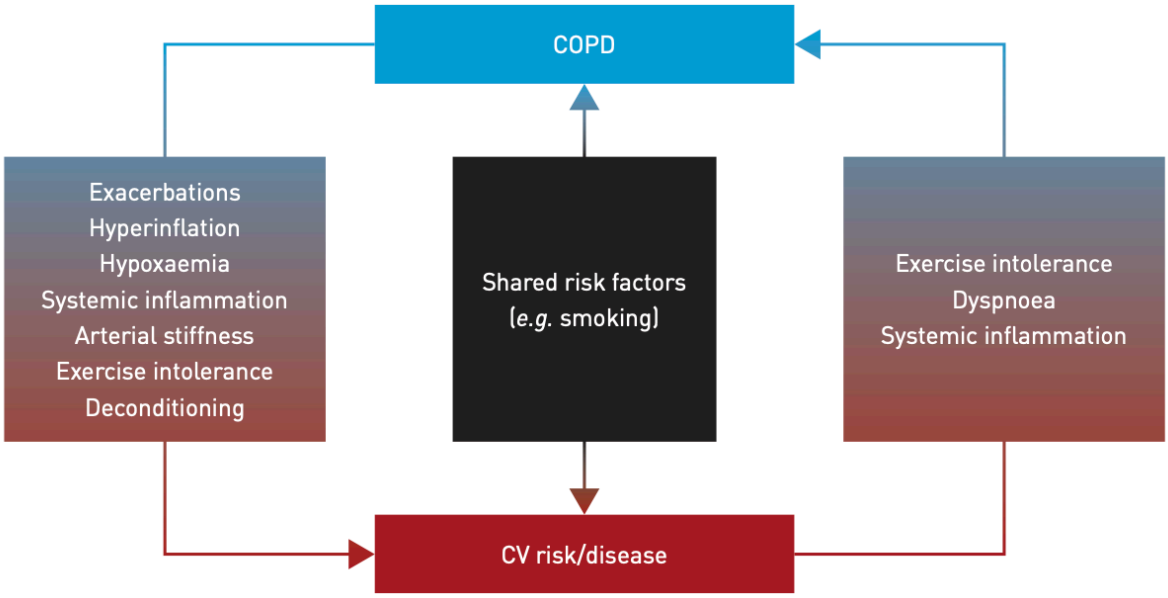


Figure 1-10: Proposed flow chart of the interactions between COPD and CV risk/CVD

Adapted from (Rabe, 2018).

A plethora of studies explains how systemic inflammation is a driving mechanism for atherothrombosis and the development of CVD in COPD (Gan, 2004; Ghoorah, De Soyza, & Kunadian, 2013). Elevated values of inflammatory markers such as CRP, IL-6, and fibrinogen, which contribute to the development of atherosclerosis and CVDs, are confirmed by the atherogenic inflammatory role (Libby, 2002).

Cigarette smoking results in an increase in cellular sources of reactive oxygen species (ROS), known as oxidative stress (Rahman, 2005). These ROSs are involved in several pathologies,

including COPD and CVD, due to their role in triggering inflammation (Austin, Crack, Bozinovski, Miller, & Vlahos, 2016). Inflammation of the lungs is a hallmark manifestation of COPD. The inflammatory infiltrate in the small airways is formed by the release of neutrophils, pro-inflammatory cytokines such as IL-6 and 8, TNF-α, CRP, fibrinogen, and monocytic chemotactic activities (MCP 1, GM-CSF) in the lungs (Hogg, 2009). During AECOPD, those inflammatory mediators further increase (Shawn D Aaron, 2001). When the balance between pro- and anti-inflammatory cytokines in the lungs is disrupted, the pro-inflammatory cytokines penetrate "spill over" into systemic circulation (Barnes, 2016), thereby contributing to atherosclerosis plaque formation (Barnes, 2009; Sinden, 2010; S. Van Eeden, 2012).

The Lung Health Study measured CRP at baseline in 4,803 subjects with mild-to-moderate COPD and studied them for one year. Reported results showed high CRP levels at baseline, predicting all-cause mortality relating to CVDs (Man, 2006). Sin and colleagues (2003) analysed data from the NHANES III study and found CRP was highly elevated with moderate to severe COPD, which increased the cardiac risk almost twofold (Sin & Man, 2003). These are in line with the Copenhagen City Heart Study data, which revealed that the ten-year risks for COPD hospitalisation and death in individuals with CRP above 3 mg/L were 54% and 57%, respectively (Dahl, 2007). In contrast, De Torres *et al.* (2008) reported a baseline serum CRP level which was not significantly associated with survival status in patients with moderate to very severe COPD (de Torres, 2008).

On the other hand, higher levels of fibrinogen are found to be associated with increased risk of exacerbations (Thomsen, 2012), presence of heart disease (J. Miller, 2013), and mortality of over three years in patients with COPD (Bartolome R Celli, 2012). It has been suggested

that fibrinogen is associated with the development of atherosclerotic disease through platelet aggregation and a prothrombotic state (Meade, 1995). A meta-analysis assessed the relationship between fibrinogen levels and the risk of vascular and non-vascular outcomes on individual participant data. It reported moderately strong associations between plasma fibrinogen levels and the risks of CHD, stroke, and other vascular mortalities (Danesh, 2005).

Intermittent hypoxia in COPD is another risk factor that might induce systemic inflammation, oxidative stress, upregulation of cellular adhesion molecules in endothelial cells and reduce vascular compliance, contributing to the progression of atherosclerosis (Linden, 2014). Furthermore, several studies have shown a rise in inflammatory mediators (CRP, IL6, fibrinogen, leucocytes, and TNFα) playing a crucial role in the development of common CVD risk factors in COPD patients including arterial stiffness (Jain, Khera, Corrales–Medina, Townsend, & Chirinos, 2014), endothelial dysfunction (ED) (Clapp, 2004), and platelet activation (Davì & Patrono, 2007).

1.2.8.1 Arterial stiffness

The rigidity of the arterial wall or elevated arterial stiffness is a surrogate marker for cardiovascular risk and is a consequence of ageing, vascular inflammation (atherosclerosis), and hypertension, which is a strong independent predictor of fatal cardiovascular events (Laurent, Alivon, Beaussier, & Boutouyrie, 2012; Mancia, 2013; Vivodtzev, 2014). The Framingham Heart Study analysed data of 2,232 participants to comprehend the correlation between the first episode of major CVD (myocardial infarction, unstable angina, heart failure, or stroke) in relation to arterial stiffness within nine years, measured by the pulse wave velocity (PWV), and augmentation index (AIx@75 % corrected for a heart rate of 75 beat/min). Reported results revealed PWV was associated with a 48% increase in CV risk

(95% CI, 1.16 to 1.91 m/s, p=0.002); therefore, the PWV marker of arterial stiffness is a valuable biomarker of CVD risk (G. F. Mitchell, 2010).

In the ERICA study (Evaluation of the Role of Inflammation in Chronic Airways Diseases), Fisk and colleagues (2018) evaluated vascular biomarkers PWV, AIx, and carotid intima-media thickness (CIMT) in the COPD with matched controls (Fisk, 2018). These biomarkers were all higher in patients with COPD even after adjustments for confounders (9.95±2.54 versus 9.27±2.41 m/s; P<0.001), (28±10% versus 25±10%; P<0.001), and (0.83±0.19 versus 0.74±0.14 mm; P<0.001), respectively, compared with controls.

Several other studies reported higher arterial stiffness indices in severe-to-very severe COPD stages (Vivodtzev, 2014). Mills *et al.* (2008) demonstrated raised markers of systemic inflammation involved in the progression of arterial stiffness and COPD severity (Mills, 2008). They conducted a prospective case-control study to identfy the mechanisms responsible for COPD association with CVDs, such as IHD and stroke. They measured augmentation pressure (AP) as a surrogate risk measurement for arterial stiffness. Results in 102 patients with stage 3 and stage 4 COPD, compared to 103 healthy controls, showed an increased AP (17 mm Hg and 14 mm Hg; p = 0.005, respectively). This elevation in arterial stiffness was correlated with an increase in blood pressure (BP) and serum CRP in the COPD group (Mills, 2008). Additionally, Sabit *et al*. (2007) studied arterial stiffness utilising PWV in 75 COPD patients compared to 43 healthy smoking control subjects free of CVD. The study observed a pronounced increase in PWV and BP in patients compared with control subjects, and this increase in the COPD group was statistically significantly correlated with predicted FEV1% (Sabit, 2007).

These results are in line with recent evidence from an Egyptian cross-sectional study that included 80 mild-to-moderate COPD patients and their healthy matched controls (Mansour, El-Shabrawy, Khalil, Eldamanhory, & Embarak, 2020). They sum up that PWV and AIx@75 % were negatively correlated with predicted FEV1%. The mechanistic role behind the increase of arterial stiffness is unidentified; however, it could be related to other mechanisms, including tissue changes in arterial CIMT (O'Leary, 1999), increased collagen (Xu, Zarins, Pannaraj, Bassiouny, & Glagov, 2000), elastin degradation (Maclay, 2012), diminished endothelial nitric oxide (NO) production, and increased matrix metalloproteases (Zieman, Melenovsky, & Kass, 2005). Maclay *et al.* (Maclay, 2012) in their seminal work on systemic elastin degradation in COPD concluded that the COPD group had increased skin elastin degradation and matrix metalloproteinase pro (MMP-9) compared to matched controls, which is related to emphysema severity and arterial stiffness. Furthermore, Mills *et al*. drew attention to a possible mechanism for the increased cardiovascular risk in COPD. In conclusion, endothelial dysfunction is a result of various pathological processes. These processes may be distinguished by an increase in large arterial stiffness, therefore provoking systolic blood pressure to raise left ventricular afterload and enabling the pathological process of hypertension (Mills, 2008).

1.2.8.2 Endothelial dysfunction

The vascular endothelium lines the blood luminal surface of the vessels. Endothelial cells (ECs) have multiple functions involving the regulation of vascular tone (mediator of endothelium-dependent vasodilation), vascular structure via inhibiting cell proliferation, and preventing interaction between blood cells and the vessel wall via inhibiting platelet adhesion, activation, and aggregation (Rajendran, 2013). The maintenance of vascular tone is accomplished by the release of vasodilators substances NO and prostacyclin (PGI_2) via ECs

as well as the release of endothelin agents to dispose of vasoconstrictor effects on vascular tone (Galley & Webster, 2004; Inoue, 1989). NO is endogenously produced via nitric oxide synthase (NOS) enzymes and plays a cardioprotective role in vascular endothelial cells and pulmonary regulatory functions via macrophage activity and pulmonary artery vasodilation (Knowles & Moncada, 1994). Disruption of NO generation via inhibition of NOS provoke functional change of endothelium (Goligorsky, 2005). Subsequently, endothelial signalling becomes impaired, which triggers various early and late cellular mechanisms of atherogenesis, such as increased endothelial permeability, platelet aggregation, increased generation of chemokines, cytokines and leukocyte adhesion (Davignon & Ganz, 2004). Reported evidence from the Framingham cohort suggested a positive correlation was observed between the severity of hypertension and impairment of endothelial function (EF) measured by flow-mediated dilation (FMD) (E. J. Benjamin, 2004). However, the controversy lies in whether ED is a cause or an effect of hypertension, as ED of pulmonary and systemic arteries has been defined by different pathological mechanisms. Preliminary *in vitro* experiments carried out in the early 1990s reported that ED occurs in severe emphysema patients in the pulmonary arteries resulting from raised exogenous acetylcholine and adenosine diphosphate (ADP) in pulmonary artery rings (Dinh-Xuan, 1991). As research evolved, it became apparent that smokers either suffering from mild or COPD or not suffering from COPD at all can develop ED in the pulmonary artery ring and increased intimal thickness of pulmonary muscular arteries, if exposed to acetylcholine and different concentrations of ADP (Polverino, Celli, & Owen, 2018). This is determined by endothelium-dependent relaxation, which is mediated by NO (Peinado, 1998).

Pre-clinical and clinical studies regarding the development of ED in COPD have suggested various mechanisms, such as smoking, pulmonary/circulatory inflammation, and high

oxidative stress levels (Polverino, 2018). Smoking and reactive oxygen species (ROS) demonstrated a pivotal role in the pathogenic mechanism of ED through increased systematic oxidative stress in the early life history of COPD (Schulz, 2011; Zhang, Venardos, Chin-Dusting, & Kaye, 2006). One mechanism suggested smoking as a trigger inflammatory mediator since excessive oxidative stress stimulates nicotinamide adenine dinucleotide phosphate oxidase ($NADPH^{+}$) to generate ROS, causing uncoupling of eNOS to release nitrotyrosine and nitric oxide ($ONOO^{-}$) and inhibit NO , thus increasing a procoagulant and pro-inflammatory lead to vascular inflammation, the early marker for hypertension and atherosclerotic diseases (Figure 1.11) (Malerba, 2017).

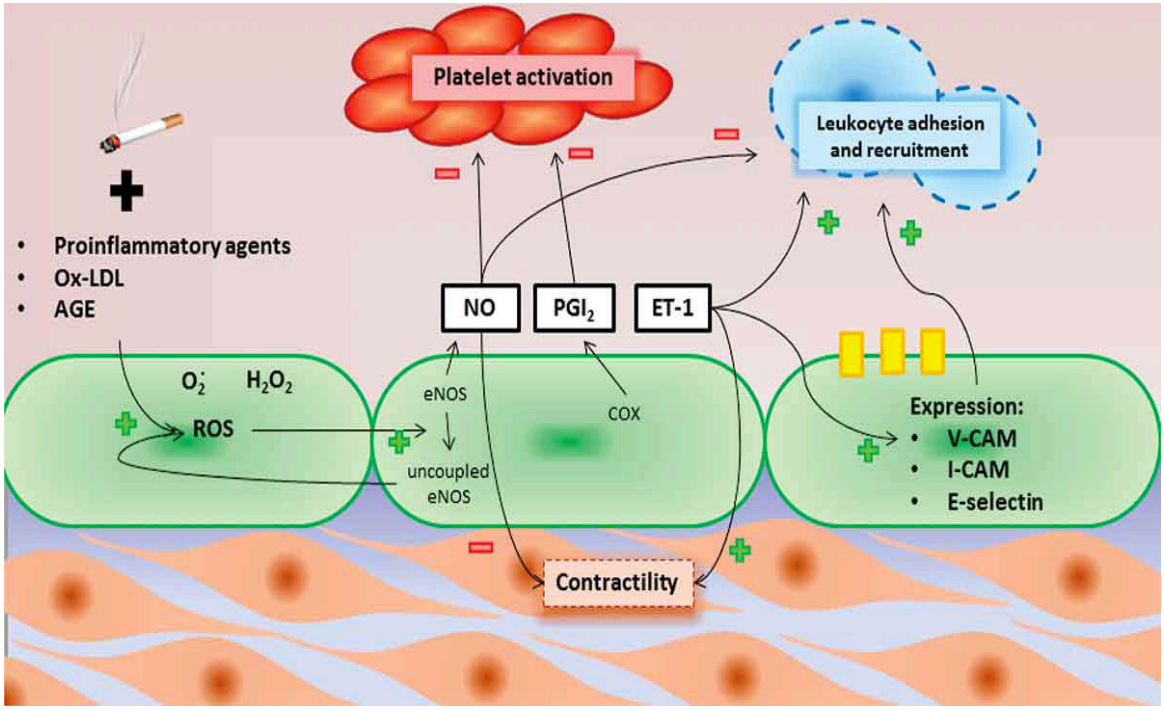


Figure 1-11: Summary of factors involved in vascular endothelial homeostasis in COPD Ox-LDL: oxidised low-density lipoprotein; AGE: advanced glycation end products; NO: nitric oxide; PGI2: prostacyclin; ET-1: endothelin-1; V-CAM: vascular cell adhesion molecule; I-CAM: intercellular adhesion molecule; ROS: reactive oxygen species; eNOS: endothelial nitric oxide synthase; COX: cyclooxygenase. Adapted from Malerba *et al.* 2017 (Malerba, 2017).

Persistent low-grade systemic inflammation in COPD patients may predispose pulmonary and systemic EC adhesion molecules E- and P-selectin, and intercellular adhesion molecule V-CAM and I-CAM expressions (Onder, Topcu, Dökmetas, Türkay, & Seyfikli, 1997).

Activated ECs release cytokines and mediators of inflammation CRP, IL-6, and fibrinogen (Polverino, 2018). In COPD, elevated CRP levels bind to damaged tissue and facilitate oxidative stress processes, causing injury and inflammation to the vascular wall (Barnes, 2009). Subsequently, dysregulation of eNOS-mediated NO production occurs by decreasing the stability of eNOS mRNA (Touyz, 2002).

ED can be measured in clinical settings noninvasively via different clinical testing methods that evaluate functional properties of normal and activated endothelium (Deanfield, 2005). The most used measure is flow-mediated dilation (FMD), a physiologic measure of endothelial reactivity to endogenous NO, which is widely used in clinical practices to correlate ED with clinical outcomes (Deanfield, 2005). Barr *et al.* (2007) and Eickhoff *et al.* (Eickhoff, 2008) measured pulmonary function and CT percentage of emphysema in 107 former COPD participants. They concluded that impaired FMD was associated with lower FEV_1 and higher CT percentage of emphysema compared to matched controls who were former smokers without COPD (Barr, 2007; Eickhoff, 2008). In clinical practice, FMD is an useful surrogate risk marker for CV events proposed as a useful adjunct to cardiovascular risk factor assessment (Anderson, 2006). Gokce *et al*. (2002) and Modena *et al*. (2002) examined the value of ED with FMD during prognosis by studying CV death and incidents such as myocardial infarction and stroke at 2 and 60 months. The findings showed a 24% CV event rate and an 11.7% stroke event rate, respectively.

Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of eNOS and a novel circulating risk biomarker measure of endothelial dysfunction (Böger, Maas, Schulze, & Schwedhelm, 2005). The ADMA physiological pathway in the human body results from the degradation of methylated proteins that were derived from protein arginine

methyltransferases (PRMTs) (Leiper & Nandi, 2011). Rising oxidative stress levels in the endothelium might increase PRMT activities, which results in increased ADMA levels associated with inhibiting NO production (Sydow & Münzel, 2003). ADMA is eliminated from the body via two mechanisms: the enzyme dimethylarginine dimethylaminohydrolase (DDAH) metabolising ADMA to citrulline and dimethylamine, or through renal excretion (MacAllister, Fickling, Whitley, & Vallance, 1994). Decreased availability of L-arginine, the enzyme's substrate of NOS, leads to a decrease in L-arginine/ADMA plasma ratio, which is associated with pathophysiology of different diseases' including COPD (Davì, 2007), hypertension (Achan, 2003), and development of atherosclerosis (Cooke, 2000). The calculation of the arginine/ADMA ratio, proposed by Bode-Böger *et al.* (2007), is an indicator that would reflect NOS imbalances caused by ADMA accumulation. A "normal" indicator would be represented by the range of 0.0044-0.0076 μmol/L (Bode-Böger, Scalera, & Ignarro, 2007).

Several pathophysiological studies *in vitro* and *in vivo* reported ADMA as a novel CV risk factor (Böger, 1998; Kielstein, 2004; Zakrzewicz & Eickelberg, 2009). In COPD, cigarette smoking and oxidative stress are important regulators of ADMA synthesis. Aydin *et al.* (2017) compared 58 patients with COPD to 30 healthy subjects to evaluate ADMA with NO serum levels (Aydin, 2017). In the COPD group, it was reported that ADMA was higher and NO was lower, and both were strongly correlated with FEV1% compared to the healthy group. Continuing, Zinellu and colleagues (2016) investigated arginine and methylated arginine levels (ADMA) plasma levels in COPD patients (Zinellu, 2016). They recruited 43 COPD patients (29 mild and 14 moderate) and 43 matched age-gender controls. Oxidative stress markers were high in COPD, increasing with severity. Arginine/ADMA ratio increased due to reduced arginine levels in COPD compared to the controls. In multiple logistic

regression analysis, both oxidative stresses and arginine/ADMA ratio were independently associated with COPD severity (Zinellu, 2016). Moreover, exacerbations may increase ADMA, or exogenous availability of arginine may reduce ADMA and result in reversed NOS function (Vögeli, 2017). Matsuoka *et al.* (1997) and Sydow *et al*. (2003) reported elevated levels of ADMA have been associated with hypertensive individuals and atherosclerotic diseases (Matsuoka, 1997; Sydow, Schwedhelm, , 2003). More *in vivo* evidence is needed to directly measure ADMA in COPD as well as to study the role of COPD pathogenesis with ADMA levels in both pulmonary and circulatory vasculature systems.

1.2.8.3 Platelet activation

Platelets are complex cells produced in the bone marrow with a short lifespan and multifunction (van der Meijden & Heemskerk, 2019). Platelets play a critical role in atherogenesis. Endothelial cells are an interface between inflammation and inappropriate activation of the blood coagulation system via different mechanisms including the release of eNOS or arachidonic acid-prostacyclin (prostaglandin I_2, PGI_2) downstream pathways (Versteeg, Heemskerk, Levi, & Reitsma, 2013). EC NOS expression activity in inflammatory disease such as COPD is decreased and subsequently stimulates endothelial E-selectin, collagen glycoprotein VI (GPVI) receptors, intercellular cell adhesion molecule (I-CAM-1), and von Willebrand factor (vWF) to induce the platelet surface expressions of P-selectin, which trigger platelet leukocyte aggregation formation (Csoma, 2019). Platelets secreting and releasing ADP and thromboxane A2 (TXA2) from granules excites platelet recruitment and platelet aggregation (Jurk & Kehrel, 2005). Another mechanism through which platelets are activated by increasing surface protein expression CD40L or through cytokines IL (interleukin)-1B and oxidised low-density lipoprotein cholesterol (O_X-LDL) (Vanhoutte, Shimokawa, Tang, & Feletou, 2009). Continuous release of IL-1B in response to chronic inflammatory conditions will lead to recruiting leukocyte and monocyte adhesion to the

endothelium. Consequently, more platelets binding to collagen and fibrinogen will predispose aggregation (Beaulieu, 2014).

NO and prostacyclin (PGI_2) synergically prevent platelet activation processes, including adhesion, aggregation, and procoagulant activity (J. A. Mitchell, Ali, Bailey, Moreno, & Harrington, 2008). ECs control platelet reactivity by converting arachidonic acid into PGI_2, and inhibit platelet aggregation by increasing intracellular cyclic adenosine monophosphate (cAMP) (Majed & Khalil, 2012). On the other hand, NO signalling the production of cyclic guanosine monophosphate (cGMP) and protein kinases, decreases intracellular Ca^{+2}, and suppressing platelet surface from binding to fibrinogen (Davì, 2007). In clinical settings, platelet aggregates and plasma P-selectins are recognised as reliable surrogate markers of platelet activation (Ferroni, 2012).

In COPD, cumulative evidence correlated with the presence of platelet activation along with risk factors such as circulating inflammatory mediators, smoking, and hypoxia, particularly in severe and very severe stages (Guzmán-Grenfell, 2011; Wang, Li, Cao, & Li, 2013). However, the underlying mechanism of enhanced platelet activation is not well understood in COPD patients. Chronic release of systemic inflammatory cells and cytokines may trigger platelet activation in response to vascular ED (Malerba, 2017). Surface receptors, such as P-selectin and CD40 production, are induced, facilitating the adhesion of platelets and forming platelet-monocyte aggregates (Merten & Thiagarajan, 2004). Harding and colleagues (2004) compared 25 healthy smokers with 25 matched healthy non-smokers on an antiplatelet regimen. They assessed the effect of smoking on the CD40/CD40L or platelet-monocyte aggregation (Harding, 2004). Their investigation concluded that smokers have increased surface expression of CD40 on monocytes, CD40L on platelets, and platelet-monocyte-

aggregates compared with non-smokers (45.9±7.7% vs 39.9±6.5%, 2.9±1.0% vs 2.3±0.6%, and 26.6±10.9% vs 19.7±8.6% respectively) (Harding, 2004). Maclay *et al*. (2011) examined platelet-monocyte aggregation in 18, 16, and 12 patients during AECOPD with stable matched controls (Maclay, 2011). They used flow cytometry to measure platelet activation markers (platelet-monocyte aggregates and platelet/soluble P-selectin expression). Reported results showed platelet-monocyte aggregates elevated in stable COPD compared with controls (mean (SD) 25.3 (8.3) % vs 19.5 (4.0)%, *P*=.01), significantly increasing during AECOPD (32.0 (11.0)% vs 25.5 (6.4)%, *P*=.03) (Maclay, 2011). P-selectin expressions did not differ between groups, and serum CRP concentrations in patients with COPD were higher compared with control subjects, supporting the hypothesis that low persistent systemic inflammation in COPD may contribute to platelet activation (Barnes, 2016).

Hypoxia or hypoxemia were hypothesised to induce platelet activation in various diseases, including COPD (Hutton, 1984; Ogawa, 1990; Onder, 1997). Some preliminary work was carried out by Wedzicha and associates (1991), who compared the platelet aggregation ratio in 23 patients with chronic airflow obstruction with and without hypoxemia and ten control subjects without respiratory disease. Reported results revealed that hypoxic stable COPD had increased platelet aggregate formation, suggesting platelet activation induce hypercapnia and increase the risk of pulmonary thromboembolism (J. Wedzicha & Tan, 1991).

The risk of developing platelet aggregation in COPD may increase with ROS production during hypoxia from mitochondria in the respiratory chain. Nana-Sinkam *et al.* (2007) reported that patients with severe emphysema showed diminished expression of prostacyclin production, which exerts important vasodilation function through the inhibition of platelet aggregation by increasing intracellular cyclic adenosine monophosphate (cAMP) (Nana-

Sinkam, 2007). Increasing age, chronic systemin inflammation, lipid accumulation in the arterial wall and vascular endothelial dysfunction complex pathological events facilitate the process of atherosclerosis and thrombotic risk in COPD (Libby, 2002). In theory, the pathogenic mechanisms behind atherosclerosis are considered complex, as they are influenced by dynamic cardiovascular risk factors.

1.2.9 Hypertension

1.2.9.1 Epidemiology

Elevated arterial blood pressure (BP), known as hypertension (HTN), is one of the significant contributing cardiovascular risks for atherosclerosis and CVDs (Alexander, 1995). It affects two-thirds of adults above the age of 50, and HTN is controlled in nearly 50% of those affected (Go, 2013). Disability-adjusted life-years (DALYs) in 2015 estimated that 874 million adults had systolic blood pressure (SBP) of 140 mm Hg or higher (Forouzanfar, 2017). It is stated in the 2015 WHO report that hypertension affects 1.13 billion people worldwide, and this is projected to negatively impact more than 1.5 billion people by 2025 (G. R. F. Collaborators, 2015). In 2015, a report published by Public Health England (PHE) indicated that one in four adults (around 12.5 million) are affected by hypertension, 31% of men and 26% of women (England, 2017).

Hypertension also poses a profound financial impact in some countries. In England, it was reported that the direct cost of diseases caused by HTN to the NHS is approximately £2.1 billion every year (England, 2017). Data from the United States estimated $47 billion as the total direct and indirect costs of HTN in health care services, medications, and absence from work (Heidenreich, 2011). The Burden of Disease report estimated that high BP was accountable for 10.7 million mortalities worldwide (Forouzanfar, 2017).

1.2.9.2 Aetiology and diagnosis

Arterial HTN is a complex disorder resulting from the interactions of many environmental and genetic factors. The genetic factors include a family history of high BP, which increases the risk of HTN in the offspring (Mancia, 2013). Additionally, there are more than 25 rare

mutation genetic variants contributing to the development of HTN (Whelton, 2018). Various traditional environmental and lifestyle exposures are associated with HTN including age; sex (women tend to have a lower BP than men); ethnicity (people of Black African and Black Caribbean origin are more vulnerable to HTN); social deprivation; lifestyle such as smoking, excessive intake of alcohol and salt, obesity, emotional stress; and lack of physical inactivity (England, 2017).

The National Institute for Health and Care Excellence (NICE) recommends that HTN diagnosis should be considered when sustained clinic systolic blood pressure (SBP) is greater than or equal to 140 mmHg, or sustained diastolic blood pressure (DBP) greater than or equal to 90 mmHg; or both (UK, 2019). The diagnosis is then confirmed with ambulatory blood pressure monitoring (ABPM) or home blood pressure monitoring (HBPM). Meanwhile, the American College of Cardiology (ACC) and the American Heart Association (AHA) have recently changed the definition for HTN to SBP >130 mmHg and DBP >80 mmHg, which suggests more individuals are considered to have hypertension (Whelton, 2018). The classification for HTN as per the NICE and ACC / AHA guidelines is given in Table 1.5.

Table 1-5: Diagnostic and classification criteria for HTN

BP Category	**ACC/AHA guidelines in Diagnostic Criteria for Hypertension in Adults (Whelton, 2018)**
Normal	<120 systolic and <80 diastolic
Elevated	120–129 systolic and <80 diastolic
Hypertension	130–139 systolic or 80–89 diastolic
Stage 1	130/80 mmHg to 139/89 mmHg
Stage 2	≥140 systolic or ≥90 diastolic
BP Category	**NICE guidelines in Diagnostic Criteria for Hypertension in Adults (UK, 2019)**
Normal	<120 systolic and <80 diastolic
Stage 1	clinic BP ranging from 140/90 mmHg to 159/99 mmHg and subsequent ABPM daytime average or HBPM average BP ranging from 135/85 mmHg to 149/94 mmHg
Stage 2	clinic BP of 160/100 mmHg or higher but less than 180/120 mmHg and subsequent ABPM daytime average or HBPM average BP of 150/95 mmHg or higher
Stage 3 or severe hypertension	clinic systolic blood pressure of 180 mmHg or higher or clinic diastolic blood pressure of 120 mmHg or higher

***Note: ACC, the American College of Cardiology; AHA, the American Heart Association; NICE, National Institute for Health and Care Excellence.**

Often, hypertension is misdiagnosed. For example, some patients' BP readings in the clinic were compared to HBPM readings and there was a discrepancy of more than 20/10 mmHg due to "white-coat" HTN phenomena (Franklin, Thijs, Hansen, O'brien, & Staessen, 2013). With masked hypertension, on the other hand, individuals showed normal BP readings in the clinic, but readings at ambulatory daytime BP or home BP above 135/85 mmHg (Pickering, Eguchi, & Kario, 2007). 90% of patients with primary HTN do not have an identifiable underlying cause, while secondary HTN, which is reported in 10% of patients, usually has a known cause: either a disease such as renal disorder, or medication use (Whelton, 2018).

1.2.10 Hypertension pathophysiology and COPD

An obscure interaction of multiple vascular effectors including sympathetic nervous system, renin-angiotensin-aldosterone system (RAAS), and inflammatory mediators, contributes to hypertension pathophysiology (Beevers, Lip, & O'Brien, 2001). Hypertension has been strongly linked with sympathetic stimulation of the heart, peripheral vasculature, and kidneys, resulting in increased cardiac output, increased vascular resistance, and fluid retention (Delacroix, Chokka, & Worthley, 2014). One proposed mechanism suggested that the elevation in sympathetic activity in hypertensive patients was associated with an increase in epinephrine (adrenaline) and norepinephrine (noradrenaline) (Dibona, 2013). Hyperstimulation of α-adrenoceptors and enhancing the release of angiotensin or endothelin are strongly connected to the increase in vascular tone (Sarzani, Salvi, Dessì-Fulgheri, & Rappelli, 2008). Peptide levels increase leads to an increase in cytosolic calcium in the vascular smooth muscle, resulting in vasoconstriction and vascular remodelling, which triggers hypertension (Dibona, 2013). In addition, the renal sympathetic nervous system has been engaged in hypertension pathophysiology through efferent and/or afferent pathways

(Mathias, 1991). In the efferent pathway, signals are carried to the kidneys from the sympathetic nervous system (SNS), causing an increase in renin release, RAAS activation, and sodium and water retention, which leads to hypervolemia and an increase in BP (Janssen & Smits, 1989). The efferent pathway contributes to increased BP by increasing renal perfusion, which triggers the afferent pathway and exacerbates sympathetic overactivity (Ciriello & de Oliveira, 2002).

The RAAS system has a major role in regulating BP by maintaining extracellular fluid volume and peripheral resistance (Sarzani, 2008). RAAS is activated in response to SNS stimulation and glomerular underperfusion. These stimuli induce renin release from the juxtaglomerular apparatus from the kidney, which is responsible for converting angiotensinogen into angiotensin I-inactive (Foëx & Sear, 2004). The endothelium-bound angiotensin-converting enzyme (ACE) then rapidly cleaves and converts it in the lung to the active and potent vasoconstrictor angiotensin II, which elevates the BP (Foëx, 2004). Angiotensin II induces aldosterone release from the adrenal gland, which promotes renal epithelial cells to increase sodium and water reabsorption of the kidney, which also leads to a further increase in BP (Cody, 1997).

Endothelial dysfunction also plays a part in hypertension pathophysiology. The positive correlation between endothelial dysfunction and hypertension severity has been indicated in many studies (Dharmashankar & Widlansky, 2010). This correlation is induced by excessive systemic OS and vascular inflammation (Alexander, 1995). The Women's Health Study demonstrated increased levels of CRP, a marker of systemic inflammation, correlating significantly with future risk of developing hypertension-associated ED (Albert, Glynn, Buring, & Ridker, 2004). Another mechanism links hypertension-induced vascular

superoxide O_2^- to endothelial dysfunction via inhibiting eNOS. This leads to a reduction in endothelium-derived relaxing factors, such as NO and prostacyclin, and enhances the production of endothelin-1 and thromboxane A2 vasoconstrictor mediators (Hermann, Flammer, & Lüscher, 2006).

Hypertension in patients with COPD is the most frequent CV risk morbidity (Rabe, 2018). Several population-based studies reported HTN occurring among COPD patients ranging from 40% to 50% (Fumagalli, 2013; David M Mannino, Thorn, Swensen, & Holguin, 2008; Rabe, 2018). In the cardiovascular risk factors in COPD (ARCE in Spanish) multicentre, a cross-sectional study reported the prevalence of cardiovascular risks in COPD patients, including hypertension (53%), obesity (27%), dyslipidaemia (26%), and diabetes (23%) (de Lucas-Ramos, 2008). Miller *et al.* (2013) in the ECLIPSE, a large observational cohort study of 2,164 COPD patients, reported cardiovascular comorbidities such as hypertension, heart failure, ischaemic heart disease, arrhythmia, and stroke as common symptoms in COPD subjects, which are associated with raised inflammatory markers (J. Miller, 2013). More recently, Perticone and associates (2021) evaluated whether COPD and hypertension together increased the risk of cardiovascular incidents, including fatal and non-fatal cardiovascular stroke, myocardial infarction, and cardiovascular death in 524 hypertensives with COPD compared to matched controls hypertensives without COPD (Perticone, 2021). After 57 months, they reported that the number of cardiovascular incidents in the COPD group was higher than in hypertensives without COPD (133 vs 72, respectively).

The pathophysiology of hypertension in COPD is complex. This might be related to several shared common modifiable and non-modifiable risk factors that influence the development of hypertension in the COPD population. These risk factors include smoking tobacco, ageing,

and chronic systemic inflammation (Müllerova, Agusti, Erqou, & Mapel, 2013). Available evidence suggests a pivotal role is played by smoking in both CVD and COPD, as it activates endothelial dysfunction, formation of atherosclerotic plaque, and hypertension by increasing oxidative stress and systemic inflammation (Corbi, 2013; Finks, Rumbak, & Self, 2020). Smoking also contributes to the development of arterial stiffness, a surrogate marker of CV risk in COPD, which becomes more severe due to AECOPD episodes, subsequently influencing systemic hypertension (Vivodtzev, 2014). Chronic low-grade systemic inflammation can lead to an increased risk of hypertension in COPD patients. Eickhoff *et al.* (year) studied COPD populations and demonstrated the severity of airway obstruction and increased systemic inflammation and how it is linked to increased risk of ED, contributing to circulatory and pulmonary hypertension (Eickhoff, 2008). An association between ED and hypertension is well established, with evidence that ED precedes hypertension, arising from mechanistic data showing that oxidative stress influences inhibitors of endothelium-derived nitric oxide synthase (eNOS) and results in a plethora of atherogenic risk factors that may lead to hypertension in humans (Sander, Chavoshan, & Victor, 1999). Observations from rat studies suggest that oxygen free radicals may be important in the pathogenesis of hypertension (Nakazono, 1991). It has also been suggested that superoxide anions (O_2^-) might trigger the development of hypertension in some models, presumably by inactivating endothelium-derived nitric oxide (NO), thus mitigating this important vasodilator mechanism (Nakazono, 1991). Pre-clinical and human studies in HTN have shown that the activation of several ROS sources and circulatory proinflammatories have contributed to different mechanisms that alter eNOS production of NO and impair EC signalling to regulate vasodilation and platelet activation (Figure 1.12), (Schulz, 2011).

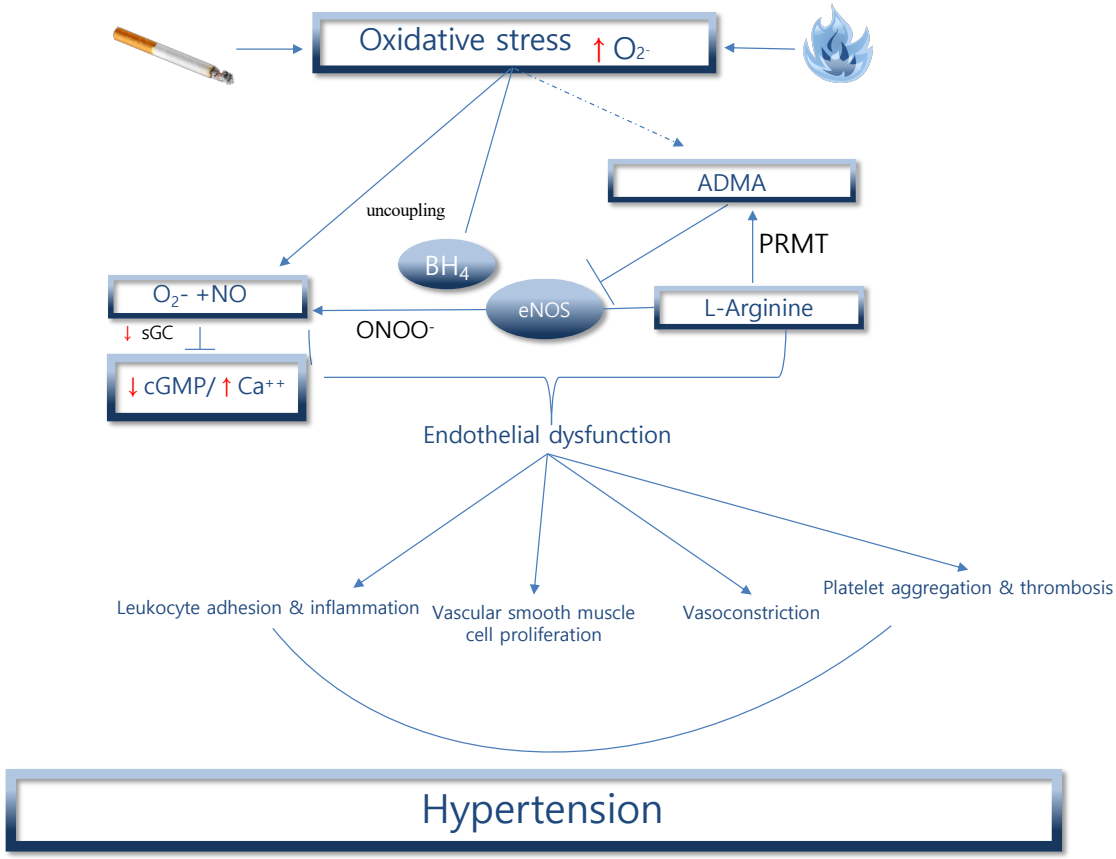


Figure 1-12: Schematic representation of the mechanisms by which COPD-associated ED develops hypertension

ROS and systemic inflammation increase oxidative stress markers within endothelium tissue, such as the NADPH and superoxide anions O_2^-. Superoxide anions O_2^- induce the formation of peroxynitrite (ONOO−) which then decreases nitric oxide (NO) bioavailability. Also, O_2^- may uncouple the eNOS- by oxidising the eNOS cofactor BH4 to BH_2, leading to an impairment of NO. Another suggested mechanism stated that O_2^- inactivating NO under the formation of $ONOO^-$ can inhibit soluble guanylate cyclase (sGC) activity and decrease cyclic guanosine monophosphate levels (cGMP), which then increase cellular Cu^{++} levels causing vasoconstriction. Another alternative mechanism proposed L-Arginine merged into protein arginine methyltransferases (PRMT), allowing the liberation of asymmetric dimethylarginine (ADMA) during proteolysis, which acts as an endogenous inhibitor of eNOS. Subsequently, risks for endothelial dysfunction will be initiated, including platelet aggregation, continued vascular inflammatory response, and vasoconstriction decreased flow-mediated dilation (FMD), which increased the risk of developing hypertension. Adapted from (Lundberg & Weitzberg, 2010)

Moreover, data from experimental studies and the Framingham community-based study showed elevated levels of brain natriuretic peptide (BNP), a biomarker associated with increased risk of hypertension which is an early sign of left ventricular hypertrophy (LVH) (Freitag, 2003; Kuroski de Bold, 1998). BNP is a hormone secreted selectively by the myocytes of the left ventricle in response to elevated wall stress that has been found to be an

independent predictor of endothelial dysfunction and hypertension (Chong, 2004). BNP regulates vascular tone via binding to the natriuretic peptide receptor-A, which induces eNOS to release NO (van der Zander, 2002). In hypoxic conditions as such with severe and very severe COPD patients, there is an inverse correlation between high BNP levels and endothelial function, contributing to the chronic hypoxia phenotype (Khatri, 2020; Pauriah, 2012). Continuous development of these risk factors may damage the arterial walls as they become susceptible to fatty plaque build-up and, consequently, lead to CVDs. This highlights the importance of the early prevention and management of hypertension and how it should be treated based on standard guidelines.

1.2.11 Treating hypertension in COPD

Although reducing BP is essential in treating patients with HTN, it is important to consider the underlying risk factors for HTN, such as lipid disorders, diabetes, and smoking (Boffa, Constanti, Floyd, & Wierzbicki, 2019; Whelton, 2018). Intervening these risks may reduce the pathophysiological process of HTN. Early focused goal to control HTN is to reduces SBP to <140mmHg and DBP to <90mmHg (Boffa, 2019). Largely, HTN can be managed with several strategies. These include non-pharmacological interventions or lifestyle changes such as smoking cessation, increasing levels of Dietary Approaches to Stop Hypertension (DASH) and physical activities, as well as reducing sodium intake, alcohol consumption and weight (Whelton, 2018). These lifestyle changes may delay in persons with prehypertensive status (120<SBP<130 mmHg; 80<DBP<89 mmHg) the administration of antihypertensive treatment regimens (Egan & Stevens-Fabry, 2015). Additionally, lifestyle change options should be included in stage 1 HTN as complementary along with antihypertensive therapy,

which might help reduce the number of antihypertensive therapies used at the same time (Elmer, 2006).

Antihypertensive therapy guidelines have not considered specific classes of antihypertensive treatments for COPD. Understanding pharmacokinetic and pharmacodynamic antihypertensive agents, pulmonary side effects, and other comorbidities is essential to managing HTN in COPD (Finks, 2020). Angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs), also known as RAAS inhibitors, exert different beneficial effects in COPD patients (Forth & Montgomery, 2003; Vasileiadis, Goudis, Giannakopoulou, & Liu, 2018). These include inhibiting angiotensin II and lung tissue pro-inflammatory mediators as well as decreasing the breakdown of bradykinin (Forth, 2003). Observational studies have reported that ACEi and ARBs may provide beneficial protection against emphysema progression and FEV1% decline. This inhibits transforming growth factor-B signalling in alveolar tissues in addition to lowering AECOPD and mortality rates (Mancini, 2006; Petersen, 2014). Additionally, RAAS inhibitors can stimulate NO bioavailability, which leads to the reduction of BP and reverse endothelial dysfunction (Ghiadoni, 2003; Imanishi, 2008). The Heart Outcome Prevention Evaluation (HOPE) revealed the use of ACEi agent Ramipril as it showed to reduces coronary events and may have prevented atherosclerosis proregression in patients who did not have left ventricular dysfunction or HF (Dagenais, 2001). On the other hand, coughs, bronchospasm, and angioedema have been reported as the most prevalent side effects of ACEi use in the general elderly population (Dicpinigaitis, 2006; Mahmoudpour, 2016; Morimoto, 2004). ARBs are tolerated well by COPD patients with minimal coughing side effects (Whelton, 2018). However, it is recommended that ACEi and ARBs should not be used together to treat COPD

patients with hyperkalaemia risk, as both may increase potassium levels (Wetmore, Yan, Horne, Peng, & Gilbertson, 2021).

Antihypertensive diuretic classes included thiazide and loop diuretics. Thiazide agents are considered safe and the first line of choice for HTN treatment in COPD (Boffa, 2019; Whelton, 2018). Their mechanism of action is through increasing renal sodium and fluid elimination (Pourafshar, Alshahrani, Karimi, & Soleimani, 2018). Evidence shows that thiazide use by COPD patients reduced AECOPD warrant hospitalisation and lower HF morbidity as well (Herrin, 2013). However, potassium level monitoring should be evaluated routinely, as electrolyte imbalance is considered a side effect that leads to hypokalaemia (Zillich, Garg, Basu, Bakris, & Carter, 2006).

There is no evidence reporting beta-blocker contraindications for COPD. However, although controversial, current guidelines recommend using cardio-selective beta-blockers agents for HTN in COPD patients with pre-existing IHD, CAD, and HF (MacNee, 2019). Cumulative evidence suggests beta-blocker therapy may benefit patients with COPD in reducing AECOPD and mortality rates (Du, Sun, Ding, Lu, & Chen, 2014). However, beta-blocker agents that cause bronchospasm are contraindicated for COPD patients who present with reactive airway diseases such asthma-COPD overlap (Dransfield, 2019). Calcium channel blockers are another antihypertensive class that decreases BP, as they reduce calcium levels in muscle cells, leading to vasorelaxation. The final antihypertensive agent class is alpha-blockers, which are safe and usually coupled with diuretics agents in the management of uncontrolled HTN (Boffa, 2019). They are also considered selective agents given for specific indications, such as in patients with prostatic hypertrophy (Biernacki & Flenley, 1989).

Nevertheless, achieving the guidelines' goal to lower BP to 130/80 mmHg or less in COPD population is challenging due to the variety of COPD disease- and patient-related factors that influence BP control. These, including COPD severity with comorbidities, require patients to take polypharmacy; however, some medications may counteract the effect of other medications. In severe COPD groups often systemic glucocorticoid therapy and intranasal decongestant sprays used as part of COPD treatment, which happens to increase SBP (Leuppi, 2013; Salerno, Jackson, & Berbano, 2005). Other patient-related factors, including obesity, depression, and substance use, may affect COPD patients' adherence to HTN management (Wu, Zhu, & Ghitza, 2018). Taken together, increasing systemic OS and low-grade systemic inflammation may link COPD pathophysiology with HTN and subsequently impact vascular endothelium functions. Impaired NO bioavailability is the driving mechanism for ED, and ED is considered an important risk factor for hypertension and CVD in COPD. Lately, cumulating evidence advocates for combining dietary NO_3^- supplementation using beetroot juice (BRJ) as a safe, natural, and affordable complementary to antihypertensive medications. In particular, BRJ might help lowering BP and attenuate hypertension-associated ED by enhancing NO availability.

1.2.12 Introduction to dietary NO_3^- supplementation and CV risk markers

1.2.12.1 Production of nitric oxide (NO) and signalling in the body

In vivo NO (nitrogen monoxide) biochemistry is known as a small diatomic free radical signalling molecule with a short half-life (< 1 second) in human circulating blood (Kelm, 1999). Seminal research showed that the endothelium released a substance that caused vasodilation. In 1987, published landmark papers labelled observational discoveries as "NO signalling molecule in the cardiovascular system". These discoveries revealed that NO plays a pivotal role in various physiological processes, including BP, platelet activation, and neurotransmission, as a mediator of inflammation, and as a host defence (Garthwaite, Charles, & Chess-Williams, 1988; Palmer, Ferrige, & Moncada, 1987; Radomski, Palmer, & Moncada, 1990). Research has since been evolving in understanding biological NO regulatory actions and mechanisms. In mammals, there are two proposed NO synthesis pathways. NO is endogenously produced via the NOS family of enzymes (Figure 1.13); (Lundberg, 2010).

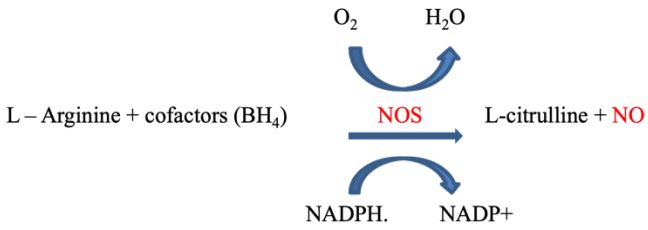


Figure 1-13: The L-arginine – nitric oxide synthase (NOS) pathway This produces NO from the substrates L-arginine and molecular oxygen.

The biophysiological mechanism of this pathway suggests that the vascular endothelium, particularly ECs, plays a central role in regulating vascular homeostasis via releasing potent biological substances NO and prostacyclin/ prostaglandin I_2 (PGI_2) (Verhamme & Hoylaerts,

2006). These substances are involved in regulating vascular dilation, immune function, and platelet activation. Seminal works on NO reported that amino acid L-arginine was catalysed by NO synthase (NOS) to produce NO and L-citrulline (Salvador Moncada & Higgs, 1993). There are three NOS families of enzymes including endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Lundberg, 2010). These enzymes catalyse the conversion of L-arginine to NO and L-citrulline in a reaction dependent on the presence of molecular oxygen and several cofactors, such as BH_4 (Alderton, Cooper, & Knowles, 2001). Generation of eNOS enzymes and prostacyclin synthase (PGIS) enzymes catalyse the production of NO from L-arginine and PGI_2 from prostaglandin H_2. NO and PGI_2 signalling cascades act synergically on relaxing the vascular smooth muscle and inhibit platelet activation through downstream pathways (Figure 1.14) (Salvador Moncada, 1993).

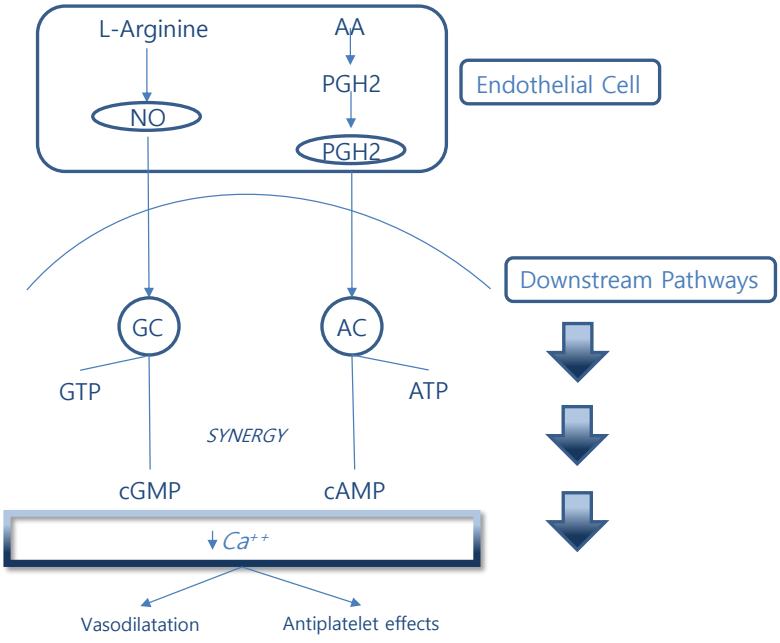


Figure 1-14: NO and PGI2 downstream signalling pathways

Shear stress and vascular inflammation stimulate eNOS to produce NO, which induces signalling to regulate downstream pathways (Cunningham & Gotlieb, 2005). NO downstream signalling pathway targets the haem side of soluble guanylyl cyclase (sGC) to stimulate the conversion of guanosine-5-triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), known as the NO/sGC/cGMP pathway (Apostoli, Solomon, Smallwood, Winyard, & Emerson, 2014). Subsequently, cGMP activates protein kinases (PKG), which phosphorylates inositol triphosphate (IP3) and binds to the calcium ion (Ca^{++}) channel on the surface of the endoplasmic reticulum, causing Ca^{++} to exit the cell, thereby relaxing the smooth muscle and suppressing platelet surface P-selectin from binding to fibrinogen (Apostoli, 2014). In addition, PGI_2 activates adenylate cyclase (AC) to stimulate the surface of the platelet or smooth muscle cells to stimulate the conversion of adenosine triphosphate to cyclic AMP. Accordingly, cAMP phosphorylates and activates protein kinases (PKA), causing Ca^{++} influx out of platelet intracellular or smooth muscle cell mitochondria (J. A. Mitchell, 2008).

In clinical practice, platelet aggregates and plasma P-selectin are recognised as reliable surrogate markers of platelet activation (Ferroni, 2012). Unlike PKG, one of PKA's potent actions is to inhibit platelet shape changes or aggregation (Hettasch & Sellers, 1991). The cGMP/cAMP pathways terminate via the phosphodiesterase enzymes PDE5 and PDE2,3, respectively (Denninger & Marletta, 1999). Another mechanism recently linked the conversion of nitrite alongside *N*-nitroso proteins, and iron-nitrosyl back to NO under hypoxic conditions (Woessner, McIlvenna, Ortiz de Zevallos, Neil, & Allen, 2017).

1.2.13 Nitrate-Nitrite-NO dietary pathway (enterosalivary circulation)

Ishiwata (1975) and Duncan (1995) were the first to describe the mechanism of enterosalivary NO^{-}_{3} circulation and its correlation to NO_3^- concentration in saliva (Duncan, 1995; ISHIWATA, 1975). NO is produced alternatively from our diet in the body via the nitrate-nitrite-NO pathway (Ysart, 1999). Ingested dietary rich NO^{-}_{3} enters the bloodstream through the stomach, with the majority being excreted renally. Approximately 25% of ingested nitrate is extracted by the salivary glands via the entero-salivary circulation (Lundberg, 2010), where concentrations may be over ten-fold greater than those measured in the plasma. It is then excreted in saliva and reduced from inert nitrate NO_3^- to bioactive nitrite NO_2^- via nitrate reductases produced by the oral commensal facultative anaerobic bacteria. Saliva rich with NO_2^- is swallowed and further reduced to NO under acidic condition of the stomach, while some of the NO_2^- enters the circulation directly. Circulating NO_2^- is reduced to NO in the blood or vasculature or muscle cells in process involves enzymatic and non-enzymatic components such as deoxyhaemoglobin, xanthine oxidoreductase (XOR), aldehyde oxidase, and vitamin C (Lundberg & Govoni, 2004). Finally, NO signals the downstream pathways by inducing sGC and cGMP for biological effects of muscle relaxation or antiplatelet effects, Figure 1.15; (N. Benjamin, 1994; J. O. Lundberg, 2004; Lundberg, Weitzberg, Lundberg, & Alving, 1994).

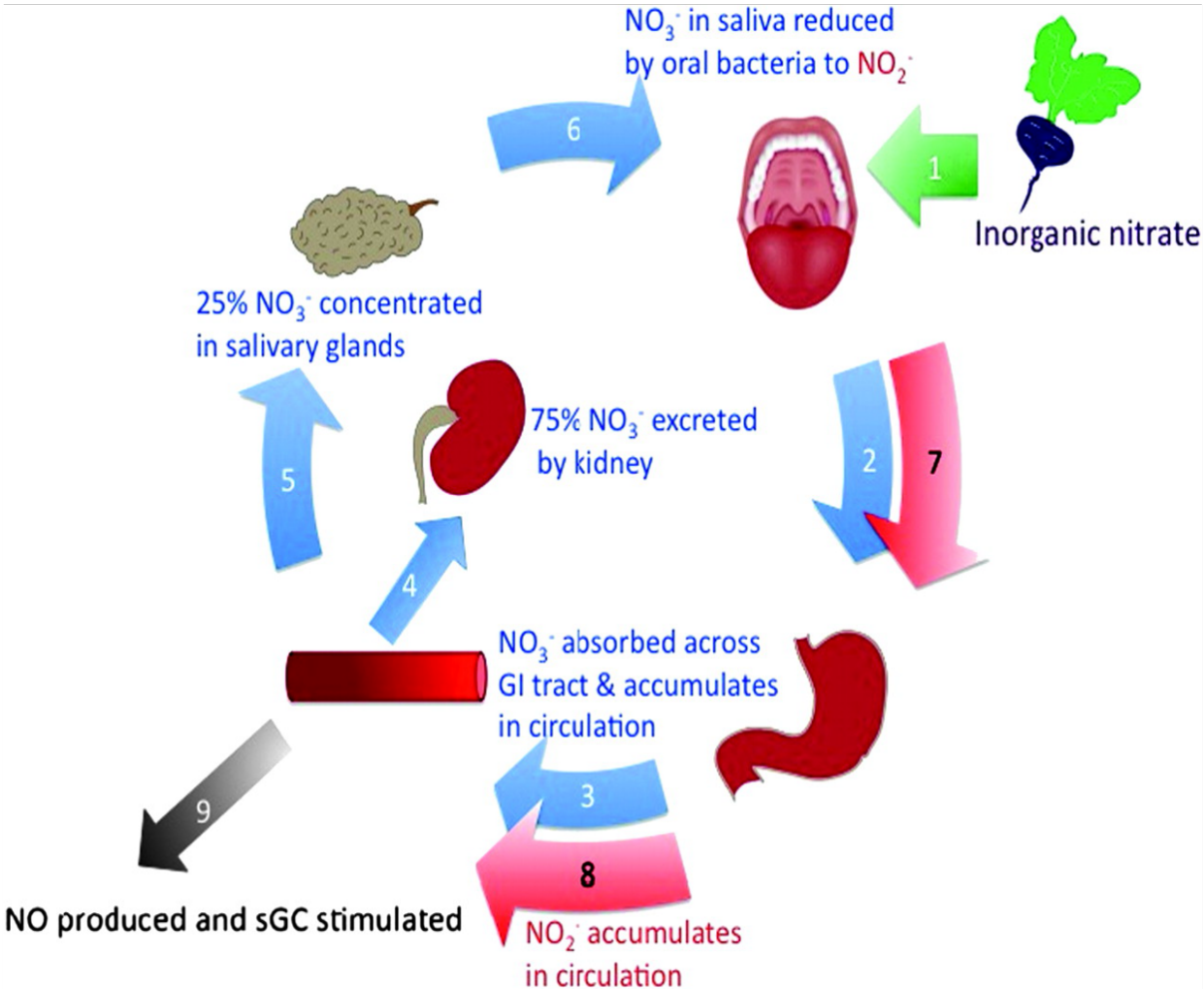


Figure 1-15: Schematic representation of the enterosalivary circulation Adapted with permission (V Kapil, Webb, & Ahluwalia, 2010).

1.2.14 Dietary NO_3^- supplementation and oral microbiome

The human oral cavity is an important habitat of oral facultative anaerobes located on the dorsal surface of the tongue (Lundberg, Weitzberg, & Gladwin, 2008). Cumulating evidence showing dietary NO_3^- may result in changes in the oral microbiome (Burleigh, 2019). These changes are indicated by 16S ribosomal RNA gene sequencing. The pathway of these changes suggests enterosalivary circulation, where bacterial conversion of diet-rich NO_3^- independently supplements NO production via nitrate reductases and participates in the regulation of endothelial vasodilation (Lundberg, Weitzberg, Cole, & Benjamin, 2004). Previous studies demonstrated the disruption of enterosalivary circulation via the use of oral

antiseptics, resulting in increased SBP (Vikas Kapil, 2013). Seminal research works suggest NO_3^- supplementation generates NO and is associated with changes in the composition of certain oral microbiomes from the genera *Granulicatella, Neisseria*, *Haemophilus,* and *Rothia* (Doel, Benjamin, Hector, Rogers, & Allaker, 2005; E. R. Hyde, 2014). In a cohort of 27 with excellent cardiovascular and oral health, Tribble *et al.* (2019) evaluated the association of tongue cleaning with antiseptic chlorhexidine wash with response to SBP and tongue microbiome composition before and after exposure, using polymerase chain reaction (PCR) amplification of 16S rRNA gene sequencing. The subjects using CHX mouthwash for seven days were associated with a significant increase in SBP after one week of use and recovery from use, which resulted in an enrichment of nitrate-reducing bacteria on the tongue (Tribble, 2019). In addition, smoking, inhaled medications, and gastro-oesophageal reflux factors influence the oral microbiota and thereby nitrate/nitrite reductase activity (Godara, Godara, & Khullar, 2011). There is scarce knowledge on the composition of the COPD oral microbiome and how it varies between subjects during dietary nitrate intake. The ability to fully acknowledge and understand the oral microbiota will lead to supporting novel probiotic approaches to improving nitrate metabolism in COPD patients.

1.2.15 Dietary NO_3^- supplementation and CV risk markers

Reported findings from pathophysiological research linked impairments of eNOS and NO production in the vascular endothelial to traditional CV risks, such as ageing, smoking, and hypertension (Committee, 2016). NO is described as “a key signalling molecule in the cardiovascular system” (M Carlström, Lundberg, & Weitzberg, 2018). There is growing evidence demonstrated ingesting a diet rich in-NO_3^- may facilitate signalling a series of cardiovascular functions in the body including BP (Eckel, 2014). High BP known as hypertension is a global public health issue due to various modifiable risk factors, including

high salt intake, poor fruit and vegetable intake, and subsequent deficiency of vitamins C and D, as well as physical inactivity (McCartney, Byrne, & Turner, 2015). Therefore, researchers and statement guidelines, including the American Heart Association (AHA), are turning to natural products and recommending Dietary Approaches to Stop Hypertension (DASH) or a Mediterranean diet, which could improve vascular health and delay or attenuate the development of HTN (Appel, 1997; McCartney, 2015). Joshipura *et al.* (2001) studied the impact of inorganic dietary-rich NO_3^- including beetroot consumption on cardiovascular health in more than 100,000 respondents free of CVDs (Joshipura, 2001). Their analyses over eight years demonstrated the benefits of increased dietary nitrate intakes from beetroot and leafy vegetables, reducing the risks of stroke by 30%, ischaemic heart disease (IHD) by 20%, and other cardiovascular diseases by 30% (Hung, 2004).

According to the World Health Organization and the European Food Safety Authority, the recommended nitrate daily intake is 3.7 mg/kg per day, or approximately 4.2 mmol per day per person weighing 70 kg (Authority, 2008; Speijers, 2003). The DASH landmark RCT in 459 participants (high-normal or Stage 1 hypertension) for 11 weeks. The team investigated two dietary interventions: fruits and vegetables only and a combination diet (fruits, vegetables, and low-fat dairy products and low in total and saturated fat), effects on BP, measured in the clinic and by 24-h ambulatory monitoring. The trial results revealed significant changes in BP parameters for both groups (SBP -2.8 vs -5.5 mm Hg; DBP -1.1 vs – 3.0 mm Hg) with a greater decrease in BP in the combination diet group (Sacks, 1999). These preventative effects of lowering BP from fruits and vegetables demonstrated a reduction of ≥2 mm Hg; SBP and DBP could reduce CHD and stroke risk by 6% and 15%, respectively (Cook, Cohen, Hebert, Taylor, & Hennekens, 1995; Stamler, 1997).

Beetroot (*Beta vulgaris*) is gaining prominence in HTN research since it is rich in NO_3^-. High-level evidence and clinical research have shown ingestion of NR-BRJ provides a reliable source of NO, which regulates and mitigates the pathophysiology behind exercise performance, HTN, ED, ischaemia/reperfusion injury, and thrombosis (Gee & Ahluwalia, 2016; Jackson, Patterson, MacDonald-Wicks, Oldmeadow, & McEvoy, 2018; McMahon, Leveritt, & Pavey, 2017). While the mechanism is not completely understood, it is possible to say that nitrate- and nitrite-containing beetroot and green leafy vegetables may improve endothelium-independent vasodilation, inhibit platelet aggregation, suppress vascular inflammation, and limit the proliferation of vascular smooth muscle cells. This NO_3^-- NO_2^--NO pathway is a fundamental component of vasodilation and BP regulation in humans (Bondonno, 2018). More specifically, NO is found to regulate blood flow and vascular tone (Gee, 2016). Augmentation of the NO_3^- and NO_2^- supply from external sources, such as BRJ, was shown to mitigate an increase in BP, suggesting that beetroot could play a vital role in BP management and control (Ahmad, 2018). Cumulating results of animal and human studies confirm the utility of using NO_3^- containing BRJ in BP.

1.2.16 Pre-clinical studies with NO_3^- supplementation and BP

The body of animal research into the effects of NO_3^- containing beetroot for hypertension constantly increases. Bhaswant *et al*. (2017) and Gheibi *et al*. (2018) tested the effects of nitrate supplementation on male rats with obesity and type 2 diabetes. The results suggest that nitrite and nitrates can reduce chronic inflammation and oxidative stress, which are deeply implicated in the pathophysiology of hypertension. (Bhaswant, Brown, McAinch, & Mathai, 2017; Gheibi, Jeddi, Carlström, Gholami, & Ghasemi, 2018). Tropea *et al.* (2020) found that beetroot juice improved endothelial function and reduced BP in pregnant mice, implying that beetroot consumption could improve cardiovascular health and outcomes during pregnancy

(Tropea, 2020). Beetroot juice rich in NO_3^- suppressed right ventricular hypertrophy and lowered arterial thickening in the lungs, thus implying that nitrate consumption could benefit patients with pulmonary hypertension (Tawa, 2021).

In a study by Gonzaga *et al.* (2020), the administration of NO_3^- to hypertensive mice reduced endothelial dysfunction, mostly due to its antioxidative effects (Gonzaga, 2020). Morris *et al*. (2019) went further to confirm that NO_3^- supplementation from beetroot was effective in reducing the levels of salt-induced HTN (Gonzaga, 2020). Guimarães *et al*. (2019) tested the utility of dietary NO_3^- consumption in rats with hypertension and found that beetroot supplementation normalised the expression of angiotensin II type-1 receptors. Sympathetic nerve activity also decreased, reducing the incidence of hypertension and optimising BP levels (Guimarães, 2019). All in all, animal studies convincingly demonstrate the advantages of NO_3^- - containing beetroot supplementation in hypertension, since it mitigates the mechanisms involved in high BP, including improving vasodilation, reducing arterial stiffness, and reducing sympathetic nerve activity, oxidative stress, and inflammation.

1.2.17 Clinical studies with NO_3^- supplementation and BP

A large study of the influence of NO_2^-- and NO_3^- - containing vegetables on 1,546 non-hypertensive subjects demonstrated that nitrate-rich food may reduce the risk of incident hypertension in 3-years (Golzarand, Bahadoran, Mirmiran, Zadeh-Vakili, & Azizi, 2016). It is also effective in adults with uncontrolled hypertension (Conor P Kerley, Dolan, James, & Cormican, 2018). Speaking of numbers, high-level meta-analyses reported NO_3^- and NO_2^- obtained from beetroot and other sources, such as spinach, reduced SBP by at least 4.80 mmHg, arterial stiffness by at least 0.23 m/s, and platelet aggregation by at least 18.9%. Duration of NO_3^-/NO_2^- intervention in the included trials ranged from 1 hour to 70 days

(Jackson, 2018). Webb and co-workers (2008) conducted one of the earliest RCTs, assessing inorganic NO_3^- effects on BP in 14 healthy volunteers. They randomised participants to drink 500 mL of active NO_3^- or water as a placebo. The active-BRJ demonstrated an acute SBP/DBP reduction (−10.4/-8 mm Hg) within 30 min. They also reported results showing that a reduction in BP outcomes correlated with increases in plasma NO_2^- concentration as well as the possibility that BRJ may prevent other CV risk outcomes, including improving FMD and altering platelet activation (Webb, 2008). Kerley *et al*. (2017) evaluated the impact of seven days of 140 ml of nitrate-rich (NR-BRJ) or placebo (nitrate- depleted) BRJni 11 subjects (controlled HTN vs uncontrolled HTN) using ABPM for 24 hours. They concluded that NR-BRJ significantly decreased both SBP and DBP by – 2.2 and – 5.9, respectively, in the uncontrolled HTN (C. Kerley, Dolan, & Cormican, 2017). In the following year, the same group used a higher NR-BRJ concentration (12.9 mmol NO_3^-) and an ambulatory BP monitor (ABPM) for 14 days with 20 uncontrolled HTN participants in a double-blind, randomised, placebo-controlled, crossover trial. Results revealed that the NR-BRJ group showed a significant reduction in 24-h SBP/ DBP at −8 mmHg/ −4 mmHg compared to the placebo (Conor P Kerley, 2018).

Kapil and colleagues conducted the longest double-blinded, placebo-controlled clinical trial of 68 patients with hypertension who received daily BRJ for four weeks (250 mL of 6.4 mmol NR-BRJ vs 250 depleted-nitrate BRJ). The primary endpoint was comparing BP change measured by various methods in clinic, ABPM, and HMBP with placebo. The NR-BRJ significantly dropped in clinic BP by7.7/2.4 mmHg (3.6–11.8/0.0–4.9, P<0.001), ABPM reduced by 7.7/5.2 mm Hg (4.1–11.2/2.7–7.7, P<0.001), and the HMBP was reduced by 8.1/3.8 mm Hg (3.8–12.4/0.7–6.9, P<0.001). They also assessed the change in vascular function measures of endothelial function using FMD and arterial stiffness. They found the

FMD at NR-BRJ group improved by ≈20% (P<0.001), and arterial stiffness aPWV reduced by 0.59 m/s (Vikas Kapil, Khambata, Robertson, Caulfield, & Ahluwalia, 2015).

1.2.18 Clinical studies with NO_3^- supplementation and other CV risk outcomes

Raubenheimer and colleagues (2017) involved 12 healthy adults to examine the acute effects 3 hours and 6 hours after a dose of 140 mL (12.9 mmol) NR-BRJ versus nitrate-depleted placebo on several CV risk factors outcomes including (SBP/DBP/MAP) and blood monocyte-platelet aggregates. A notable reduction seen in BP parameters was −7.9 mmHg/ −5.7 mmHg/ −6.4 mmHg, respectively, as well as the percentage of platelet aggregates significantly decreased (Raubenheimer, 2017). Velmurugan and colleagues (2016) investigated the impact of BRJ on vascular and platelet function in volunteers with hypercholesteremia. A randomised group was given a once-daily dose (250 mL) of NR-BRJ (James Whites Drinks) or a placebo for 6 weeks. It was shown that active-BRJ caused an increase in FMD of 1.1% and a decrease of 0.3% in the placebo. Nitrate-rich BRJ was also shown to inhibit platelet-monocyte aggregates with a reduction of 7.6% compared to an increase of 10.1% in the placebo, along with suppressing stimulated P-selecting expression (Velmurugan, 2016).

A recent systematic review and meta-analysis by Bahrami *et al.* (2021), including RCTs, indicated NO_3^- -containing BRJ modulated arterial stiffness PWV mean by 0.27 m/s, and improved endothelial function FMD by 0.62% (Bahrami, Arabi, Feizy, & Rezvani, 2021). Furthermore, in a recent double-blind, crossover RCT, Walker and associates (2019) used 140 ml of BRJ (800 mg NO_3^-) or placebo in 15 healthy male elderly individuals to assess the acute impact on AIx%, FMD, and PWV. After three hours of BRJ intake, there was a

significant reduction in the mean AIx% by −8.7% and an increase in FMD by +1.18% in the active-nitrate BRJ group compared to the placebo, while PWV was unchanged in both groups (Walker, 2019). However, a crossover double-blind, placebo-controlled RCT recruited 14 healthy volunteers to receive either 8 mmol KNO3- or matched placebo and found no acute change effects on FMD, while other vascular functions and SBP exerted the active-nitrate effects on both PWV and SBP by .4 m/s and -4 mmHg, respectively (Bahra, Kapil, Pearl, Ghosh, & Ahluwalia, 2012).

Cardiac brain natriuretic peptide (BNP) is a hormone expressed in ventricular myocytes in response to elevated pressure, which enhances cardiac wall stress. Normally, BNP levels in plasma are low, and it has been shown to be a reliable diagnostic and prognostic marker in HF patients (van der Zander, Houben, Kroon, & de Leeuw, 1999). Recent studies show that BNP acts on the endothelium by influencing the activity of eNOS and iNOS, which has us questioning a possible unknown mechanism that may exist between natriuretic peptides and endothelial function (van der Zander, 2002). Breidthardt *et al.* (2010) concluded that a high-dose nitrate strategy on top of standard therapy for BNP is safe and notably accelerates cardiac recovery in patients with HF (Breidthardt, 2010).

Overall, evidence is accumulating from animal and human studies, both reporting promising results of dietary nitrate supplementation in lowering BP, improving endothelial function, and modifying platelet aggregation. Yet, important factors such as optimal dosage, frequency, time of drinking, and other patients' characteristics need further investigation. More research is needed to confirm the mechanism behind the beetroot- NO_3^-—NO_2^--NO pathway and its downstream signalling role in regulating vasodilation, arterial stiffness, and antiplatelet effects in COPD patients.

1.2.19 Clinical studies with NO_3^- supplementation and COPD

A scoping search for existing trials evaluating the effects of BRJ on CV risk outcomes such as BP, FMD, arterial stiffness, and platelet activation in COPD patients yielded a handful of evidence with inconsistent results. Curtis *et al.* (2015) studied the acute effect of single but high doses of BRJ on COPD patients and reported that DBP fell by 7 ± 8 mmHg while there were no significant differences detected in SBP and MAP (Curtis, 2015). Another study in the COPD patients, administrating doses of 4.8 mmol NO_3^- BRJ (70 mL) twice daily in a span of three days, found a 10 mm Hg reduction in SBP (Leong, 2015). Kerley *et al*. (2015) reported acute effect of 12.9 mmol NR- BRJ on SBP, DBP, and MAP by −12 ± 19, −1.6 ± 16, and −5 ± 4 mm Hg respectively (Conor P. Kerley, 2015). At the same year, Berry *et al.* used a single dose of 7.58 mmol NR-BRJ on 15 mild-to-moderate COPD patients, and found SBP was dropped by 8 mm Hg lower and DBP by 3 mmHg (Berry, 2015).

Another short cross-over RCT over 7 days evaluated the effect of (140 mL) NR-BRJ on 15 sever-to-very sever COPD patients and they observed a lowering effect on DBP only by 5 mmHg (Friis, Steenholt, Løkke, & Hansen, 2017). However, Shepherd *et al.* (2015) and Leong *et al.* (2015) provided 6.77 mmol twice a day for 2.5 and 3 days and found no differences in all BP measurements (Leong, 2015; Shepherd, 2015). More recently, our research group conducted an RCT (ON-EPIC trial), evaluated the effects of twice-weekly pulmonary rehabilitation (PR) combined with BRJ intake at a dose of 140 ml (12.9 mmol) NR-BRJ or a placebo of 140 ml depleted-nitrate Pl-BRJ. The group consumed NR-BRJ shows an improvement in exercise capacity, BP, and endothelial function as compared to the PR only group (Pavitt, 2020). Their results revealed drop on SBP, DBP, and MAP by -5.0 mm Hg (-5.0, -0.5), -5.0 mm Hg (-5.0, 0.0), and -5.0 mm Hg (-5.0, -1.0), respectively. Additionally, an increase was observed in the FMD % change in (n = 20) subgroups (10

active-BRJ vs 10 placebo BRJ) +6.6% (0.6, 17.6) vs −4.7% (−21.5,11.8), respectively. However, whether this effect is from both PR and BRJ or BRJ alone is unclear and requires further investigation. While BRJ was promising in healthy and other non-COPD diseases, the effect on CV risk outcomes in the COPD population is inconclusive. Few small studies have emphasised the effects of acute/short-term BRJ effect on CV risk outcomes in the COPD population. Currently, there is insufficient evidence to support or refute the use of longer-time BRJ for any effect on BP.

The evidence proposed potential mechanisms of NO effect on BP are summarised in the following schematic diagram (Figure 1.16).

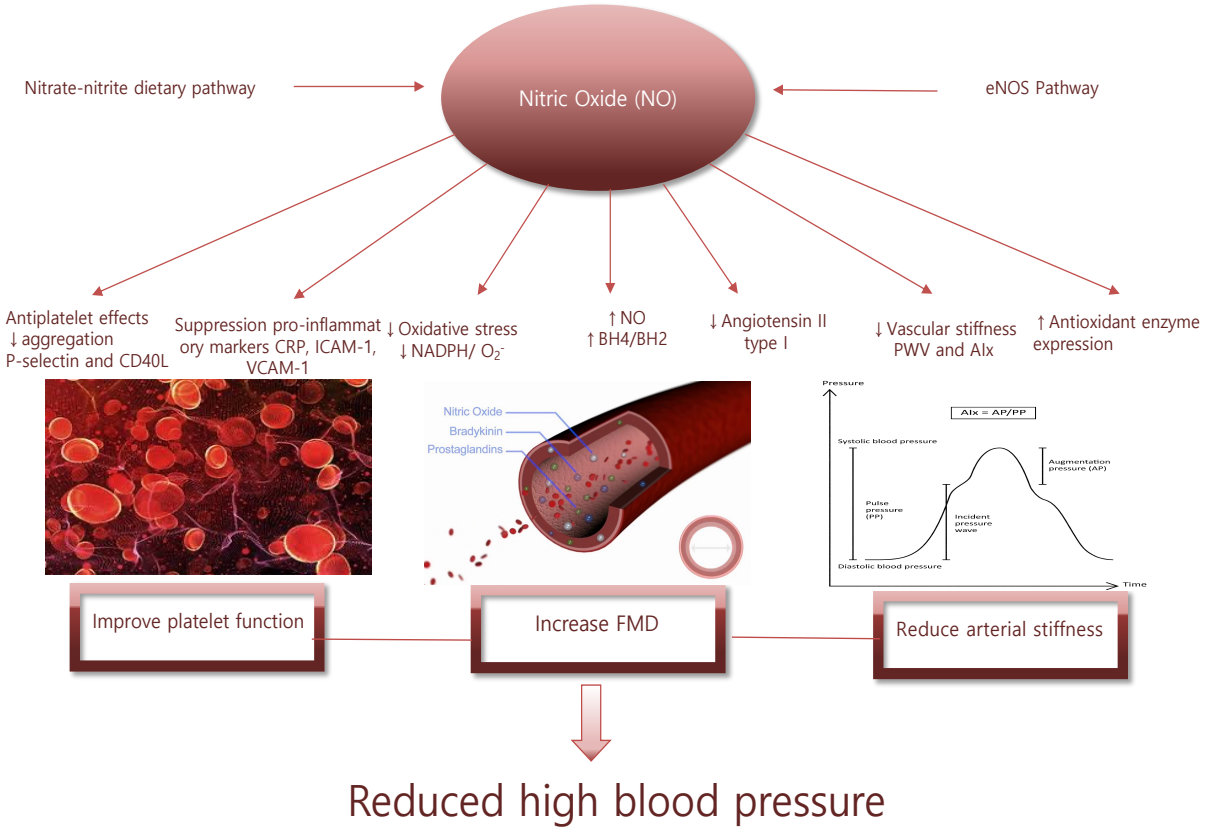


Figure 1-16: proposed mechanisms for lowering BP via endogenous eNOS pathway and exogenous dietary nitrate-nitrite dietary pathway

1.3 Rationale

CVDs are the most frequent comorbidities and a significant cause of mortality in COPD. Hypertension is a strong traditional CV risk that frequently occurs in COPD patients. Its presence is associated with an increased risk of hospitalisation, stroke and major adverse CVD events (Engström, Hedblad, Valind, & Janzon, 2001). Endothelial dysfunction is a common pathophysiology element in COPD and HTN which is linked with impaired endogenous NO bioavailability. Hence, early detection of ED and reverse impaired NO bioavailability in COPD patients could prevent HTN, atherosclerosis, and CVDs. Existing evidence suggests dietary NO_3^-, in the form of BRJ, revealed a potential ergogenic benefit on exercise tolerance, muscle oxygen uptake, as well as on CV outcomes during exercise in COPD individuals. There is no systematic review summarising and quantifying the BRJ effect on CV risk outcomes in patients with COPD. To the best of our knowledge, there is insufficient evidence to support or refute the longer-term BRJ effect on BP and other CV markers, such as endothelial dysfunction and platelet activation. Endothelial dysfunction frequently occurs in individuals with COPD. There is a possibility that changing a modifiable risk factor, such as diet, by including BRJ in their diet regimen, may attenuate endothelial function, thereby lowering high BP, which could reduce the risk of developing CVD.

Therefore, the overall aim of this thesis was to investigate the effect of prolonged daily consumption of beetroot juice (BRJ) on CV risk outcomes in patients with COPD over three months.

Acquiring this information is achieved by integrating and employing a variety of research methods. First, I carried out a comprehensive systematic review and meta-analysis exploring the existing RCTs and evaluated the effects of dietary NO_3^- supplementation on CV risk

outcomes in COPD for summaries, quantification of efficacy, and to help us to tailor safe intervention. Then I conducted a randomised, double-blind, parallel-group, placebo-controlled trial, allowing us to investigate the effects of prolonged BRJ treatment on SBP as the primary endpoint. Finally, I further investigated BRJ effects on vascular function markers, including endothelial function assessed by peripheral arterial tonometry (PAT), augmentation index, plasma ADMA and L-arginine levels, and platelet activation biomarkers. This thesis has generated novel contributions to the clinical knowledge area of treating CV risks in COPD.

1.4 Aims and hypotheses

Accordingly, Chapters 3, 4, 5 and 6 will address the following research questions and hypotheses:

I. In patients with chronic lung disease, does the literature support a role for dietary NO_3^- supplementation on CV risk outcomes? (Chapter 3)

- *Research aim*: To conduct a systematic review and meta-analysis of human experimental trials assessing the effects of dietary nitrate supplementation on a variety of CV risk outcomes, including BP, endothelial function, arterial stiffness indices, and platelet aggregation in patients with COPD.

II. In patients with COPD, what are the prolonged (three months) effects of a daily single dose of 70 ml (400 mg—nitrate), in the form of BRJ, on SBP at rest? (Chapter 4)

- The following *hypotheses* are tested in Chapter 4:
  a) In patients with COPD, daily exposure to a BRJ 70mls (400 mg—nitrate) dose for three months will produce a sustained fall in the SBP at rest compared to a placebo.

b) In patients with COPD, daily exposure to a BRJ 70mls (400 mg—nitrate) dose for three months will improve exercise capacity performance compared to a placebo.

c) In patients with COPD, daily exposure to a BRJ 70mls (400 mg—nitrate) dose for three months will elevate plasma [NO_3^-] and [NO_2^-] levels compared to a placebo.

d) In patients with COPD, daily exposure to BRJ 70mls (400 mg—nitrate) dose for three months will improve health-related quality of life compared to a placebo.

e) In patients with COPD, daily exposure to a BRJ 70mls (400 mg—nitrate) dose for three months will increase fractional exhaled NO (FeNO) compared to a placebo.

f) In patients with COPD, daily exposure to a BRJ 70mls (400 mg—nitrate) dose for three months will reduce serum BNP levels compared to a placebo.

g) In patients with COPD, daily exposure to BRJ 70mls (400 mg—nitrate) dose for three months will be an acceptable treatment (>80% of participants comply with treatment, defined as >80% doses consumed) compared to a placebo.

III. In patients with COPD, what are the prolonged (three months) effects of a daily single dose of 70 ml (400 mg—nitrate), in the form of BRJ, on the markers of vascular function, endothelial function, arterial stiffness, and the endogenous nitric oxide synthase inhibitor – plasma asymmetric dimethyl L-arginine (ADMA) levels? (Chapter 5)

- The following *hypotheses* are tested in Chapter 5:

h) In patients with COPD, daily exposure to BRJ 70mls (400 mg—nitrate) dose for three months will improve endothelial function compared to a placebo.

i) In patients with COPD, daily exposure to BRJ 70mls (400 mg—nitrate) dose for three months will reduce the augmentation index at HR 75bpm compared to a placebo.

j) In patients with COPD, daily exposure to BRJ 70mls (400 mg—nitrate) dose for three months will reduce ADMA levels compared to a placebo.

k) In patients with COPD, daily exposure to BRJ 70mls (400 mg—nitrate) dose for three months will raise the plasma L-arginine levels compared to a placebo.

IV. What are the prolonged (three months) effects of a daily single dose of 70 ml (400 mg—nitrate), in the form of BRJ, on platelet aggregate percentage? (Chapter 6)

- The following *hypotheses* are tested in Chapter 6:

l) In patients with COPD, daily exposure to BRJ 70mls (400 mg—nitrate) dose for three months will lower the circulating platelet aggregate percentage compared to a placebo.

Chapter 2: Description of Methods

2.1. Overview

To achieve the thesis's general aim, we carried out four studies. The first study was a systematic review and meta-analysis to identify the available studies examining the efficacy of dietary NO_3^- supplementations on CV risk outcomes in patients with COPD (Chapter 3). The second study was an RCT to investigate the effect of prolonged BRJ consumption on BP in individuals with stable COPD (Chapter 4). The third and fourth studies were mechanistic-based. We investigated whether prolonged BRJ treatment influences measures of vascular function: endothelial reactive hyperaemia assessed by PAT, augmentation index, plasma ADMA, plasma arginine, and plasma BNP levels compared to matched placebo BRJ (Chapter 5). Then, we conducted another lab-based substudy, using *ex vivo* whole blood 96-well plate and flow cytometry, to assess the prolonged BRJ treatment effect on platelet activation biomarkers: platelet aggregation counts and P-selectin expression percentage compared to a matched placebo BRJ (Chapter 6).

2.2. Method of reporting the ON-BC trial

We followed the Consolidated Standards of Reporting Trials (CONSORT) checklist in reporting this RCT aimed to study the effect of dietary nitrate supplementation using BRJ on BP and vascular function in individuals with COPD (Boutron, Altman, Moher, Schulz, & Ravaud, 2017).

2.3. Ethical approval

The ON-BC trial was granted full ethical approval by the Health and Care Research Wales (HCRW) following Research and Ethics Committee (REC) reference (19/LO/1660) (see Appendix 1). All participants gave their written informed consent before participating, and the research was conducted in accordance with the Declaration of Helsinki and Good Clinical

Practice guidelines. The ON-BC trial is registered in the ISRCTN registry (ISRCTN47839214 https://doi.org/10.1186/ISRCTN47839214).

2.4. Subjects studied

Before screening for potential participants, we decided to implement stratification sampling based on whether the participants were treated with antihypertensive drugs or not (prehypertension). I screened the profile of anticipated participants for eligibility criteria and then contacted them. We identified participants with stable COPD who attended our clinic and had a clinical SBP ≥130 mmHg or stated in their medical record that they were diagnosed with HTN. We also looked into existing research/audit registers of patients, including those who had completed pulmonary rehabilitation. Additionally, advertisement flyers were distributed to hospital departments and through the BLF network of Breath Easy Groups (Figure 1). All subjects gave informed written consent (Appendix 2).

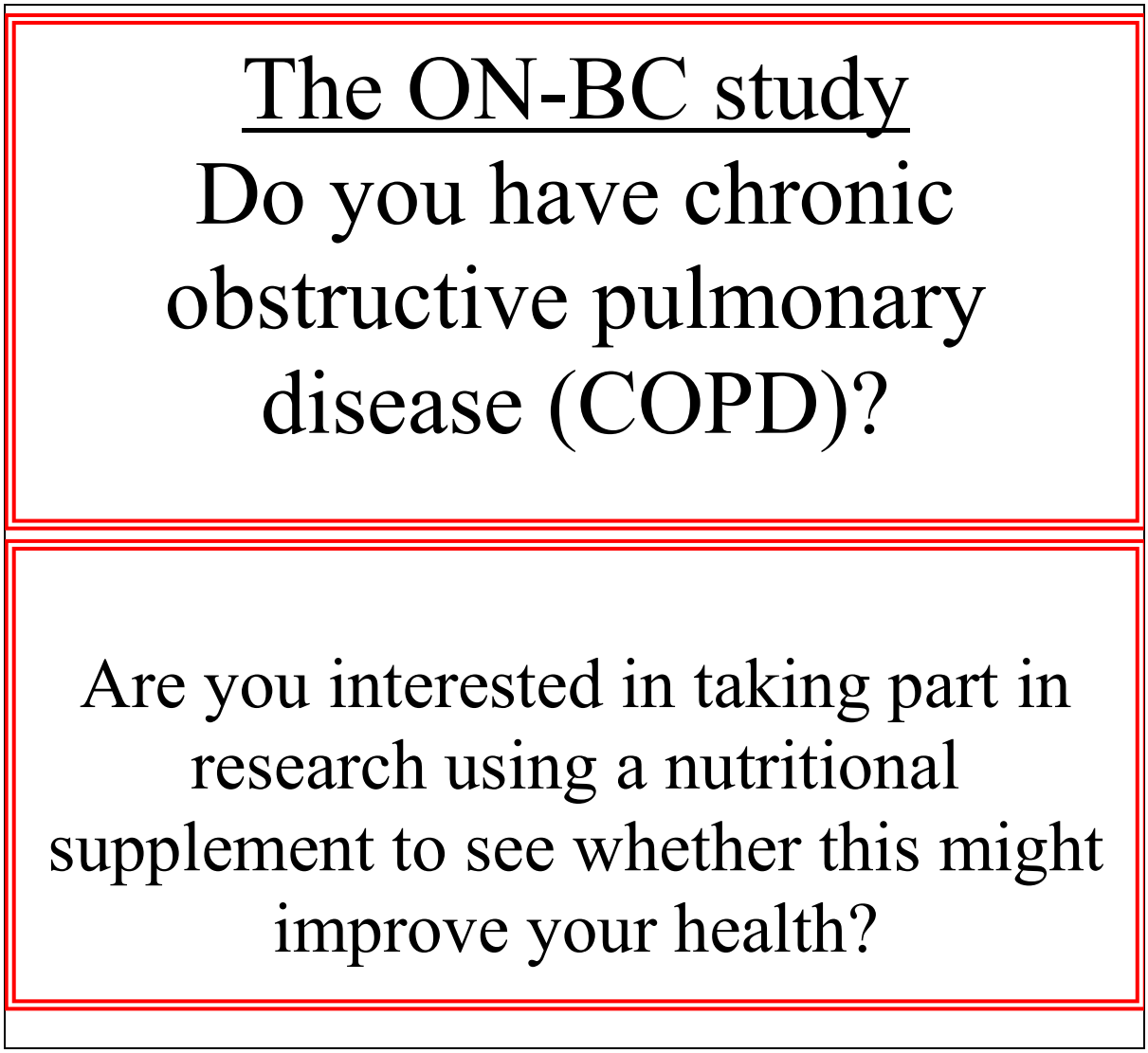


Figure 2-1 Study advertisement flyer

2.5. Eligibility criteria

Most of the patients in this study were recruited from outpatient clinics at The Royal Brompton Hospital. Clinic notes of anticipated participants were screened for eligibility criteria, and then I contacted them. Participants needed a clinical diagnosis of COPD

according to the British Thoracic Society (NICE guideline, 2019) with largely irreversible airflow obstruction defined as an FEV1<80% predicted, and an FEV1/FVC ratio of <70%. Other criteria are as follows:

INCLUSION CRITERIA

- Patients diagnosed with COPD based on the GOLD criteria
- Both males and females between 18 and 85 years of age
- Able to understand and comply with protocol requirements, instructions, and protocol-stated restrictions
- Blood pressure meeting criteria based on SBP ≥ 130 mmHg via home BP monitor

EXCLUSION CRITERIA

- Patients unable to provide informed consent
- Patients had AECOPD within one month
- Patients having significant comorbidity limiting exercise tolerance
- Patients having a significant renal impairment (estimated glomerular filtration rate (eGFR) <30 ml. min1)
- Hypertensive patients using > three antihypertensive medications
- Patients changed their BP medication in the previous month
- Use of nitrate-based medications influencing the plasma nitrate/nitrite levels (Table 2.1)
- Use of BRJ (Beet) Shots within one month.

Table 2-1 List Oral nitrate-based Medications

Medication class	Brand name
Glyceryl Trinitrate	Nitro-Dur®, Minitran®, Transiderm-Nitro®, Deponit®, and Percutol®
Isosorbide Mononitrate	Imdur®, Ismo Retard®, Isotard®, Monomil®, XL Chemydur®, 60XLModisal®, XLMonomax®, XLIsib®, 60XLMonosorb®, XL60 Zemon®, Isodur®, and Elantan® LA
Nicorandil	Ikorel®

2.6. Interventions

Participants were randomly allocated in a parallel-group manner to consume either NR-BRJ or nitrate-depleted Pl-BRJ (70 mL BEET-IT® SPORT Shot, James White Drinks, Ipswich, UK), which were identical in appearance, taste, smell, and nutrient composition (Figure 2.2). The NR-BRJ contained 400 mg of NO_3^- mixed with organic lemon juice (2%), whereas the NO_3^- was removed by the manufacturer to make the Pl-BRJ juice. The Pl-BRJ was generated by the standardised method of passing the juice, before pasteurisation, through an ion-exchange column containing Purolite A520E ion exchange resin, which selectively removes NO_3^- (Lansley, 2011). The person responsible for dispensing the juice was not involved in the study testing or analysis. After the randomisation process, the allocated juice was delivered directly from the juice factory to the patient's home and administered in a double-blind fashion. Participants were then asked to drink their intervention juice, one beverage daily, at

9 a.m. (+/- 2 h) for 90 days. Participants were reminded 48 hours before attending Visit 1 (baseline) to avoid NO_3^- rich food for 48 hours. Participants were requested to match their caffeine consumption to avoid the known ergogenic effect of caffeine for 24 hours before the intervention visits (Warren, Park, Maresca, McKibans, & Millard-Stafford, 2010). Participants were also asked to avoid strenuous exertion, given the known effect on the vasoconstrictor response of the endothelium (Elliott, Alsalahi, & Fisher, 2018). On the day of Visit 2 (post-intervention), I reminded them to drink the juice at least three hours before attendance.

Figure 2-2 BEET-IT® SPORT Shot, James White Drinks, Ipswich, UK

2.7. Sample size

The study was powered for the primary outcome measure of change in SBP. Data from the ON-EPIC trial showed a fall of a (mean ± SD) 5 ± 3.7mm Hg (Pavitt, 2020). I powered the study to test the hypothesis that the systolic BP after 12-wk BRJ intakes is less than before BRJ intakes by a clinically meaningful difference of 3 mmHg. The INTERSALT epidemiological study (1991), which included results of BP parameters from more than 10,000 men and women, suggested that a 3mmHg reduction of SBP in the population would result in an 8% overall reduction in mortality due to a stroke, a 5% reduction in mortality due to CHD, and a 4% decrease in all-cause mortality. Taking a power of 90% and a significance level of 0.05, I would require 32 patients within each group to identify a 3 mmHg fall with treatment. To allow for a 10% dropout rate, therefore, I needed to recruit 72 patients in total. For the exploratory outcomes (endothelial function, blood tests, microbiome), I analysed at least 30 subjects. This is based on the number that produced a positive result in our previous trial (ON-EPIC) investigating the effect of nitrate supplementation and pulmonary rehabilitation on flow-mediated dilatation. Additionally, a previous study by Webb *et al.* (2008) found a difference of FMD in healthy volunteers by 25% before and after seven days of nitrate supplementation; a sample size of 20 was needed to detect a difference with a power of 90% at a significance level of $\alpha = 0.05$ and SD ± 2.5 (Webb, 2008). We maximised adherence to the research protocol via follow-up calls and completed each participant's data on the same day of their visit to limit any loss of data.

2.8. Randomisation procedure

As mentioned earlier in Section 2.4, before screening for potential participants, we decided to implement stratification sampling based on whether the participant was treated with antihypertensives or not (prehypertension). Then, in the randomisation of the allocation

process, we randomised the allocation of participants based on a block size of ten. By doing this, we sought to analyse the differential effect of BP medication use on the treatment within subgroups. This study was a double-blind parallel-group placebo-controlled trial. Consenting participants were allocated randomly using a computer-generated system, from SealedEnvelope™ (https://www.sealedenvelope.com), to the NR-BRJ group or Pl-BRJ group.

2.9. Statistical analysis

The data were tested for normality using the Shapiro-Wilk test, and data were expressed as a frequency (%) or mean ± SD or median (IQR or range). I compared categorical variables between groups using the Chi-square test. I compared continuous variables with an independent *t*-test in the case of normally distributed data, or the Mann-Whitney U test in the case of non-normally distributed data. For within-group (repeated measures) analysis, we used a paired *t*-test for normally distributed data with Wilcoxon's test. Correlations were assessed using Pearson's or Spearman's rank correlation coefficients. Analyses were performed using SPSS for Windows (version 27.0, IBM Corp., Armonk, NY). Further, the analyses of the following pre-specified exploratory outcomes, changes in platelet aggregation, were performed using two-way ANOVA with Bonferroni post hoc test in GraphPadTM Prism software version 9.0 for Mac. Significance was accepted at a p-value <0.05.

2.10. Study protocol

The original study protocol involved participants attending the clinical research facility for three visits. The COVID-19 pandemic made it extremely difficult for some participants to attend for several reasons, including: (1) the hospital becoming a COVID-19 in-patient

centre; (2) some of the participants did not complete the two required COVID-19 vaccinations; (3) some participants received a recommendation from the NHS to shield during the pandemic; (4) others feared that attending the clinic might expose them to a higher risk of infection.

Consequently, the primary endpoint SBP measured with a home BP monitor was taken remotely as planned, without the face-to-face visit.

The two intervention visits began at the same time of day (+/- 2h), with a minimum of 88 days and a maximum of 90 days between the two visits. The study BP log sheets, juice adherence log sheets, and quality-of-life (QoL) questionnaires were mailed to remote participants, including a reply-paid envelope for return. Participants then mailed back their home BP recordings and the questionnaires within one week. Participants who agreed to attend the face-to-face elements of the trial had extra tests including endothelial function using the EndoPAT2000 device, ECG, FeNO, blood tests, and 6MWT. These are described in subsequent chapters.

An overview of the study protocol flow diagram is given in Figure 2.3.

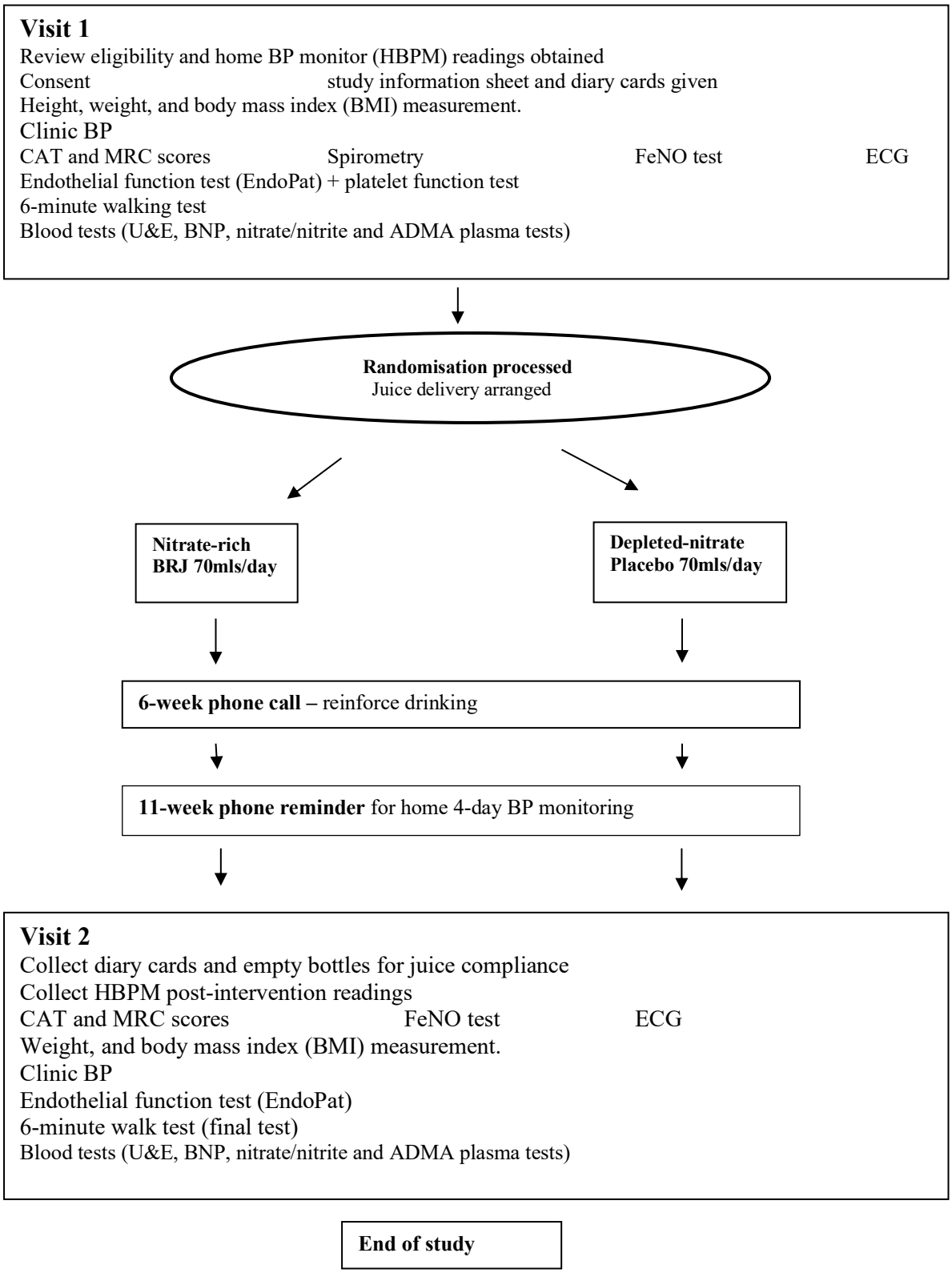


Figure 2-3 **The study protocol flow diagram**

2.11. Office BP

We performed the clinic BP in accordance with the British Hypertension Society's standard operating procedure (Williams, 2004), using an Omron M3 Comfort BP monitor (HEM-7134-E - Omron Health Care. Inc., Japan) after the subject sat quietly for a minimum of 5 min before measurement. The subject's arm was supported. Two measurements (one minute apart) were performed, and I recorded their mean average. The mean arterial pressure (MAP) was calculated using the following equation:

$MAP \approx (SBP + (DBP \text{ X } 2))/3$

2.12. Oxygen saturation (SpO_2)

Oxygen saturation (SpO_2) is estimated by continuously measuring the absorption of two different wavelengths of light through the tissue by pulse oximetry (Toffaletti & Rackley, 2016). We measured the SpO_2 at the end of the five-minute rest period using a digital finger pulse oximeter (Mommed Pulse Oximeter, Ltd.).

2.13. Anthropometric measurements

Subjects' height was measured (cm) without shoes with a wall-mounted measure, as well as weight (kg) using standardised scales. Then, we calculated the body mass index (BMI) using the following equation: $BMI = \text{weight (kg)}/\text{height (m)}^2$

2.14. Spirometry measurements (FEV1, FVC, and FEV1/FVC)

Participants were referred to our clinic with spirometry, Vyntus One (Vyaire Medical, Germany), post-bronchodilator results confirming the diagnosis of COPD according to the American Thoracic Society/ European Respiratory Society (M. R. Miller, 2005). The patient was asked to position the nose clips on their nose, place the mouthpiece in their mouth creating a tight seal, and breathe normally. They were then instructed to breathe into total lung capacity (TLC) and exhale as hard and as fast as they possibly could until they reached residual volume (RV). At this point, they were instructed to inhale as hard and as fast as possible until they were back at TLC. Tests were repeated three times to conform to published quality-assurance criteria. We recorded the following variables: FEV1, FEV1%, FVC, FVC%, FEV1/FVC, and FEV1/FVC%.

2.15. Health status and Quality-of-Life questionnaires

The researcher conducted a structured interview with the participants to gather data on the following:

(i) Smoking status

(ii) Smoking amount (pack/year)

(ii) Current respiratory medications

(iii) Other medical diagnoses

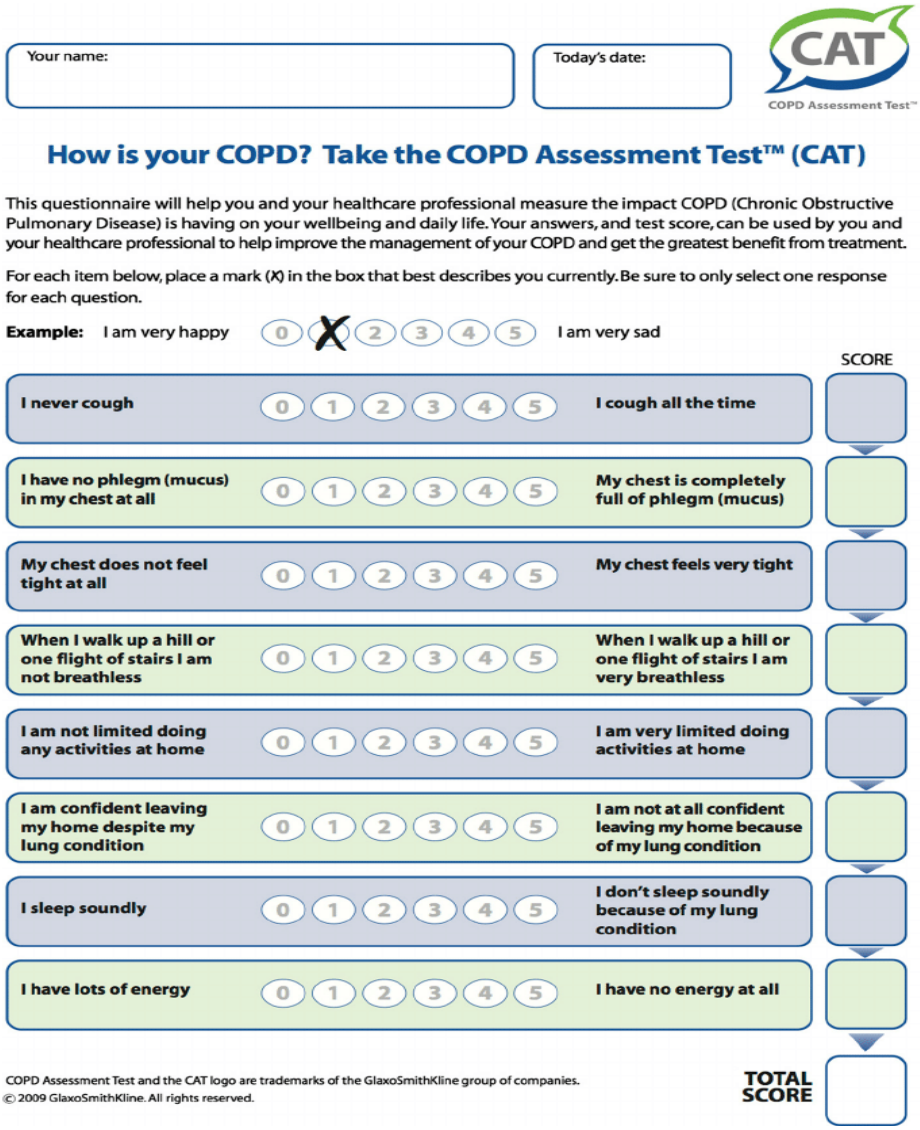
(iv) Current antihypertensive medications

(iv) Use of antiseptic mouthwash (which can affect oral microbiota)

(v) Exacerbation history in the past 12 months

2.15.1. COPD assessment (CAT)

This eight-item symptom score has been validated and is responsive for both exacerbations and pulmonary rehabilitation (Dodd, 2011; P. W. Jones, 2009). A change of two points is considered the minimum clinically important difference (MCID) (Kon, 2014). Each item is scored 0–5, giving a score from 0 to 40, with 40 being the worst possible health status (Figure 2.4).



Your name:

Today's date:

CAT
COPD Assessment Test™

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 X 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	

Figure 2-4 COPD Assessment Test (CAT) questionnaire. From **(https://www.catestonline.org/patient-site-test-page-english.html).**

2.15.2. Medical Research Council (MRC) breathlessness score

Dyspnoea is the cardinal symptom of COPD and develops during physical activity at an early stage. Its impact is associated with a reduction in exercise performance and quality of life (Stenton, 2008). The dyspnoea/ breathlessness scale has been in use for many years for grading the effect of breathlessness on daily activities. This scale measures perceived respiratory disability. Patients were asked to select from one to five statements that describe the entire range of respiratory disability (Figure 2.5) (Stenton, 2008).

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 2-5 Medical Research Council (MRC) dyspnoea scale. From Stenton *et al.*, 2008 with permission (12)

2.16. Home self-activated BP monitoring

I sent each participant a home BP monitor (HBPM), the Omron M3 Comfort (HEM-7134-E - Omron Health care. Inc., Japan) (Figure 2.6).

Figure 2-6 Home BP monitor Omron M3 (Omron Health Care. Inc. Japan). From (https://medaval.ie/device/omron-m3-comfort-hem-7134-e/).

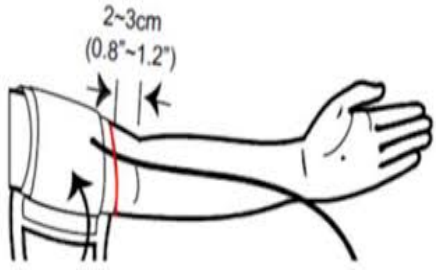
for screening to confirm he/she SBP>130 mmHg. If the readings confirm SBP>130 I included them and consider this measurement as a baseline or pre-intervention reading. Participants recorded their BP measurements twice, three times daily over four days at baseline and then again during the last week before end of the study. The BP values used for analysis were taken as a mean of all measures recorded on days 2 to 4 during the assessment

period. An instruction guide for the use of HMBP according to the NICE recommendations was given to each participant via mail and confirmed by telephone (Boffa, 2019).

These were the step-by-step instruction guides for the use of HMBP taught to the participants.

1. Kindly sit in a chair with a backrest and feet on the floor for at least five minutes and stay relaxed without speaking. The arm should be supported at the level of the heart, resting on a cushion, pillow, or armrest. Ensure that no tight clothing constricts the arm, as seen in the following image:

2. Wrap the cuff at least one finger above your elbow line, aligning with the ‘artery black mark’. During inflation, the cuff wrap guide will signal if the cuff is on correctly, as seen in the following image:



Source: https://www.omron-healthcare.co.uk/

3. Press "START" to take a reading, and the cuff will begin to inflate as it measures your BP, as seen in the following image:

Source: https://www.omron-healthcare.co.uk/

4. Take two BP readings (one minute apart) in the morning, afternoon, and evening for four days at the beginning (one week before drinking juice) and at the end of the study (last week of drinking juice). The device will keep the measures in its memory so they are available when you bring the monitor back.

2.17. Electrocardiogram (ECG)

A 12 lead ECG was performed after 5 min of rest on a couch using (GE Healthcare MAC 5500 HD Resting ECG System - Marquette 12SL, LAN). We obtained the following parameters: heart rate, PR, QRS, QT, and QTc intervals.

2.18. Endothelial function by (PAT) test

The flow-mediated vasodilatation (FMD) method assessed by brachial artery ultrasound is the most frequent non-invasive measurement method used extensively in vascular research (Corretti, 2002). It is reliable and valid; however, the operator is required to have experience with specialised training; therefore, the results are based on operator reliability. In the past decade, computerised analysis of endothelial function via peripheral arterial tonometry (PAT), a digital microvascular response to induced reactive hyperaemia, has evolved to reduce FMD limitations and to help physicians in the clinic save time and space. The EndoPAT^{TM}2000 device is non-invasive, easy to use, user-independent, and uses pulse wave amplitude (PWA) of the index finger after reactive hyperaemia to measure PAT (Figure 2.7).

Figure 2-7 EndoPat device setting in the ON-BC study

The method principle of the EndoPAT2000 device is based on recording endothelium-mediated changes using sensors that measure blood flow via digital PWA signals and are reported as reactive hyperaemia index (RHI) score arbitrary units. Pulse amplitude response to hyperaemia is automatically calculated from the hyperaemia in the finger of the experimental arm as a ratio of the post-deflation average pulse amplitude to the baseline average pulse amplitude. This result was divided by the corresponding ratio from the contralateral control hand to obtain the RHI score.

In addition, the EndoPat® software is automatically able to provide an augmentation index (AIx75), a surrogate measure of arterial stiffness, by estimating the timing and magnitude of the pulse wave reflection (Heffernan, 2010). It is calculated from the average baseline resting

PWA data over 3.5 min before cuff occlusion following this formula: (P1–P2/ P1) × 100, where P1 = systolic peak pressure and P2 = the reflected peak pressure. Then, the values were adjusted to a standard heart rate of 75 beats/min (AIx75). Lower AIx75% values compared to the baseline are considered to be an improvement, and AIx75% values < 10% (including negative results) reflect healthy arterial elasticity (G. F. Mitchell, 2004).

Participants, upon arrival to do the test, rested for 10 min in a quiet, temperature-controlled (21–24°C) room with dimmed lights and were asked to remain as still as possible and silent during the entire measurement period. Each recorded test consisted of 5 min of baseline measurement, 5 min of occlusion measurement, and 5 min post-occlusion measurement (hyperaemic period). Occlusion of the brachial artery was performed on the non-dominant upper arm. The occlusion pressure was at least 60 mmHg above the systolic blood pressure (minimally 200 mmHg, and maximally 300 mmHg). A computerised automated algorithm was generated to automatically calculate the RHI value by dividing post-PWA by the pre-occlusion value of PWA of the same arm, normalised to the control arm, and then multiplied by the baseline correction factor (Patvardhan, 2010). Absolute endothelial dysfunction was defined as an RHI score of less than 1.67 (Figure 2.8). An RHI value of 1.67 was used as a cut-off value to diagnose endothelial dysfunction in patients with coronary microvascular endothelial dysfunction (Bonetti, 2004). The measurements protocol was carried out according to the published manufacturing protocol (Appendix 5).

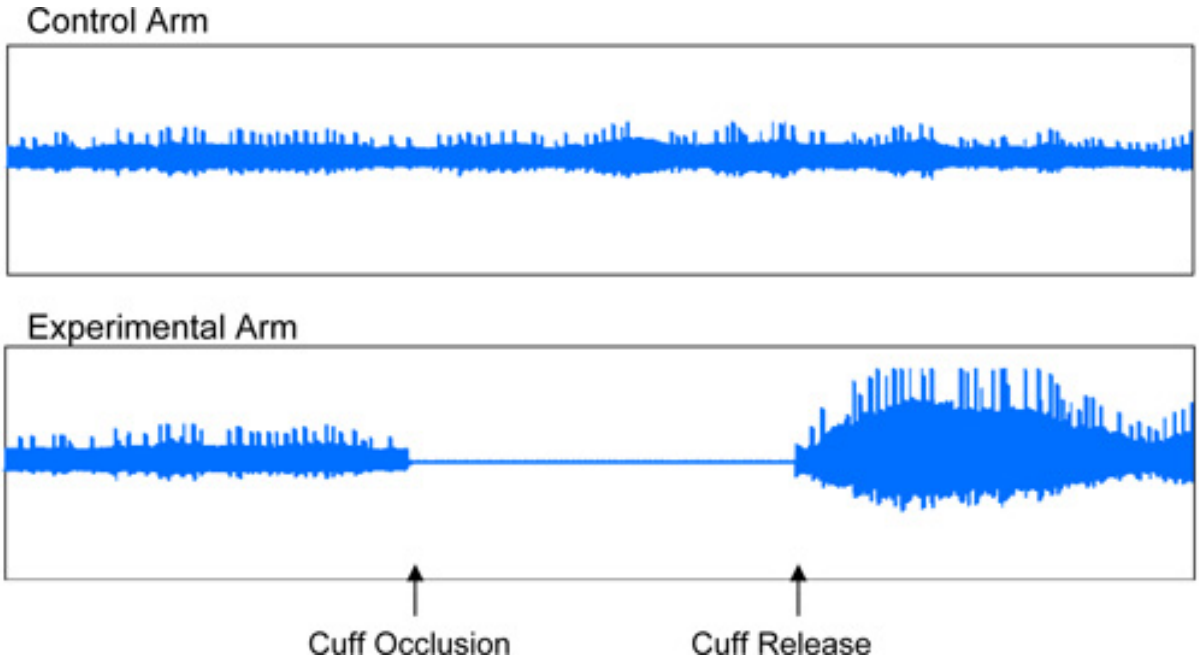


Figure 2-8 Representative tracing of the peripheral arterial tonometer (PAT)

2.18.1. Validity of EndoPAT measurements in healthy subjects

I conducted a reliability study to determine the repeatability of endothelial function measure reactive hyperaemia index RHI using the EndoPAT^{TM} – 2000 device. Ten healthy adult members of the laboratory staff participated in this study. I asked the subjects to avoid any strenuous exertion and to abstain from caffeine consumption in the 1 h preceding period (Table 2.3). The primary outcome was to determine the interrater reliability of repeated RHI measurements on day-to-day measurements performed at the same time of the day on two consecutive days. An interrater reliability test method reflects the variation of data measured by one rater across two or more trials, using the intraclass correlation coefficient (ICC) (Koo & Li, 2016). A coefficient of variation was also calculated to identify the variation between repeated measurements (Table 2.4). The ICC for the operator within the repeated measures of RHI was 0.89, indicating an "almost perfect agreement" between the first and second visits

(Table 2.5). We analysed the correlation of measurements with Bland–Altman plots. The Bland– Altman plots (Figure 2.9) demonstrated no evidence of systematic error. In conclusion, the main finding is that the measurements of RHI using EndoPat (Itamar Medical Systems) are highly reliable and repeatable. The demonstrated agreement between the measurements on different occasions indicates that any future significant change in the main project would likely represent a true change in RHI and not operator error when measuring with the device.

Table 2-2 Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
AGE	10	26.00	65.00	36.5	13.18459
GENDER	10	9 (Male)	1 (Female)		
VISIT1	10	1.14	2.52	1.67	0.38
VISIT2	10	1.41	2.60	1.90	0.38

Table 2-3 Mean, standard deviation, coefficient of variation of RHI

VISIT 1	VISIT 2	MEAN	SD	CV
1.31	**1.83**	**1.57**	**0.36**	**0.23**
1.14	**1.41**	**1.27**	**0.19**	**0.14**
1.71	**1.76**	**1.73**	**0.03**	**0.02**
1.77	**1.53**	**1.65**	**0.16**	**0.10**
1.31	**1.68**	**1.49**	**0.26**	**0.17**
1.47	**1.59**	**1.53**	**0.08**	**0.05**
1.63	**1.68**	**1.65**	**0.03**	**0.02**
2.52	**2.60**	**2.56**	**0.05**	**0.02**
2.45	**2.17**	**2.31**	**0.19**	**0.08**
1.87	**1.69**	**1.78**	**0.12**	**0.07**

* **Abbreviations: SD: Standard Deviation, CV: Coefficient of Variation**

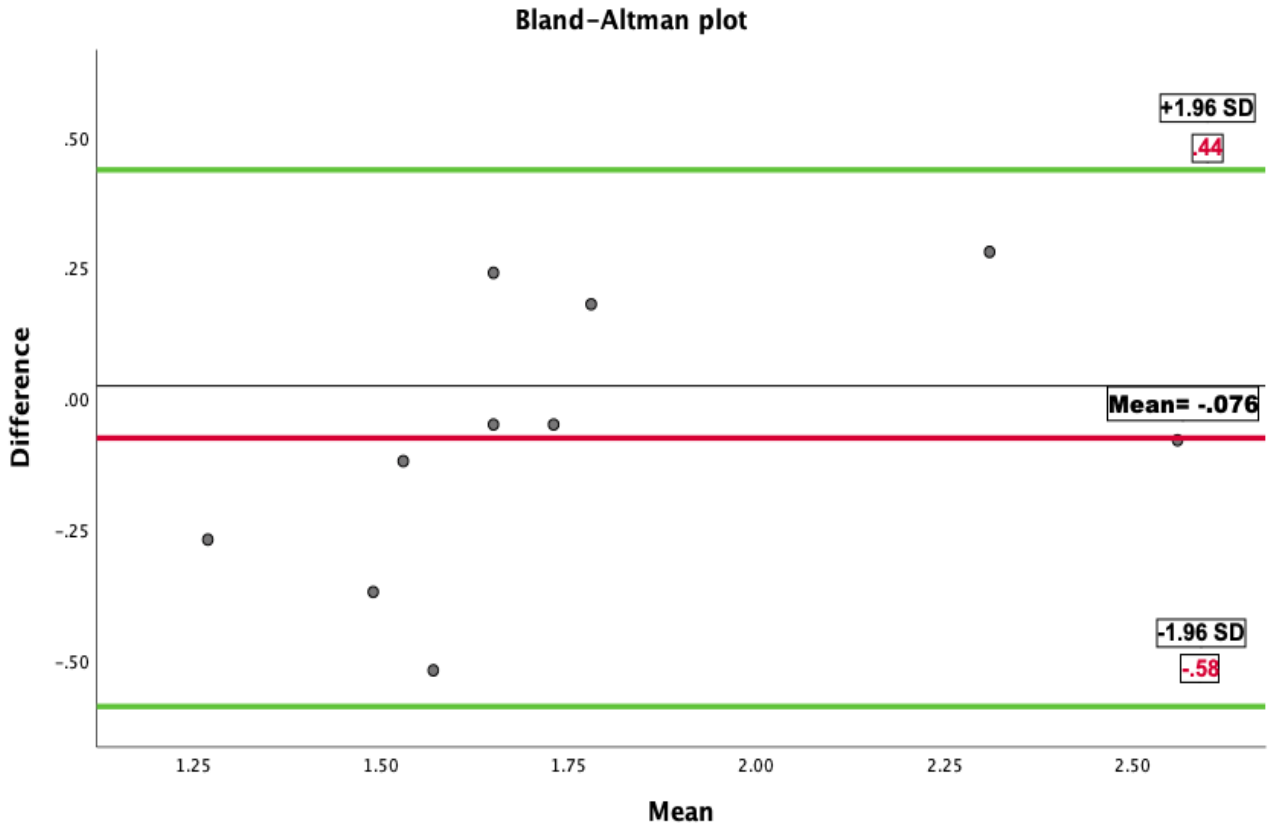


Figure 2-9 Bland–Altman plot of the reactive hyperaemia index values on two occasions

Table 2-4 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	.799[a]	.403	.945	8.828	9	9	.002
Average Measures	.888	.574	.972	8.828	9	9	.002

A 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.

2.19. Exhaled nitric oxide (FeNO)

Nitric oxide in the exhaled air (FeNO) was measured using the NIOX VERO system NIOX VERO® device (Circassia, London, UK) (see Figure 2.10). FeNO is a marker of airway inflammation and it is a helping identifies eosinophilic airway inflammation in asthmatic patients (Miskoff, Dewan, & Chaudhri, 2019). FeNO levels are lowered by smoking. The purpose of using this test in our study was to establish a baseline FeNO before participants drank the BRJ for subsequent measurements while drinking the juice. According to the stated guidelines, FeNO levels of < 25 of parts per billion (ppb) are considered normal, 25 ppb–50 ppb are considered intermediate, and > 50 ppb are considered high (Dweik, 2011). An increase change in FeNO level by >10 ppb for values under 50 ppb at the follow up visit from the baseline visit is considered significant in asthmatic patients (Dweik, 2011). To make a measurement, a filter was placed on the mouthpiece. The subject exhaled steadily for approximately 10 s and was guided by a visual monitor on the device screen to ensure the exhalation was uniform and steady. The exhaled NO values of the three measured were averaged and reported in parts per billion (PPB).

Figure 2-10 NIOX VERO® device.

2.20. 6-minute walk distance test (6MWD)

Each participant performed the 6MWD test at Visit 1 (baseline) and Visit 2 (post-intervention) in an enclosed flat corridor, 30 m in length, following the American Thoracic Society guidelines (Enright, 2003). Patients were instructed to cover the longest distance possible in six minutes, with or without pause. The test performed twice at each visit with the greatest distance of the two tests reported. standardised encouragement was given to patients. I calculated the total distance covered as follows:

Total metres = (number of lapses X 30 m) + extra metres when patient stopped

2.21. Blood sampling

Blood samples (a total of 20mls) were drawn at baseline and again after 11 weeks of treatment. First and foremost, written or printed labels for every participant were attached to all blood sample tubes, which included patient ID, study code, date and time, and my initials (AMA). I was always the one who drew blood from the participants during this study. Some of these blood tests were analysed by the hospital laboratory, such as blood biochemistry (urea and electrolytes U&E), CRP, LFT, iron studies, and brain natriuretic peptide (BNP).

Furthermore, the following blood analyses were conducted in collaboration with experts in these tests:

2.21.1. Measurement of plasma [NOx]

NO has a very short half-life, and one method to assess total NO production alternatively is by calculating the sum of both (NO_3^- and NO_2^-) in the plasma, denoted by NOx. Plasma NOx concentrations were assessed by the Griess method characterised by high reproducibility and minimised interferences by plasma constituents (Giustarini, Rossi, Milzani, & Dalle-Donne, 2008). I utilised the nitrate/nitrite colorimetric assay kit from Cayman Chemical (Ann Arbour, MI, USA). All samples in EDTA were defrosted and subjected to ultrafiltration using a 10–kDa filter from Merk Millipore Ltd. (Tullagreen, Ireland). The total nitrate/nitrite concentrations were determined by two-step processes: (a) the conversion of nitrate to nitrite by NADH-dependent nitrate reductase enzyme, and (b) the addition of Griess reagents, which convert nitrite to a deep purple azo product. The absorbance at 540 nm due to the azochromophore was measured by a microplate reader (TECAN Infinite M200PRO, Grödig, Austria). All absorbance was then plotted on the standard curves. Nitrate was calculated by subtracting the nitrite from [NO_x]. Typical standard curves for the analysis of total [NOx] and

[NO_2^-] are displayed in Figures 2.11–2.12. Photographs of the NOx plate post-test are presented in Figure 12.3.

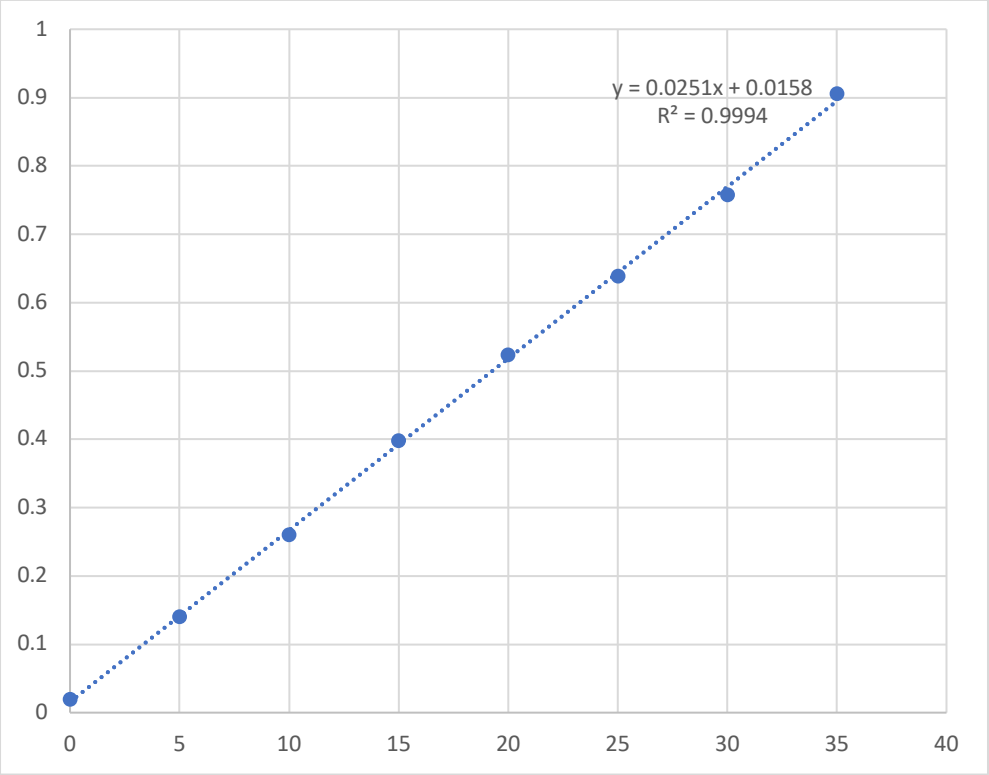


Figure 2-11 Standard curve for determination of total NOx levels by Griess reagent

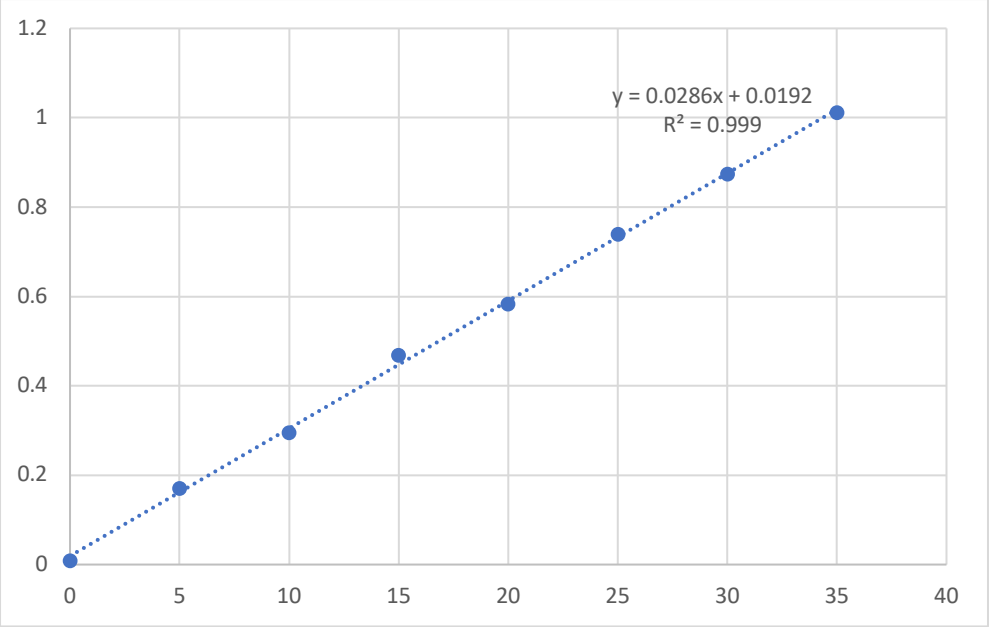


Figure 2-12 Standard curve for determination of total NO_2^- levels by Griess reagent

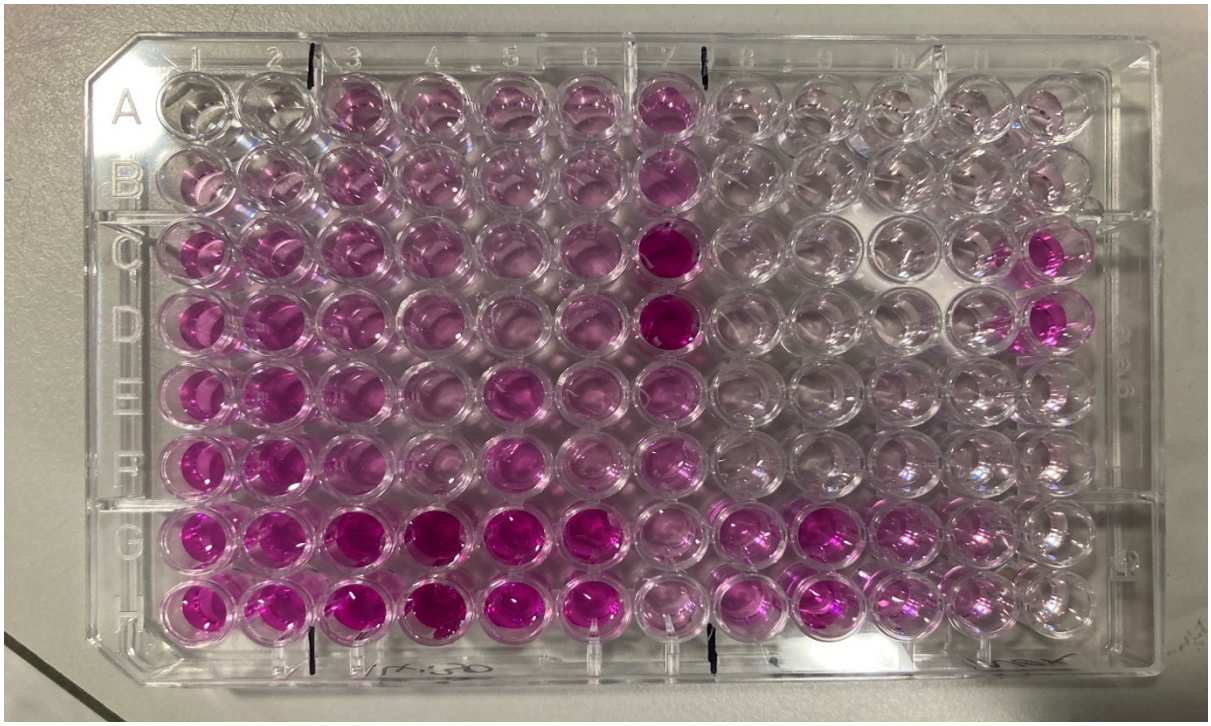


Figure 2-13 Photograph of plasma NO_x plate

2.21.2. Analysis of plasma asymmetric dimethylarginine (ADMA) and plasma L-Arginine

Plasma samples were obtained through a 21-gauge butterfly needle inserted into an antecubital vein in a labelled (4 ml) EDTA vacutainer blood tube. ADMA plasma was measured by competitive ELISA (DLD Diagnostika GmbH, Hamburg, Germany). Aliquoted samples (50 ml) of the pre-treated standards, controls, and samples were pipetted into the wells of the microtitre plate, and antiserum solution (50 ml) was added to each well. The plate was then shaken for 10 min on a horizontal shaker, and the plate was covered with adhesive foil. The plate was incubated for 90 min at room temperature (20 – 25 °C). After incubation, the solution from each well was removed and the wells were washed four times with wash buffer (250 ml). Subsequently, enzyme conjugate solution (100 µL) is added to each well, and the plate was incubated for 30 min at room temperature on a horizontal shaker. Then, the wells were again washed four times with the wash buffer. After washing, a substrate solution (100 µL) was pipetted into the wells and the plate was incubated for 20–30 min on a horizontal shaker. The reaction was stopped with a stopping solution, and the optical density was read at 450 nm (reference wavelength 570–650 nm) using a microtiter plate reader (Sunrise; TECAN, Crailsheim, Germany). The detection limit was 0.05 µmol/L with a standard range from 0.1 to 6.0 µmol/L. The intra-assay coefficients of variation were 7.5% at 0.81 µmol/L and 4.5% at 1.76 µmol/L; the interassay coefficient of variation ranged from 8.3% to 10.3%.12 (Schnabel, 2005; Schulze, 2004). Typical standard curves for the analysis of ADMA and arginine are displayed in Figures 2.14, 2.16. Photographs of plasma ADMA and arginine plates post-test are illustrated in Figures 2.15, 2.17.

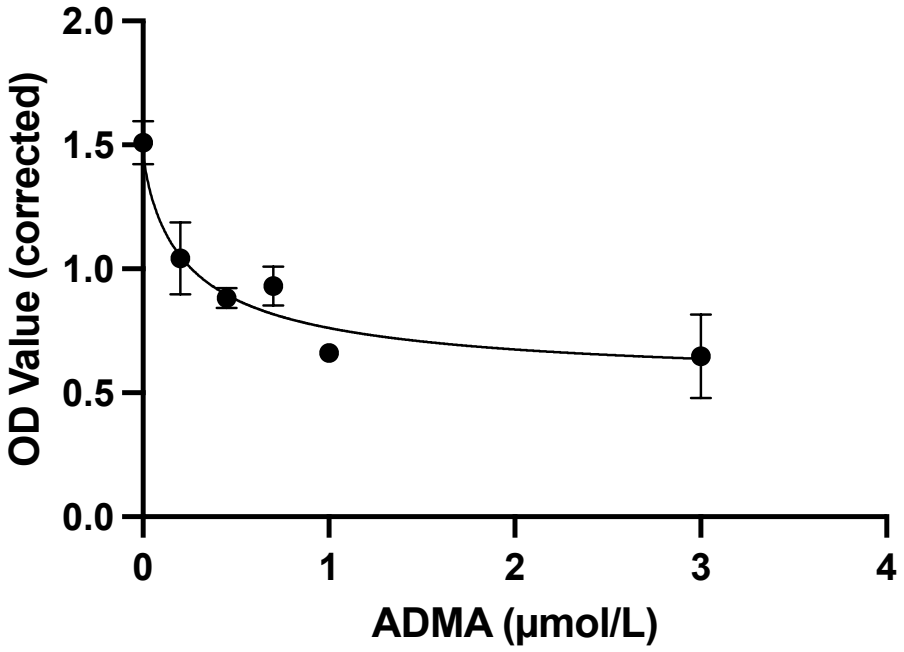


Figure 2-14 Standard curve for determination of plasma ADMA levels by ELISA (DLD Diagnostika)

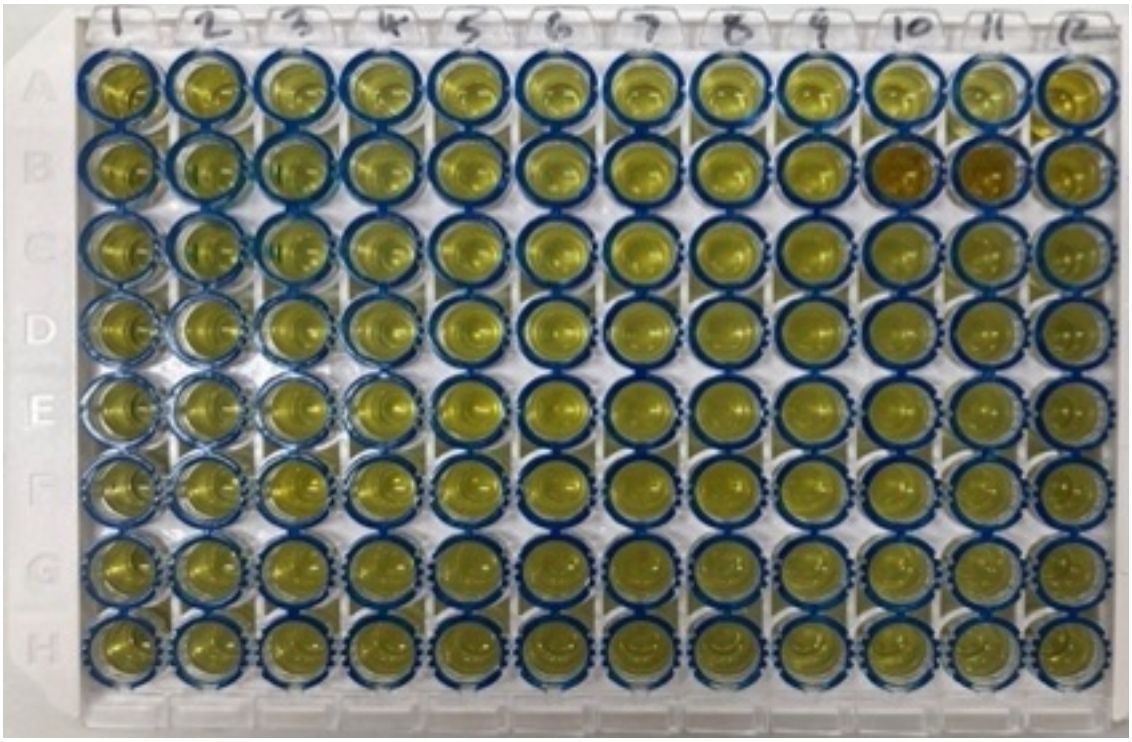


Figure 2-15 Photograph of plasma ADMA plate

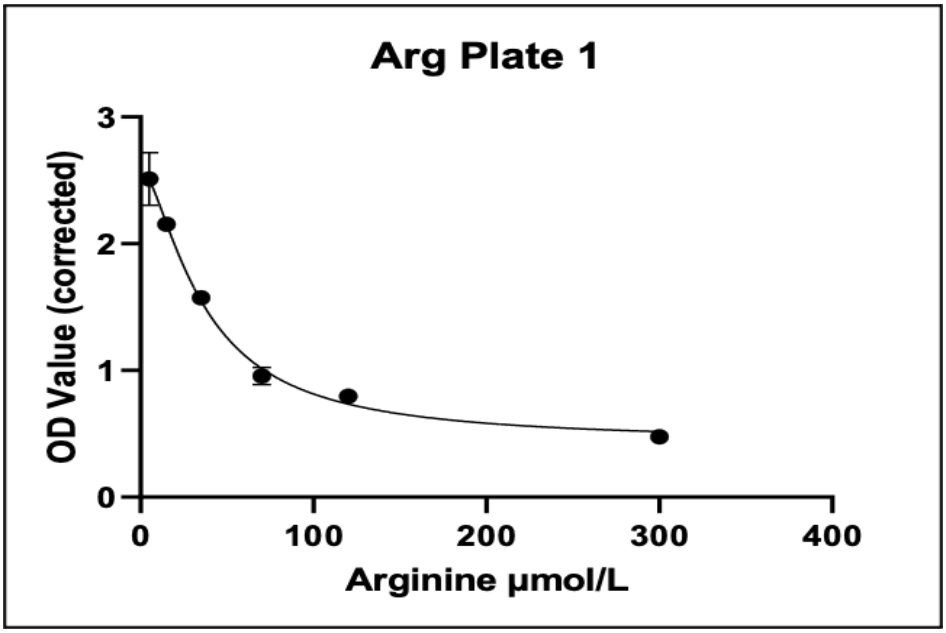


Figure 2-16 Standard curve for determination of plasma arginine levels by ELISA (DLD Diagnostika)

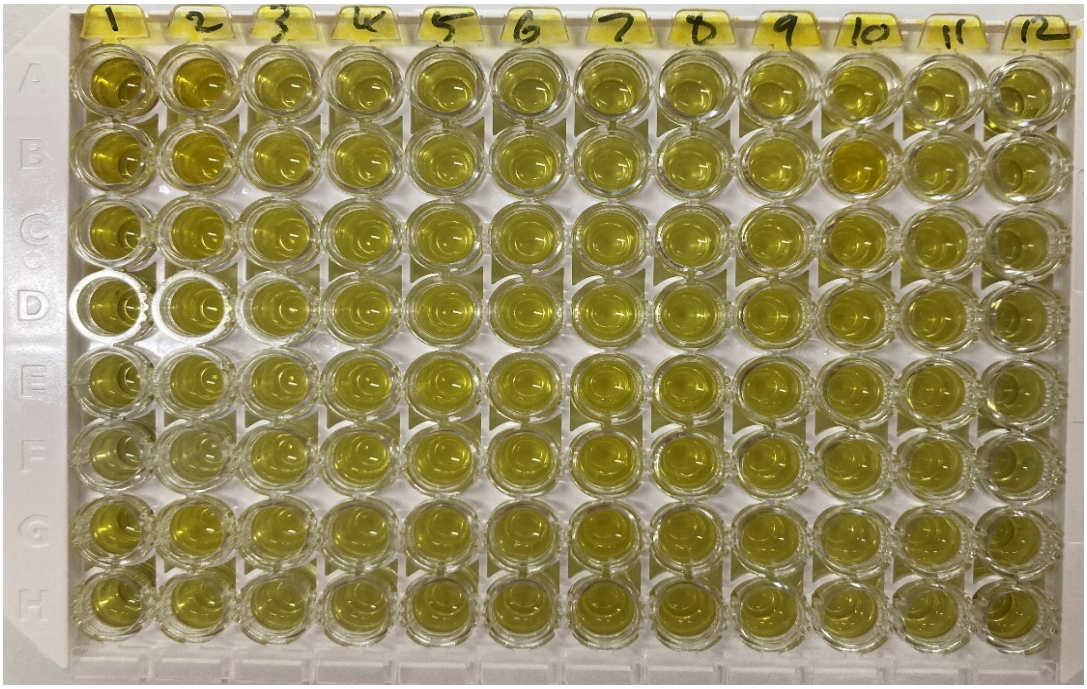
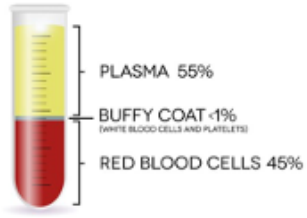


Figure 2-17 Photograph of plasma arginine plate

I used the following protocol to prepare the plasma ADMA and arginine samples:

1. I drew 4 mL venous blood samples into a labelled (4 ml) EDTA vacutainer blood tube from each subject during Visit 1 (pre-randomisation) and Visit 2 (post-intervention) through a 21-gauge butterfly needle inserted in an antecubital vein.
2. I gently inverted the tube 4–5 times.
3. Then, I centrifuged the samples within 5 min. If there was a delay, I placed the EDTA tube at 2-8°C before processing.
4. I centrifuged at 860xg for 10 min at 4 °C to obtain sample separation, as shown below:



5. I removed the plasma carefully and transferred it into a 1.5 ml Falcon tube.
6. Finally, I transferred the aliquoted plasma to dry ice and stored it at -80 °C in the lab.
7. I analysed both plasma nitrate/nitrite and plasma ADMA in cooperation with Professor Jane Mitchell's cardiovascular biology lab at Imperial College London.

2.21.3. Analysis of platelet activation biomarkers

I tested the following biomarkers: platelet aggregator expression and P-selectin expression % on *ex vivo* whole blood 96-well plate measured via flow cytometry as a service. Most of this test process and analysis was done by me under guidance from Professor Tim Warner, vascular research, Queen Mary University. This method incorporates a range of agonists at different

concentrations, which will individually test multiple pathways of platelet activation (Figure 2.18).

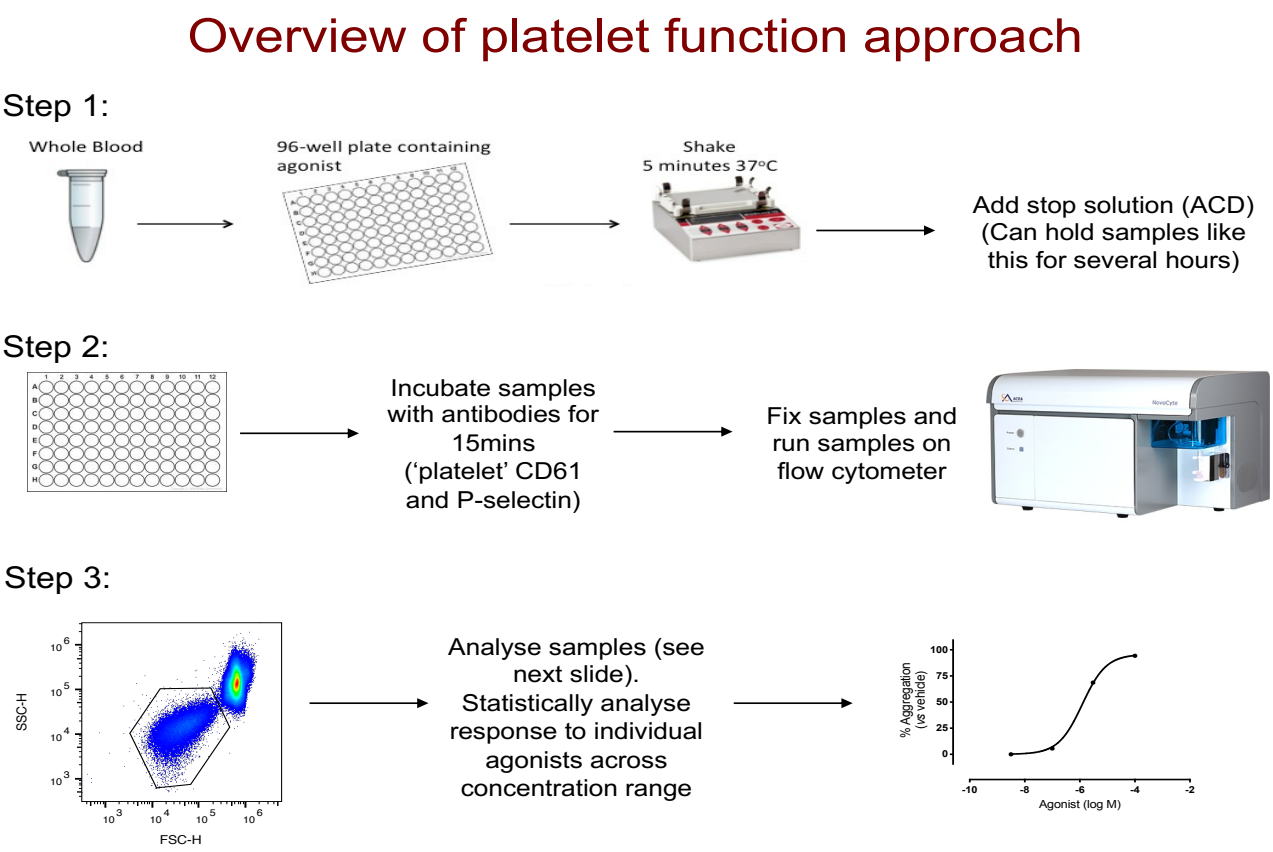


Figure 2-18 Platelet function test approach

During both visits, I collected participants' blood in a 3.2% sodium citrate blood collection container through a 21-gauge butterfly needle inserted into an antecubital vein. Platelet aggregation was determined in an ex vivo whole blood 96-plate aggregometry assay. Plates were pre-prepared with a variety of freeze-dried agonists at a range of concentrations (including vehicle), packaged in a vacuum-sealed environment, and stored at room temperature until needed. The plate vacuum sealing of the prepared plates maintained the plates in such a way that all reagents were stable for three months (at room temperature) from the date of preparation, allowing for bulk manufacture, which reduces intra-assay variability. Following venepuncture,

blood was pipetted into agonist-containing wells to initiate aggregations. This was completed within 5 min to maximise the influence of the vascular environment upon platelet responses. Aggregation was stimulated by arachidonic acid (0.03, 0.1, 0.3, 0.6µM), collagen (0.1, 0.3, 1, 3µg/ml), thrombin PAR1-receptor activating peptide (TRAP; 0.1, 0.3, 1, 3µM) and thromboxane A_2 mimetic U46619 (0.1, 0.3, 1, 3µM).

On the day of the study, blood was pipetted (40µl) into each agonist-containing well of the prepared plate. Then, the plate is placed on a plate shaker (BioShake iQ, Q instruments) for 5 min at 1000 rpm and 37°C. After 5 min, 160µl of pre-prepared acid citrate dextrose (ACD) solution was added to each well. This solution acts as a 'stop-solution' to preserve aggregation and prevent any further activation. Samples in this state were stable for up to six days before further processing. Aggregation was determined using flow cytometry. 10µl of each experimental well was transferred into a separate well containing 10µl anti-CD61 (1:25; clone VI-PL2, Biolegend) monoclonal antibody conjugated to allophycocyanin (APC) in PBS to label and identify the platelet population. Samples were stained at room temperature for 30 min before fixing with 180µl of a BSA/dextrose saline solution containing 0.1% formalin (Sigma-Aldrich). Platelets were identified by forward and side scatter and CD61 positivity using a Novocyte 3000 flow cytometer (ACEA), and totals were counted in 10µl of each experimental well sample.

- **Measuring P-selectin expression %**

Whole blood collected from individuals was placed in prepared wells containing EDTA and either vehicle (PBS), ADP (0.5µM or 40µM), or TRAP-6 (40µM). Then, the plate was placed on a plate shaker (BioShake iQ, Q instruments) for 5 min at 1000 rpm and 37°C. After 5 min, 160µL

of pre-prepared acid citrate dextrose (ACD) solution was added to each well. Samples in this state were stable for up to six days before further processing.

As for aggregation samples, 10µl of each experimental well was transferred into a separate well containing 10µl 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) for the identification of platelets and quantification of P-selectin expression. Samples were analysed using a Novocyte 3000 flow cytometer (ACEA), with expression levels calculated from 10,000 acquired platelet events. The results were expressed as the percentage of platelets positive for P-selectin.

2.22. Confidentiality

All participants' data were kept confidential. A study code and number were set for storing all confidential information. In the study, the original paper questionnaires, assessment forms, and participants' contact information were stored in accordance with the patient consent form and the patient information sheet for this study. These data were kept in a locked space at Respiratory Muscle Lab, Royal Brompton Hospital – Fulham Wing, London, SW3 6JY. Mr Ali Alasmari (primary researcher) Imperial College London acted as the data controller, processed and stored the original paper questionnaires, assessment forms and participants' contact information in accordance with all applicable legal and regulatory requirements.

2.23. Quality assurance

This study was performed in accordance with good clinical practice (GCP).

2.24. Record keeping and archiving

At the end of our study, all essential files and data were securely archived by the research team and will be stored for a minimum of ten years. This documentation will be available for audit by authorised auditors upon request.

2.25. Finance

This is a PhD research study and has been funded by the student's sponsor, Taibah University, via the Saudi Arabian Cultural Bureau in London (SACB).

CHAPTER 3: The effect of dietary NO_3^- supplementation on cardiovascular risk outcomes in patients with COPD: A systematic review and meta-analysis.

Preface:

The data presented in this chapter were taken from our article published in *Thorax*, BMJ Open Respiratory Research. For the full article, please see Appendix 5 with permission of BMJ Open Respiratory Research Press. It is important to note that I have contributed to all the processes of this review. In particular, I was involved in developing the search keys with assistance from an expert librarian. Then, I searched the databases and screened titles and abstracts. I have also extracted detailed data from the final retrieved articles that are relevant to the studies' characteristics and the impact of NO_3^-supplementation on BP measurements, HR, endothelial function, plasma NO_3^-/ NO_2^-, and FeNO. Also, I conducted a meta-analysis of these data and presented them in forest plots. I wrote this chapter on my own, and I just used the forest plots graphs with permission from the journal.

The following is the reference to this publication:

Alsulayyim AS, Alasmari AM, Alghamdi SM, Polkey MI, Hopkinson NS. 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 Sep 1;8(1): e000948.

3.1. Introduction

3.1.1. Background and rationale

As I have described in Chapter 1, low-grade chronic systemic inflammation in COPD suggested to contribute to the progression of atherosclerosis along with other CV risks, including HTN, endothelial dysfunction (ED), and platelet aggregation (Pike, Lindenmaier, Sin, & Parraga, 2014). Dietary NO_3^-supplementation, in the form of beetroot, is gaining prominence in cardiovascular health research since it is rich in nitrate concentration (Lundberg, 2008). In addition, I have previously discussed in Chapter 1 key animal and human studies on healthy and chronic diseases indicating an association between nitrate supplementation and improvement of CVD risk outcomes including BP, FMD, and platelet aggregation.

Building on the findings from the literature review in Chapter 1, it was obvious there was an unmet need for a comprehensive systematic review of RCTs to produce more robust evidence on the effect of NO_3^-supplementation on CVD risk outcomes in people with COPD.

3.1.2. Objectives

This review achieved the following objectives:

- Primary objective: examining the effects of NO_3^-supplementation on BP parameters (SBP, DBP, and MAP) in patients with COPD.
- Secondary objective: assessing the effect of NO_3^-supplementation on other CV risk measurements used in the RCTs, including HR, EF, arterial stiffness, and platelet aggregation in patients with COPD.

3.2. Methods

The review protocol was published online on PROSPERO2019 (CRD42019130123) (Booth, 2012). I reported this review according to the PRISMA statement, which aims to assist authors in enhancing the reporting of systematic reviews and meta-analyses. The PRISMA statement consists of a checklist (27 items) and a flowchart (four phases) (Moher, Liberati, Tetzlaff, Altman, & Group, 2009). The checklist items focus on the essential items for the reporting of a clear and transparent systematic review. The flowchart illustrates the four phases of the selection process (identification, screening, eligibility, and inclusion) (Moher, 2009).

3.2.1. Eligibility criteria

The selection of the studies was performed electronically by two independent reviewers, AMA and Abdullah Alsulayyim (ASA), in two stages. The first stage was screening titles and abstracts in line with the inclusion criteria. To facilitate the collection of data, I utilised web-based software (COVIDENCE), which allowed the reviewers to compare any discrepancies over any paper electronically. A third reviewer (NSH) was available to resolve by consensus any disagreements with regard to inclusion/exclusion criteria. The second stage was a full-text review. A PRISMA flow diagram was used to document the study selection process, and a record was kept of the reasons for the exclusion of studies (see Appendix 2). All included studies fulfilled the following **P**articipants, **I**nterventions, **C**omparators, **O**utcomes, and **S**tudy Design (PICOS) criteria outlined below (Table 3.1).

Table 3.1 PICOS criteria for inclusion of studies

PICOS criteria	Eligibility criteria for studies
Participants	Adults (≥18 years); COPD diagnosed with emphysema or bronchitis either undergoing usual care or taking part in pulmonary rehabilitation.
Interventions	Nitrate and/or nitrite supplementation the participants drank.
Comparators	Control/Placebo.
Outcomes	BP, HR, endothelial function, arterial stiffness indices, platelet aggregation.
Study design	Parallel/Crossover RCTs.

I excluded results that fit the following exclusion criteria in our search strategy:

1) Studies examining children under 18 years
2) Any papers written in a language other than English
3) Any conference abstracts or not published in a peer-reviewed journal, unpublished data, editorials, guidelines, or reviews. However, reviews were utilised for citation checking.

3.1.1 Search strategy

I constructed the SR's search terms with a facilitated help from Imperial College librarian. I developed the search strategies using index terms and keywords related to my PICO element. I

performed a comprehensive electronic literature search from “Inception” up to “June 2020” which was repeated on 01 Nov 2020 to identify recently published articles in the following electronic databases: Cochrane Systematic Reviews, Cochrane Controlled Trials Register, MEDLINE, EMBASE, and CINAHL (Cumulative Index to Nursing and Allied Health Literature). Additionally, I searched through reference lists from all identified studies as a secondary search. Databases were accessed via the Imperial College London – The National Heart and Lung Institute Library, London, UK. I modified the MEDLINE search strategy when applied to other databases (Appendix 4). In addition, the searches were restricted to just including the English language in the initial search.

3.2.2. Risk-of-bias assessment

All included studies were RCTs, and the quality and risk of bias were assessed using the Cochrane Collaboration’s risk-of-bias tool (Higgins, 2011). The tool included seven domains. All domain questions were answered either as 0 (low risk of bias) or 1 (high risk of bias, or bias unclear). There were no cut-offs published with these tools. However, I coded the answers to give a semi-quantitative measure of the studies in line with the assessment tool, assigning a total score between 0 and 7, in which a higher score corresponds to a higher risk of bias. The authors also utilised the five GRADE considerations (study limitations, consistency of effect, imprecision, indirectness and publication bias) criteria to assess the total of evidence for pre-specified endpoints (G. H. Guyatt, 2008). Subsequently, the authors would contendingly provide recommendations for clinical practice based on these certainty levels: very low, low, moderate and high (G. Guyatt, 2013).

3.2.3. Data extraction

The final selected studies were imported and stored in Endnote software (Hupe, 2019). I developed a standardised extracted data sheet that was piloted using Microsoft Excel spreadsheet form (Microsoft Corp., Redmond, WA, USA). I extracted the data by myself and had it independently verified by my co-author ASA. Then, I resolved any disagreements through discussion. I extracted detailed data related to the study characteristics, population characteristics, interventions, and study outcomes (Table 3.2). The form included data extracted on **P**opulation characteristics: age, sex, and GOLD stage, study sample (n), study designed RCTs double-group trials or RCT crossover; **I**ntervention: dietary NO_3^- protocol (e.g., dose, duration, frequency, and delivery method); **C**omparison placebo protocol (e.g. dose, duration, frequency, and delivery method); **O**utcome: the primary outcome was considered changes on SBP, DBP, and MAP following NO_3^- supplementation measured via resting BP, home-monitored BP and 24 hours ambulatory BP (see Table 3). I considered EF measures (FMD and/or RHI), arterial stiffness (PWV and/or AIx), HR, and platelet aggregation as secondary outcomes.

3.2.4. Measurement of treatment effect and meta-analysis

I conducted the meta-analysis to quantify the effects of the NO_3^- supplementation group compared to the placebo group on the following CV risk markers: resting BP, home-monitored BP, 24-hour ambulatory BP, HR, endothelial function measures, arterial stiffness indices, and platelet aggregation at the end of each trial. However, meta-analysis was not appropriate for FMD, given there is only one study that reported it. Also, out of all included studies, no study has included arterial stiffness and platelet aggregation in their outcomes. Additional outcomes

included in the meta-analysis were plasma NO_3^-/NO_2^- plasma level, and FeNO. We pooled pre-specified results from the included trials in the meta-analysis by calculating mean differences (MDs) and 95% CI (Higgins, 2011). The differences were combined using pooled weighted MD. A random-effect model was performed to provide a more conservative estimate of the pooled effect size when at least two studies reported the same outcome. Forest plots were also generated to estimate the pooled effects size of secondary outcomes. The χ^2 test was used to test for heterogeneity, and the I^2 was used to quantify heterogeneity (Higgins, Thompson, Deeks, & Altman, 2003). All meta-analyses were undertaken using the Cochrane Review Manager (RevMan version 5.4.0).

3.3. RESULTS

3.3.1. Main search

I identified 2,113 articles for title and abstract from all databases, and after the removal of duplicates (n = 559), 1,554 were screened for eligibility. A total of ten experimental RCT studies met our inclusion criteria. Figure 3.1 illustrates the flowchart of the study selection process. Of the ten included, two were parallel trials (Behnia, Wheatley, Avolio, & Johnson, 2018; Pavitt, 2020), and eight were crossover trials (Beijers, 2018; Berry, 2015; Curtis, 2015; Friis, 2017; Conor P. Kerley, 2015; Conor P Kerley, James, McGowan, Faul, & Cormican, 2019; Leong, 2015; Shepherd, 2015).

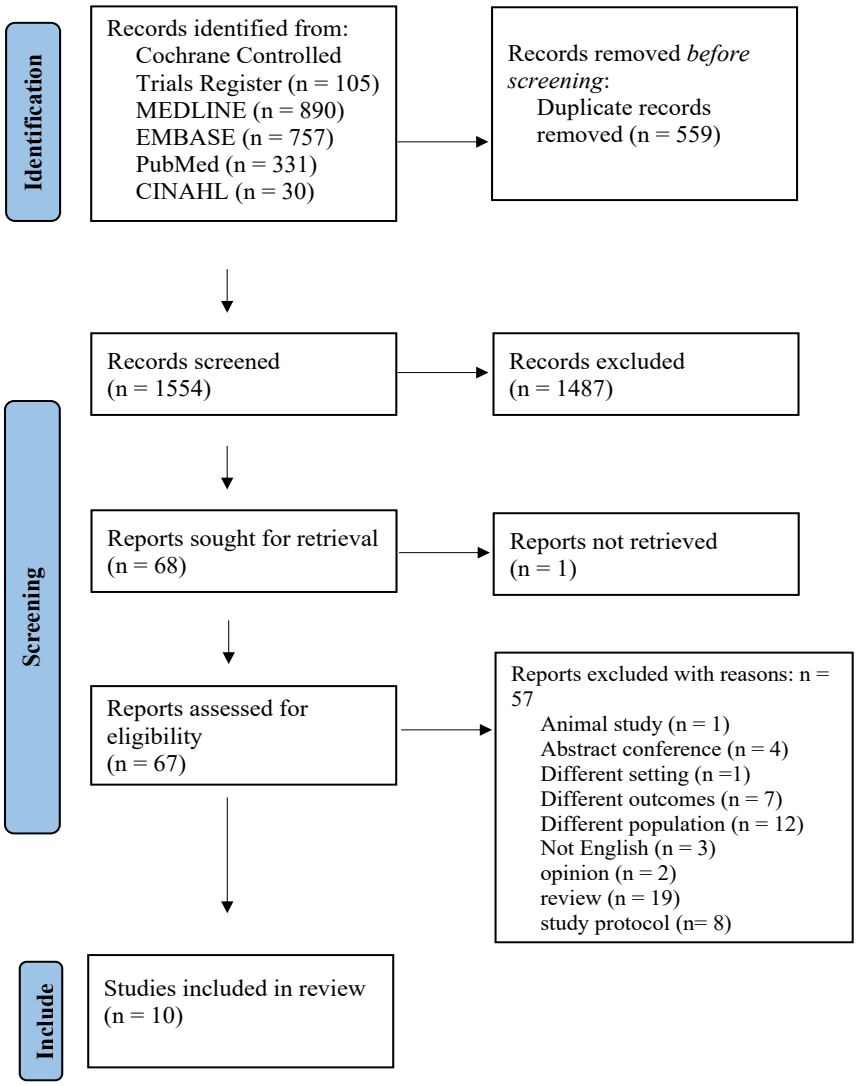


Figure 3-1 Flowchart of study selection (PRISMA flow diagram)

3.3.2. Study characteristics

The included ten trials were published between 2015 and 2020. The majority of these trials were conducted in Europe, along with two trials located in the United States of America, and one in Australia. A total of 813 participants were included in this review (mean number of participants per study, 20; range, 7–69), and 267 participants with various COPD classifications (GOLD stages 1–4) and an average of 27 participants per individual study (range 8–122) were included in the meta-analysis. Moreover, there were more men than women (152/115) in the included trials. The age range of the participants in the included studies was less variable (63–70 years). I categorised the ten trials based on the reported duration of NO_3^- supplementation as "acute effect" (one dose of NO_3^- supplementation) (n=3), "short-term" (>3 months in usual care) (n = 7) or "in addition to pulmonary rehabilitation" (n=1). Descriptions of the studies' main characteristics included in the systematic review are reported in Table 3.2.

Table 3.2 Detailed description of the included studies

Authors and Study design	**Study sample**	**Baseline BP measures: (clinic/ABPM /HBPM)**	**Details of DNS intervention (type/dose/duration)**	**Results (Following nitrate vs placebo)**
Behnia *et al.*, 2018/RCT	N = 25 GOLD stages 1–4 Age (y): 68±9 Sex (M/F): 13/12	SBP/DBP (clinic); during exercise	Active-BRJ (70ml) NO3 + Black currant juice (180 ml) Placebo: water (70ml) + black currant juice (180ml) Duration: 8 D	- Significant change in SBP: 11mmHg ($p<0.05$) at peak exercise in the nitrate group only compared to pre-nitrate - Significant increase in FeNO (ppb) in the 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 FEV1% = 69.2	SBP/DBP (clinic); during exercise	Active-$NaNO_3$ (496 mg or ~8 mmol) + water (140 m) Placebo: NaCl (680 mg) Duration: Acute & 7D washout period 7 D	- Significant increase in plasma NO_3 level on Day1 and Day7 (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 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	SBP/DBP (clinic); during exercise	Active-BRJ (140 ml) (7.58 mmol) NO3. Placebo: (163 ml) prune juice (0.01 mmol nitrate) NO3)	- 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 in end exercise DBP: 5.6 mmHg (p=0.08)

	FEV1% = 61.8		Duration: Acute washout period 7 D	- No effect on HR, p=0.70
Curtis *et al.*, 2015/RXT	N = 21 GOLD stage 2–4 Age (y): 68±7 Sex (M/F): 16/5 FEV1% = 50.1	SBP/DBP/MAP (clinic); during exercise	Active-BRJ (140 ml) (12.9 mmol) NO3. Placebo: ND-BRJ (140 ml) NO3 Duration: Acute and 7 D washout period 7 D	- Significant increase in plasma NO_3 level ($p<0.0001$) - No effect on SBP - Significant change in DBP: 7 mmHg ($p = 0.01$) - No effect on MAP ($p=0.07$) - No effect on heart rate ($p=0.06$)
Friis *et al.*, 2015/RXT	N = 15 GOLD stage 2–4	SBP/DBP (clinic); during exercise	Active-BRJ (140 ml) (600 mg) NO3 Placebo: ND-BRJ (140	- Significant increase in plasma NO_2 level ($p<0.01$) - No effect on SBP ($p=0.80$) - Significant reduction in DBP ($p<0.05$) - No effect on heart rate ($p=0.86$)

	Age (y): 63±13 Sex (M/F): 9/6 FEV1% = 44.7		ml) 600 mg) NO3 Duration: 7 D washout period 7 D	
Kerley *et al.*, 2015/RXT	N = 11 GOLD stage 2–4 Age (y): 69±7 Sex (M/F): 5/6 FEV1% = 43.4	SBP/DBP/MAP (clinic); during exercise	Active-BRJ (140 ml) (12.9 mmol) NO3 + 200 ml blackcurrant cordial Placebo: 140 ml water + 200 ml blackcurrant cordial Duration: Acute washout period 7 D	- Significant increase in plasma NO_3 level (p=0.000005) - Significant increase in plasma NO_2 level (p<0.01) - Significant change SBP: 12 mmHg (p=0.03) - Significant change in DBP: 2 mmHg (p=0.045) - Significant change MAP: 5 mmHg (p=0.018) - No effect on heart rate (p=0.19)

Kerley *et al.*, 2019/RXT	N = 8 GOLD stage 1–3 Age (y): 63±7 Sex (M/F): 5/3 FEV1% = 55	SBP/DBP (ABPM)	Active-BRJ (140 ml) (12.9 mmol) NO3 Placebo: ND-BRJ (140 ml) (.5mmol) NO3 Duration: 14 D washout period: none	- Significant increase in plasma NO_3 level (p=0.015) - Significant increase in plasma NO_2 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	SBP/DBP (clinic); during exercise	Active-BRJ (140 ml) (9.6 mmol) NO3 Placebo: ND-BRJ (140 ml) (0.0056 – 0.020 mmol) NO3	- Significant change SBP in the 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 1 hr standing (p = 0.966) and 2.7 mmHg at 4 h standing (p = 0.352)

	FEV1% = 62		Duration: 3 D washout period: 4 D	
Pavitt *et al.*, 2020/RCT During PR	N = 122 GOLD stage 2–4 Age (y): 68±10 Sex (M/F): 69/53 FEV1% = 49	SBP, DBP, MAP, FMD% (During PR)	Active-BRJ (140 ml) (12.9 mmol) NO3 Placebo: ND-BRJ (140 ml) NO3 Duration: 56 D washout period: none	- 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 % ($p= 0.046$)
Shepherd *et al.*, 2015/RXT	N = 13 GOLD Stage 1–2 Age (y): 65±8	SBP/DBP (clinic); during exercise	Active-BRJ (140 ml) (12.9 mmol) NO3 Placebo: ND-BRJ (140 ml) (0.004 mmol) NO3	- Significant increase in the plasma NO_3 level ($p=0.002$) - No effect on SBP ($p=0.91$) - No effect on DBP ($p=0.25$)

	Sex (M/F): NR FEV1% = 57		Duration: 2.5 D washout period: 7 D	

3.3.3. Risk of bias in included studies

I used the Cochrane risk-of-bias assessment tool (Higgins, 2011), and overall, the included studies showed considerable variation in the risk of bias. Lack of allocation concealment, blinding, and incomplete reporting of data were not reported in many of those trials, thereby an unclear level of bias was assigned for these three risk categories. Details about risk-of-bias judgements are reported in Figure 3.2.

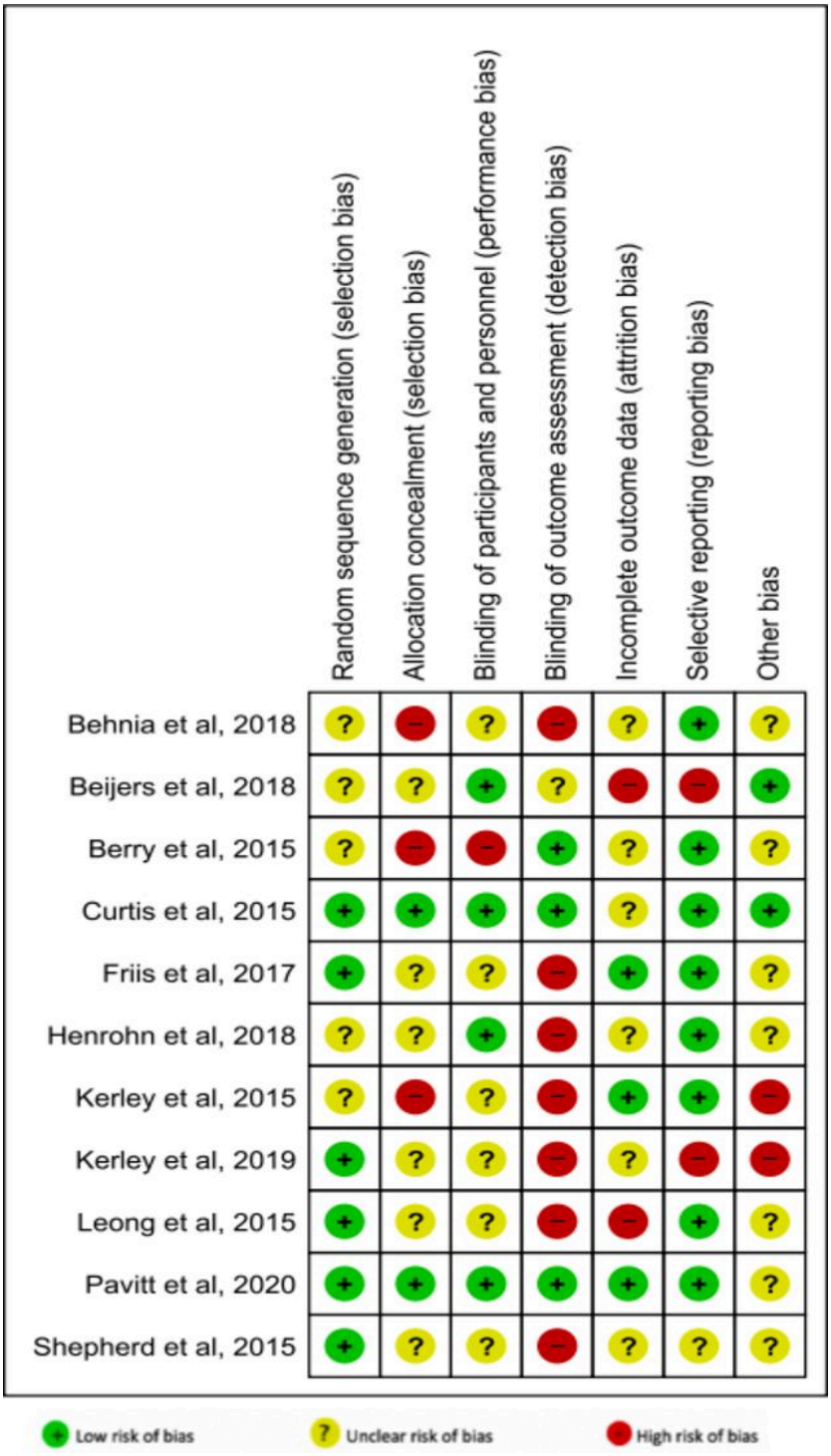


Figure 3-2 Forest plot for risk of bias of included studies. From Alsulayyim (2021) with permission.

3.3.4. Outcomes

3.3.4.1. BP

Five studies showed a significant reduction in SBP (Behnia, 2018; Berry, 2015; Conor P. Kerley, 2015; Leong, 2015; Pavitt, 2020), whereas a significant change in the DBP was also reported in five studies (Berry, 2015; Curtis, 2015; Friis, 2017; Conor P. Kerley, 2015; Pavitt, 2020). In addition, MAP outcome was included in three studies, and all detected a significant change (Curtis, 2015; Conor P. Kerley, 2015; Pavitt, 2020). Beetroot juice supplementation had a greater effect than nitrate salt (Sodium NO_3^-) on all BP parameters. The beneficial effects of beetroot juice supplementation on clinic BP were during exercise intervention, while changes in BP parameters were not confirmed when ambulatory 24-h BP monitoring was used (Conor P Kerley, 2019).

The pooled weighted mean difference from 5 RCTs included in the present meta-analysis showed nitrate supplementations associated with greater changes in SBP, with a total of 189 subjects [-3.39 mm Hg (-6.79 to 0.01), p=0.05; Fig. 3A], than both DBP with a total of 174 subjects [−2.20 mm Hg (−4.36 to −0.03), p=0.05; Fig. 3B], and MAP with a total of 89 subjects [−4.40 mm Hg (−7.49 to −1.30), p=0.005; Figure. 3.3].

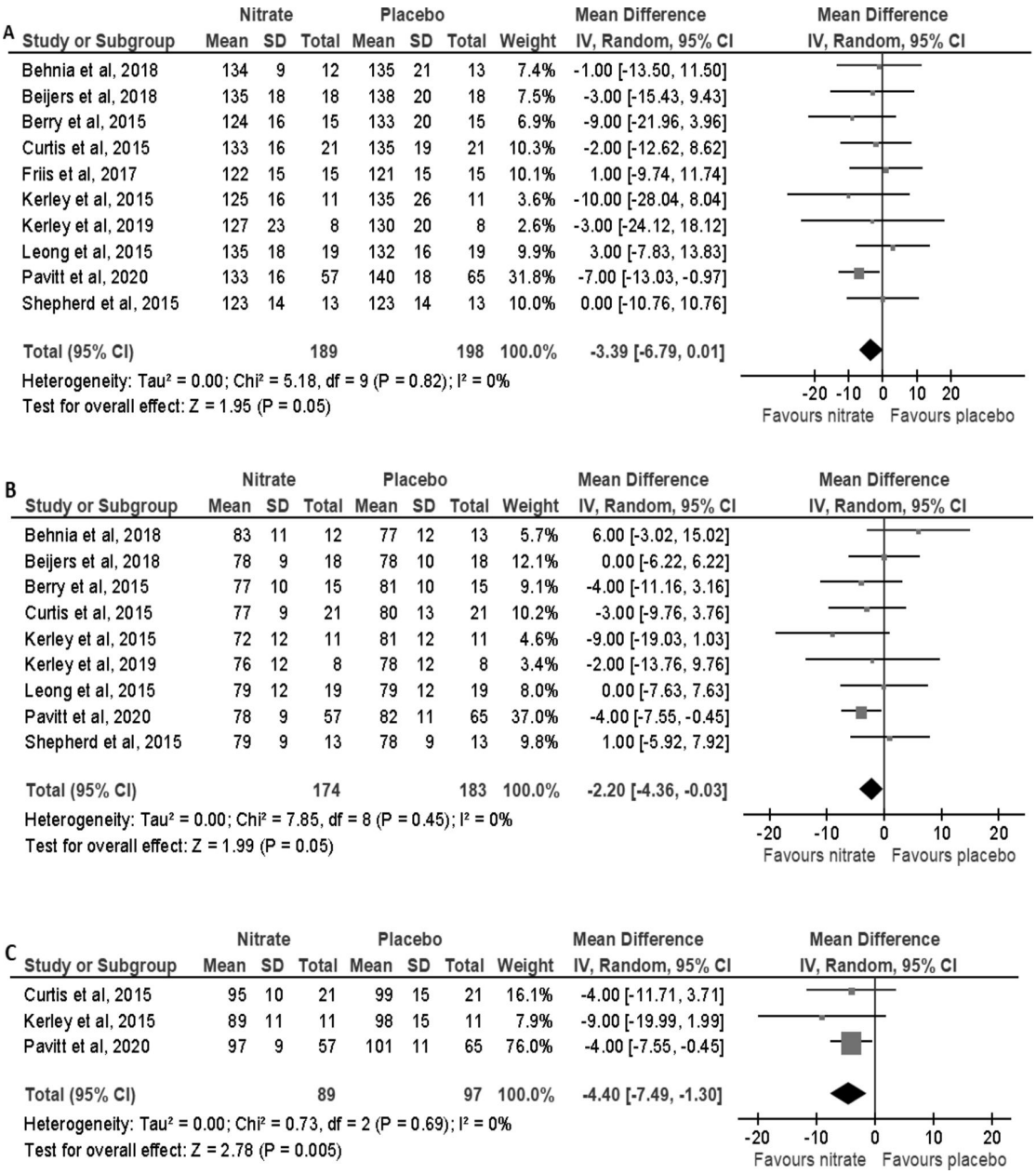


Figure 3-3 Forest plot of RCTs assessing the effect of NO_3 supplementation on SBP, DBP, and MAP Data reported as WMDs with 95% CIs. From Alsulayyim (2021) with permission.

3.3.4.2. Endothelial function

There was only one study showed an improvement in vascular function with dietary nitrate supplementation (Pavitt, 2020) in the context of a pulmonary rehabilitation programme. NR-BRJ for eight weeks improved endothelial function, assessed by flow-mediated dilation (FMD) in the treatment group (n = 10) nitrate-rich BRJ group +6.6% (0.6, 17.6) versus placebo BRJ (n=10) −4.7% (−21.5, 11.8).

3.3.4.3. Heart rate

A meta-analysis of 3 RCTs with 50 subjects that reported the impact of NO_3^- supplementation on HR showed no change in HR compared with placebo (Beijers, 2018; Curtis, 2015; Conor P. Kerley, 2015). The pooled weighted MD (95% CI) was [0.23bpm (−3.58 to 4.03), p = 0.91; Figure 3.4].

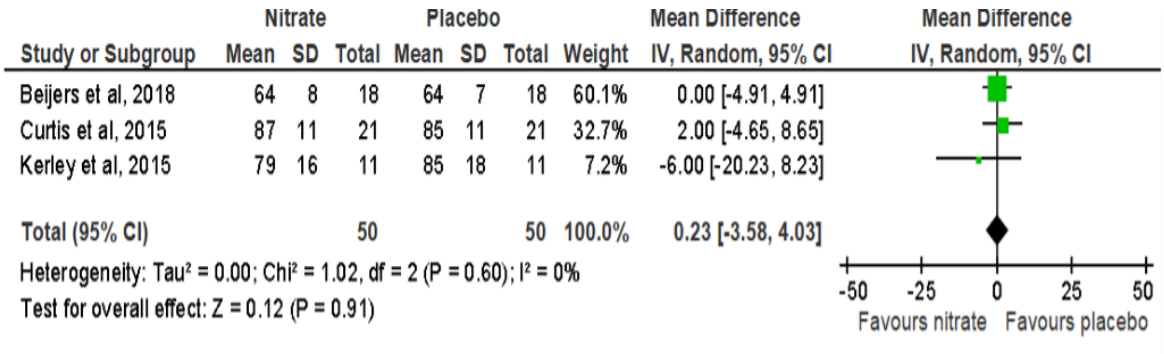


Figure 3-4 Forest plot of randomised clinical trials assessing the effect of NO_3^- supplementation on HR at rest (bpm) From Alsulayyim (2021) with permission.

3.3.4.4. Plasma NO_3^- and NO_2^- levels

Six studies that assessed the change of plasma NO_3^- and NO_2^- levels following nitrate supplementation in the treatment group compared to the placebo group were included in the meta-analysis. The pooled weighted MD (95% CI) in these studies showed a significant increase in both plasma NO_3^- and NO_2^- levels analysis [445.61mM (254.69 to 636.53), p<0.00001; Fig. 3-A], and [367.07 mM (232.87 to 501.27), p<0.00001; Figure 3.5, A and B], respectively.

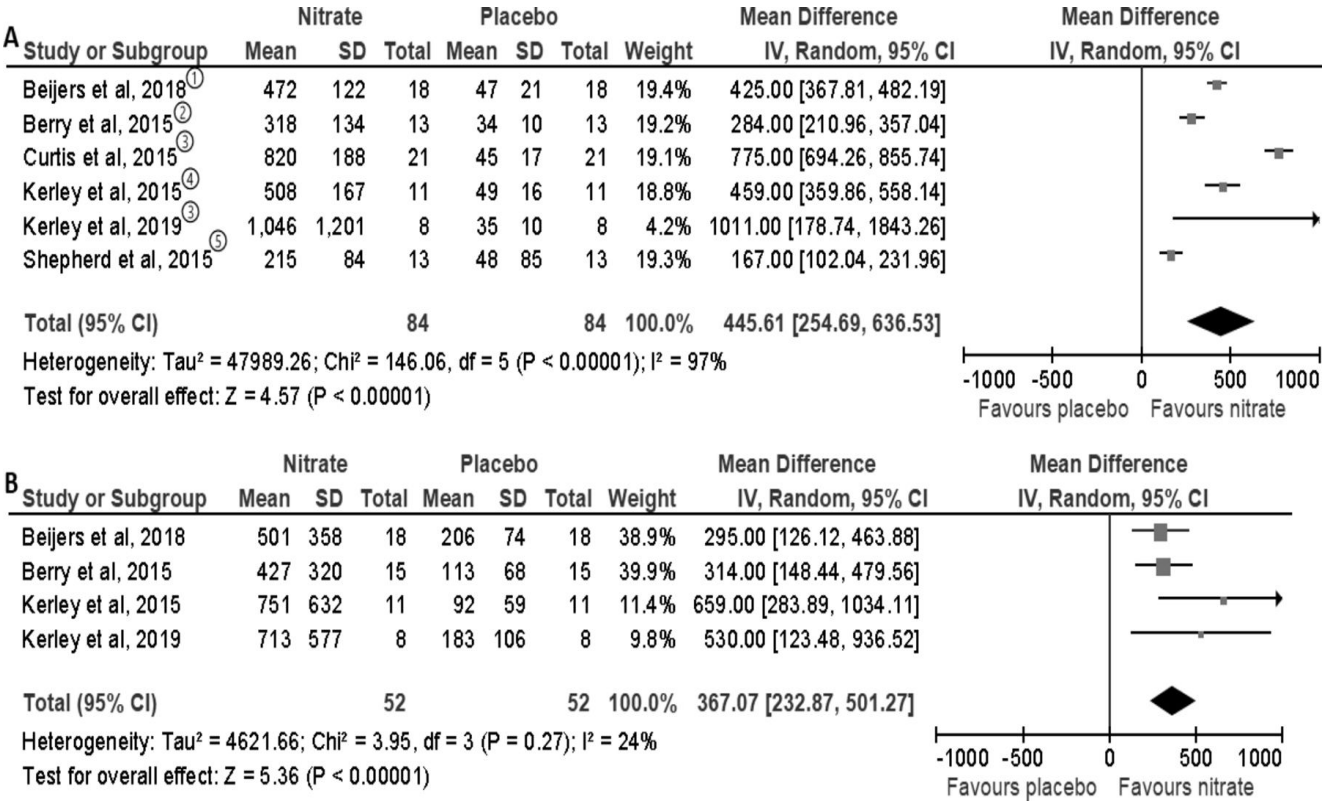


Figure 3-5 Forest plot of RCTs assessing the effect of NO_3^- supplementation on (A) plasma nitrate (μM) and (B) plasma nitrite (nM) levels.(1) sodium nitrate (8 mmol); (2) beetroot juice (BRJ) (7.58 mmol); (3) BRJ (12.9). From Alsulayyim (2021) with permission.

3.3.4.5. Fractional exhaled NO

The effects of NO_3^- supplementation on FeNO were assessed in two studies, which reported inconsistent results in FeNO following the intervention in treatment groups compared to placebo(Behnia, 2018; Conor P Kerley, 2019). Pooled analysis of MD (95% CI) was [17.23 (-3.35 to 37.80) ppb, p=0.10; Figure 3.6].

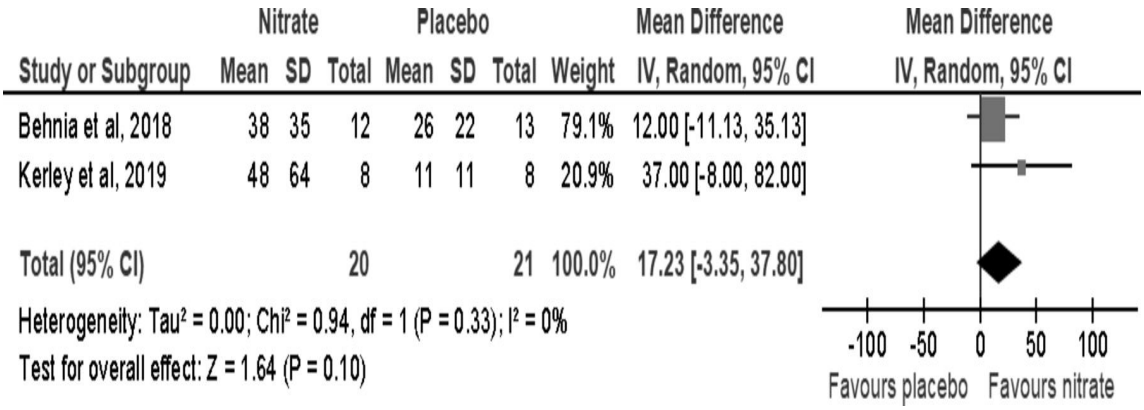


Figure 3-6: Forest plot of randomised clinical trials assessing the effect of NO_3^- supplementation on FeNO (ppb). From Alsulayyim (2021) with permission.

3.3.5. Adverse events, tolerance, and compliance

Five studies involving 110 participants reported information about adverse events encountered with NO_3^- intervention (Curtis, 2015; Conor P. Kerley, 2015; Conor P Kerley, 2019; Pavitt, 2020; Shepherd, 2015). The supplements were well tolerated among participants with no adverse events; the only side effect reported in the BRJ trials was beeturia. Reported compliance indicated that participants were compliant with drinking the intervention (BRJ) or assessed before conducting the measurements in all studies (Watson, Luke, & Inall, 1963).

3.4. DISCUSSION

This review of the literature found that in people with COPD, dietary NO_3^- supplementation lowered BP, improved endothelial function, increased plasma NO_3- and NO_2- concentrations, and elevated exhaled FeNO levels. These results were similar to previous studies conducted on healthy subjects and people with other chronic diseases (Bahrami, 2021; Jackson, 2018; F. Larsen, Ekblom, Sahlin, Weitzberg, & Lundberg, 2006; Siervo, Lara, Ogbonmwan, & Mathers, 2013).

The findings from the meta-analysis show BRJ exerted acute (at 3 hours postintervention effect) or short-term (from 2 days up to 2 months) effect on lowering BP, during exercise training. Nevertheless, testing the effects of BRJ on BP and endothelial function at rest in the absence of exercise training has not been thoroughly investigated. Dietary nitrate supplementation using BRJ has emerged as a potential nutritional agent for the prevention and treatment of hypertension and other CVD risks. Systematic reviews included clinical research have shown that ingestion of nitrate-rich BRJ may provide a reliable source of NO, which regulates and mitigates the pathophysiology behind exercise performance, HTN, ED, ischaemia/reperfusion injury, and thrombosis (Gee, 2016; Jackson, 2018; McMahon, 2017). These findings are consistent with current review and meta-analysis revealed that BRJ compared to nitrate salt intervention demonstrated significant decreases in BP and EF (Jackson, 2018). The pooled data from meta-analyses showed an overall reduction in SBP and MAP by -3.39 mm Hg and -4.40), respectively, with a modest decrease (−2.2 mm Hg) in DBP (Jackson, 2018).

Improving endothelial function has been the suggested mechanism for BRJ effects on BP via an increase in downstream cGMP activity (Denninger, 1999). This is very effective for this review since the risk of hypertension is prevalent among COPD patients (Rabe, 2018). Ageing is another risk factor in COPD patients, as it is associated with a decline in NO, which negatively affects the physiological function of large arteries (Hermann, 2006). This variation in results of BP could be related to the heterogeneity among COPD groups or the obvious variation in the studies' protocols, such as nitrate-content BRJ, volume, and BP measurement methods.

In addition, nine trials used BRJ as a nitrate source (Behnia, 2018; Berry, 2015; Curtis, 2015; Friis, 2017; Conor P. Kerley, 2015; Conor P Kerley, 2019; Leong, 2015; Pavitt, 2020; Shepherd, 2015), whereas one study tested nitrate salt (sodium nitrate - $NaNO_3$-) (Beijers, 2018). However, the daily quantity of BRJ consumed, and the nitrate concertation and volume, varied between studies. Nitrate concertation ranged from ~ 7.6 to 13.5 mmol and 70 ml to 140 ml per day, respectively, while 680 mg of $NaNO_3$- contained ~8 mmol of nitrate consumed orally per day (20). Furthermore, the placebo or control type was also varied between trials and included equivalent volumes of nitrate-removed BRJ (Behnia, 2018; Curtis, 2015; Friis, 2017; Conor P Kerley, 2019; Leong, 2015; Pavitt, 2020; Shepherd, 2015), water and/or juice (Beijers, 2018; Berry, 2015; Conor P. Kerley, 2015), or sodium chloride solution NaCl (20). The duration of the interventions and washout period for crossover trials ranged from 1.5 h to 28 days and 7 to 28 days, respectively. Overall, the meta-analysis showed that NO metabolites nitrate/nitrite increased following dietary nitrate supplementation, which is in line with findings on healthy and chronic diseases (Jackson, 2018). FeNO used in two studies reported a significant increase in FeNO levels after ingestion BRJ (Behnia, 2018; Conor P Kerley, 2019). Traditionally, FeNO is

used in clinical practice and research as a diagnostic test and monitoring for asthma progression. Studies of supplementation of NO3- intervention measured the bioavailability of NO indirectly by measures of plasma NO3- and NO2- concentrations. The guidelines for the measurement of exhaled nitric oxide recommend the avoidance of NO3--rich foodstuffs (ATS/ERS 2005) as it has been established that NO3--rich food can increase the measures exhaled nitric oxide (Dweik, 2011). Hence, it may be FeNO level can be used as a biomarker to monitor compliance in therapeutic trials of nitrate supplementation in people with COPD, warranting investigation.

This raised the following question: Do we need more trials on BRJ and CV risks in COPD? Recently available trial findings have shown that different BRJ protocols (doses and duration) are feasible for patients with COPD. The studies included in this review had a median intervention of three weeks. Given that CVD prevention and control should be based on a long-term perspective, researchers need to gather more data on the effects of BRJ on CV risk outcomes over longer periods. Also, future research should pay more attention to defining whether there are different COPD subgroups categorised as responders or non-responders to dietary NO_3^- supplementation. More evidence on the cost-effectiveness following dosing with BRJ on reported CVD incidents in COPD patients is needed to inform policy decisions.

3.4.1. Quality of evidence

I carried out the Grading of Recommendations, Assessment, Development and Evaluations (GRADE) tool and criteria to further rate the quality of evidence related to BP and endothelial function outcomes (Higgins, 2011). The majority of studies that included BP parameters in their outcomes were small and short term, which limited the precision of estimates. Furthermore,

intervention characteristics among the included studies were inconsistent (e.g., doses, duration, and delivery method), as well as the majority of the studies used the clinical BP measurement method. In clinical practice, home BP monitors and 24-hour ambulatory BP monitors are regarded by many hypertension guidelines as more reliable methods to measure and monitor BP parameters compared to the in-clinic BP measurement method (Sega, 2005). Hence, the quality of the studies' evidence supporting the effects of dietary NO_3^- supplementation on BP, by GRADE, was generally moderate. However, regarding the endothelial function outcomes, only one large high-quality RCT reported positive FMD results. Hence, there is an urgent unmet need for more evidence-based standardised BRJ intervention, utilising reliable BP measurement methods, and including more CV risk outcomes in patients with COPD.

In the body, NO is produced through two suggested mechanisms. One mechanism is endogenous via the L-arginine NOS system, which reacts with several enzymatic and non-enzymatic factors in the blood and is oxidised almost completely to NO_2^- and NO_3^- (Lundberg, 2010). The second is exogenous, from a diet including nitrate-containing beetroot and green leafy vegetables via the nitrate-nitrite-NO pathway under acidic and hypoxic conditions and the presence of enzymatic and non-enzymatic factors such as deoxyhaemoglobin in the blood (J. O. Lundberg, 2004).

3.4.2. Strength and limitations

A large-scale literature search and consistency in following a predefined protocol are major strengths of this systematic review, along with fortunate contact with the authors of the studies included, as they provided some of information in addition to data that had already been retrieved.

To the best of our knowledge, this is the first systematic review and meta-analysis that demonstrates the beneficial effects of nitrate supplementations on CVD risk outcomes in people with COPD. Nevertheless, a potential limitation of this review was the exclusion of non-English trials.

3.5. Conclusion

I have systematically reviewed and produced a meta-analysis of the effect of dietary NO_3^- on CVD risk outcomes in COPD patients. The available evidence from this meta-analysis demonstrates moderate acute and short-duration effects of NO_3^- supplementations using NR-BRJ on lowering BP among patients with COPD. Whether this effect of BRJ on the BP is sustained for a longer period is unknown. Hence, in the next chapter, I conducted a long-term randomised clinical trial to address this research question.

Chapter 4: The effect of dietary NO_3^- supplementation on BP in COPD patients: A 3-month randomised, double-blind, placebo-controlled trial (the ON-BC study).

4.1 Introduction

4.1.1 Background

As outlined in the introduction of Chapter 1, individuals with COPD are at much higher risk of hospitalisation and death due to ischaemic heart disease and CVD (Rabe, 2018). Hypertension is regarded as the most frequent CVD risk among COPD population (Finks, 2020). Endothelium-derived nitric oxide (NO) impairment is thought to play a key role in the pathological process of hypertension (Lundberg, Carlström, Larsen, & Weitzberg, 2011). The systematic review presented in Chapter 3 suggests that consumption of dietary NO_3^- supplementation using BRJ can exert significant BP-lowering effects and improve endothelial FMD in people with COPD, although studies to date have been short term or combined with exercise intervention. Therefore, in this chapter, I sought to determine whether 12 weeks of daily BRJ intake lowers BP and improves endothelial function in patients with COPD. The effects of BRJ on exercise capacity and quality of life were also evaluated.

The effects on endothelial function outcome and other measures of vascular function are described in Chapter 5.

4.2 Subjects and methods

4.2.1 Patient selection

All participants provided written informed consent before enrolment in the study, which was conducted in line with the principles of the Declaration of Helsinki. The trial was registered prospectively on a publicly accessible database (www.controlled-trials.com) reference ISRCTN47839214 and approved by the Wales Health Care Research and Ethics Committee (REC reference 19/LO/1660).

Study inclusion criteria:

- Patients diagnosed with COPD based on the GOLD criteria
- Both males and females between 18 and 85 years of age
- Able to understand and comply with protocol requirements, instructions, and protocol-stated restrictions
- Blood pressure meeting criteria based on SBP ≥ 130 mmHg via home BP monitor

Exclusion criteria:

- Patients unable to provide informed consent
- Patients having had AECOPD within one month
- Patients having significant comorbidity limiting exercise tolerance
- Patients having a significant renal impairment (estimated glomerular filtration rate (eGFR) <30 ml. min1)
- Hypertensive patients using > 3 antihypertensive medications
- Patients having changed their BP medication in the previous month
- Use of nitrate-based medications influencing the plasma nitrate/nitrite levels

- Use of BRJ (Beet Shots) within one month.

4.2.2 **Study design**

The study was conducted at a single site (Royal Brompton Hospital, London) as a double-blind, randomised, placebo-controlled trial. The randomisation of patients was stratified by the use of antihypertensive medication. The randomisation list was generated by an independent statistician via computer software: https://www.sealedenvelope.com/, using block randomisation (block size 10). Both the subjects and the study coordinator (I) who were consenting/administering and performing clinical outcome measurements were blinded to the randomisation process, treatment allocation and throughout the data analysis.

Potential subjects were found from clinic patient lists, advertisements made within the hospital, the British Lung Foundation Breathe Easy group, and social media. They were contacted via phone and screened verbally for study inclusion eligibility. If the subjects met our study eligibility criteria and wished to participate, I sent them a home BP monitor (Omron Healthcare, HEM-7155-E-M3 Comfort; see Chapter 2) with instructions to take measurements for four days. During each day, the subjects were told to measure their BP three times (morning/afternoon/evening). At each time of the day, they measured their BP twice (a minute apart) and recorded the results in the sent BP measurements diary card. After completing four days, self-reported values of BP measurements were sent to us, and I calculated the average SBP. If SBP was $\geq$130 mmHg, the subject was eligible.

4.2.3 **Intervention**

Participants were randomly allocated in a parallel-group manner to consume either NR-BRJ or placebo (nitrate-depleted) Pl-BRJ (70 mL BEET-IT® SPORT Shot, James White Drinks, Ipswich, UK), which were identical in appearance, taste, smell, and nutrient composition. The NR-BRJ contained 400 mg of NO_3^- mixed with organic lemon juice (2%), whereas the NO_3^- was removed by the manufacturer to make the Pl-BRJ juice. The Pl-BRJ was generated by the standardised method of passing the juice, before pasteurisation, through an ion-exchange column containing Purolite A520E ion exchange resin, which selectively removes NO_3^- (Lansley, 2011). The person responsible for dispensing the juice was not involved in the study testing or analysis. After the randomisation process, the allocated juice was delivered directly from the juice factory to the patient's home and administered in a double-blind fashion. Participants were then asked to drink their intervention juice, one beverage daily, at 9 am (+/- 2 h) for 90 days. Participants were reminded 48 hours before attending Visit 1 (baseline) to avoid NO_3^- rich food for 48 hours. Participants were also requested to match their caffeine consumption for 24 hours before the intervention visits. Participants were also asked to avoid strenuous exertion, given the known effect on the vasoconstrictor response of the endothelium (Elliott, 2018). On the day of Visit 2 (post-intervention), I reminded them to drink the juice at least three hours before attendance.

4.2.4 **Study protocol**

The original study protocol involved participants attending the clinical research facility for three visits. The COVID-19 pandemic made it extremely difficult for some participants to attend for several reasons, including: (1) the hospital became a COVID-19 in-patient centre; (2) some of the participants did not complete the two required COVID-19 vaccinations; (3) some participants

received a recommendation from the NHS to shield during the pandemic; (4) others feared that attending the clinic may expose them to a higher risk of infection.

Consequently, the primary endpoint (SBP) was taken remotely by the home BP monitor as planned, without the need for face-to-face visits. Meanwhile, some participants agreed to come to our lab to conduct the secondary and exploratory outcome measurements, including 6MWD, blood tests, measurements of vascular function, and platelet aggregation tests.

The two intervention visits began at the same time of day (+/- 2h), with a minimum of 88 days and a maximum of 90 days between the two visits. BP log sheets, 90-day juice drinking time log sheets, and the study quality-of-life (QoL) questionnaires for pre- and post-interventions were mailed to remote participants, including a reply-paid envelope for return. Participants then mailed back their home BP readings and questionnaires within one week. Participants who agreed to attend the face-to-face sessions had extra tests, as mentioned above and described in subsequent sections.

An overview of the study protocol flow diagram is provided in Figure 4.1.

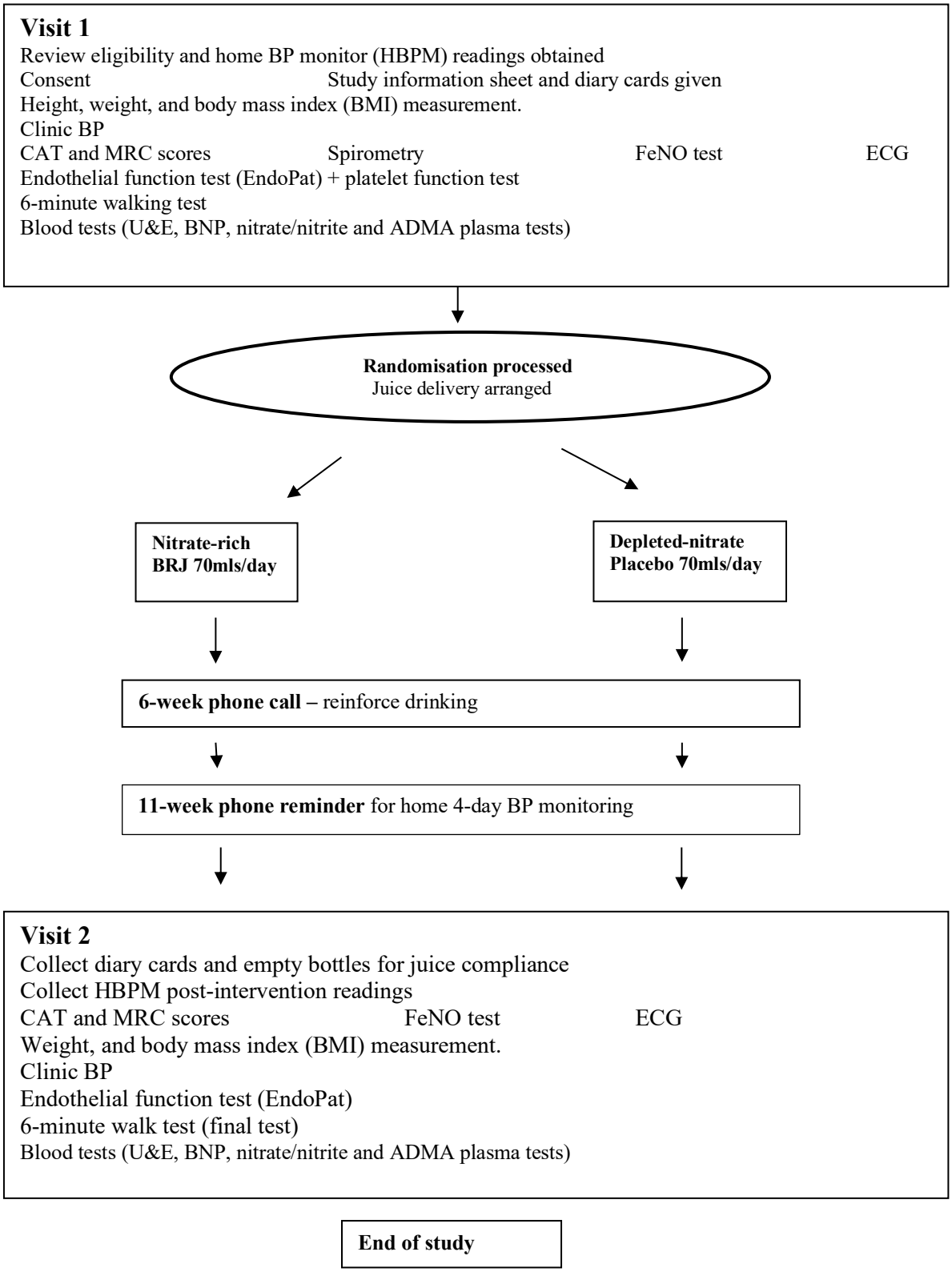


Figure 4-1. The study protocol flow diagram

4.2.5 **Primary and secondary outcome measures**

4.1.1

Primary endpoint

- Comparing the change from baseline at 12 weeks on SBP between treatment groups.

Secondary endpoints

- Comparing the change from baseline at 12 weeks on 6MWT distance (6MWD) between treatment groups.
- Comparing the change from baseline at 12 weeks on health-related quality of life assessed by the COPD Assessment Test (CAT) score between treatment groups.

4.2.6 **Exploratory endpoints**

- Comparing the change from baseline at 12 weeks on DBP between treatment groups.
- Comparing the change from baseline at 12 weeks on MAP between treatment groups.
- Comparing the change from baseline at 12 weeks on HR between treatment groups.
- Comparing change from baseline at 12 weeks on plasma NO_x metabolites (NO_3^- /NO_2^-) concentrations between treatment groups.
- Comparing the change from baseline at 12 weeks on FeNO (ppb) levels between treatment groups.
- Comparing the change from baseline at 12 weeks on endothelial function between treatment groups (the results are reported in Chapter 5).
- Comparing the change from baseline at 12 weeks on BNP blood levels between treatment groups (the results are reported in Chapter 5).
- Comparing the change from baseline at 12 weeks on plasma arginine, plasma ADMA levels, and plasma L-arginine levels between treatment groups (the results are reported in Chapter 5).
- Comparing the change from baseline at 12 weeks on platelet aggregation % between treatment group groups (the results are reported in Chapter 6).

4.3 Sample size, data analysis, and statistics

4.3.1 Sample size

The study is powered for the primary outcome change in SBP response at 12 weeks from baseline. Data from the ON-EPIC trial showed a fall of a (mean ± SD) 5 ±3.7mmHg (Pavitt, 2020). Taking a power of 90% and a significance level of 0.05, we would require 32 patients within each group to identify a (3 mmHg) fall with treatment. I assumed a dropout rate of 10%; therefore, our anticipated recruiting target was 72 patients in total.

4.3.2 Data analysis and statistics

Baseline demographic, clinical anthropometrics, symptoms, and physiological variables are summarised for each group of the study in Table 4.1. Continuous variables are presented as mean and standard deviation (SD) or median and interquartile range (IQR), and categorical variables are presented as frequency and percentages. I compared the outcomes between the groups according to the type of variables. Categorical variables were compared using the Chi-square test. Dependent continuous variables were compared using either the independent t-test for normally distributed data or the Mann-Whitney U test for non-normally distributed data. For the evaluation of the potential connection between the change in SBP and baseline variables or covariates, I employed Pearson or Spearman's correlation tests for continuous variables, as appropriate. One-way between groups ANOVA (Kruskal-Wallis Test) was used to assess significant changes in SBP between stratified subgroups. I assessed the correlation between dichotomous variables and changes in HSBP using the point-biserial correlation. Furthermore, I

built a multiple linear regression model adjusting for confounding factors on the variance of change in HSBP. The quality of the model was described by *R* squared. I considered a p-value of < 0.05 to indicate a statistically significant difference. Statistical analysis was performed on a per-protocol basis. Data analyses were performed using the Statistical Package for the Social Sciences v.27 (SPSS, Chicago, IL, USA), and figures were prepared using GraphPad Prism version 9.0 for Mac (GraphPad Software, San Diego, California, USA)

4.4 Results

4.4.1 Recruitment process

Recruitment to the trial started in October 2020 and was completed in August 2021. I screened a total of 123 COPD subjects with COPD, but 38 (31.0%) of them were excluded from recruitment for not fulfilling the inclusion criteria, declining to participate, or withdrawing before randomisation. Further details of the recruitment process are presented in the CONSORT diagram (Figure 4.2). It is to be noted that I enrolled 85 subjects, which was more than I anticipated based on our sample size calculation (see Section 4.3.1). Of the 85 subjects enrolled, 4 (4.7%) withdrew of consent before randomisation, and 11 (13.0%) participants withdrew after randomisation. Finally, 70 participants completed the trial.

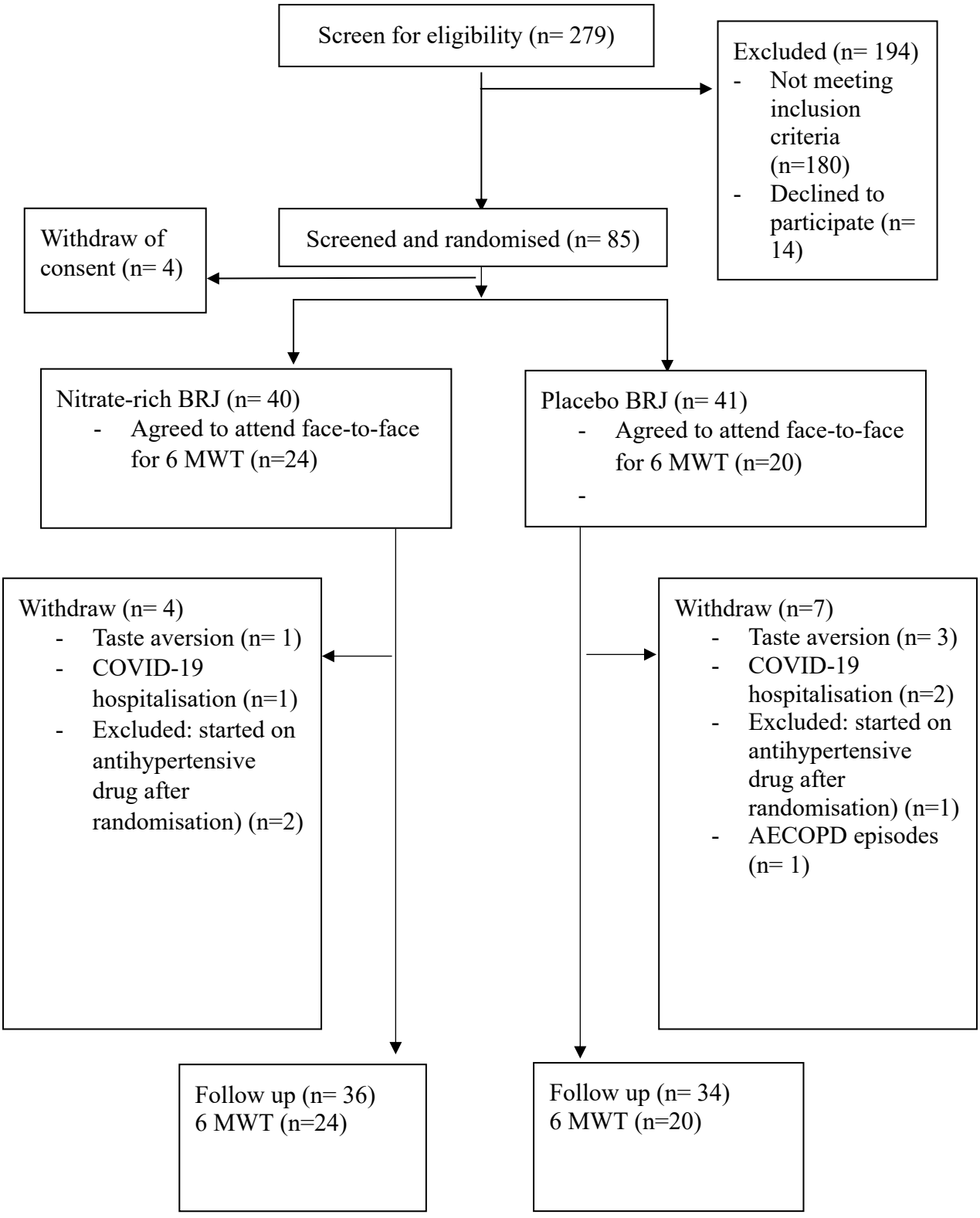


Figure 4-2 CONSORT flow diagram for the ON-BC trial

4.4.2 **Baseline characteristics of the treatment and placebo groups**

Overall, participants in both groups were similar in baseline characteristics. The participants were typical of all COPD (GOLD – classifications) regarding median age (65 vs 66 years), gender distribution (72% vs 68% male), BMI (26.8 vs 26.7 kg/m^2), and FEV1% (44.0 vs 41.7). Moreover, the distribution of people on antihypertensive medications across groups was 15 vs 17 (p =.477). Most participants were of Caucasian ethnicity, except for three Afro-Caribbeans and one Asian. The baseline characteristics of both groups compared are presented in Table 4.1.

Table 4.1 Demographic, and baseline clinical characteristics of the subjects (n = 70).

Variable	Pl-BRJ n (34)	NR-BRJ n (36)	P-value
Sex, Male n (%) *	23 (67.6)	26 (72.2)	0.68
Age (years)**	66 (40, 82)	65 (48, 76)	0.81
Ethnicity, n (%)			0.42
White Caucasian	32 (94.1)	34 (94.4)	
Black	1 (2.9)	2 (5.6)	
Asian	1 (2.9)	0 (00)	
BMI (kg/m^2)	26.7 (23.9, 30.1)	26.8 (23.0, 30.7)	0.87
Smoking status (n)			0.44
Ex- smoker	28 (82.4)	32 (88.9)	
Current smoker	6 (17.6)	4 (11.1)	
Smoking Hx. (pack-years) ***	31.9 (17)	34.0 (11.1)	0.53
FEV1 (L)	1.2 (.86, 1.6)	1.1 (.75, 1.8)	0.86
FEV1 (% predicted)	40.7 (31.4, 57.2)	44.0 (27.3, 61.1)	0.91
Gold grade, n (%)			0.06
COPD I	1 (2.9)	8 (22.2)	
COPD II	5 (14.7)	8 (22.2)	
COPD III	15 (44.1)	12 (33.3)	
COPD IV	13 (38.2)	8 (22.2)	
AECOPD last year			0.25
0	13 (38.2)	20 (55.6)	
1	17 (50.0)	9 (25.0)	
2	3 (8.8)	4 (11.1)	
3	0 (0.0)	1 (2.8)	
>3	1 (2.9)	2 (5.6)	
CV comorbidities, n (%)			
Hypercholesterolaemia	7 (20.6)	9 (25.0)	0.78
Hypertension	17 (50.0)	15 (41.7)	0.63
IHD	6 (17.6)	8 (22.2)	0.77
COPD inhalers, n (%)			
LAMA	16	14	0.49
LABA/LAMA	5	4	0.65
LABA/ICS	18	18	0.81
LABA/LAMA/ICS	9	11	0.70
HTN medications, n			
ACEi/ARBs	10	9	0.79
CCBs	4	8	0.35
β-blockers	3	7	0.31
Diuretics	2	7	0.15
Completed PR, n (%)	20 (55.6)	22 (64.7)	0.43
Mouthwash-use/week, n			0.91
1-3/wk	8	6	
4-7/wk	7	8	
FeNO ppb, (n=20 vs. 24)	23.5 (±13.7)	19.6 (±6.9)	0.26
eGFR mL/min, (n=20 vs. 24)	90 (80, 90)	83 (71, 90)	0.07
BNP (ng/L), (n=20 vs. 24)	36.1 (17)	33.5 (19)	0.77

Abbreviations: BMI, body mass index; GOLD, Global Initiative for Chronic Obstructive Lung Disease; FEV1, forced expiratory volume in 1 s; IHD, ischaemic heart disease; LABA, long-acting beta-agonists; LAMA, long-acting muscarinic antagonists; ICS, inhaled corticosteroids; ACEi, angiotensin-converting enzyme inhibitors; ARBs, angiotensin receptor blockers; CCB, calcium channel blockers; β-blockers, beta-blockers; PR, Pulmonary rehabilitation; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; BNP, brain natriuretic peptide.
Note: Data reported as number (%) *, median (IQR)**, and mean ±SD***.

4.4.3 **Intervention tolerance and compliance**

The intervention was well tolerated by both groups. Almost all the participants drank the intervention juice for 90 days ± 2 days. The participants' compliance with the intervention was assessed through diary card returns. There was no significant difference in treatment compliance between groups (96% vs 97%; $p = 0.78$). In addition, no serious adverse events were reported by the participants during the study. Two subjects from the Pl-BRJ group and one subject from the NR-BRJ group withdrew soon after the first week of juice arrival because they did not like the taste of BRJ. Moreover, five participants reported a common and expected reaction to the BRJ, which was beeturia (three from NR-BRJ and two from Pl-BRJ). Nonetheless, these five participants completed the study.

4.4.4 **Effects of BRJ on BP and HR**

Dietary NO_3^- supplementation lower SBP, MAP, but not DBP, as determined by the home BP monitor (Figure 4.3). The NR-BRJ group was associated with a significant drop in SBP, the primary endpoint measure, compared to the placebo group (median difference [95% CI]; -3 mmHg [-6 to-3], $p < 0.0001$). The median differences in DBP showed no significance between groups: -1 mmHg [-4 to 1], $p = 0.17$). MAP, on the other hand, was associated with a significant change in NR-BRJ compared to the placebo group (median difference, -3 mmHg [-4 to 0], $p = 0.03$). Resting heart rate appeared to be unaffected by BRJ. There were no significant differences

found between groups (median difference, -1.5 bpm [-3 to 2], $P = 0.52$; Figure. 4.4. Table 4.2 presents further details on BP and HR data in each group.

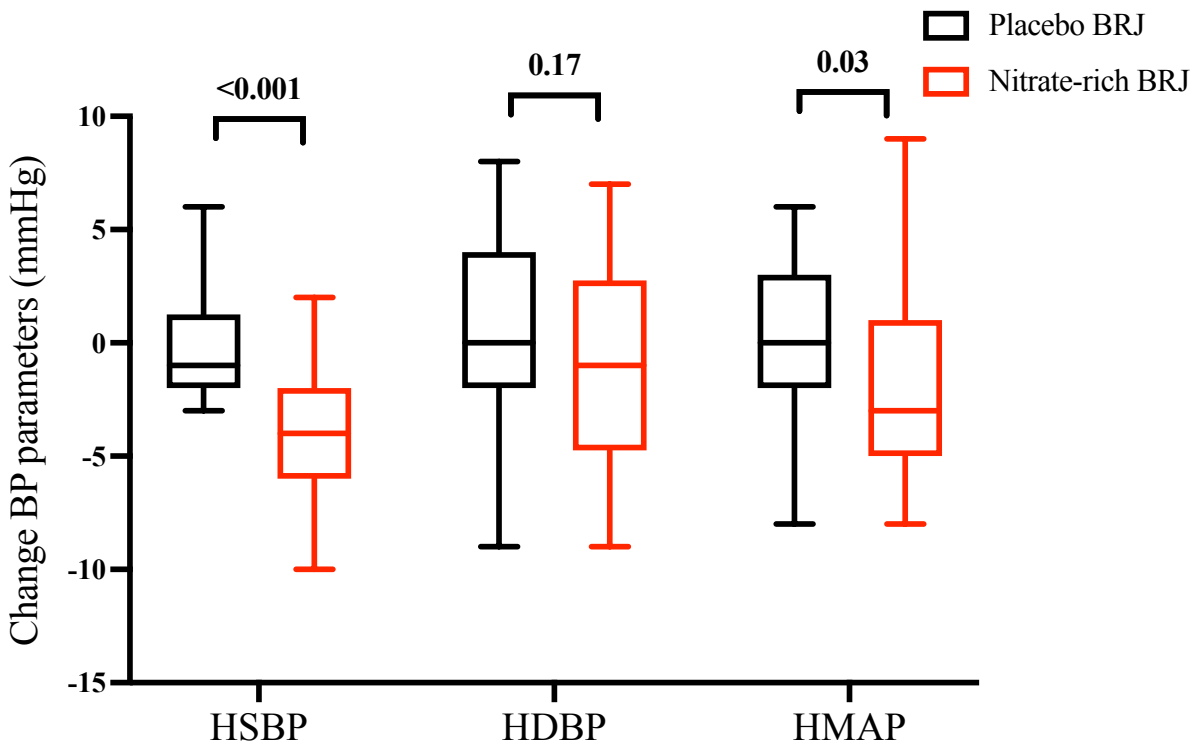


Figure 4-3 Effect of dietary NO_3^- supplementation on change BP parameters in Pl-BRJ (n = 34) and NR-BRJ (n = 36) COPD individuals

Change in home-monitored BP parameters in the placebo BRJ (n = 34) vs nitrate-rich BRJ (n = 36) in subjects with COPD. Data are presented as the 25th–75th percentile, with the solid line representing the median value and whiskers the minimum to maximum values. Comparisons between groups were made using the Mann-Whitney U Test. BP was measured via a home monitor at rest for four days, three times every day, and each time, was measured twice, one minute apart. Then, the average of the last three days' value is reported. I found a significant reduction in systolic, mean arterial, but not diastolic blood pressure between dosing conditions. Change in HSBP in placebo BRJ-1.0 mm Hg (−2.0, 1) vs nitrate-rich BRJ group −4.0 mm Hg (−6.0, −2.0), p<0.001; change in HDBP in placebo BRJ 0.0 mm Hg (−2.0, 4.0) vs nitrate-rich BRJ group −1.0 mm Hg (−5.0, 3.0), p = 0.17. Change in HMAP in placebo BRJ 0.0 mm Hg (−2.0, 3.0) vs nitrate-rich BRJ group -3.0 mm Hg (−5.0, -1.0), p=0.03. P<0.05 is considered significant. Change within group calculated as post value – pre-value. Negative change in HMAP and HDBP. And HMAP denotes a better outcome. Abbreviations: HSBP – home systolic blood pressure; HDBP – home diastolic blood pressure; HMAP – calculated home mean arterial pressure.

Table 4.2 Impact of dietary NO_3^- supplementation on home BP parameters at baseline and three months

	Pl-BRJ (n = 34)			NR-BRJ (n = 36)			Effect size (95% CI)	Between groups *p*-value
Variables	Pre	Post	Change	Pre	Post	Change		
HSBP (mmHg)	136 (132, 140)	134 (131, 140)	-1 (-2, 1)	135 (130, 143)	131 (127, 139)	-4 (-6, -2)	-3 (-6 to -3)	<0.0001
HDBP (mmHg)	86 (82, 90)	87 (80, 92)	0 (-2, 4)	87 (79, 91)	84 (79, 90)	-1(-5, 3)	-1 (-4 to 1)	0.17
HMAP (mmHg)	103 (100, 106)	103 (98, 106)	0 (-2, 3)	103 (97, 107)	99 (96, 106)	-3 (-5, 1)	-3 (-4 to 0)	0.03
HHR (bpm)	78 (68, 84)	78 (70, 87)	2 (-1, 5)	79 (72, 85)	79 (73, 86)	1 (-2, 5)	-1 (-3 to 2)	0.52
Pre- to post-intervention data and change illustrated as median (IQR); between-group effect size estimates presented as the median of differences (95% CI). *p*-value <0.05 is considered significant. The Mann-Whitney U test was used to compare groups.								

I carried out a subgroup analysis to further understand the effects of our interventions between participants treated with antihypertensive medication and untreated (prehypertension) using a one-way ANOVA. The results, as shown in Figure 4.5, indicate no difference in the effects on HSBP between drug-naïve and treated hypertensive participants in both the Pl-BRJ and NR-BRJ groups (Pl-BRJ: no-med (n = 18) and yes-med (n = 16); NR-BRJ: no-med (n = 15) and yes-med (n = 21); $p = 0.915$ vs $p = 0.221$, respectively.

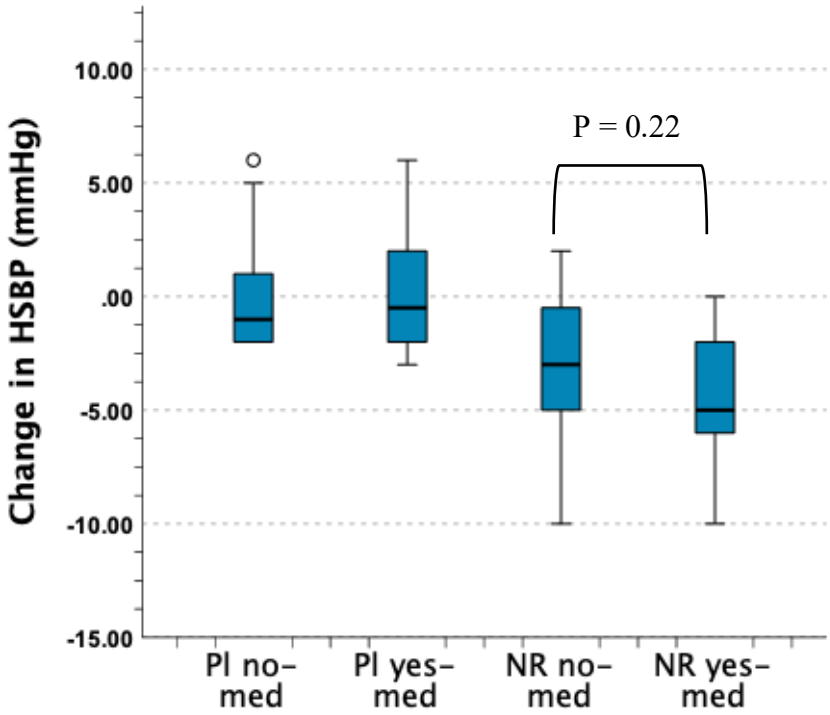


Figure 4-4 One-way ANOVA effect of dietary NO_3^- supplementation on SBP mmHg of stratified subgroups no-med (n = 18), and yes-med (n = 16); NR-BRJ no-med (n = 15), and yes-med (n = 21).

Also, I observed that people in the NR-BRJ group who were using ACEi (n = 9) had a greater drop in SBP (median: - 6 mmHg) compared with people who were not using ACEi (n=27; median: -3 mmHg). In contrast, the median change of HSBP of the patients treated with ACEi compared to those not treated in the Pl-BRJ group showed a similar change (-1 mmHg vs -1 mmHg).

4.4.5 Effects of BRJ on quality-of-life

Participants' quality-of-life (QoL) assessments measured by CAT score and MRC dyspnoea scale questionnaires did not differ between groups, with median differences (95% CI), 1 [-3, 0],

p =.09) and 0 [-1, 1], *p* =.46, respectively. Table 4.3 illustrates the QoL data at baseline and post-intervention for each treatment group.

Table 4.3 Effects of dietary NO_3^- supplementation on quality-of-life questionnaires from baseline to post-intervention

	Pl-BRJ (n = 34)			NR-BRJ (n = 36)			**Effect size (95% CI)**	**Between groups *p*-value**
Variables	Pre	Post	Change	Pre	Post	Change		
CAT score	22 (17, 27)	19 (16, 27)	0 (-2, 1)	22 (17, 26)	19 (14, 25)	-2 (-5, 1)	1 (0 to 3)	0.09
MRC scale scores	4 (2, 4)	4 (3, 4)	0 (0, 0)	3 (2, 4)	3 (2, 4)	0 (-1, 1)	0 (0 to 1)	0.46
Pre- to post-intervention data and change illustrated as median (IQR); between-group effect size estimates presented as the median of differences (95% CI). *p*-value <0.05 is considered significant. The Mann-Whitney U test was used to compare effect size between groups.								

4.4.6 **Effect of BRJ on exercise capacity (6MWT)**

There were (n = 44) participants, (n = 20) in the placebo arm vs (n = 24) in the nitrate-rich arm, from the total enrolled participants (n = 81), who were able to attend our research laboratory during COVID-19 lockdowns and conduct the 6MWT. They performed the 6MWT at baseline (Visit 1) and 12 weeks post-intervention (Visit 2). There was a clinically significant increase in 6MWD (metres) following nitrate-rich beetroot juice compared to placebo, mean difference (95% CI), 30.04 metres [15.7 to 44.4], $p < 0.0001$; Figure 4.6).

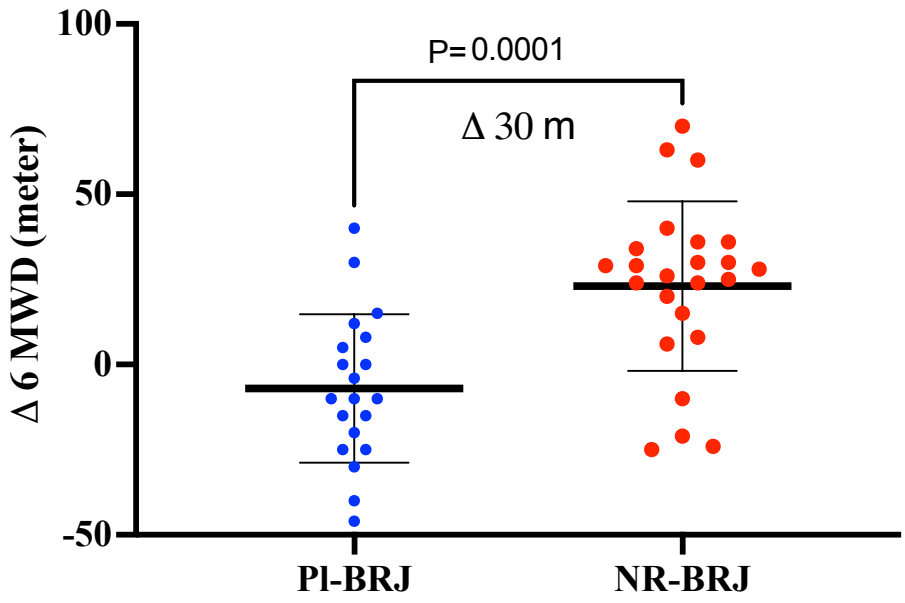


Figure 4-5 Effect of dietary NO_3^- supplementation on 6MWD (metres) in Pl-BRJ (n = 20) and NR-BRJ (n = 24) COPD individuals

The blue dots represent individual change, with the solid line representing the group mean value, and whiskers (±SD) values. Unpaired t-test was used to compare the change in 6MWT distance covered between treatment groups, and calculated treatment effect: Δ30.04 metres [15.7 to 44.4], $p < 0.0001$.

Further details about pre-to-post data and changes in 6MWD between groups are presented below in Table 4.4.

Table 4.4 Effects of dietary NO_3^- supplementation on 6MWD from baseline to post-intervention

	Pl-BRJ (n = 20)			NR-BRJ (n = 24)			Effect size (95% CI)	Between groups *p*-value
Variables	Pre	Post	Change	Pre	Post	Change		
6MWD (metre)	362.5 (±91.4)	356 (±98.0)	-7 (±21.8)	384.5 (±79.0)	407.5 (±72.4)	23 (±24.9)	30.0 (15.7 to 44.4)	<0.0001
Pre- to post-intervention data and change illustrated as mean (±SD); between-group effect size estimates presented as the mean difference (95% CI). *p*-value <0.05 is considered significant. Unpaired t-test was used to compare effect size between groups.								

4.4.7 Effect of BRJ on plasma NO_x concentrations

Data on plasma [NO_3^-] and [NO_2^-] concentrations were available for 44 subjects. As shown in Figure 4.7 A-B, dietary NR intake was associated with significant rises in the plasma [NO_3^-] concentration at 12 weeks, but a modest rise observed, with plasma [NO_2^-] concentration not reaching significant compared to Pl, mean differences (95% CI), Δ 72.4 μM, (60.4 to 84.3) $p <$ 0.0001, and Δ 0.30 μM, (-0.01 to 0.58) $p = 0.059$, respectively. Analysis of the baseline plasma [NO_3^-] concentrations between treatment groups showed there was no difference, NR-BRJ vs Pl-BRJ (8.42 ±4.8 μM vs. 8.13 ±7.8 μM, $p = 0.88$). It is noteworthy that baseline data for plasma [NO_2^-] revealed low nitrite concentrations in both arms below the quantifiable limit (0.2 μM) in many baseline samples. Hence, I corrected these values to the assay manufacturing recommended quantifiable limit value (0.02 μM).

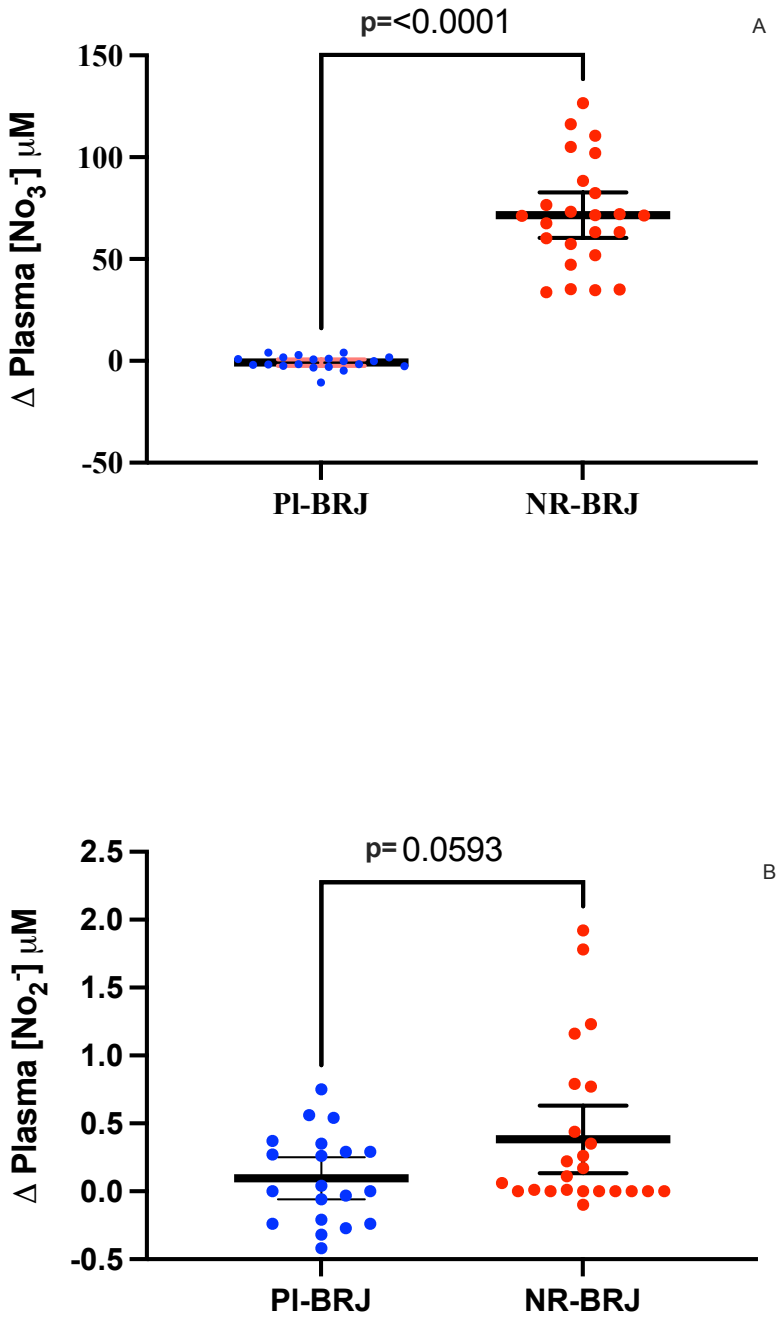


Figure 4-6 Effect of dietary NO_3^- supplementation on plasma [NO3-] and [NO_2^-] (μM) concentrations in individuals with COPD

The blue dots represent individuals' change, Pl-BRJ (n=20) and NR-BRJ (n=24), with the solid line representing the group mean value and whiskers (±SD) values. An unpaired t-test was used to compare change in changes in [NO3-] and [NO_2-] concentration between treatment groups, mean differences (95% CI), Δ 72.4 μM, (60.4 to 84.3) $p < 0.0001$ and Δ 0.30 μM, (-0.01 to 0.58) $p = 0.059$, respectively. Abbreviations: NO_3-, nitrate; NO_2-, nitrite.

4.4.8 **Effect of BRJ on FeNO levels**

There was a statistically significant difference observed in FeNO levels following consumption of NR-BRJ compared to the Pl-BRJ, mean difference (95% CI), Δ 12.0 ppb (3.57 to 20.5), p = 0.006 (Figure 4.9). Within the treatment conditions in the NR-BRJ group there was a statistically significant increase on FeNO levels; 23.5 (±13.7) ppb to 38.4 (±22.2) ppb; estimated mean change (95% CI), 15 (6.5, 23.3) ppb. In the placebo BRJ group, there was no significant increase change on FeNO levels 19.6 (±6.8) ppb to 22.8 (±6.9) ppb; estimated mean change (95% CI), 3 (0.24,6.5) ppb.

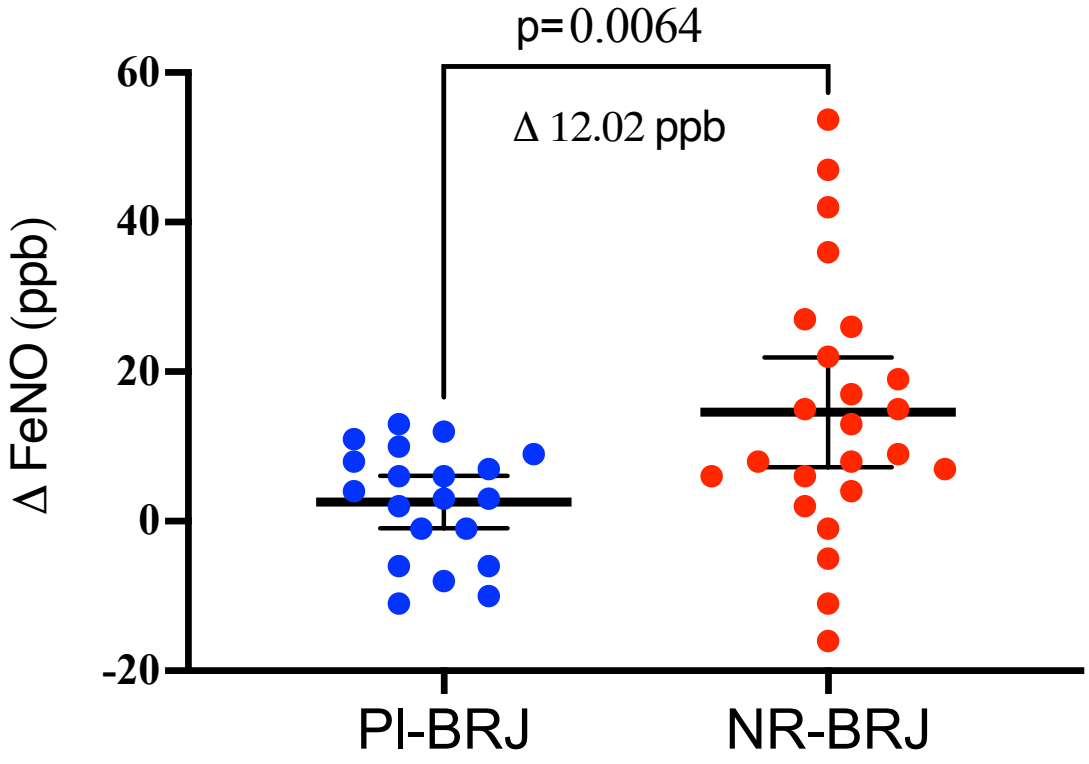


Figure 4-7 Effect of dietary NO_3^- supplementation on exhaled NO (FeNO) level in the Pl-BRJ (n=20) and NR-BRJ (n=24) in individuals with COPD

The blue dots represent individual change, with the solid line representing the group mean value, and whiskers (±SD) values. An unpaired t-test was used to compare the change in FeNO levels between treatment groups, mean differences (95% CI), Δ 12.0 ppb (3.57 to 20.5), p = 0.006. Abbreviations: FeNO, fractional exhaled nitric oxide; ppb, parts per billion.

4.4.9 Correlations of change in SBP and plasma [NO_3^-] in NR-BRJ group

I ran Spearman's rank-order correlation to define the relationship between post plasma [NO_3^-] concentration and the main endpoint change home SBP in the NR-BRJ group (n = 36). There was a strong significant negative correlation between change in home SBP and change plasma [NO_3^-] (rho = -0.558 (95% CI: -0.9, -0.2); p= 0.005) (Figure 4.10).

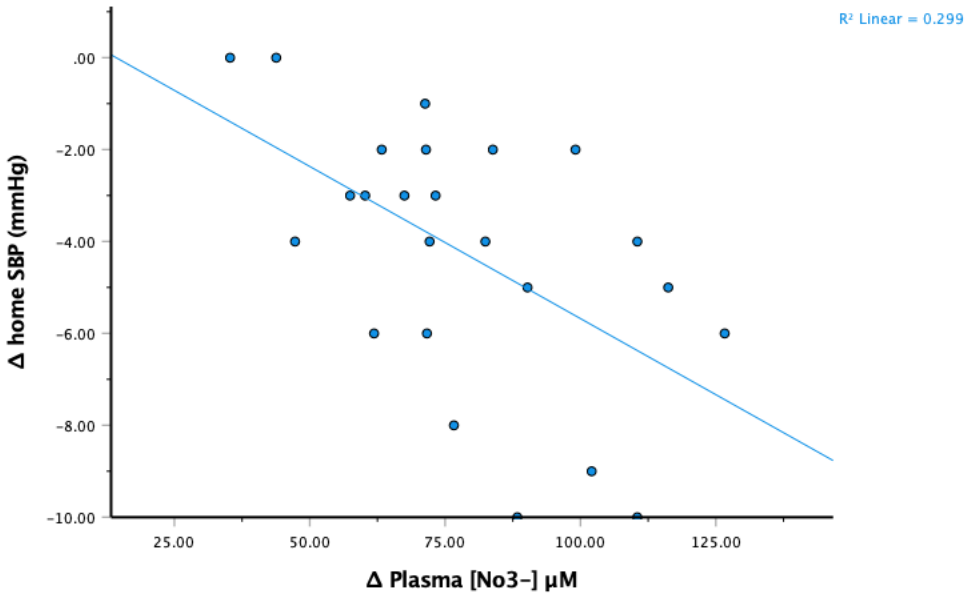


Figure 4-8 Correlation of absolute changes in resting SBP and change plasma [NO3-] concentration in the NR-BRJ group (n = 24).

Finally, I conducted a multiple linear regression analysis to assess the association between our intervention and the change in HSBP after adjusting for possible confounding variables. I found our intervention to be associated with lowering SBP (β= -0.607, t= -6.6, *p*=<0.001) after adjusting for treatment, FEV1%, number of exacerbations in the past 12 months, treatment with ACEi, and diagnosis with HTN. The result shows that 45.0% of the variance in the changed HSBP can be explained by the model in Table 4.7; F (5, 64) = 10.5, p < 0.001.

Table 4.5 Multiple linear regression model to control for confounding variables on change in HSBP

Parameters	**Regression Coefficient β**	**P**
Change in HSBP		
Variables included: intervention, FEV1%(pred), number of exacerbations in past 12 months, treated with ACEi, and diagnosed with HTN (R^2=0.45, P<0.001)		
Intervention treatment	-.607	<0.001
FEV1%(pred)	.019	0.86
Number of exacerbations in the past 12 months	.193	0.05
Angiotensin-converting enzyme inhibitors (ACEi)	-.274	0.03
Hypertension	.118	0.23

4.5 Discussion

4.5.1 Summary of results

The main finding of the study is that daily single-dose ingestion of NR-BRJ for 12 weeks lowers SBP compared to Pl-BRJ in individuals with COPD with high BP. In addition, I observed an improvement in the 6MWD and a significant elevation in FeNO level associated with a substantial rise in circulating nitrate. I found also a significant negative correlation between change in home SBP and change plasma [NO_3^-] in the active BRJ group only. These results suggest that chronic consumption of BRJ could be a potential preventative and therapeutic strategy to lower BP, subsequently reducing the risk of developing CVD and improving exercise capacity.

4.5.2 Significance of the results

4.5.2.1 Nitrate-rich BRJ exerts a sustained lowering effect on SBP

The current study findings demonstrate that longer-term supplementation of NR-BRJ exerted a sustained lowering effect on resting SBP associated with increased circulatory nitrate concentration compared to Pl-BRJ treatment. This is the first demonstration of such a permanent SBP-lowering benefit via long-term supplementation (12 weeks) of BRJ compared to placebo to date, in COPD or any population. These results suggest that longer-term consumption of BRJ may potentially have important implications for the primary and secondary prevention of CV risks in individuals with COPD. The INTERSALT epidemiological study (1991), which included results of BP parameters from more than 10,000 men and women, showed a 3-mmHg reduction of SBP in the population would result in an 8% overall reduction in mortality due to a stroke, a 5% reduction in mortality due to CHD, and a 4% decrease in all-cause mortality (Stamler, 1997). The Heart Outcome

Prevention Evaluation (HOPE) study also showed that a modest reduction of SBP by 3.3 mm Hg and DBP by 1.4 mmHg was associated with a 22% reduction in relative risk of cardiovascular mortality, myocardial infarction, and stroke (Dagenais, 2001).

The finding in this chapter further supports the potential advantageous effects of BRJ on BP reported by previous studies (detailed in chapter 1). My result shows a drop in the BP in the NR-BRJ group similar to that reported by Kapil and Pavitt (Vikas Kapil, 2015; Pavitt, 2020). However, the key strengths of this study are its long duration (12 weeks) study and being without PR. Whereas Kapil's study was a 4-week study and Pavitt's study added BRJ intervention to PR program in both arms of the study which was not the case in my study. Kapil et al., (2015) reported that dietary nitrate intake over four weeks in hypertensive groups with no manifest CVD was associated with a significant reduction in home SBP, 8.1 mmHg (3.8–12.4, P<0.001), in both subgroups, drug-naïve and treated hypertensives (Vikas Kapil, 2015). Pavitt and colleagues (2020) conducted the largest double-blind parallel-groups RCT over eight weeks in the context of a PR programme (±800 mg nitrate) using BRJ supplementation. The intervention group had a median difference of 5 mmHg drop in SBP compared to the placebo (Pavitt, 2020) In general, it seems that BRJ intervention in our study might have BP lowering effect even without exercise programs.

Our findings are also very similar to the overall findings from meta-analyses by Jackson *et al.* (2018), Ashor *et al.* (2015), and He *et al.* (2021) (Ashor, Lara, & Siervo, 2017; He, 2021; Jackson, 2018). Their meta-analyses findings indicated NR-BRJ/inorganic nitrate was associated with a significant reduction in clinical resting SBP, MD (95%CI), *p-value*: -4.1 mm Hg (-6.1 to -2.2), *P* <0.001; -4.80 mm Hg (-6.59 to -3.01), *P* <0.0001; -3.90 mmHg (-5.23 to -2.57, P < 0.001), respectively. However, the authors of the meta-analyses conducted

subgroup analyses and reported that the reduction in SBP was only significant when measured at rest in the clinic, as compared to ABPM and HBPM methods. More notably, only a small number of RCTs were included in those meta-analyses using the ABPM and HBPM to measure BP. A possible explanation for this may be that although ABPM and HBPM are accurate and recommended measurements for diagnosing and monitoring BP compared to the clinical method, their applicability in clinical settings or research is very sparse due to being expensive method (Sega, 2005).The number of participants in the current study (n = 70) was high compared to other studies that investigated BRJ on BP in patients with HTN (Jajja, 2014; Vikas Kapil, 2015).

Our findings contrast with a previous study by Bondonno and colleagues (2015), which found no reducing effects of BRJ on home SBP in subjects (n = 27) treated for hypertension (p = 0.62) (Bondonno, 2015). However, this study is different from our study, as the participants of Bondonno's study were mostly females (63%) and had a lower baseline SBP (mean ± SD: 132.9 ± 11.8). There are some methodological issues for obtaining BP measurements in clinics, such as bias resulting from the white-coat syndrome, standardisation of protocol, or operator bias (Pickering, 2005). Also, it has been hypothesised that the effect of BP-lowering drugs in patients is proportional to pre-treatment BP level (Law, Wald, Morris, & Jordan, 2003). Gilchrist and colleagues evaluated the effect of (±465 mg) NR- BRJ on ABPM over 14 days in (n = 27) subjects and concluded there was no effect (Gilchrist, 2013). A possible explanation for this might include subjects already taking vasoactive medications or the timing at which the participants consumed the BRJ during the evening meal. Unlike Gilchrist study inclusion criteria, I excluded participants on vasoactive agents, and I instructed them to drink the BRJ at 9:00 in the morning.

Nevertheless, contrary to our one-way ANOVA findings in Table 4.2, Kapil and co-workers (2015) subgroups analysis revealed that people consuming NR-BRJ and using BP-lowering drugs experienced a significantly great drop in BP outcomes as compared to people consuming NR-BRJ only. (Vikas Kapil, 2015). This study drove us to examine the relationship between antihypertensive drug types and changes in HSBP. Subsequently, I conducted a multiple linear regression (Table 4.5), and the regression slope for ACEi was (β -.213, P =0.02). I then conducted a subgroup analysis to compare the subjects using ACEi with those not using ACEi in the NR-BRJ group. I found approximately twice the reduction in the NR-BRJ subjects' HSBP when using ACEi (-3 mmHg vs -6 mmHg, P = 0.007). Our result, to some extent, is consistent with the Kapil study (Vikas Kapil, 2015), although the degree of change was smaller in our study. Interestingly, the result appears not to be related to the baseline SBP, as both groups of subjects with and without ACEi had a similar baseline (p = 0.117). Evidence shows that the angiotensin-converting enzyme (ACE) biologically lowers the effects of the vasoactive peptide, bradykinin, which mediates the release of the NO (Brown, Blais Jr, Gandhi, & Adam, 1998). ACEi agents, on the other hand, decrease ACE levels (Tojo, 1996). Consequently, bradykinin activity resumes, leading to an increase in NO activity and vessel dilatation. Also, previous laboratory work on animals has shown that induced hypertension in rats reduces NOS activity, which was reversed by ACEi drugs (Tojo, 1996).

I found a statistically significant lowering effect on MAP between conditions with estimated median differences (-3 mmHg, (P =0.03), Figure. 4.2). This finding matches the three studies included in the meta-analysis reported in Chapter 3 (Curtis, 2015; Conor P. Kerley, 2015; Pavitt, 2020).

On the other hand, the current study did not find a significant difference in change in DBP at 12 weeks between NR-BRJ and Pl-BRJ; median of differences (IQR)) -1 mm Hg (-5, 3, P = 0.17; Figure 4.2). Although this result did not reach a significant level, our meta-analysis in Chapter 3 showed that BRJ could improve all three parameters of BP (SBP, DBP, and MAP). Our results in this chapter showed a difference (not significant) in DBP, but it is possible that repeating this study on a larger sample size might improve the power and help detect the between-group difference. Similar findings were reported by studies including hypertensive and type 2 diabetes (Bondonno, 2015; Gilchrist, 2013). Bondonno *et al.* (2015) reported that short-term intakes of BRJ (~400 mg/d) compared to the placebo did not result in significantly lowering DBP measured via 24-hr ABPM in the treated hypertensive group MD (95% IC) 73.0 (69.8, 76.3; P = 0.51) (Bondonno, 2015). Gilchrist *et al.*, (2013), in a crossover RCT of individuals with type 2 diabetes, observed no effect on 24-hr ABPM DBP between placebo and active-BRJ groups, MD (95% IC), 1.9 mm Hg (−0.4 to 4.1, P =0.11) (Gilchrist, 2013). A possible explanation for this may be related to heterogeneity among COPD participants, which led to non-responders to NO_3^- supplementation in the current study. Additionally, there are several confounding factors, including ageing-related change in oral microbiota, change in gastric redox environment, or change in the efficiency of enzymatic reductase activity (i.e., deoxyhaemoglobin, or xanthine oxidoreductase) (M Carlström, 2018). Other explanations might be that the COVID-19 lockdown attenuated the participants' behaviours of physical activity to presumably less active. Another possible behaviour change might alter to potential benefits of NR-BRJ on BP is the excessive drinking of alcohol above the guidelines. The UK government released a report emphasising the increased amount of alcohol consumption among citizens during shielding (Kilian, 2022). Alcohol may increase vascular reactivity due to an increase in intracellular calcium levels and stimulation of angiotensin II, leading to inhibition of NO production (Husain, Ansari, & Ferder, 2014).

4.5.2.2 **Nitrate-rich BRJ effect on exercise capacity**

Another important clinically relevant finding in the current results suggests that prolong NR-BRJ supplement may induce clinically meaningful improvement in exercise capacity (6MWT) in patients with COPD. The 6MWT is a valid and reliable assessment tool for functional exercise capacity in patients with COPD (Hernandes, 2011). The results, as shown in Table 4.4, indicate that the NR-BRJ significantly ($P < 0.001$) improved the distance covered in metres at 12 weeks during the 6MWT as compared to Pl-BRJ (23±24.9 m vs -7±21.8 m). The estimated treatment effect of NR-BRJ on the six-minute walk distance (6MWD) is 30.4 m (± 7.1). This treatment effect in our study exceeded the reported minimal important difference (MID) for 6MWD in individuals with COPD (Holland, 2010). This finding broadly supports the work of other studies in this area reported in our published meta-analysis linking NR-BRJ with the improvement of exercise capacity in COPD (Alsulayyim, Alasmari, Alghamdi, Polkey, & Hopkinson, 2021).

Recently, Pavitt and colleagues found NR-BRJ treatment augmented exercise capacity improvements during PR program compared to placebo; median (IQR) change in ISWT distance +60 m (10, 85) vs +30 m (0, 70), with estimated treatment effect 30 m (95% CI 10 to 40); p=0.027 (Pavitt, 2020). Furthermore, studies from animals and healthy subjects demonstrated that dietary nitrate supplementations have been shown to optimise mitochondrial efficiency and blood flow in skeletal muscles (F. J. Larsen, 2011). BRJ was also found to reduce the O_2 consumption in healthy participants by 10% and 19% during submaximal exercise (Vanhatalo, 2010). Another possible explanation for this improvement might be related to the use of ACEi agents. Curtis and colleagues' reported RCT findings indicated that ACEi agents may augment the effect of exercise during the PR programme in COPD patients (Curtis, 2016). They suggested that ACEi may induce bradykinin, thereby

enhancing NO activity and capillary vasodilation in the skeletal muscle of individuals with severe COPD.

In contrast, previous RCTs by Friis (2017) and Shepherd (2015) reported NR-BRJ supplementation as compared to Pl-BRJ during short durations (2.5 to 7 days), did not increase 6MWD using the cycle, participants might reach a ceiling effect , (515±35 m vs PL: 520±38 m, P = 0.46 and 456±86 vs 449±79m; P=0.37, respectively (Friis, 2017; Shepherd, 2015). A possible explanation for this inconsistency might be due to a variation in doses and the timing of assessment. Also, individuals with COPD are at a high risk of falls due to imbalance; hence, walking tests may be a more appropriate choice than the cycle test in this group. The number of participants in these trials were low, and they included mostly mild-to-moderate COPD, excluding severe and very severe patients who are characterised by severe hypoxemia. It is proposed that the exogenous NO pathway involved the reduction of NO_3^- to NO_2^- to NO enhanced during hypoxia on the body (Castello, David, McClure, Crook, & Poyton, 2006). Accordingly, further trials are needed to assess whether this outcome can be replicated in severe and very severe COPD stages during a longer duration, as well as whether increasing daily dosing of BRJ could induce a greater benefit in 6MWD.

4.5.2.3 **Effect of nitrate-rich BRJ on circulatory plasma [NO_x] concentrations**

Our findings have shown plasma levels of NO_3^- rising substantially following supplementation in the NR-BRJ, compared to very low levels in Pl-BRJ. This suggests NO_3^- and NO_2^- as a biomarker for NO bioavailability (Kleinbongard, 2003). An explanation for this increase in plasma NO_3^- and NO_2^- after 12 weeks of NR-BRJ intake was indicative of an increased nitrate metabolism through the enterosalivary nitrate-nitrite-NO pathway

(Lundberg, 2011). Another possible explanation is that daily intake of concentrated NO_3^- in BRJ for 90 days may modulate the oral microbiome bacteria that reduce nitrate to nitrite. On the other hand, although NO_2^- levels increased numerically following NR-BRJ, the difference was not statistically significant compared to Pl. It should be noted that plasma NO_2^- concentration fell below the limit of quantifiability in the majority of baseline samples. An explanation for this is that the samples were not pre-treated with a thiol-blocking agent before centrifugation. Such treatment ensures there is no flux of NO_2^- across cell membranes, which may lower plasma concentrations following uptake by erythrocytes and white cells during processing (Tsikas, 1997).

4.5.2.4 **Effect of nitrate-rich BRJ on FeNO levels**

Another important clinical finding was the increase in FeNO observed following NR-BRJ dosing compared to the placebo. This finding is in line with previous evidence on healthy individuals and concludes that dietary nitrate supplementations are associated with an increase in FeNO and are proportional to the increase in salivary nitrate and nitrite (Zetterquist, Pedroletti, Lundberg, & Alving, 1999). Pavitt (2020) and Behnia (2018) found that their results indicated a significant increase in FeNO in the NR-BRJ compared to the placebo (22 vs. 11, $P = 0.002$, 38 vs. 26, $p = < 0.05$, respectively). There are several possible explanations for this result. Suggested mechanisms state that increased levels of NO in exhaled air may be determined by increased action of inducible NOS, located in the airways following diffusion of NO into the capillary circulation (R. W. Hyde, 1997). Furthermore, a strong positive correlation was noticed between changes in FeNO levels and plasma NO_3^- levels ($rho = .473$, $P < 0.001$), suggesting that this could be used as a potential biomarker to monitor participants' compliance in intervention trials.

4.5.3 **Possible mechanisms of action**

The method of action of food content-nitrate in reducing BP is currently unclear, although several have been proposed. It is likely that the BP-lowering effects of BRJ I observed is due to endogenous sequential reduction of NO_3^- to NO via the enterosalivary nitrate-nitrite-NO pathway which stimulating the vasodilatory action of the arteries (Duncan, 1995). Physiologically, ingested dietary rich with NO^-_3 dietary rich NO^-_3 enters the bloodstream through the stomach, with the majority being excreted renally. Approximately 25% of ingested nitrate is extracted by the salivary glands via the entero-salivary circulation (Lundberg, 2010), where concentrations may be over ten-fold greater than those measured in the plasma. It is then excreted in saliva and reduced from inert nitrate NO_3^- to bioactive nitrite NO_2^- via nitrate reductases produced by the oral commensal facultative anaerobic bacteria. Saliva rich with NO_2^- is swallowed and further reduced to NO under acidic condition of the stomach, while some of the NO_2^- enters the circulation directly. Circulating NO_2^- is reduced to NO in the blood or vasculature or muscle cells in process involves enzymatic and non-enzymatic components such as deoxyhaemoglobin, xanthine oxidoreductase (XOR), aldehyde oxidase, and vitamin C (J. O. Lundberg, 2004). Finally, NO signals the downstream pathways by inducing sGC and cGMP for biological effects of muscle relaxation or antiplatelet effects (N. Benjamin, 1994; J. O. Lundberg, 2004; Lundberg, 1994).

Nevertheless, the increase in exercise capacity observed in participants' 6MWD following BRJ consumption may contributed to improved endothelial function (A. M. Jones, Thompson, Wylie, & Vanhatalo, 2018). Functional endothelium enhance the blood flow to muscles, thereby possibly facilitating skeletal muscle O_2 delivery, a reduction in the O_2 cost

required for mitochondrial ATP synthesis, and an increase in the efficiency of ATP contraction coupling (Bailey, 2009; F. J. Larsen, 2011).

4.5.4 **Critique of method**

At 12 weeks, the ON-BC is the longest RCT in any population that I have identified, evaluating the effects of BRJ on individuals with elevated BP. Unlike other studies of COPD, I did not exclude people based on GOLD stages, which makes our study more representative of the COPD population. I also used an at-home BP monitor to collect the BP data. In clinical practice, home BP monitors are regarded by many hypertension guidelines as a more reliable method to measure and monitor BP parameters compared to the in-clinic method (Sega, 2005). There was also a low dropout rate, suggesting that dietary nitrate supplementation, in the form of BRJ and placebo, are well tolerated. Although this is the longest study to date, longer studies are needed to examine the effects of BRJ at CVD event rates and establish the effect of increased exercise capacity on daily life. It would be beneficial if Future RCT include measuring the SBP in weekly bases during the trial duration to understand whether the BRJ effect sustained the same from day 1 to day 90. Also, we should take in consideration measuring the post intervention SBP measurement after different time point (0 time, in 2 weeks, in 4 weeks) washout.

Nitrate-deplete BRJ is an ideal placebo given its identical taste and appearance(Kelly, 2013). However, BRJ may contain several bioactive compounds, such as vitamin C, carotenoids, phenolics, and betalains, that could also have biological effects (Hoffman, 2011). Although the use of NO_3^--depleted BRJ as a control allows us to distinguish the effect of NO_3^- itself, this may have underestimated the benefits of beetroot juice if other factors are important.

An important feature of the study is that about 35% of the subjects participated entirely remotely, because of the COVID-19 pandemic. This meant that it was not possible to capture some secondary endpoints in all participants. However, numbers of face-to-face vs remote were similar in both arms mean that this is not likely to have influenced the primary endpoint. Also, the results of this study should be interpreted with caution, as the majority of the participants were whites of European extraction.

Plasma NO_2^- concentration fell below the limit of quantifiability in the majority of baseline samples. I did not pre-treat the samples with a thiol-blocking agent before centrifugation. Such treatment ensures there is no flux of NO_2^- across cell membranes, which may lower plasma nitrite concentrations following uptake by erythrocytes and white cells during processing (Tsikas, 1997).

4.6 Conclusion

Our results show that NR-BRJ is associated with sustained reduction in SBP and an improvement in 6MWD as compared to placebo in COPD people with SBP of ≥130 mm Hg. BRJ is therefore a promising, safe, and low-cost potential therapy to reduce vascular risk in this population. The improvement in exercise capacity seen is also notable.
Although this study has shown an improvement in BP, it is not clear what other effects BRJ has on the vascular system. In the next chapter, I describe further investigations to explore the impact of NR-BRJ on measures of vascular function.

Chapter 5: Effects of dietary NO_3^- supplementation on vascular function in COPD: a mechanistic substudy

5.1 Introduction

Previously, in Chapter 4, I found a significant association between NR-BRJ and the reduction in SBP. In addition, only one study investigated the effect of BRJ on endothelial function (EF) during the pulmonary rehabilitation exercise programme, measured by flow-mediated dilation (FMD) in patients with COPD (Pavitt, 2020). In vivo endothelial (vasodilator) function is mediated by vascular release of NO signaling molecules. As such, reduction of endothelial derived NO via modulation or inhibition of eNOS activity is a contributory factor of endothelial dysfunction (Sibal, C Agarwal, D Home, & H Boger, 2010). As outlined earlier, accumulation of endogenous asymmetric dimethylarginine added (ADMA) is a competitive inhibitor of the eNOS enzyme and considered to be a risk for developing essential hypertension and CVD (Vallance & Leiper, 2004).

Nonetheless, studies to date have been short term, and no study has thoroughly investigated further the molecular mechanism of the improvement observed in endothelial function following dietary NO_3^- supplementation in any clinical trial. Accordingly, I carried out a mechanistic substudy to investigate whether the reduction I observed in Chapter 4 on SBP following NR-BRJ is associated with changes in endothelial function and plasma ADMA levels.

5.2 Subjects and methods

5.2.1 Patient selection

All the participants in this chapter were drawn from the ON-BC study described in Chapter 4 and had provided written, informed consent prior to enrolment. In this substudy, the aim was to include all the subjects recruited in the previous chapter (n = 70). Due to the COVID-19

pandemic, it became extremely difficult to have participants come to the clinic for several reasons that I have explained in the previous chapter.

In total, 44 participants (placebo BRJ (n = 19) vs nitrate-rich BRJ (n = 21)) enrolled face-to-face in the ON-BC study and were able to attend for vascular endothelial function assessment using the EndoPat® device. The assessment inclusion criteria were as follows:

- Participants diagnosed with COPD based on GOLD criteria
- Male or female between 18 and 85 years of age
- Able to understand and comply with protocol requirements, instructions, and protocol-stated restrictions
- An SBP of ≧130 mmHg (determined via home BP monitoring)

Exclusion criteria:

- Participants with finger deformities or injuries
- Unable to provide informed consent
- Within one month of diagnosis of AECOPD
- Having significant peripheral artery disease in the arms
- Having significant renal impairment (estimated glomerular filtration rate (eGFR) <30 ml. min1)
- Taking oral nitrate medications influencing plasma nitrate/nitrite levels
- Used Beet Shots within one month

5.2.2 Trial protocol

Participants and assessors were both blinded to the allocation of treatment (treatment vs placebo). The participants attended two experiment visits (pre- and post-intervention), separated by 11 weeks (90 ± 2 (SD) days). An overview of the study protocol flow diagram is given in Figure 5.1.

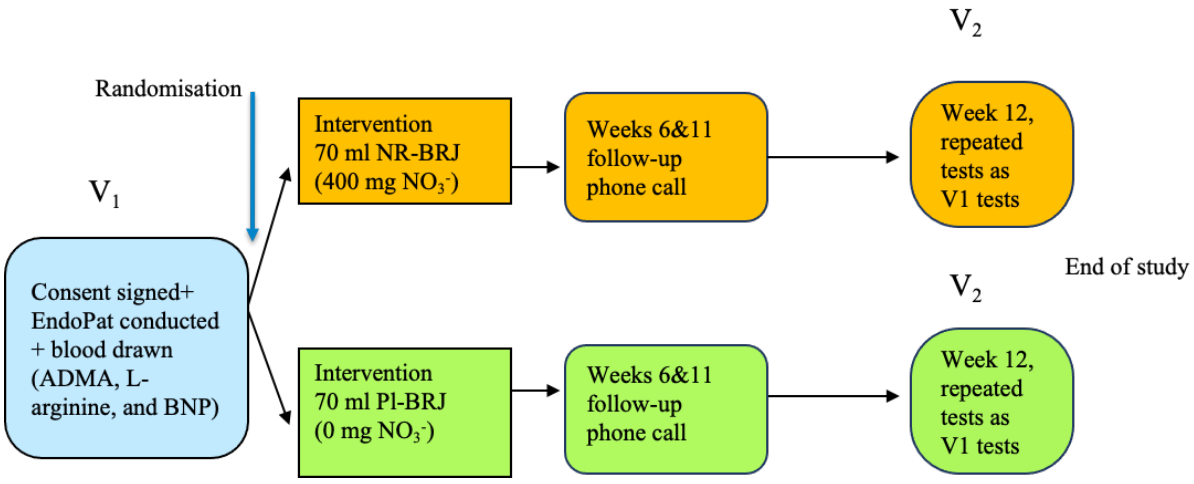


Figure 5-1 the study protocol flow diagram

Visit 1 (pre-intervention): I standardised the procedures in both visits so participants did the same steps at both visits

- Before attending the visits, participants were instructed to avoid vigorous exercise, caffeine, and alcohol for 24 hours before each visit. They were also instructed to avoid eating high nitrate-content foods or juices provided in a list for 48 hours before each experimental visit. The participants underwent the following baseline demographics, anthropometrics, and physiological outcomes.

- Upon arrival, participants rested quietly in a seated position for 10 min before brachial BP was measured twice, one minute apart from each other, using the Omron M3 Comfort (HEM-7134-E - Omron Health Care Inc., Japan).
- The participants then moved to a supine position on a bed. Following another 10 min rest, vascular function (endothelial dilatation function and arterial stiffness) were assessed by measuring the RHI and AIx@75, respectively.
- Participants then moved back to the seated position, and a blood sample was drawn from an antecubital vein for analysis of plasma ADMA and arginine/ADMA, blood BNP, and plasma NO3- and NO_2- concentrations.

Visit 2 (post-intervention) measurements were carried out in the same order and timing as the pre-intervention visit.

5.2.3 Measurements and procedures

5.2.6.1 Peripheral arterial tonometry (PAT)

I assessed the vascular endothelial function via reactive hyperaemia index (RHI) score as well as I measured marker of arterial stiffness (AIx75%) using the finger plethysmography peripheral arterial tonometry (PAT) (EndoPAT2000 - Itamar Medical Ltd., Caesarea, Israel). Further details about the methods and the measurement protocols can be found in Chapter 2: Description of Methods.

5.2.6.2 Plasma ADMA and arginine concentrations

Plasma samples were obtained through a 21-gauge butterfly needle inserted into an antecubital vein in a labelled (4 ml) EDTA vacutainer blood tube. Plasma ADMA and arginine were measured by competitive ELISA (DLD Diagnostika GmbH, Hamburg,

Germany). Further details on the analysis process can be found in Chapter 2: Description of Methods.

5.2.4 Primary endpoint

- Compare the change of endothelial RHI score between NR-BRJ treatment and placebo.

5.2.5 Exploratory endpoints

- Compare the change in arterial stiffness using AIx75% between NR-BRJ treatment and a placebo.
- Compare the change in plasma ADMA levels between NR-BRJ treatment and a placebo.
- Compare the change in plasma arginine between NR-BRJ treatment and a placebo.
- Compare the change in plasma Arginine/ADMA between NR-BRJ treatment and a placebo.
- Compare the change in plasma BNP levels between NR-BRJ treatment and a placebo.

5.2.6 Sample size, data analysis, and statistics

5.2.6.1 Sample size

Data from the ON-EPIC trial, found that a sample size of 20 was needed to detect a difference with a power of 90% at a significance level of $\alpha = 0.05$ and increase FMD by 6%

SD ± 2.5. However, in our study we sought to conduct the EndoPat RHI on all of the participants.

5.2.6.2 Data analysis and statistics

Baseline demographic, clinical anthropometrics, symptoms, and physiological variables were summarised for each group of the study. Continuous variables were presented as mean ±SD or 95% confidence intervals, or median and interquartile range (IQR). Categorical variables were presented as frequencies and percentages. Normality was assessed through a histogram and Shapiro-Wilk tests. I examined the change in RHI (change = post-intervention—baseline) in each treatment allocation and compared the mean change between the BRJ group and the placebo group. I used an unpaired t-test comparison of the change response in all outcomes between groups while a paired t-test was used for within-group comparisons. To further explore the relationship between treatment intervention groups and the two variables, I specifically intended to examine changes in RHI and AIx75. I conducted a Pearson correlation with possible confounding factors. Confounding factors were selected based on previous literature and biological plausibility. Confounding factors include gender, age, smoking history, FEV1% (pred), HR, height, and ACEi. Accordingly, I used a multiple linear regression for change in RHI, adjusting for those confounding factors. The quality of the models was described by R squared. I considered a p-value < 0.05 to indicate a statistically significant difference. Statistical analysis was performed on a per-protocol basis. Data analysis was performed using the Statistical Package for the Social Sciences v.27 (SPSS, Chicago, IL, USA), and figures were prepared using GraphPad Prism version 9.0 for Mac (GraphPad Software, San Diego, California, USA

5.3 Results

5.4.1 Participants' clinical characteristics

More than half (43 out of 70) of the participants (21 in the NR-BRJ group vs 19 in the Pl-BRJ group) from the ON-OBC study underwent assessment at baseline for RHI, and the same individuals were reassessed at the completion of the study. Out of these 43 participants, 40 were included in the final analysis. Further details of the recruitment process are presented in the CONSORT diagram (Figure 5.2). All baseline characteristics for both subgroups are shown in Table 5.1. Overall, participants in the NR-BRJ group and Pl-BRJ group were well matched at baseline measurements.

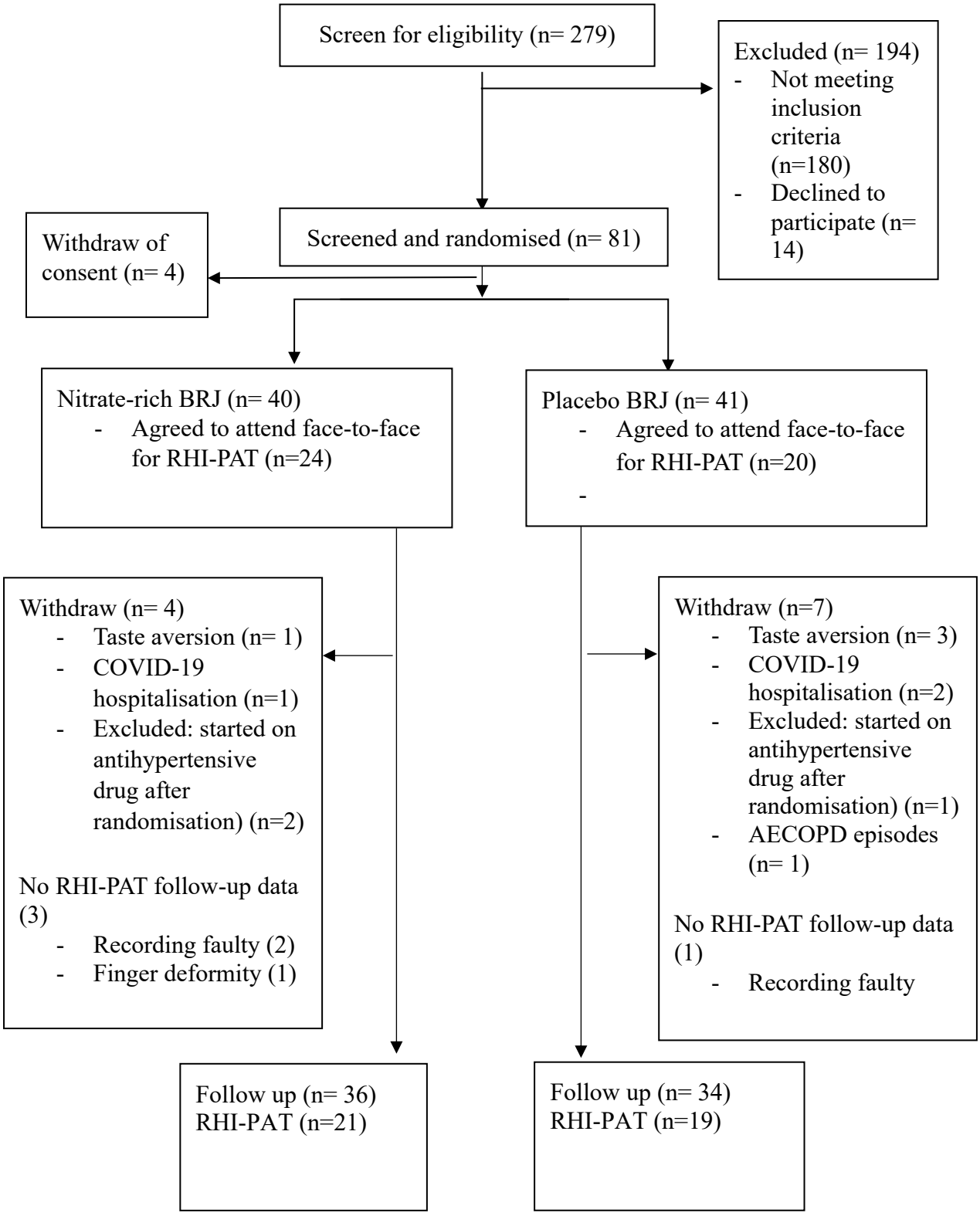


Figure 5-2 CONSORT flow diagram

Table 5.1 Demographic and baseline clinical characteristics of the subjects

	Pl-BRJ n (19)	**NR-BRJ n (21)**	**P-value**
Sex, Male n (%) ***	12 (63.2)	15 (71.4)	0.74
Age (years)**	66 (59, 68)	65 (57, 72)	0.96
Ethnicity, n (%)			
White Caucasian	19 (100)	20 (95)	1.00
Black	0 (00)	1 (5)	
Height, cm*	172 (9.6)	171 (9.3)	0.77
Weight, kg	77.1 (16.6)	76.6 (14.0)	0.91
BMI (kg/m^2)	26.0 (4.6)	26.4 (4.1)	0.76
Smoking status n (%)			
Ex- smoker	14 (74)	17 (81)	0.71
Current smoker	5 (26)	4 (19)	
Smoking Hx. (pack-years) *	33.4 (15.1)	34.6 (9.9)	0.78
FEV1 (% predicted)	43.5 (18.3)	47.6 (19.0)	0.50
GOLD grade, n (%)			0.47
COPD I	2 (10.5)	5 (23.8)	
COPD II	3 (15.8)	5 (23.8)	
COPD III	7 (36.8)	7 (33.3)	
COPD IV	7 (36.8)	4 (19.0)	
Comorbidities, n (%)			
Hypertension	7 (42.1)	8 (38.1)	0.99
Hypercholesterolaemia	4 (21.1)	3 (19.0)	1.00
IHD	3 (15.8)	5 (14.3)	0.88
OSA	2 (10.5)	2 (9.5)	1.00
Hypertension meds. n			
ACE-i/ARBs	5	4	0.71
CCBs	3	4	1.00
β-blockers	1	3	0.61
Diuretics	0	2	0.49
MRC dyspnoea score	3 (2, 4)	2 (2, 4)	0.10
CAT score	21 (7.3)	21 (6.7)	0.75
Clinic BP*			
SBP, mmHg	141 (17.9)	139 (11.8)	0.74
DBP, mmHg	75 (10.3)	79 (8.7)	0.13
Endothelial RHI score*	2.08 (0.51)	2.09 (0.40)	0.96
BNP (ng/L) *	27.5 (18, 44)	36.5 (24, 50)	0.32
AIx@75 (%)	23.2 (12.0)	21.3 (14.3)	0.65
ADMA (μmol/L) **	0.54 (0.41, 0.66)	0.47 (0.42, 0.62)	0.64
Arginine (μmol/L) **	97.7 (86.1,109)	99 (81.6, 119)	0.56
Arg./ADMA	178.1(111, 272)	172 (133, 266)	0.87

Note: Data shown are mean ±SD*, median (IQR)**, and number (%)***. *P* values are for Mann-Whitney U test or independent *t*-test between groups.

Abbreviations: BMI-body mass index; GOLD-Global Initiative for Chronic Obstructive Lung Disease; FEV1-forced expiratory volume in 1 s; IHD-Ischaemic heart disease; OSA-Obstructive sleep apnoea; ACEi-Angiotensin- converting enzyme inhibitors; ARBs-Angiotensin receptor blockers; CCB- calcium channel blockers; MRC-Medical Research Council (MRC) dyspnoea scale; CAT- COPD Assessment Test; SBP- systolic blood pressure; DBP- diastolic blood pressure; MAP- mean arterial pressure; HR- heart rate; RHI-reactive hyperaemia index; BNP-B-type natriuretic peptide; AIx@75 - augmentation index corrected for HR @75 bpm; ADMA- Asymmetric dimethylarginine.

5.4.2 Effect of NR-BRJ on vascular function measures

5.4.2.1 Effects on endothelial function (RHI score)

As illustrated in Figure 5.3, the NR-BRJ treatment showed significant improvements in the RHI score compared to the Pl-BRJ treatment (MD [95%CI]; 0.32 [0.01 to 0.63], p = 0.04). The reported data in Table 5.2 demonstrate the pre-to-post mean change of each treatment arm's RHI.

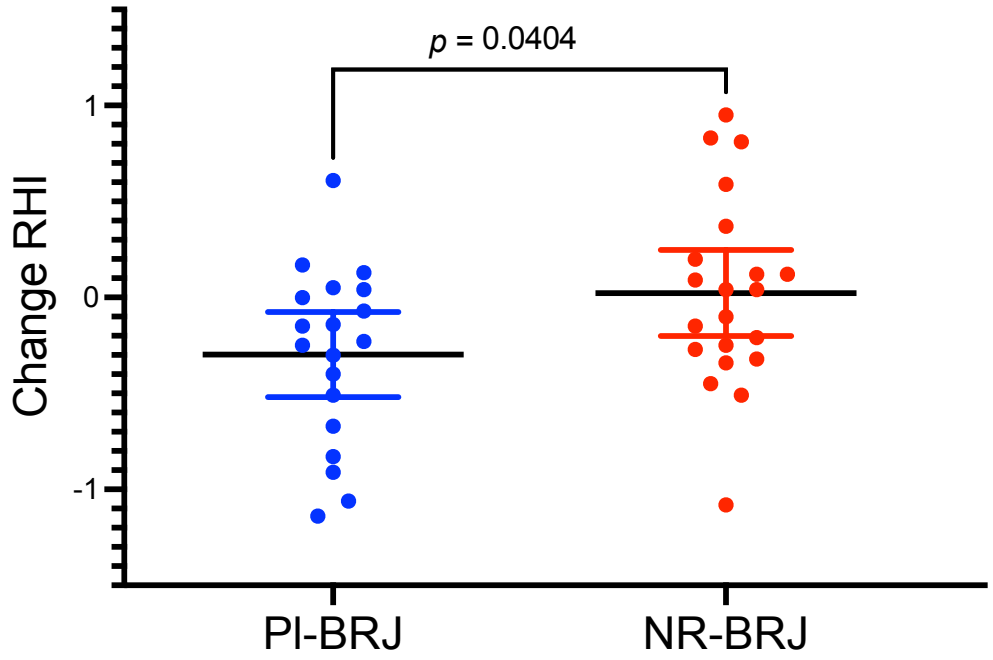


Figure 5-3 Effect of dietary NO_3^- supplementation on endothelial RHI score

Data presented in blue and red dots represent individual change in Pl-BR (n = 19) compared to NR-BRJ (n = 21). The black solid line represents the group mean value, and whiskers refer to (95% IC) values. **p*-value shown is for comparisons of the change between groups using the unpaired t-test with treatment effects Δ0.32 [0.01 to 0.63], p = 0.04).

5.4.2.2 Effects on arterial stiffness (AIx75)

The NR-BRJ treatment was associated with significant improvement in AIx75% compared to the placebo (MD [95%CI]; 7.61% [-14.3 to -0.95], p =0.03) (Figure 5.4). Further details of the pre-to-post mean change of each treatment arm are presented in Table 5.2.

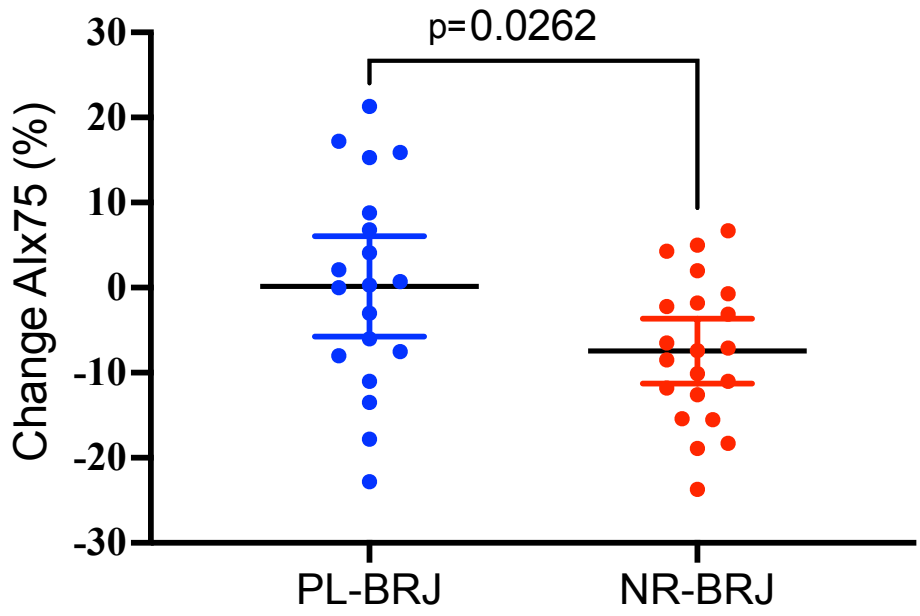


Figure 5-4 Effect of dietary NO3- supplementation on change in (AIx75 %)

Data presented in blue and red dots represent individual change in Pl-BR (n = 19) compared to NR-BRJ (n = 21). The solid line represents the group mean value and whiskers (95% IC) values. The P-value shown is for comparisons of the change between groups using the unpaired t-test with treatment effects Δ 7.61% [-14.3 to -0.95], p =0.03.

Table 5.2 Effects of dietary NO_3^- supplementation on RHI in treatment groups

	Pl-BRJ (n = 19)			NR-BRJ (n = 21)			Effect size (95% CI)	Between groups *p*-value
Variables	Pre	Post	Change	Pre	Post	Change		
RHI (score)	2.08 (±0.51)	1.91 (±0.49)	- 0.18 (±0.52)	2.09 (±0.40)	2.12 (±0.43)	0.03 (±0.46)	+ 0.32 (0.01 to 0.63)	0.04
AIx75 (%)	21.31 (±14)	21.46 (±10)	+0.15 (±13)	23.2 (±12)	15.7 (±9)	-7.46 (±8)	- 7.61% (-14.3 to - 0.95)	0.03

Change within group calculated as post value – pre-value. *The data of pre-and-post presented as mean (SD). Paired t-test for comparisons change within groups analysis. Between-group effect size estimates presented as mean differences (95% CI). *p*-value <0.05 is considered significant. A positive change in RHI score denotes a better outcome. Abbreviations: Pl-BRJ, placebo beetroot juice; NR-BRJ, nitrate-rich beetroot juice.

5.4.2.3 Effect of BRJ on plasma ADMA, Arginine, Arginine/ADMA, and BNP

A statistically significant reduction was observed in the plasma ADMA levels of the NR-BRJ group as compared to the Pl-BRJ group (MD [95%CI]; Δ-0.13 μmol//L [-0.37 to -0.01], p =0.03, Figure 5.5 A). Additionally, an improvement associated with plasma arginine levels was observed in the NR-BRJ group as compared to the Pl-BRJ group (MD [95%CI]; Δ17.1 μmol//L [8.60 to 31.6], p < 0.001, Figure 5.5 B). There was an improvement in the calculated arginine/ADMA ratio at 12 weeks in the NR-BRJ group (MD [95%CI]; Δ98.7 [28.20 to 140.5], p = 0.003) Figure 5.5 C). Further details about pre-to-post data and the mean change of each treatment arm are found in Table 5.4.

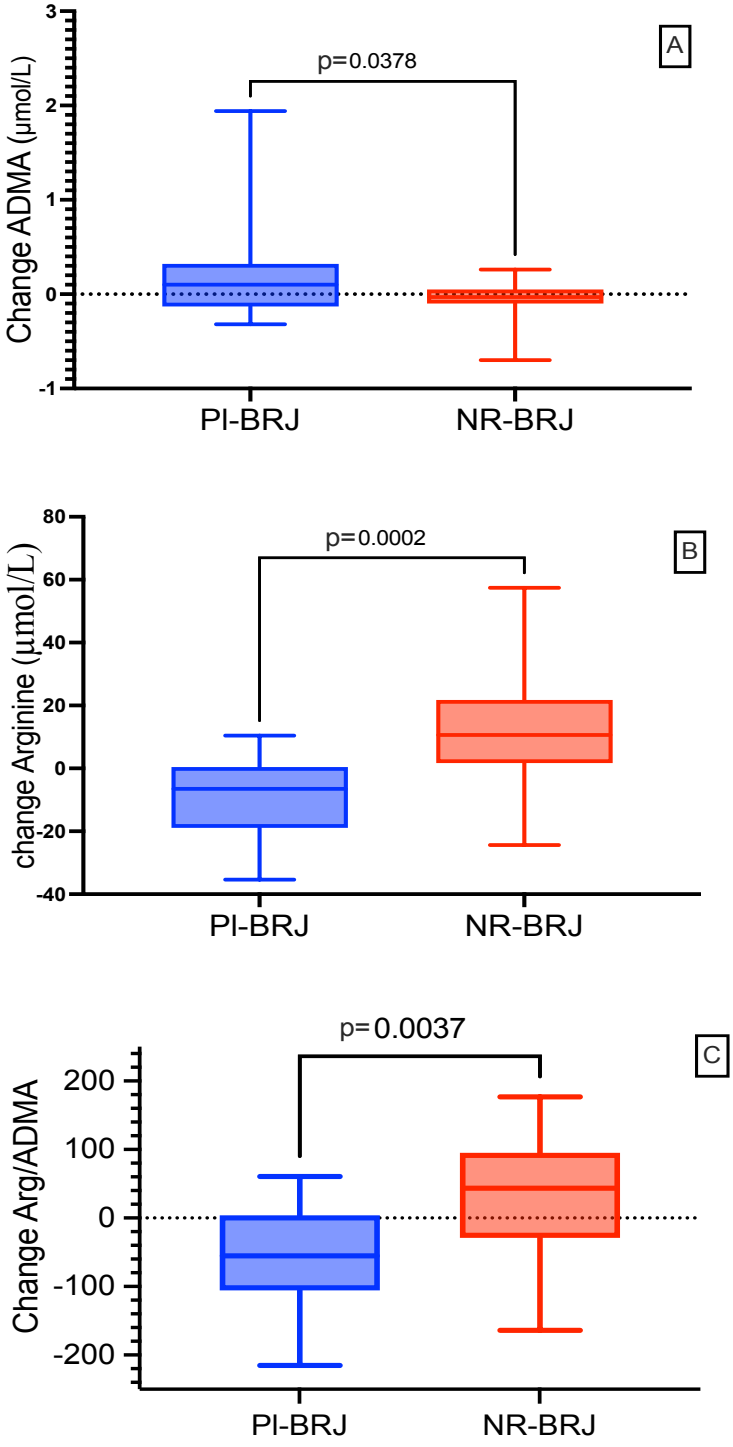


Figure 5-5 Effect of dietary NO_3^- supplementation on plasma ADMA (A), arginine (B), and arginine/ADMA (C)

The line in the box centre represents the groups' median change in Pl-BR (n = 18) compared to NR-BRJ (n = 18). The box shows 25% and 75% (IQR), and error bars show the minimum and maximum values. P-value shown is for comparison with the change in ADMA, arginine, Arg/ADMA between groups using the Mann-Whitney U test with median differences Δ-0.13 µmol//L [-0.37 to -0.01], p =0.03, Δ17.1 µmol//L [8.60 to 31.6], p <0.001, and Δ98.7 [28.20 to 140.5], p =0.003, respectively.

Table 5.3 Effects of dietary NO_3^- supplementation on plasma ADMA, arginine, Arg/ADMA, and BNP in treatment groups

	Pl-BRJ (n = 18)			NR-BRJ (n = 18)			Effect size (95% CI)	Between groups *p*-value
Variables	Pre	Post	Change	Pre	Post	Change		
ADMA (μmol/L)	0.52 (.41, .78)	0.69 (.43, 1.13)	0.10 (-0.13, 0.32)	0.54 (0.42, 0.71)	.50 (0.42, 0.63)	-0.03 (-0.10, 0.05)	- 0.13 (-0.37 to -0.01)	0.03
Arginine (μmol/L)	99 (81.6, 119)	118 (89, 140)	-6.5 (-18.9, 0.40)	97.7 (86.1,109)	87.5(70.9, 105)	10.6 (1.7, 21.8)	+ 17.1 (8.60 to 31.6)	<0.0001
Arg/ADMA	178 (111, 272)	128 (76, 216)	-55.0(-113, 3.8)	172 (133, 266)	236 (147, 320)	43.7(-29.0, 95.1)	+ 98.7 (28.20 to 140.5)	0.003
BNP (ng/L)	36.5 (24.0, 50.0)	33.5 (26.0, 44.8)	-2.5 (-12.0, 9.0)	27.5 (18.3, 43.8)	36.0 (15.3, 46.0)	-1(12.3, 9.8	-1.54 (-12.4 to 9.36)	0.44

Change within group calculated as post value – pre-value. *The data of pre-and-post are presented as median (IQR). Wilcoxon Signed-Rank Test for comparisons of change within-group analysis. Between-group effect size estimates are presented as a median of differences (95% CI). *p*-value <0.05 is considered significant. The Mann-Whitney U test was used to compare effect size between groups. Negative changes in ADMA denote better outcomes, while positive changes in arginine and arg/ADMA denote better outcomes. Abbreviations: Pl-BRJ, placebo beetroot juice; NR-BRJ, nitrate-rich beetroot juice.

5.4.3 Correlations of change in endothelial RHI score and plasma [NO3-] in NR-BRJ group

I ran Spearman's rank-order correlation to define the relationship between post plasma [NO3-] concentration and the primary endpoint change in RHI score in the active treatment group. I observed a strong significant positive correlation between change in RHI score and change in plasma [NO3-] (rho = 0.501 (95% CI: 0.08, 0.77); p = 0.02) (Figure 5.6).

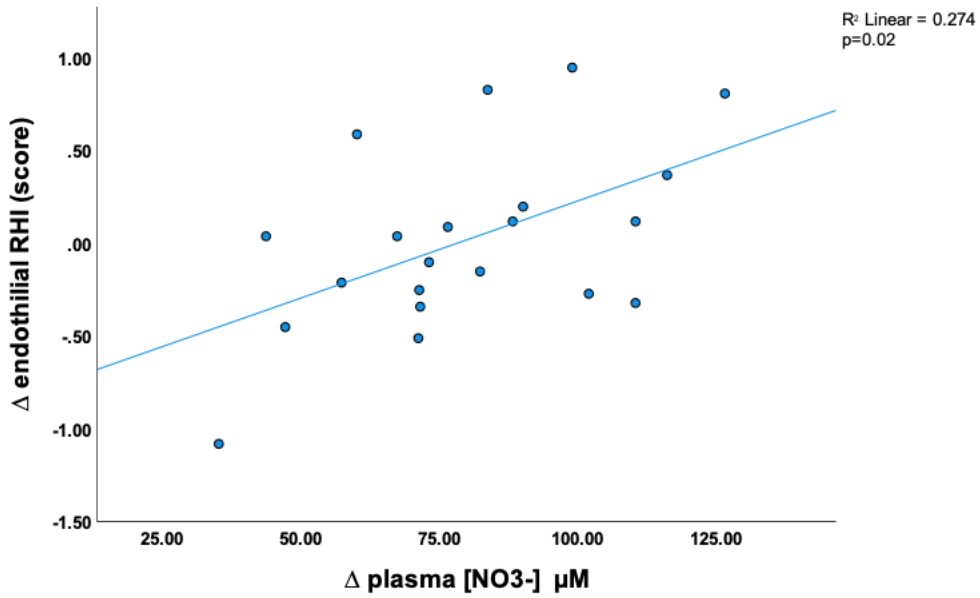


Figure 5-6 Correlation of absolute changes in endothelial RHI score and change plasma [NO3-] concentration in NR-BRJ group (n=21)

5.4 Discussion

5.4.1 Summary of results

The initial aim of this chapter was to identify whether the reported significant reduction in SBP in Chapter 4 following ingestion of NR-BRJ as compared to a placebo in COPD participants was accompanied by a change in vascular function measures. I found a significant difference in RHI scores following NR-BRJ intake (7.5%) compared with Pl-BRJ (–8.8%). I also observed a statistically significant reduction in AIx75%, circulating plasma ADMA, and increased plasma arginine in the NR-BRJ group compared to the placebo group. There was no evidence that plasma BNP levels changed in either group. The improvement observed in endothelial function after nitrate-rich BRJ supplementation may potentially have implications for the prevention of atherosclerosis and cardiovascular diseases.

5.4.2 Significance of the results

5.4.2.1 Nitrate-rich BRJ effect on endothelial RHI score

Longer-term supplementation of NR-BRJ is associated with a significantly improved endothelial function in COPD individuals with high BP. Endothelial dysfunction is a validated risk marker of CVD among patients with COPD (Barr, 2007; Moro, 2008). An effect likely attributed to the substantial increase in circulating nitrate-derived NO was observed in Chapter 4. It was found that improving endothelial function (measured via FMD) by 1% would reduce the risk of developing CVD by 10% (Ras, Streppel, Draijer, & Zock, 2013). Our findings are consistent with previous results from animal and human studies that confirm the utility of using NR-BRJ in endothelial dysfunction.

Bhaswant *et al.* (2017) and Gheibi *et al.* (2018) tested the effects of nitrate supplementation on male rats with obesity and type 2 diabetes. The results suggest that nitrites and nitrates can

reduce chronic inflammation and oxidative stress, which is deeply implicated in the pathophysiology of endothelial dysfunction (Bhaswant, 2017; Gheibi, 2018). Tropea *et al.* (2020) found that BRJ improved endothelial function and reduced BP in pregnant mice, implying that beetroot consumption could improve cardiovascular health and outcomes during pregnancy (Tropea, 2020).

These observations add to novel findings and to the work of others that noticed substantial FMD improvements following dietary nitrate intervention in healthy subjects (Vikas Kapil, 2010; Webb, 2008), and other clinical populations, including untreated hypercholesterolemia (Velmurugan, 2016), and hypertension (Vikas Kapil, 2015; Rammos, 2014). In COPD, there is only one RCT found a significant association between NR-BRJ consumption and improvements in FMD compared to placebo, with an estimated treatment effect of 20.3% (Pavitt, 2020). Dietary modulation with nitrate-rich vegetables may increase circulating nitrite-mediated NO, resulting in the relaxation of vascular smooth muscle cells and improving vascular endothelial dysfunction.

In contrast, Bahra *et al*. (2012) found an acute single dose of KNO_3 did not affect FMD in healthy young adults (Bahra, 2012). A possible explanation for their results might be that nitrate supplements in young healthy adults (27 ± 1.8) years with a low baseline SBP (115± 3.1) mmHg, have no effect as compared to the elderly population. Jackson and colleagues (2018) explained in their meta-analyses that 10 RCTs out of 11 reported the effectiveness of dietary nitrate supplementation on endothelial FMD measures in the elderly (Jackson, 2018). Hence, young healthy adults might be less responsive to improvements in endothelial function compared to the elderly at high CVD risk.

5.4.2.2 Nitrate-rich BRJ effect on arterial stiffness (AIx75%)

Dietary nitrate-rich consumption has been shown to lower AIx75%, a surrogate marker measure of arterial stiffness and a valid predictor for cardiovascular events (G. F. Mitchell, 2010). This is the first study evaluating the effect of dietary nitrate supplementation using BRJ on vascular stiffness measures among the COPD population. Our findings corroborate the results of previous work in healthy young adults and the elderly clinical population, demonstrating that dietary nitrate supplementation can acutely improve measures of arterial stiffness (Bahra, 2012; Vikas Kapil, 2015; Rammos, 2014; Velmurugan, 2016).

These effects are in line with evidence suggesting that nitrite-derived NO via the enterosalivary nitrate-nitrite-NO pathway stimulates vasodilation, leading to reduced arterial stiffness (Vikas Kapil, 2010). While the exact underlying mechanism for this change in arterial stiffness is unclear, reports from animal-mechanistic studies suggest induced sodium nitrite therapy reduces oxidative stress and advanced glycation end products that are associated with arterial stiffening in aged mice (Fleenor, 2012; Sindler, 2011). Arterial compliance is suggested to be determined by both the distending pressure and the intrinsic wall properties (Laurent, 2006). Hence, long-term dietary nitrate-rich supplementation may alter structural properties. However, the improvement in endothelial function measured by FMD may play a role in vascular remodelling, hence improving AIx75%.

5.4.2.3 Nitrate-rich BRJ effect on plasma ADMA, plasma L-arginine

The effect of BRJ on the novel cardiovascular risk marker plasma, ADMA, has not been previously demonstrated in humans *in vivo*. I found that NR-BRJ lowered circulating ADMA levels compared to placebo, was accompanied by an increase in plasma arginine raising the arginine/ADMA ratio. This is consistent with the results found when studying the effects of

dietary inorganic nitrate supplementation in rat models of renal and cardiovascular disease. Carlström and colleagues (2011) reported nitrate treatment (1 mmol NO_3^- kg/day daily over eight weeks) in salt-induced hypertension rat models attenuates levels of ADMA and oxidative stress biomarkers (MDA, 8-OHdG) and increases levels of L-arginine compared with controls (Mattias Carlström, 2011). Furthermore, pre-clinical studies showed increased plasma arginine concentration following dietary NO_3^- supplementation suppressed tissue arginase activity, which redirects L-arginine flux from ornithine/urea production to NO/citrulline formation and enhances NO bioavailability (Ashmore, 2014).

Mechanistically, plasma ADMA is an endogenously produced competitive inhibitor of eNOS enzyme, which reduces endothelial function (Cooke, 2004). ADMA is generated from the modification of arginine residues and catalysed by a group of enzymes known as arginine N-methyltransferases (PRMTs) (Böger, 2000). When the proteins undergo proteolysis, free methylarginines are released to form ADMA and SDMA. Dimethylarginine dimethylaminohydrolase (DDAH) is an enzyme that metabolises > 90% of the endogenous plasma ADMA to citrulline (Murray-Rust, 2001).

In this study, I cannot clarify the molecular mechanisms of the downregulation of ADMA and the upregulation of arginine in COPD patients. However, as mentioned earlier, DDAH is a critical regulator of ADMA. I hypothesise that modulation of DDAH and increased DDAH repression could restore the physiological eNOS activity via manipulating plasma ADMA concentrations, thereby increasing NO production. There are several *in vitro* and *in vivo* studies demonstrating that overexpression of DDAH-I is sufficient to reduce ADMA levels and increase NOS activity (Dayoub, 2003; Leiper, 2011). Hence, future clinical trials should

investigate the mechanism of plasma ADMA reduction following NR-BRJ. In addition, measuring oxidative stress biomarkers and NOS would be very advantageous.

5.4.3 Critique of method

To the best of our knowledge, this was the first study to examine the long-term effects of BRJ on endothelial function using EndoPat in the COPD population. One advantage of the EndoPat system is that the contralateral arm serves as its internal control that can be used to correct for any systemic drift in vascular tone during the test (Moerland, 2012). This study has many strengths, including the randomised double-blinded parallel-group study design, strict inclusion and exclusion criteria, and high supplement adherence.

Limitations in this work should be taken into consideration for future trials. There was wide inter-individual variation in RHI scores in response to study supplementation. Another explanation for the wide inter-individual variation of our RHI scores might be the sensitivity of probes to movements, which may have resulted in excessive signal noise exposure (Hansen, Butt, Holm-Yildiz, Karlsson, & Kruuse, 2017). Although the current sub study was based on a small sample of participants, I was able to observe significant differences between treatments.

5.5 Conclusion

Our findings provide for the first-time evidence that long-term consumption of NR-BRJ improves measures of vascular function, including endothelial dysfunction and vascular stiffness. Other novel findings from this mechanistic substudy were that NR-BRJ intake over a longer period reduced plasma ADMA and increased plasma L-arginine in individuals with COPD.

CHAPTER 6: Beetroot Juice Effects on Platelet Activation Markers in Patients with COPD

6.1 Introduction

6.1.1 Background

As discussed in Chapter 1, patients with COPD exhibit low-grade systemic inflammation, which is pathogenically linked to endothelial dysfunction (Barnes, 2009). Endothelial cells (EC) are an interface between inflammation and inappropriate activation of the blood coagulation system via different mechanisms, including the release of nitric oxide (NO) or arachidonic acid-prostacyclin (prostaglandin I_2, PGI_2) downstream pathways (Versteeg, 2013). ECs' NO signalling activity in inflammatory diseases such as COPD is decreased (J. Wedzicha, 1991). This subsequently stimulates endothelial E-selectin and intercellular cell adhesion molecules (I-CAM-1) to induce the platelet surface expressions of P-selectin, triggering platelet leukocyte aggregation formation (Csoma, 2019). However, in the past decade, a sequential reduction of NO_3^- to NO pathway from dietary nitrate-rich vegetables such as BRJ has been proposed as an alternative system that acts to maintain NO generation and suppress platelet aggregation (Vikas Kapil, 2013). For more details about related literature, please see Chapter 1.

6.1.1 Rationale and study aim

In Chapter 5, I observed that prolonged consumption of BRJ improved endothelial function, as assessed by the EndoPat associated with increased circulatory nitrate concentrations. Growing evidence from pre-clinical and clinical studies revealed that dietary nitrate or nitrate salt supplementation lowers BP, improves vascular function, and attenuates platelet activation markers via nitrite-mediated (NO)-dependent mechanisms (Jackson, 2018; Velmurugan, 2013). However, the effects of dietary nitrate supplements using BRJ on stimulated platelet aggregation in the COPD population remain unclear. Platelet activation is independently

associated with long-term mortality and cardiovascular events (Harrison & Mumford, 2009). Therefore, in the present substudy, I aimed to examine whether, compared to a placebo, consumption of dietary nitrate supplementation using NR-BRJ over 12 weeks attenuates stimulated *ex vivo* platelet activation in people with COPD.

6.1.2 Study objectives

To evaluate the effects of BRJ on platelet reactivity in COPD patients using two separate measures:

1) Platelet aggregatory profiles in response to a range of agonists.
2) The ability of platelets to degranulate P-selectin in response to increasing levels of stimulation.

6.2 Methods

6.2.1 Participants

The participants in this substudy are the same as in Chapter 5. Only 44 out of the 81 participants enrolled in the ON-BC trial agreed to attend face to face due to the COVID-19 pandemic. In the preliminary analyses, I analysed 20 plates (Pl-BRJ) vs 24 plates (NR-BRJ). I excluded 1 plate (Pl-BRJ) vs 2 plates (NR-BRJ) for technical reasons. In the final stage, I sought to exclude plates of participants who were on antiplatelet therapy (n = 2) Pl-BRJ vs NR-BRJ (n = 3). Therefore, the final plates analysed were a total of 17 (Pl-BRJ) vs 19 (NR-BRJ) (Figure 6.1).

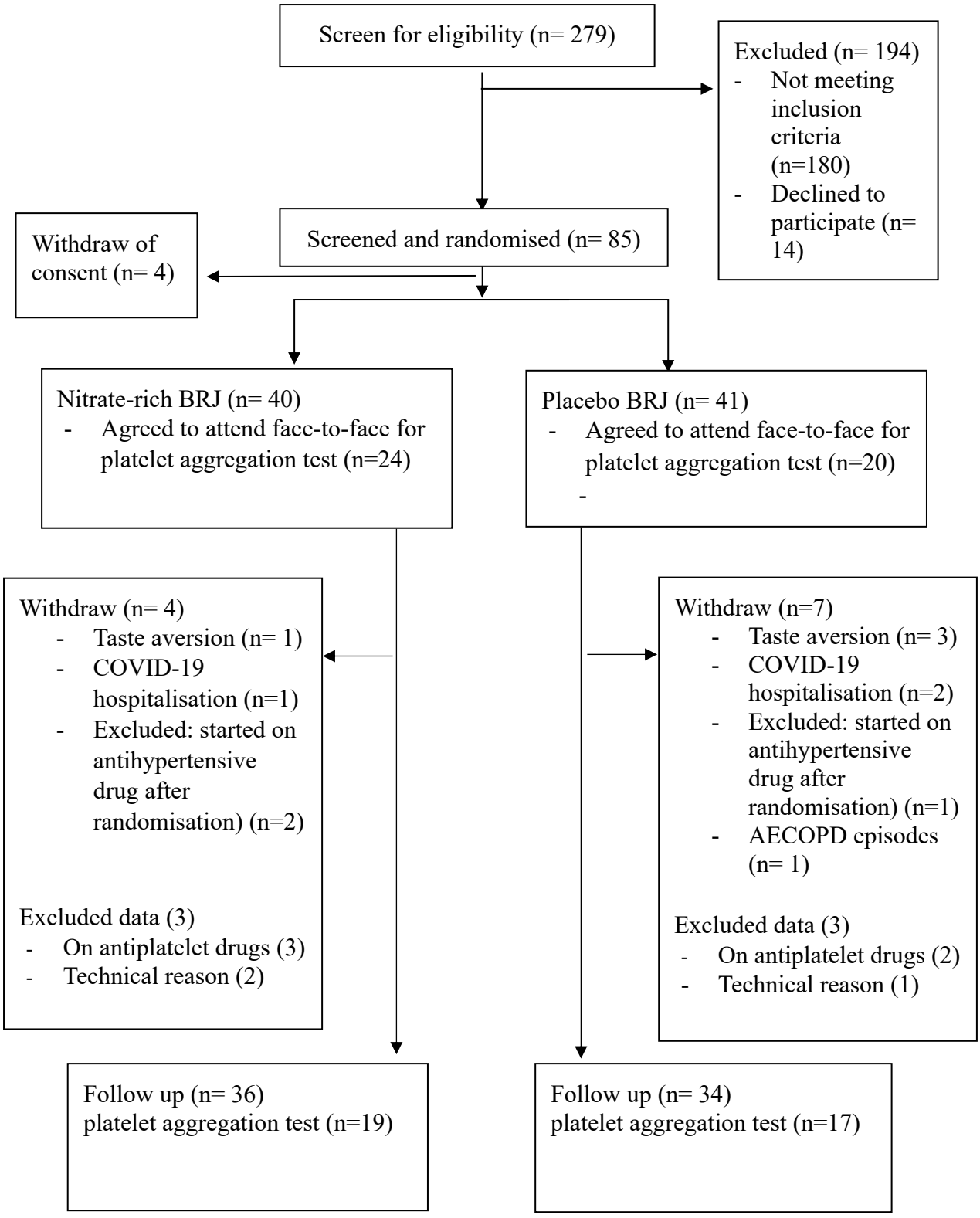


Figure 6-1 CONSORT flow diagram

6.2.2 Study protocol

6.2.2.1 Lab visits

During the trial, participants came twice for pre- and post-intervention measures, and visits were separated by 90 ± 2 (SD) days. During the initial visit (baseline visit), participants came to the research lab and peripheral blood was drawn for the platelet aggregation assay test. Visit 2 (post-intervention) was carried out in the same order and timing as the baseline visit.

6.2.2.2 Measurements

Blood Collection: A 2 ml blood sample was collected from each participant into a (2.7-mL) 3.2% buffered sodium citrate vacutainer (Becton Dickinson, Franklin Lakes, NJ), through a 21-gauge butterfly needle inserted into an antecubital vein. The samples underwent two processing phases.

The effects on platelet aggregation and P-selectin were assessed using a novel whole blood 96-well platelet aggregation assay test (Armstrong, 2015). I processed the samples in collaboration with Professor Timothy Warner's research lab at William Harvey Research Institute, Queen Mary University of London (QMUL), United Kingdom. A further description of the method and procedure is available in Chapter 2.

6.2.3 Data analysis and statistics

The platelet activation analyses were pre-specified as exploratory endpoints. Dose-response curves were presented as mean ±SEM in GraphPad Prism (version 9.3.1, GraphPad Software for Mac, La Jolla, California, USA). Two-way ANOVA repeated measures with Bonferroni post-tests were applied for comparisons between the Pl-BRJ and NR-BRJ groups. Effect sizes

(i.e., differences between groups) are shown as means (95% CI). Values of $p < 0.05$ were considered significant. All other results, such as baseline demographic, clinical anthropometrics, and physiological variables, were summarised for each group of the study and presented as mean ± standard deviation (SD).

6.3 Results

6.3.1 Participant characteristics

Platelet aggregation was assessed in 36 subjects: the Pl-BRJ group (n = 17) vs the NR-BRJ group (n = 19), who were enrolled face-to-face in the ON-BC study. All baseline characteristics for both subgroups are shown in Table 6.1. Overall, participants in the Pl-BRJ group and NR-BRJ group were well matched at baseline measurements. Most randomised patients were male (69%), and 95% were of White Caucasian ethnicity. There were no significant differences in the demographics, prevalence of diseases, concurrent medications, or haematology parameters between the groups.

Table 6.1 Participant characteristics.

	Placebo BRJ n (17)	Nitrate-rich BRJ n (19)	P-value
Sex, Male n (%) ***	12 (63.2)	15 (71.4)	0.74
Age (years)**	66 (59, 68)	65 (57, 72)	0.96
Ethnicity, n (%)			
White Caucasian	19 (100)	20 (95)	1.00
Black	0 (00)	1 (5)	
Height, cm*	172 (9.6)	171 (9.3)	0.77
Weight, kg	77.1 (16.6)	76.6 (14.0)	0.91
BMI (kg/m^2)	26.0 (4.6)	26.4 (4.1)	0.76
Smoking status, n (%)			
Ex-smoker	14 (74)	17 (81)	0.71
Current smoker	5 (26)	4 (19)	
Smoking Hx (pack-years) *	33.4 (15.1)	34.6 (9.9)	0.78
FEV1 (% predicted)	43.5 (18.3)	47.6 (19.0)	0.45
GOLD grade, n (%)			0.47
COPD I	2 (10.5)	5 (23.8)	
COPD II	3 (15.8)	5 (23.8)	
COPD III	7 (36.8)	7 (33.3)	
COPD IV	7 (36.8)	4 (19.0)	
Comorbidities, n (%)			
Hypertension	8 (42.1)	8 (38.1)	0.78
Hypercholesterolaemia	4 (21.1)	4 (19.0)	0.63
IHD	3 (15.8)	3 (14.3)	0.77
Hypertension medications, n			
ACE-i/ARBs	5	4	0.71
CCBs	3	4	1.00
β-blockers	1	3	0.61
Diuretics	0	2	0.49
Antithrombotic therapy, n			0.13
aspirin,	2	3	
clopidogrel	2	1	
Clinic BP			
SBP, mm Hg	141 (17.9)	139 (11.8)	0.74
DBP, mm Hg	75 (10.3)	79 (8.7)	0.13
Endothelial RHI (score)	2.08 (±0.51)	2.09 (±0.40)	0.90
Haematology parameters			
Platelet count (10^9/L)	277 (70)	260 (70)	0.41
Haemoglobin (g/L)	146 (16.5)	148 (9.8)	0.85
Leukocytes ($\times 10^9$/L)	7.6 (6.7, 10.1)	7.4 (6.2, 8.5)	0.32

Note: Data expressed as mean ±SD*, median (IQR)**, and number (%)***. *P* values are for the Mann-Whitney U test or independent *t*-test between groups.

Abbreviations: BMI-body mass index; GOLD-Global Initiative for Chronic Obstructive Lung Disease; FEV1-forced expiratory volume in 1 s; IHD-Ischaemic heart disease; OSA-Obstructive sleep apnoea; ACEi-Angiotensin-converting enzyme inhibitors; ARBs-Angiotensin receptor blockers; CCB- calcium channel blockers.

6.3.2 Nitrate-rich BRJ and platelet aggregation

After 12 weeks of dietary nitrate intake, no significant reduction in NR-BRJ platelet aggregation levels were found compared to the placebo in response to AA, collagen, TRAP6, and U4 agonists, -1.2% [-13 to 12; $p = 0.85$], 9.5% [-8.9 to 28; $p = 0.30$], 11% [-2.0 to 24; $p = 0.09$], and 0.44% [-13 to 14; $p=0.94$], respectively (Figure. 6.2, A–D).

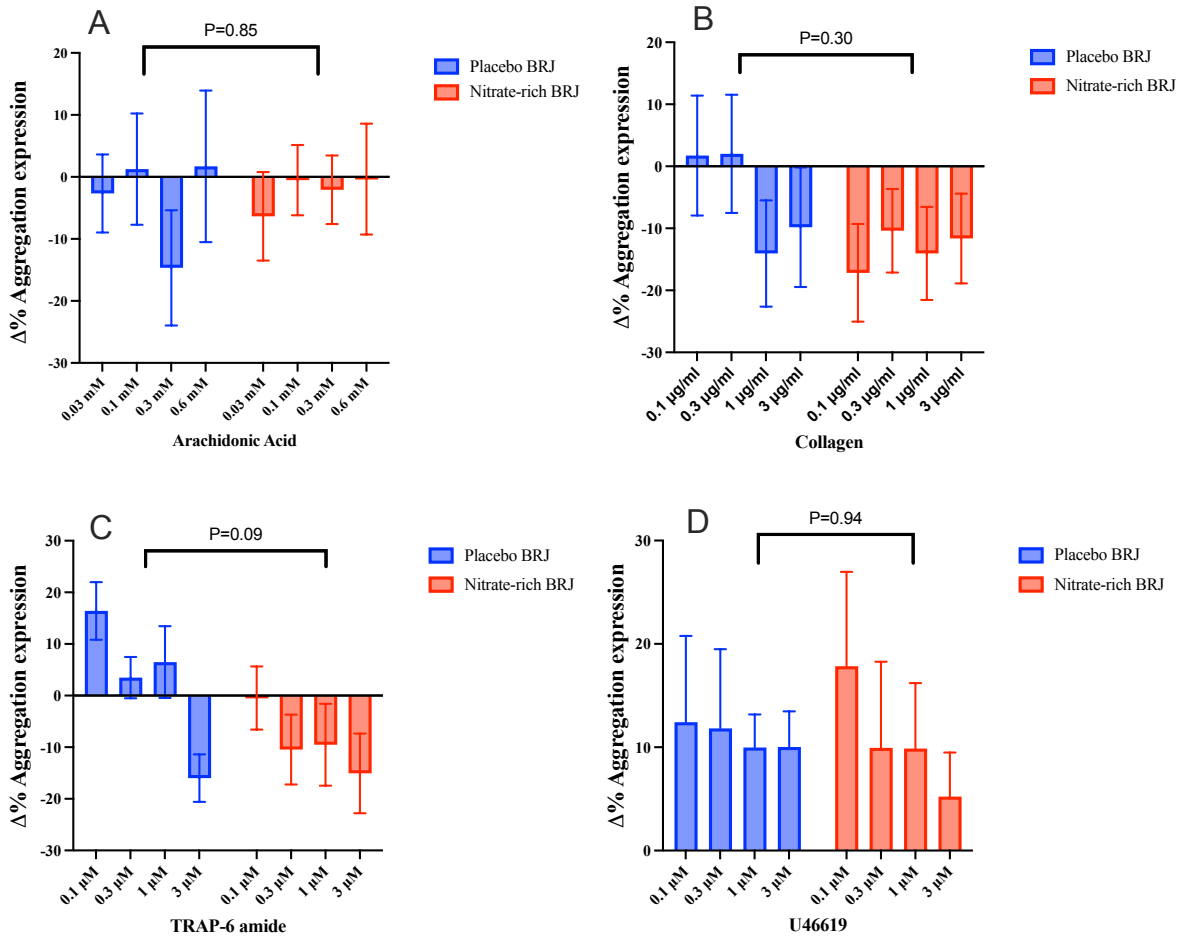


Figure 6-2 (A-D) Change in % platelet aggregation formation following dietary NO_3^- supplementation

Data shown are placebo BRJ (n = 17) vs 70 mL NR-BRJ (n = 19) expressed mean ± SEM. *Ex vivo* stimulation of platelets in small volumes of whole blood in 96-well plates measured with flow cytometry in response to the following agonist concentrations: arachidonic acid (0.03–0.6 µM), collagen (0.1- 3 µg/ml), TRAP-6 amide (0.1- 3 µM), and thromboxane A2 mimetic U46619 (0.1- 3 µM). P-value for the comparison between groups using two-way ANOVA with Bonferroni post-tests.

6.3.3 Nitrate-rich BRJ and P-selectin expression

Similarly, there were no differences in P-selectin fluorescence expression percentage between the groups, 1.7% [-5.1 to 8.5; p = 0.63; Figure 6.3].

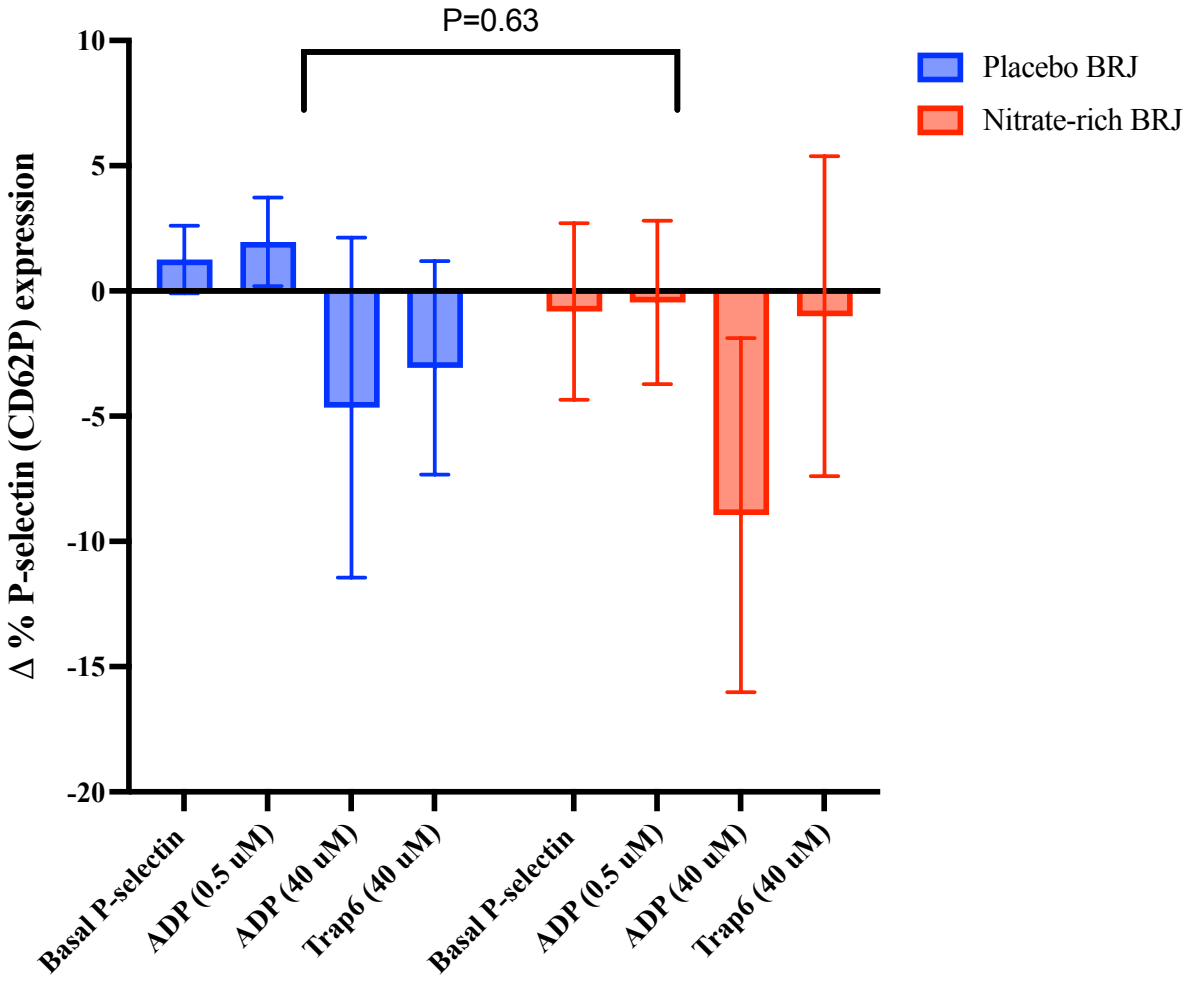


Figure 6-3 Change in platelet P-selectin expression following dietary NO_3^- supplementation

Data shown are placebo BRJ (n = 17) vs 70 mL NR-BRJ (n = 19) expressed mean ± SEM. *Ex vivo* stimulation of P-selectin in response to following agonists vehicle, ADP 0.5µM, ADP 40µM TRAP-6 40µM. P-value for the comparison between groups using two-way ANOVA with Bonferroni post hoc tests.

6.3.4 Correlations of COPD severity and platelet aggregation

I ran Spearman's rank-order correlation to define whether platelet aggregation is associated with disease severity markers such as FEV_1%. I observed a moderately negative correlation between the participants FEV_1% (pred) and baseline induced platelet aggregation % (rho = -0.359 (95% CI: -0.63, -0.01); p = 0.04) (Figure 6.4).

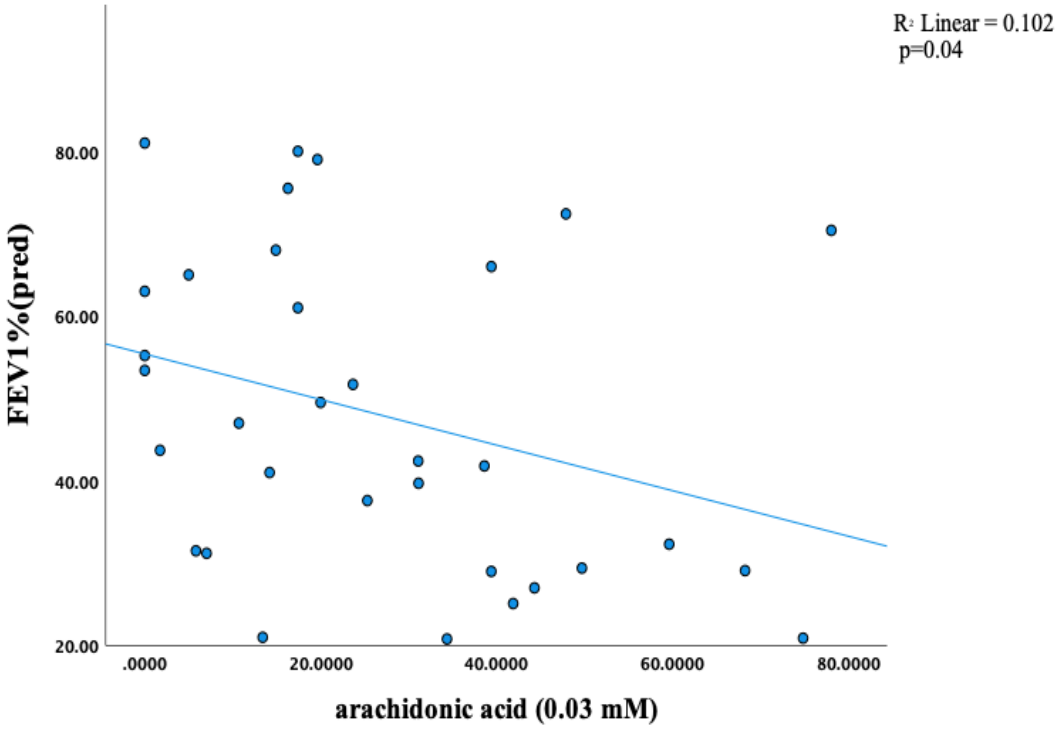


Figure 6-4 Correlation of FEV_1% and baseline platelet aggregation induced by arachidonic acid (n= 36)

6.4 Discussion

In the present study, I examined the effect of a single daily dose of NR-BRJ on platelet aggregation and P-selectin over a period of 12 weeks. I did not observe a reducing effect of NR-BRJ on *ex vivo* platelet aggregation that was stimulated to different agonists' concentrations compared to the placebo in people with COPD. However, I found baseline platelet aggregation % inversely correlated to the participants FEV_1%.

Consumption of NR-BRJ has been demonstrated as having a reducing effect on platelet aggregation compared to placebo on healthy people and people with chronic conditions (Vikas Kapil, 2015; Velmurugan, 2016; Velmurugan, 2013). In contrast, our findings were not in line with the above-mentioned studies (Vikas Kapil, 2015; Velmurugan, 2016; Velmurugan, 2013). This may be due to the different methods used. They utilised the platelet-rich plasma (PRP) method for the assessment of the effect of BRJ on platelet aggregation. The effect of nitrate in these observations was suggested to be related to nitrate bioactivation via the enterosalivary circuit to nitrite-mediated NO as well as due to elevation of cGMP. In this study, I could not identify meaningful effects of BRJ on aggregation and therefore was not able to clarify the molecular mechanisms of the downstream NO-cGMP pathway and platelet NO release to understand these results.

I measured platelet aggregation using a novel *ex vivo* whole blood 96-well plate testing method that is reliable to test in mice and in humans (Armstrong, 2015). This test has many advantages, including requiring no specific laboratory training and the use of small volumes of blood (≈40 µL) (Armstrong, 2015). The whole blood assay approach utilised in this study was initiated within 5 min of drawing blood. This was done to minimise any delay, as far as logistically possible, as compared to PRP-based assays. However, this assay, as with other

platelet ex vivo aggregation tests are performed in blood or processed blood (i.e., PRP) outside of the body. The effects of NO on platelets is short lived. NO has a short half-life (seconds) and its second messenger (cGMP) is rapidly metabolised by phosphodiesterase enzymes. This means that platelet aggregation studies performed ex vivo do not capture well the events occurring at the vessel wall mediated by endogenous NO. It should be considered that I confirmed that nitrate was increased in the blood of patients post BRJ (see chapter 4). If nitrate was active on platelet aggregation, this test would have indicated a reduction in aggregation in the BRJ group. This is in line with studies from Schafer and co-workers showing that inorganic nitrate (up to 60 uM) does not affect human platelet aggregation in vitro (Schafer, Alexander, & Handin, 1980). In chapter 4, I found that blood contained approximately 70 uM, which is the same order of magnitude as used in the Schafer study. Therefor the key difference between my study and that of Velmurugan and Vikas is that I have investigated responses in COPD patients where perhaps the local in vivo environment makes NO related signalling even shorter lived ex vivo.

Nevertheless, the significant correlations finding I observed is consistent with previous cohort has suggested COPD severity characteristics (FEV1) inversely related to platelet aggregation and higher compared to matched healthy (8.3)% vs. (4.0)% (Maclay, 2011). Based on my other results and what is known in the field, the effects of COPD on ex vivo platelet aggregation are likely due to fundamental pathological changes in the platelet – which are not short live (i.e., as the effect in vivo of NO would be). This could be the result of upregulation of receptors, down regulation of inhibitor pathways or the presence in blood of pro-aggregatory hormones.

6.5 Conclusion

Although, this study has been unable to demonstrate effect for BRJ on platelet aggregation using whole blood flow cytometry. Participants' baseline platelet aggregation % was inversely correlated with COPD disease severity. Further larger clinical trials examining the effect of BRJ in COPD population platelets' activation are needed using the gold standard method of light transmission aggregometry (LTA), to draw a firm conclusion

Chapter 7: General discussion, future research and conclusion

7.1 Overview

This chapter will discuss the main findings and clinical implications of the thesis. Also, I will suggest some future research, strengths and limitations.

People with COPD and CVD share common risk factors. As detailed in Chapter 1, CVD is a major cause of morbidity and mortality in COPD. Hypertension is a traditional CVD risk marker that frequently occurs in COPD. Emerging short-term studies of healthy and clinical populations demonstrate that daily dietary nitrate supplementation improves CV risk outcomes, including BP, endothelial function, and platelet aggregation.

In COPD, previous studies on the effects of dietary nitrate using BRJ supplement reported improvement in exercise capacity, muscle oxygen uptake, and CV risk outcomes including BP, and endothelial function FMD during exercise intervention in COPD individuals. These studies also had obvious limitations including intervention duration (acute/short), small sample sizes, and selection biases among COPD patients, which necessitates more research on BRJ and CV risk outcomes in COPD patients.

Therefore, the overall aim of the present research was to examine the effects of daily nitrate-rich BRJ versus placebo BRJ beverages on CV markers in the COPD population over a three-month period.

I carried out a meta-analysis, a clinical trial, and a mechanistic substudies to achieve this aim.

7.2 Main findings, clinical implications, and future studies

7.2.1 Systematic review and meta-analysis

The systematic review and meta-analysis results showed that dietary nitrate supplementation reduced BP parameters, improved endothelial FMD, increased circulatory plasma NO_3^- and NO_2^- concentrations, and elevated exhaled FeNO levels.

However, I found that the effectiveness of BRJ on BP in people with COPD was investigated for a relatively short duration, with several methodological limitations. I concluded that the promising reported results from these RCTs suggest that dietary nitrate supplementation, using BRJ, is affordable safe treatment, can augment the improvement in BP, endothelial function, and exercise capacity produced by exercise interventions including PR. Yet, it is unclear whether this nitrate-lowering effect from BRJ can be sustained over a longer duration on BP without exercise.

Therefore, I hypothesised that daily nitrate-rich BRJ could lower high BP in COPD patients over long term (12 weeks).

7.2.2 BRJ and BP in COPD: RCT

For the first time, I demonstrated that daily single-dose ingestion of NR-BRJ for 12 weeks lowers SBP compared to Pl-BRJ in individuals with COPD with high BP. This effect was apart from other confounding factors mentioned in previous studies in COPD, such as exercise. In addition, I observed increased exercise performance (6MWD) and a substantial rise in circulating nitrate. The reduction in BP and increase in exercise capacity observed in

this RCT might be caused by an improvement in endothelial function. Therefore, I explored the effect of BRJ on vascular function.

7.2.3 BRJ and vascular function in COPD: mechanistic substudies

I also conducted a mechanistic substudy of the RCT to identify whether NR-BRJ improved vascular function markers. I found that longer-term supplementation of NR-BRJ significantly improves endothelial function (RHI) and reduces arterial stiffness marker (Aix75%). Further significant and novel findings of this substudy were the reduction in plasma ADMA and the rise in plasma arginine levels. Long-term drinking of NR-BRJ supplement may alter vascular structural properties. However, the remarkable reduction I observed in the plasma ADMA was not studied previously in vivo. The only studies that explored the effect of nitrate supplementation on ADMA were rat models, which reported that daily nitrate supplementation (1 mmol NO_3^- kg/day over a short period) attenuates levels of ADMA and oxidative stress biomarkers (MDA, 8-OHdG) as well as increasing levels of L-arginine in rats with salt-induced hypertension (Mattias Carlström, 2011).

As explained in Chapter 1, circulating ADMA levels are increased in people with hypertension and CVD. Evidence widely recognized increased ADMA levels as a mechanism for the development of endothelial dysfunction which inhibiting the formation of eNOS enzyme (Cooke, 2004). Plasma ADMA is endogenously produced, an analogue of the amino acid L-arginine, from the modification of arginine residues and catalysed by a group of enzymes known as arginine N-methyltransferases (PRMTs) (Dowsett, 2020). When the proteins undergo proteolysis, free methylarginines are released to form ADMA and SDMA. Dimethylarginine dimethylaminohydrolase (DDAH) is a critical regulator of ADMA. I

speculate that modulation of DDAH and increased DDAH expression could restore physiological eNOS activity via manipulating plasma ADMA concentrations, thereby increasing NO production.

However, in this study, I could not clarify the molecular mechanisms of the downregulation of ADMA and the upregulation of arginine in COPD patients. Future clinical trials should investigate the mechanism of plasma ADMA reduction following NR-BRJ. In addition, measuring oxidative stress biomarkers and eNOS activity would be very advantageous. Finally, NR-BRJ treatment was unable to attenuate platelet aggregation formation in response to different platelet agonists using the novel *ex vivo* whole blood 96-well plate essay method in people with COPD. Further larger clinical trials examining the effect of BRJ in the COPD population's platelet activation are needed, using appropriate gold standard methods like light transmission aggregometry (LTA), which would help to draw a firm conclusion.

7.3 Strengths and limitations

One major strength of the main study of this thesis was that I conducted the longest (12 weeks) double-blind, parallel-group RCT in any population evaluating the effects of BRJ. Unlike other studies of COPD, I did not exclude people based on GOLD stages. Therefore, our study is representative of the COPD spectrum seen clinically. I had a low dropout rate and high adherence to treatment. I also used an at-home BP monitor to collect the BP data. Home BP monitors are regarded by many hypertension guidelines as a more reliable method to measure and monitor BP parameters compared to the in-clinic method (Sega, 2005). Another major strength is that I assessed numerous CVD risk biomarkers, such as plasma ADMA and arginine/ADMA ratio, for the first time in human trials.

On the other hand, the limitations of this work should be considered for future trials. The use of peripheral arterial tonometry (PAT) for the assessment of endothelial function shows wide inter-individual variation in RHI scores. An explanation for the wide inter-individual variation in RHI scores might be related to the sensitivity of probes to the movements of participants during the test, which may have resulted in excessive signal noise exposure (Hansen, 2017). The results of this study should be interpreted with caution, as the majority of the participants were of White European ethnicity. Therefore, similar studies on different ethnic groups are needed.

7.4 Clinical implications

The reduction in SBP suggests that the longer-term consumption of BRJ may potentially have important clinical implications for the primary and secondary prevention of CV risks in individuals with COPD. The INTERSALT epidemiologic study (1991), which included results of BP parameters from more than 10,000 men and women, showed a 3-mmHg reduction of SBP would result in an 8% reduction in mortality due to a stroke, a 5% reduction in mortality due to CHD, and a 4% decrease in all-cause mortality (Stamler, 1997). The Heart Outcome Prevention Evaluation (HOPE) study also showed that a reduction of SBP by 3.3 mm Hg and DBP by 1.4 mmHg was associated with a 22% reduction in relative risk of cardiovascular mortality, myocardial infarction, and stroke (Dagenais, 2001). Further, it was found that improving endothelial function (measured via FMD) by 1% would reduce the risk of developing CVD by 10% (Ras, 2013).

Our results support advocating the dietary nitrate approach using BRJ as an available, safe, and cheap supplement to tackle the burden of hypertension and CVD in the COPD population. Larger clinical trials in clinical practice may facilitate the translation of dietary

nitrate supplementation into COPD treatment guidelines. It is proposed that treatment of hypertension should start before the disease has evolved in individuals with "prehypertension" as a preventative strategy (Julius, 2006).

7.5 Future research

Although this is the longest study to date, longer studies are needed to examine the effects of BRJ at CVD event rates and establish the effect of increased exercise capacity on daily life. The European Food Safety Authority recommends a single daily dose of 3.7 mg of NO_3^- .kg body weight-1.day-1, equivalent to 4.2 to 4.7 mmol of NO_3^- for a 70 to 80 kg individual (Hord, Tang, & Bryan, 2009). However, long-term NR-BRJ intakes associated with the risk of CVD-related outcomes on adverse events are not currently known. Further, larger clinical trials in clinical practice may facilitate the translation of dietary nitrate supplementation into COPD treatment guidelines. It is proposed that treatment of hypertension should start before the disease has evolved in individuals with "prehypertension" as a preventative strategy (Julius, 2006). Also, I suggest that future molecular mechanistic studies should examine the effect of BRJ on platelet cGMP levels, platelet NO release, and P2Y12 receptors.

7.6 Conclusion

In summary, I found BRJ supplementation to exert a long-term reduction in BP, improvement in exercise capacity, and vascular function in COPD subjects with high BP. Further novel findings were that NR-BRJ decreased plasma ADMA and increased plasma L-arginine levels in COPD subjects. These findings suggest that NO_3^- supplementation is a candidate intervention for future health strategies to lower BP and prevent hypertension in people with COPD.

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Appendices

Appendix 1: HRA approval letter

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Dr Nicholas Hopkinson
Respiratory Muscle Lab
Royal Brompton Hospital
Fulham Rd
SW3 6NP

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

10 January 2020

Dear Dr Hopkinson

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

Study title:	**The ON-BC study: Oral Nitrate supplementation and Blood pressure in COPD – a randomised clinical trial**
IRAS project ID:	**271589**
Protocol number:	**The ON-BC study**
REC reference:	**19/LO/1660**
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 2: Consent form

Royal Brompton & Harefield NHS
NHS Foundation Trust

Imperial College London

CONSENT FORM

The ON-BC study: Oral Nitrate supplementation and Blood pressure in COPD – a randomised clinical trial
Name of Principal Investigator: Dr NS Hopkinson

Patient Identification Number for this trial: **Please initial box**

I confirm that I have read and understood the information sheet for this study (Version 2) dated 13-11-19 and have had the opportunity to ask questions which have been answered fully.	
I understand that my participation is voluntary and I am free to withdraw at any time, without giving any reason and without my medical care or legal rights being affected.	
I understand that sections of any of my medical notes may be looked at by responsible individuals from Imperial College London, from Royal Brompton NHS Trust or from regulatory authorities where it is relevant to my taking part in this research.	
I give / do not give **(delete as applicable)** consent for information collected about me to be used to support other research in the future, including those outside of the EEA.	
I give / do not give **(delete as applicable)** consent for samples collected during this study to be used in future ethically approved studies. I give permission for my samples to be sent to other organisations, including those outside of the EEA.	
I give / do not give **(delete as applicable)** consent to being contacted to potentially taking part in other research studies.	
I consent to take part in the above study.	

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

(COPIES: 1 for patient, 1 for researcher, and 1 to be kept with hospital notes)

Appendix3: EndoPAT test protocol

1. Prior to the study in the phone I 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. I measure the blood pressure using the control arm (the arm that is not occluded during the EndPAT test.
3. I 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. I kindly 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. for the test I always used the index finger as recommended; however, if this finger is unsuitable, I 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. Then, I place the occlusion cuff pressure on the arm to be occluded during the test. I applied it without excess pressure.
8. At the step, we click the "Patient Information" icon on the tool bar to create a new patient file. Then, I enter the participant information including study ID, age, gender, height (cm), weight (kg), SBP/DBP.
9. I select two new PAT probes and connect to the pneumo-electrical tubing.

10. Then I place the connected probes into the sockets of the arm-supports and press the "Deflate" button on the top of the device and then I ask the participants to insert index fingers completely into the probes, and I press the "Inflate" button on the top of the Endo-PAT 2000 device.
11. I place 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 I position 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. I explain 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. I then click 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 artifactual signals.
15. Then I click 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, I tell the patient that I are going to inflate the cuff for the occlusion phase and participant should stay relaxed and not move the fingers.
17. I rapidly inflate the cuff pressure to a supra-systolic pressure of 60mmHg above the patient's systolic pressure, whichever is higher, and I start the stopwatch again for

minutes. To confirm occlusion, I 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, I deflate the cuff and I tell the subject I are 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 I 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 I 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 (see figures 3.6, 3.7).

Appendix4: Data bases search strategies of the systematic review.

Ovid MEDLINE(R) (1946 to June 2020)

#	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	Cardiovascular/ or Endothelial/ or Platelet/ 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
40	limit 39 to dt=20190531-20200615

Embase (1947 to June 2020)

#	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	Cardiovascular/ or Endothelial/ or Platelet/ 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
40	limit 39 to dd=20190531-20200615

CINAHL (EBSCO) (1960 to March 2020)

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

Appendix5: Systematic review publication

Respiratory research

BMJ Open Respiratory Research

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

Abdullah S Alsulayyim ,[1,2] Ali M Alasmari,[1,3] Saeed M Alghamdi,[1,4] Michael I Polkey,[1] Nicholas S Hopkinson [1]

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For numbered affiliations see end of article.

Correspondence to
Dr Nicholas S Hopkinson; n.hopkinson@ic.ac.uk

ABSTRACT

Background Dietary nitrate supplementation, usually in the form of beetroot juice, may improve exercise performance and endothelial function. We undertook a systematic review and meta-analysis to establish whether this approach has beneficial effects in people with respiratory disease.

Methods A systematic search of records up to March 2021 was performed on PubMed, CINAHL, MEDLINE (Ovid), Cochrane and Embase to retrieve clinical trials that evaluated the efficacy of dietary nitrate supplementation on cardiovascular parameters and exercise capacity in chronic respiratory conditions. Two authors independently screened titles, abstracts and full texts of potential studies and performed the data extraction.

Results After full-text review of 67 papers, eleven (two randomised controlled trials and nine crossover trials) involving 282 participants met the inclusion criteria. Three were single dose; seven short term; and one, the largest (n=122), done in the context of pulmonary rehabilitation. Pooled analysis showed that dietary nitrate supplementation reduced systolic blood pressure (BP), diastolic BP and mean arterial pressure (mean difference (95% CI), −3.39 mm Hg (−6.79 to 0.01); p=0.05 and −2.20 mm Hg (−4.36 to −0.03); p=0.05 and −4.40 mm Hg (−7.49 to −1.30); p=0.005, respectively). It was associated with increased walk distance in the context of pulmonary rehabilitation (standardised mean difference (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).

Conclusion Dietary nitrate supplementation may have a beneficial effect on BP and augment the effect of pulmonary rehabilitation on exercise capacity. Short-term studies do not suggest a consistent benefit on exercise capacity.

PROSPERO registration number CRD42019130123.

Key messages

- Does dietary nitrate supplementation improve cardiovascular parameters and exercise capacity in people with chronic respiratory disease?
- We found moderate evidence to support the hypothesis that dietary nitrate supplementation lowers blood pressure. There was low Grading of Recommendations, Assessment, Development and Evaluations evidence to support an improvement in exercise capacity in people with chronic obstructive pulmonary disease.

Why read on?

- This review systematically evaluates the available evidence regarding the impact of dietary nitrate supplementation on exercise capacity and cardiovascular parameters in individuals with respiratory disease.

INTRODUCTION

Exercise limitation is a common feature in individuals with chronic respiratory disease (CRD) despite optimum medical treatment including pulmonary rehabilitation (PR) and pharmacotherapy.[1–3] Factors contributing to breathlessness and reduced physical activity include altered pulmonary mechanics and cardiovascular function as well as skeletal muscle impairment.[4–7] Nitric oxide (NO) is a ubiquitous signalling molecule with a key role in endothelial function, and a relationship between plasma nitrite (NO_2^-) levels and exercise performance has been identified.[8 9] Dietary NO_3^- supplementation, which increases NO availability via a NO_3^-–NO_2^-–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[10 11] and evidence suggests that NO_3^- supplementation with beetroot juice (BRJ) can reduce oxygen consumption