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NITRATE-RICH BEETROOT JUICE AND ITS EFFECTS ON CARDIOVASCULAR HEALTH AND COGNITION IN YOUNGER AND OLDER ADULTS

A thesis presented in partial fulfilment of the requirements for the degree of

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in
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Abstract

Background: Evidence suggests supplementation with nitrate-rich beetroot juice (BR) may improve cardiovascular responses and cognition in male and female, younger and older adults. However, there is still limited research in this area, particularly in older adults and with regards to measures of endothelial and cognitive function where equivocal findings have been observed. In addition, it is unclear whether age-related differences impact on the benefits from dietary nitrate supplementation. Older adults tend to have an increase in blood pressure (BP) and decline in cognition with aging, and thus may respond more favourably to BR supplementation compared to younger adults. Additionally, the effects of dose and duration (acute versus chronic) of supplementation with dietary nitrate are still not well known.

Overall aims: To 1) investigate the effects of varying doses of nitrate-rich BR on cardiovascular responses and cognition in older adults, and 2) examine the effects of acute and chronic supplementation with BR on cardiovascular responses and cognition in the two age groups.

Methods: The "Dose-response study" (Chapter 4) examined the effects of acute supplementation with varying doses of BR (0.15 (placebo; PL), 2.5, 4.9, and 9.8 mmol nitrate) on cardiovascular and cognitive responses in older adults (50-80 years; n=11) in a randomised, double-blind, crossover designed trial. At each visit, resting blood samples for plasma nitrate and nitrite, BP, mean arterial pressure (MAP), systemic vascular resistance (SVR), heart rate (HR), resting metabolic rate, and cognitive function were completed pre- and 2.25 h post-supplementation. The "Acute study" (Chapter 5) was a randomised, double-blind, crossover study. Twenty-four participants, 13 younger (18-30 years) and 11 older (50-70 years), completed resting blood samples, BP, HR, cognitive function, and mood and perceptual measurements pre- and post-supplementation with nitrate-rich BR (10.5 mmol) or placebo (1 mmol). The "Chronic study" (Chapter 6) was a double-blind, randomised control trial investigating acute, acute + chronic, chronic + acute (exercise performance) and chronic supplementation with either nitrate-rich BR (10.5 mmol) or PL (0.15 mmol) over 28 days in 21 younger (18-30 years) and 22 older (50-80 years) adults. On days 0, 14, and 28 resting blood samples, BP, MAP, SVR, HR, and cognitive performance measures were completed pre- and 2.25 h post-supplementation, while oxygen uptake ($\dot{V}O_2$) and time trial performance were measured 2.5 h post-supplementation.

Results: Ingestion of varying doses of dietary nitrate in the Dose-response study increased plasma nitrate and nitrite concentrations in a dose-dependent manner, with a significant difference shown between each treatment for plasma nitrate ($p < 0.001$). However, plasma nitrite was only significantly increased following consumption of the 9.8 mmol treatment compared to PL ($p = 0.004$). Systolic blood pressure (SBP), diastolic blood pressure (DBP), and MAP were reduced 2.25 h post-supplementation with the 9.8 mmol treatment compared to the PL, 2.5, and 4.9 mmol treatments ($p < 0.05$). Following

supplementation with the 9.8 mmol dose, Corsi span (cognitive function) was improved during the Corsi block tapering test (CBT) compared to PL (p = 0.013) and 2.5 mmol (p = 0.004) treatments.

In the Acute study, supplementation with nitrate-rich BR also significantly increased plasma nitrate (p < 0.001) and nitrite (p = 0.003) concentrations in younger and older adults relative to PL. Systolic BP was reduced in both younger and older adults (p < 0.001) 2.25 h post-supplementation with BR compared to PL, while DBP was only reduced in older adults (p = 0.013). Older adults had a greater increase in plasma nitrite (p = 0.038) and reduction in DBP (p = 0.005) compared to younger adults. Cognitive performance measures were also improved in younger and older adults following acute supplementation with nitrate-rich BR, with a reduction in reaction time observed during the Stroop test (p = 0.045).

The Chronic study showed a significant reduction in SBP, DBP, and MAP following acute supplementation with nitrate-rich BR on day 0 in older adults (p < 0.001) and following an acute dose in the context of chronic supplementation (acute + chronic) on day 28 in both older (p < 0.01) and younger (p < 0.05) adults. Fourteen days' chronic supplementation with nitrate-rich BR, in the absence of an acute dose, also reduced SBP (p = 0.019), DBP (p = 0.004), and MAP (p = 0.005) in older but not younger adults. Older compared to younger adults also had a greater reduction in SBP, DBP, and MAP following acute supplementation with nitrate-rich BR on day 0 (p < 0.05) and following chronic supplementation on day 14 (p < 0.05). Acute + chronic supplementation with nitrate-rich BR reduced SVR in older adults on days 14 (p = 0.032) and 28 (p = 0.016), with a greater reduction in older compared to younger adults observed on day 14 (p = 0.015) and a trend for greater reduction in older adults observed on day 28 (p = 0.056). Acute (on day 0) and acute + chronic (on day 28) consumption of nitrate-rich BR improved reaction time during the Stroop test in older adults (p = 0.042 and 0.006, respectively), while older (relative to younger) adults also showed a greater improvement in reaction time during the Stroop test following chronic supplementation on days 14 (p = 0.016) and 28 (p = 0.02) despite no effect of treatment on these days. Older adults also showed a greater reduction in $\dot{V}O_2$ at 20% completion of the cycle time trial following acute supplementation with BR on day 0 (p = 0.044), despite no effect of treatment at this time point.

Conclusion: The results from this PhD project showed that acute and daily supplementation with nitrate-rich BR can improve BP and cognitive function in younger and older adults, with greater benefits observed in the older cohort. Furthermore, chronic supplementation with nitrate-rich BR in older adults can improve BP independent of an acute dose. These results suggest that daily supplementation with nitrate-rich BR may have a role in clinical settings for helping maintain healthy cardiovascular and cognitive function. Future research should investigate the use of nitrate-rich BR as a potential preventative therapy for cardiovascular and cognitive diseases such as hypertension and dementia.

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Abbreviations

ADP	Adenosine diphosphate
ANT	Adenine nucleotide translocase
ATP	Adenosine triphosphate
BFV	Blood flow velocity
BP	Blood pressure
BR	Beetroot Juice
CBT	Corsi block tapering
CBF	Cerebral blood flow
cGMP	Cyclic guanosine monophosphate
CKD	Chronic kidney disease
COPD	Chronic obstruction pulmonary disease
CRT	Choice reaction test
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
GC-MS	Gas chromatography-mass spectroscopy
GLP-1	Glucagon-like peptide-1
FAS	Felt arousal scale
FMD	Flow mediated dilation
fNIRS	Functional near infrared spectroscopy
FS	Feeling scale
GTP	Guanosine triphosphate
HFpEF	Heart failure with preserved ejection function
HHb	Deoxyhemoglobin
HPLC	High-performance liquid chromatography
HR	Heart rate
iNOS	Inducible nitric oxide synthase
MAP	Mean arterial pressure
MRI	Magnetic resonance imaging
NIRS	Near infrared spectroscopy
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide

NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
O_2Hb	Oxyhemoglobin
P/O	Phosphate to oxygen
PAD	Pulmonary arterial disease
PCr	Phosphocreatine
Pi	Inorganic phosphate
PL	Placebo
POMS	Profile of mood states
PWV	Pulse wave velocity
RH	Reactive hyperaemia
RRI	Renal resistance index
RVIP	Rapid visual information processing
SBP	Systolic blood pressure
SD	Standard deviation
SVR	Systemic vascular resistance
TPR	Total peripheral resistance
TVR	Total vascular resistance
$\dot{V}O_2$	Oxygen uptake

Chapter 1
Introduction

This chapter provides background for this thesis and outlines the rationale and objectives of the PhD study. The chapter concludes with an outline of the thesis structure.

1.1 Background

An individual's health and performance (both mental and physical) can be influenced by many factors ranging from their genetic predisposition to daily habits and lifestyle choices (e.g., dietary intake, activity levels, and sleep quality) (Clements et al., 2014). Some of these factors are more controllable and have greater impact on health and performance outcomes than others; for example, dietary habits have been indicated as a dominant influencer of health and performance, and hence can be easily improved to promote beneficial outcomes (Seeram, 2008). For this reason, in the last 15 years more research has begun to focus on food-based supplements that contain key bioactive compounds purported to promote health benefits (Carter et al., 2020, Clifford et al., 2015). Additionally, the percentage of the aging population is increasing; people are living longer with more morbidities and thus a reduced quality of life (Ministry of Health, 2016). This results in the need for medical treatment of these morbidities, which puts more strain on the medical system and increases health care costs (Jackson, 2011). Therefore, food-based supplements have become important as an effective tool to improve quality of life via the potential to slow or reduce common morbidities such as cardiovascular and neurological disorders.

In 2012, the New Zealand Trade and Enterprise survey showed that New Zealand's natural products industry reached over NZ$1 billion in total revenue with most of this income coming from health supplements, functional ingredients, and functional foods. This made New Zealand's natural products industry one of the top revenue makers in the country and it is expected to continue to grow rapidly, with predictions to reach NZ$5 billion by 2025. Additionally, older adults appear to be interested in natural methods to help maintain their health and quality of life which helps to drive this market further (Karelakis et al., 2020). Therefore, the use of food-based supplements shown to provide health and performance benefits have become a major part of today's society.

One key nutritional compound that has gained recent attention is inorganic dietary nitrate (NO_3^-). Nitrate is found in high concentrations in green leafy vegetables and root vegetables such as beetroot. Nitrate is a precursor for nitric oxide (NO), a bioactive molecule, which has many functions in the human body ranging from regulation of neurotransmission and blood flow to alterations in oxygen consumption and has been shown to have health (Berry et al., 2015, Kenjale et al., 2011), cognitive (Wightman et al., 2015, Stanaway et al., 2019) and physiological performance (Breese et al., 2013, Lansley et al., 2011a) benefits. There are two major pathways for NO production in the human body; the first is through the conversion of L-arginine to NO by the enzyme nitric oxide synthase (NOS) and secondly, via reduction of dietary NO_3^- to NO_2^-, then to NO (Bailey et al., 2012). There has been greater research interest into improving health and performance by using dietary

NO_3^- (particularly in the form of beetroot juice) as a food-based supplement to increase NO via this second pathway (Kemmner et al., 2017, Lansley et al., 2011b, Stanaway et al., 2019).

Previous studies have shown acute and chronic dietary NO_3^- supplementation can result in positive health-related outcomes, including reduced blood pressure (BP) (Kelly et al., 2013, Larsen et al., 2006, Stanaway et al., 2019) and improved blood flow and oxygenation to the muscle and brain (Kenjale et al., 2011, Presley et al., 2011, Wightman et al., 2015). Specifically, three days of supplementation with NO_3^--rich beetroot juice (9.6 mmol·day^{-1}) reduced systolic (-10 mmHg) and diastolic (-4 mmHg) BP in older adults compared to the control (Kelly et al., 2013), while two days of supplementation with a high NO_3^- diet and beetroot juice (12.4 mmol) increased cerebral perfusion to the brain in older adults (Presley et al., 2011). In contrast, one study in younger adults showed no improvement in BP following six days supplementation with 8 mmol of NO_3^--rich beetroot juice (Cermak et al., 2012). Nonetheless, these findings are indicative of potential cardiovascular and cerebrovascular benefits, which may result in lower risk of cardiovascular disease and dementia, which are major contributors to health loss (the loss of healthy life due to disability, illness, or death) in older adults (Ministry of Health, 2016).

Furthermore, it has been shown that dietary NO_3^- supplementation can significantly improve cognitive performance, enhancing reaction time (Gilchrist et al., 2014, Stanaway et al., 2019) and increasing speed of mental processing capability (Wightman et al., 2015). However, investigations into the benefits of dietary NO_3^- supplementation on cognitive performance are scarce and present mixed findings, with some studies showing no improvements in cognition (Kelly et al., 2013, Thompson et al., 2014). Studies have shown that both acute and prolonged supplementation with dietary NO_3^- can result in a significant reduction in the oxygen cost of exercise (Bailey et al., 2009, Larsen et al., 2007), reduced energy utilisation (Bailey et al., 2010), and increased time to fatigue and time trial performance (Eggebeen et al., 2016, Kenjale et al., 2011, Lansley et al., 2011a), thus promoting improved exercise performance. However, one study showed no improvement in 50-mile time trial performance in younger adults following acute supplementation with NO_3^--rich beetroot juice (6.2 mmol) (Wilkerson et al., 2012).

The differing results for the potential benefits mentioned above may be due to methodological factors which have been suggested to influence the effects of dietary NO_3^- supplementation on cardiovascular and cognitive responses (Stanaway et al., 2017). In particular, the dose of dietary NO_3^- used, the age of the participants (younger versus older adults), the timing of supplementation, the participants' health status (e.g., having hypertension, diabetes, chronic obstructive pulmonary disease etc.), and duration of supplementation may impact the magnitude and significance of the

studies outcomes. Currently, there is limited research into variations between key methodological factors, for example many studies only use one dose rather than investigating the effects of varying doses. In addition, there are limited studies investigating any possible age-related (younger versus older adults) differences in responses to dietary nitrate supplementation, and only a handful of studies have used supplementation durations of longer than 15 days. These are important methodological factors that require further experimental exploration.

1.2 Rationale for this PhD Study

A review of the literature showed that there is limited research investigating the potential benefits of dietary NO_3^- supplementation on cardiovascular and cognitive health and performance in older adults, with even less examining the potential differences in older compared to younger cohorts. Only two previous studies have investigated differences in cardiovascular measures between older and younger adults following NO_3^--rich beetroot juice intake (Hughes et al., 2016, Rogerson et al., 2022), however cognitive and physiological performance were not explored. Additionally, no previous studies have investigated the dose-response relationship of dietary NO_3^- supplementation in older adults on cardiovascular and cognitive measures. There is also limited research examining chronic supplementation with dietary NO_3^- for longer than 15 days and the potential mechanisms of action are still not well known. Therefore, four key areas of dietary NO_3^- supplementation research which required further investigation were determined, namely, the dose-response relationship in older adults, the acute effects on cardiovascular responses and cognition in older versus younger adults, the effects of chronic supplementation on cardiovascular responses and cognition, and investigation into potential mechanisms of action.

1.3 Overall Aims and Research Questions

The overall aims of this thesis were to firstly examine the effects of supplementation with varying doses of dietary NO_3^- in older adults and secondly to examine the effects of acute and chronic nitrate supplementation (in the form of beetroot juice), on cardiovascular responses and cognition in younger (18-30 y) and older (50-80 y) adults.

The four research questions to address these overall aims are noted in Sections 1.3.1 – 1.3.4.

1.3.1 Research Question 1

Does supplementation with dietary NO_3^- improve cardiovascular, cerebrovascular, and metabolic health, and physiological and cognitive performance in older adults?

Human clinical trials investigating the effects of dietary NO_3^- supplementation on cardiovascular, cognitive, and cerebrovascular responses in older adults are evaluated and reviewed in a systematic review. The aim of the systematic review is to summarise the efficacy of dietary NO_3^- supplementation for conferring health benefits and enhancing performance in older adults.

1.3.2 Research Question 2

Does acute supplementation with different doses of dietary NO_3^- improve cardiovascular health and cognitive function in older adults (50 – 80 y) in a dose dependant manner?

An acute, double-blind, placebo-controlled, crossover study (the Dose-response study) has been designed to investigate the effects and dose-response relationships of acute supplementation with dietary NO_3^- at dosages of 0.15, 2.5, 4.9 and 9.8 mmol on plasma [NO_2^-] and [NO_3^-], cognitive performance, blood pressure, heart rate, systemic vascular resistance, and resting metabolic rate in older adults.

1.3.3 Research Question 3

Does acute supplementation with NO_3^--rich beetroot juice improve cardiovascular and cognitive health and performance in younger compared to older adults?

To determine the cardiovascular and cognitive health and performance effects of acute supplementation with NO_3^--rich beetroot juice for younger and older adults, a randomised, double-blind, crossover study (the Acute study) has been designed. This study aims to investigate the acute effects of supplementation with dietary NO_3^- on plasma [NO_2^-] and [NO_3^-], blood pressure, cognitive performance, mood, and heart rate and to examine potential differences in effects between younger (18 – 30 y) and older (50 – 80 y) adults.

1.3.4 Research Question 4

Does chronic supplementation with NO_3^--rich beetroot juice improve cardiovascular health and performance, and cognition in younger compared to older adults?

To investigate this research question, a chronic, double-blind, randomised control trial (the Chronic study) has been designed. This study examines the effects of prolonged (14- and 28-day) supplementation with dietary NO_3^- on plasma [NO_2^-] and [NO_3^-], blood pressure, systemic vascular resistance, cognitive performance, and heart rate in younger and older adults and investigates how this compares to effects of acute supplementation. Additionally, effects on cycle performance and oxygen uptake (during a work-to-completion trial), potential differences in effects between younger

(18 – 30 y) and older (50 – 80 y) adults, and any effect of duration of supplementation between the two age groups are examined following chronic supplementation with NO_3^--rich beetroot juice.

1.4 Thesis Structure

This PhD project follows the format of thesis by publication. An overview of the thesis chapters is presented below with identification of those chapters that have been published, submitted for publication, or in preparation for publication.

Chapter 1 provides the background and rationale for this PhD study. It also introduces and describes the overall aims and four research questions to be addressed in this PhD project.

Chapter 2 is a literature review and provides an overview of dietary nitrate and nitrite and how they act in the human body via their reduction to nitric oxide. This chapter also discusses the clinical research around four key areas of dietary nitrate supplementation, namely the appropriate dose, the potential health benefits, the potential performance benefits, and the potential benefits for older adults. Research around proposed mechanisms of action of dietary nitrate supplementation is also discussed.

Chapter 3 is an extension and magnification of the literature review (Chapter 2) and addresses Research question 1 (Section 1.3.1). This chapter is a systematic review of the clinical evidence of the effects of supplementation with nitrate-rich beetroot on cardiovascular and cognitive health and performance in older adults. This study was published in *Nutrients* in 2017 and is entitled, "Performance and Health Benefits of Dietary Nitrate Supplementation in Older Adults: A Systematic Review".

Chapter 4 presents the first experimental chapter of this PhD project, focusing on the dose-response effects of dietary nitrate supplementation on cardiovascular and cognitive responses in older adults, and addresses Research question 2 (Section 1.3.2). This study has been prepared for submission to *Nutrition, Metabolism & Cardiovascular Diseases*.

Chapter 5 is the second experimental chapter which investigates the acute effects of dietary nitrate supplementation on cardiovascular responses, cognition, and mood in older compared to younger adults and addresses Research question 3 (Section 1.3.3). This study, published in *Nutrients* in 2019, is entitled, "Acute Supplementation with Nitrate-Rich Beetroot Juice Causes a Greater Increase in Plasma Nitrite and Reduction in Blood Pressure of Older Compared to Younger Adults".

Chapter 6 is the third experimental chapter which investigates the effects of chronic supplementation with dietary nitrate on cardiovascular and cognitive responses in older and

younger adults and addresses Research question 4 (Section 1.3.4). This research will be prepared for submission to *The American Journal of Clinical Nutrition*.

Chapter 7 discusses the main findings and highlights the main conclusions of the PhD project, incorporating all the studies. It also presents the limitations of the research and the recommendations for future investigations into the health and performance benefits of dietary nitrate supplementation.

The Appendix section contains supplementary files and supporting documents for this thesis and includes research outputs associated with this PhD study, including a list of publications, oral and poster presentations, and awards (Appendix 1). It also includes copies of the published journal articles with the corresponding DRC 16 forms (Appendices 2 and 3), and templates of the participant information sheets, consent forms, remaining DRC 16 forms, and other relevant information for the three experimental studies (Dose-response, Acute, and Chronic studies; Appendices 4-6).

Chapter 2
Literature Review

This chapter begins with an overview on nitrate, nitrite, and nitric oxide and their effects in the human body, followed by a review of the literature on the effects of dietary nitrate supplementation on cardiovascular and cognitive health and performance.

2.1 Nitrate and Nitrite: An Overview

2.1.1 What are Nitrate and Nitrite?

Nitrate (NO_3^-) is a polyatomic anion containing a single nitrogen atom surrounded by three oxygen atoms; it is abundant in the environment, being found in the air, soil, water, and foods; predominately within green leafy vegetables as they absorb NO_3^- from the soil as a by-product of the nitrogen cycle and subsequently store high concentrations in their leaves (Clifford et al., 2015). This stored NO_3^- is reduced using energy from photosynthesis to produce amino acids, then proteins used for growth (Bedale et al., 2016). Nitrate accumulation is especially evident in vegetables (such as beetroot) that have low light exposure as they convert less NO_3^- to amino acids because of limited photosynthesis (Gilchrist et al., 2010).

Nitrite (NO_2^-) is a symmetrical anion containing a single nitrogen atom surrounded by two oxygen atoms of equal bond length. Both NO_3^- and NO_2^- can be produced endogenously in the human body via oxidation of L-arginine in the presence of O_2 by enzymes from the nitric oxide synthase (NOS) family which forms NO, where NO can then be oxidised to NO_2^- by ceruloplasmin and NO_3^- via reaction with oxyhemoglobin (Bailey et al., 2012). In the soil most NO_2^- is oxidised to NO_3^- by bacteria, thus NO_3^- is the stored form in plants.

2.1.2 Dietary Sources of Nitrate and Nitrite

Nitrate and nitrite are mainly acquired via oral consumption, with greater than 80% of an individual's daily NO_3^- intake coming from their diet. Daily NO_2^- intake is also obtained exogenously either directly from food consumption or indirectly via dietary NO_3^- conversion to NO_2^- (Hord et al., 2009). The majority of NO_3^- ingestion comes from vegetables (such as lettuce, spinach, and beetroot) which account for approximately 60–80% of an individual's daily intake (Bailey et al., 2012). Another major source of dietary NO_3^- intake comes from man-made sources, predominately cured meats, where nitrates (in the forms of sodium or potassium nitrate) are regularly used for their anti-microbial and anti-fungal properties as well as their ability to maintain the flavour and colour of red meat (Bedale et al., 2016). Small amounts of NO_2^- can also be found in vegetables and some breakfast cereals; however, the major dietary source of NO_2^- is cured meats such as bacon, luncheon sausage, and sausages, where NO_2^- is used as a preservative (Clements et al., 2014). Whilst green leafy vegetables are known to have high concentrations of NO_3^- and some NO_2^-, levels can also vary dramatically within individual sources, for example organic vegetables are likely to have lower NO_3^- and NO_2^- levels as they are not grown with the aid of nitrogen-containing fertiliser. Genotype, storage processes and transport conditions can also affect the NO_3^- content of vegetables (Hord et al., 2009).

2.1.3 Nitrate and Nitrite in the Human Body

Once inorganic NO_3^- is consumed it is rapidly absorbed from the stomach and duodenum of the small intestine where it then enters the blood stream (Gilchrist et al., 2014). About 20 - 25% of the NO_3^- absorbed from the circulation is concentrated (10 to 20-fold) in the salivary glands and then enters the entero-salivary cycle (Bedale et al., 2016). From here entero-salivary anaerobic bacteria located in the crypts of the tongue reduce NO_3^- to NO_2^- which is then swallowed again (Clifford et al., 2015). A small amount of this NO_2^- will be reduced to NO in the acidic environment of the stomach, while most re-enters the systemic circulation and is transported to specific locations in the body (predominately the vasculature) where it can be reduced to NO via various catalysis such as deoxyhemoglobin and xanthine oxidoreductase (Bailey et al., 2012, Gilchrist et al., 2014, Hord et al., 2009).

The rate and efficiency with which NO_3^- is reduced to NO relies predominately on the individual's oral microbiota composition (Clements et al., 2014). Hyde et al. (2014) conducted a metagenomic analysis of six healthy participants' oral cavities to investigate the bacterial communities and establish which bacteria were predominately associated with NO_3^- reduction. They found that five bacterial species (*Veillonella, Prevotella, Neisseria, Haemophilus, and Actinomyces*) were the major NO_3^- reducers and that the concentration of these bacteria varied between individuals. With this in mind, it is important to note that the use of chlorhexidine-based mouthwashes should be avoided when investigating potential benefits from dietary NO_3^- supplementation as they have been shown to alter the oral microbiome and inhibit NO_3^- reducing capacity (Kapil et al., 2010).

In comparison to the process following NO_3^- consumption, ingested NO_2^- can be immediately converted to NO in the stomach or absorbed into the systemic blood stream from the small intestine, where it can be directly transported to various tissues of the body for reduction to NO (Bailey et al., 2012). Once reduced to NO it can stimulate various functions in the body depending on the location at which NO is produced (Jones, 2014).

2.1.4 History of Nitrate and Nitrite Supplementation

The use of dietary nitrate in the form of beetroot dates back to the Roman period, where it was used for medicinal purposes in controlling hypertension-derived symptoms (Clifford et al., 2015). Despite this, research in the area of NO_3^- and NO_2^- supplementation was not established until the mid-1900s when Furchgott and Bhadrakom (1953) investigated the effects of various drugs on rabbit aorta and found that supplementation with sodium nitrate resulted in vasodilation of a preconstricted aorta. Following this, the potential for beneficial effects of NO_3^- and NO_2^-

supplementation were further established by Lundberg et al. (1994) who found that NO is produced in the acidic environment of the stomach following ingestion of dietary NO_3^- and NO_2^-, and thus could be used to regulate NO levels. This was a milestone for the discovery of the NO_3^- - NO_2^- - NO pathway in the human body. Later, it was found that there was an alteration to the NO_2^- arterial-venous gradient, resulting in greater usage of NO_2^- despite the known reduction in the rate of the NOS pathway when exercising (Gladwin et al., 2005). Based on these outcomes Gladwin et al. (2005) went on to suggest that NO_2^- is the likely substrate for the production of NO in humans.

The NO_3^- - NO_2^- - NO pathway was then further explored in humans when Larsen et al. (2006) investigated the effects of NO_3^- supplementation on blood pressure and found that it significantly reduced systolic and diastolic values in healthy individuals. Moreover, Larsen et al. (2007) were the first to report reduced oxygen demand following oral supplementation with NO_3^- (in the form of sodium nitrate) during exercise. Others have subsequently examined the effects of NO_3^- supplementation (in the form of beetroot juice) on mitochondrial efficiency and muscle contractility (Bailey et al., 2010), $\dot{V}O_2$ response (Lansley et al., 2011b, Wylie et al., 2016), and time trial performance (Hoon et al., 2014, Lansley et al., 2011a). More recently, studies have examined the effects of NO_3^- supplementation on cognition and mood, while also looking at the potential health benefits in the older population (Kelly et al., 2013, Stanaway et al., 2019, Wightman et al., 2015). These later findings provide evidence for the use of beetroot juice supplementation in manipulation of the NO_3^- to NO_2^- to NO pathway to promote health and performance benefits.

2.2 What is Nitric Oxide and why is it Important?

Nitric oxide (NO) is a heteronuclear diatomic molecule with one oxygen molecule bound to a nitrogen molecule; it is a clear coloured gas that is a free radical (due to its unpaired electron) and is toxic in high concentrations. However, at low concentrations, NO has many beneficial functions within the human body.

Endogenous NO was first discovered in 1980 and originally named endothelium-derived relaxing factor (EDRF). This name was given as the compound was originally identified due to its vasodilatory effects following release from arteries and veins (Furchgott and Zawadzki, 1980). Seven years later Ignarro et al. (1987) showed that EDRF was in fact NO. Since then, investigations into the effects of NO in the human body have increased; it is now known that NO has multiple physiological roles when present at sufficient concentrations (Gilchrist et al., 2014). The main proposed functions of NO in the human body include increased vasodilation and blood flow (Kenjale et al., 2011, Presley et al., 2011), increased oxygen delivery and muscle contractility (Bailey et al., 2010), enhanced

neurotransmission (Martina et al., 2012), improved learning and memory (Gilchrist et al., 2014, Wightman et al., 2015), and stronger immune defence (Martina et al., 2012).

The two major pathways for NO production in humans are shown in Figure 2.1. The first pathway is endogenous, via the oxidation of L-arginine by nitric oxide synthase (NOS) of which there are three major isoforms; one located in the endothelium of vasculature endothelial cells (eNOS), one produced by inducible macrophages (iNOS), and one in the neural tissue of the brain (nNOS) (Ellis et al., 1998, Martina et al., 2012). Consequently, the NOS pathway has important roles in the cardiovascular system (predominately in vasodilation of smooth muscle), the immune system (acting as a toxic compound to infectious agents and causing apoptosis of foreign organisms), and the nervous system (acting as a neurotransmitter and vasodilator) (Clements et al., 2014). The three NOS isoforms (eNOS, iNOS, and nNOS) are also located in other parts of the body and act as a signalling molecule in many pathways and contributing to the multiple functions of NO within the body (Clements et al., 2014, Ellis et al., 1998). The second pathway for NO production is exogenously, via the reduction of dietary NO_3^- to NO_2^-, which occurs in the mouth via lingual anaerobic bacteria (Kelly et al., 2013) as described in Section 2.1.

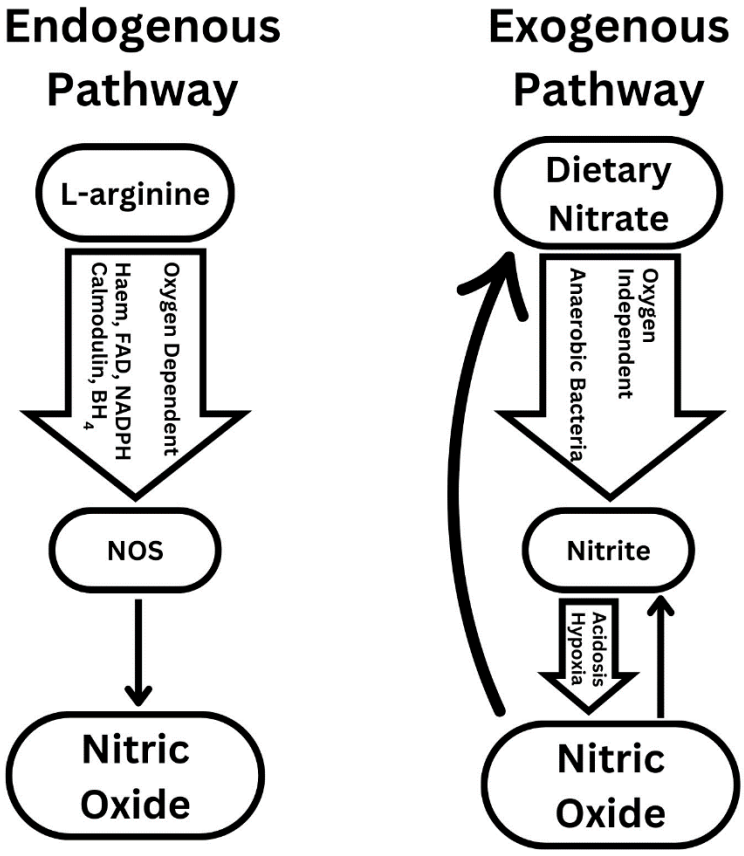

Figure 2.1. The two main pathways of nitric oxide (NO) production in humans. The pathway on the right of the diagram represents the exogenous pathway of NO production from dietary nitrate which is reduced to nitrite, which is in turn reduced to NO. The pathway on the left represents the endogenous pathway of NO production via enzymatic reduction of L-arginine by nitric oxide synthases (NOS) to NO. FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; BH_4, tetrahydrobiopterin.

2.3 Appropriate Nitrate Supplementation Dose

Due to the increasing interest in dietary NO_3^- supplementation because of its potential health and performance benefits, it is important to be able to establish an optimal effective dose for supplementation. Previous research has used a variety of NO_3^- doses ranging from as low as 4.2 mmol (Wylie et al., 2013) to as high as 16.8 mmol (Wylie et al., 2013) and have utilised various study protocols with a focus on the benefits to cardiovascular and cerebral function. A summary of some of these significant studies are presented in Table 2.1. The diversity of benefits from NO_3^- supplementation have also been shown in previous studies and will be discussed below. However, there is limited research investigating the dose-response relationship of dietary NO_3^- (Capper et al., 2022, Hoon et al., 2014, Wylie et al., 2013), with only one study examining the effects in older adults (Capper et al., 2022). One study investigated the dose-response effects of acute supplementation with placebo, 4.2 (low), 8.4 (moderate), and 16.8 (high) mmol of dietary NO_3^- on plasma [NO_2^-] and [NO_3^-] and exercise performance in healthy young men and showed plasma [NO_2^-] and the oxygen cost of moderate intensity exercise changed dose-dependently (Wylie et al., 2013). Later, Hoon et al. (2014) found that acute supplementation with a placebo, single (4.2 mmol), and double (8.4 mmol) dose of dietary NO_3^-, in well trained male rowers, also increased plasma [NO_2^-] and [NO_3^-] dose-dependently, with the double dose providing the greatest improvement in the time to completion of a 2000-m row. Recently, a study investigating acute supplementation with varying amounts of cooked beetroot (100 g / ~4.5 mmol, 200 g / ~8.9 mmol, and 300 g / ~13.4 mmol NO_3^-) demonstrated an increase in plasma, urinary, and salivary NO_3^- concentrations in a dose-dependent manner in both younger and older adults (Capper et al., 2022). These studies indicate the potential benefits for optimal dosing of dietary NO_3^- for health and performance parameters. However, with limited studies in this area and no studies that have looked at cognitive health benefits such as cognitive function, further research is needed. Additionally, there are no dose-response studies in older adults investigating cardiovascular and cognitive responses. Older adults are a potential key population for beneficial effects of dietary NO_3^- supplementation due to their age-related decrease in NO production and utilisation, and endothelial function (Hughes et al., 2016, Sindler et al., 2011, Stanaway et al., 2017), thus further research is also needed in this area.

Table 2.1. Summary of studies investigating the effects of dietary nitrate supplementation on health and performance and their key findings based on dose.

Reference	Participants	Study Design	Acute/chronic	NO_3^- Dose (mmol)	NO_3^- Dose (mg)	Key Findings
Low Dose (≤ 6.9 mmol) (≤ 426 mg)						
Bailey et al. (2009)	8 healthy, recreationally active males (age 26 ± 7 y)	Double-blind, placebo-controlled crossover	Chronic 6 days' supplementation	5.6 per day	342	↑ Plasma $[NO_2^-]$ ↓ SBP ↓ $\dot{V}O_2$ during 4 Mod-I work rates ↑ High-I cycling time to faitgue (16%)
Bailey et al. (2010)	7 recreationally active males (age 28 ± 7 y)	Randomised, double-blind crossover	Chronic 6 days' supplementation	5.1 per day	311	↑ Plasma $[NO_2^-]$ ↓ SBP ↓ DBP ↓ Muscle ATP turnover rate ↓ Muscle ADP accumulation ↓ Muscle Pi accumulation ↓ Muscle PCr breakdown
Eggebeen et al. (2016)	20 HFpEF patients (m/f 3/17) (age 69 ± 7 y)	Randomised, double-blind crossover	Acute and 7 days' supplementation	6.1	372	↑ Plasma $[NO_2^-]$ ↑ Plasma $[NO_3^-]$ ↓ SBP ↑ Time to exhaustion during submaximal cycle exercise
Kemmner et al. (2017)	17 older adults with CKD (m/f 7/10) (age 72 ± 6 y)	Randomised, open-label crossover	Acute	4.9	300	↑ Plasma $[NO_3^-]$ ↓ SBP ↓ DBP ↓ MAP ↓ RRI
Lansley et al. (2011a)	9 competitive male cyclists (club level) (age 21 ± 4 y)	Randomised, crossover	Acute	6.4	390	↑ Plasma $[NO_2^-]$ ↓ SBP ↑ 4 km TT performance. (2.8%) ↑ 16.1 km TT performance. (2.7%)
Lansley et al. (2011b)	9 physically active men (age 22 ± 4 y)	Randomised, double-blind crossover	Chronic 6 days' supplementation	6.4 per day	390	↑ Plasma $[NO_2^-]$ ↓ SBP (4%) ↓ $\dot{V}O_2$ during walking, Mod-I, and High-I running ↑ High-I running time to exhaustion

Thompson et al. (2014)	16 recreationally active males (age 24 ± 4 y)	Randomised, double-blind crossover	Acute	5	305	↑ Plasma [NO_2^-] ↓ $\dot{V}O_2$ at 50% $\dot{V}O_{2\ Max}$ ↑ Exercise tolerance ↔ Cognitive tests ↓ SBP ↑ Cerebral oxygenation (NIRS)
Wightman et al. (2015)	40 healthy adults (aged 18 – 27 y)	Randomised, double-blind, placebo-controlled	Acute	5.5	336	↑ Plasma [NO_2^-] ↑ Serial 3s subtraction performance ↑ Cerebral oxygenation (NIRS) / CBF ↔ SBP and DBP
Wylie et al. (2013)	2 groups of 10 healthy recreationally active males (age 23 ± 5 y) and (age 22 ± 5 y)	Balanced, crossover	Acute	4.2	256	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-] ↔ Steady state O_2 uptake Mod-I exercise ↔ Time to task failure ↓ SBP
Moderate Dose (7 – 9.9 mmol) (427 – 604 mg)						
Berry et al. (2015)	15 COPD patients (m/f 12/3) (age 69.6 ± 8.5 y)	Randomised, single-blind crossover	Acute	7.58	462	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-] ↑ Time to exhaustion during submaximal cycle exercise ↓ SBP ↓ DBP
Breese et al. (2013)	9 recreationally active males (n = 4) and females (n = 5) (age 30 ± 6 y)	Randomised, double-blind crossover	Chronic 6 days' supplementation	8 per day	488	↑ Plasma [NO_2^-] ↑ Rate of muscle HHb (NIRS) ↓ $\dot{V}O_2$ response time Mod-I to High-I ↑ High-I cycling time to faitgue (22%)
Cermak et al. (2012)	9 well trained, male cyclists or triathletes (age 31 ± 3 y)	Double-blind, repeated measures, crossover	Chronic 6 days' supplementation	8 per day	488	↑ Plasma [NO_2^-] ↔ SBP and DBP ↑ 10-km TT performance (1.2%) ↑ Mean power output (2%)
Gilchrist et al. (2013)	27 older adults with type 2 diabetes (m/f 18/9) (age 67.2 ± 4.9 y)	Randomised, double-blind crossover	Chronic 14 days' supplementation	7.5 per day	458	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-] ↔ BP ↔ MAP ↔ Endothelial function

						↔ Insulin sensitivity
Gilchrist et al. (2014)	27 older adults with type 2 diabetes (m/f 18/9) (age 67.2 ± 4.9 y)	Randomised, double-blind crossover	Chronic 14 days' supplementation	7.5 per day	458	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-] ↓ Simple reaction time (improvement)
Kelly et al. (2013)	6 male and 6 female older adults (age 64 ± 4; 63 ± 2 y)	Randomised, double-blind crossover	Chronic 3 days' supplementation	9.6 per day	586	↑ Plasma [NO_2^-] ↓ $\dot{V}O_2$ response time from rest to walking ↔ Cognitive tests ↓ SBP ↓ DBP
Kenjale et al. (2011)	4 male and 4 female PAD patients (age 67 ± 13 y)	Randomised, open-label, crossover	Acute	9	549	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-] ↓ DBP ↑ Walking duration longer before onset of pain (18%). ↑ Maximum walking time (17%). ↑ Tissue blood flow (NIRS) ↓ DBP ↓ SBP and HR during recovery
Wylie et al. (2013)	2 groups of 10 healthy recreationally active males (age 23 ± 5 y) and (age 22 ± 5 y)	Balanced, crossover	Acute	8.4	512	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-] ↓ Steady state O_2 uptake Mod-I exercise (1.7%) ↑ Time to task failure (14%) ↓ SBP ↓ DBP
High Dose (≥ 10 mmol) (≥ 605 mg)						
de Oliveira et al. (2016)	20 older adults with risk factors for cardiovascular disease (m/f 7/13) (age 70.5 ± 5.6 y)	Randomised, double-blind crossover	Acute	12.2	744	↑ Urinary [NO_2^-] ↑ Urinary [NO_3^-] ↑ Endothelial function ↔ BP ↔ Arterial stiffness

Presley et al. (2011)	14 healthy older adults (Not Stated) (age 74.7 ± 6.9 y)	Placebo-controlled, crossover	Chronic 2 days' supplementation	12.4	756	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-] ↑ Cerebral Perfusion (MRI)
Shepherd et al. (2016)	15 healthy older adults (m/f 8/7) (age 59.2 ± 6 y)	Randomised, double-blind crossover	Acute	11.91	727	↑ Plasma [NO_2^-] ↔ BP ↔ Hepatic blood flow (MRI) ↔ Plasma glucose ↔ C-peptide ↔ [Incretin]
Siervo et al. (2016)	19 healthy older adults (m/f 9/10) (age 64.7 ± 3 y)	Randomised, double-blind crossover	Chronic 7 days' supplementation	12 per day	732	↑ Plasma [NO_3^-] ↑ Urinary [NO_3^-] ↔ BP ↔ O_2 Consumption ↔ Hand-grip strength ↔ Up and go test ↔ Repeated chair raising test ↔ 10-m walking test
Stanaway et al. (2019)	13 healthy younger adults (m/f 9/4) (age 25 ± 3) and 11 healthy older adults (m/f 3/8) (age 56 ± 6 y)	Randomised, double-blind crossover	Acute	10.5	641	↑ Plasma [NO_2^-] in both age groups, but a great increase in older adults ↑ Plasma [NO_3^-] ↓ SBP in both age groups ↓ DBP in older adults only ↓ Stroop test reaction time in both age groups
Thompson et al. (2015)	16 male team-sports players (age 24 ± 5 y)	Randomised, double-blind crossover	Chronic 7 days' supplementation	12.8 per day	781	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-] ↑ Total work done during sprint test ↑ Reaction time in second half of sprint test
Wylie et al. (2013)	2 groups of 10 healthy recreationally active males (age 23 ± 5 y) and (age 22 ± 5 y)	Balanced, crossover	Acute	16.8	1025	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-] ↓ Steady state O_2 uptake Mod-I exercise (3%) ↑ Time to task failure (12%) ↓ SBP ↓ DBP

↑, significant increase; ↓, significant decrease; ↔, no significant difference; [NO_2^-], nitrite concentration; [NO_3^-], nitrate concentration; m/f, male/female; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; $\dot{V}O_2$, oxygen uptake; ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate; PCr, creatine phosphate; P/O ratio, phosphate/oxygen ratio; TT, time trial; Mod-I, moderate-intensity; High-I, high-intensity; CBF, cerebral blood flow; NIRS, near infrared spectroscopy; MRI, magnetic resonance imaging; [HHb], deoxyhemoglobin concentration; PAD, peripheral arterial disease; RRI, renal resistive index.

2.4 Health in NZ: Potential Benefits of Increasing Nitrate Intake

The average life expectancy in New Zealand has continued to increase significantly over the past few decades and, as a result, the rate of morbidity has also increased (Ministry of Health, 2016). This is exaggerated by the ageing population of New Zealanders ('baby boomers' born 1945 – 1965), as the older population is now living longer with multi-morbidities and associated disability (Ministry of Health, 2016). Older adults make up one-eighth of the population but account for over one-third of all health loss (loss of years lived in good health), with a large portion of this coming from cardiovascular and neurological disorders (Ministry of Health, 2016). As a result, health costs are expected to increase exponentially, which will place further strain on the social security and welfare budget to fund the increasing public health care costs as more people require treatment (Jackson, 2011). Therefore, development of methods to help reduce negative cardiovascular and cognitive deteriorations (such as atherosclerosis, hypertension, and dementia) is critical. As one major contributing factor to most of these chronic diseases is dysfunction of the endothelium and failure to maintain NO homeostasis, NO_3^- supplementation (as a NO stimulator) has gained recent attention for its potential to attenuate these diseases (Clements et al., 2014). Table 2.2 presents a summary of the studies supporting the health benefits of dietary NO_3^- supplementation, and specific benefits to cardiovascular and cerebrovascular health will be discussed in Sections 2.4.1 and 2.4.2.

Table 2.2. Summary of studies investigating the effects of dietary nitrate supplementation on health-based, cognitive performance, and physiological performance outcomes.

Reference	Participants	Study Design	Supplementation Protocol	Testing Protocols	Blood Measures (NO Indices)	Health-based Outcomes	Cognitive Performance Outcomes	Physiological Performance Outcomes
Bailey et al. (2009)	8 healthy, recreationally active males (age 26 ± 7 y)	Double-blind, placebo-controlled crossover	6 days' supplementation 500 ml·day^{-1} BR (5.6 mmol·day^{-1} NO_3^-)	4 Mod-I and 2 High-I cycle exercise bouts	↑ Plasma [NO_2^-]	↓ SBP	NT	↓ $\dot{V}O_2$ during 4 Mod-I work rates ↑ High-I cycling time to faitgue (16%)
Bailey et al. (2010)	7 recreationally active males (age 28 ± 7 y)	Randomised, double-blind crossover	6 days' supplementation 500 ml·day^{-1} BR (5.1 mmol·day^{-1} NO_3^-)	6 Low-I and 3 High-I, two-legged knee-extensor exercise bouts	↑ Plasma [NO_2^-]	↓ SBP ↓ DBP	NT	↓ Muscle ATP turnover rate ↓ Muscle ADP accumulation ↓ Muscle Pi accumulation ↓ Muscle PCr breakdown
Breese et al. (2013)	9 recreationally active males (n = 4) and females (n = 5) (age 30 ± 6 y)	Randomised, double-blind crossover	6 days' supplementation 140 ml·day^{-1} BR (8 mmol·day^{-1} NO_3^-)	Double-step cycling protocol, unloaded to Mod-I, Mod-I to High-I	↑ Plasma [NO_2^-]	↑ Rate of muscle HHb (NIRS)	NT	↓ $\dot{V}O_2$ response time Mod-I to High-I ↑ High-I cycling time to faitgue (22%)
Cermak et al. (2012)	9 well trained, male cyclists or triathletes (age 31 ± 3 y)	Double-blind, repeated measures, crossover	6 days' supplementation 140 ml·day^{-1} BR (8 mmol·day^{-1} NO_3^-)	Low-I cycling followed by 10-km TT on cycle ergometer	↑ Plasma [NO_2^-]	↔ SBP and DBP	NT	↑ 10-km TT performance (1.2%) ↑ Mean power output (2%)
Gilchrist et al. (2013)	27 older adults with type 2 diabetes (age 67.2 ± 4.9 y)	Randomised, double-blind crossover	14 days' supplementation 250 ml·day^{-1} BR (7.5 mmol NO_3^-)	NT	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	↔ BP ↔ MAP ↔ Endothelial function ↔ Insulin sensitivity	NT	NT
Gilchrist et al. (2014)	27 older adults with type 2 diabetes (age 67.2 ± 4.9 y)	Randomised, double-blind crossover	14 days' supplementation 250 ml·day^{-1} BR (7.5 mmol NO_3^-)	5 cognitive tests on E-Studio software	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	NT	↑ Simple reaction time	NT
Jones et al. (2019)	18 healthy older adults (age 63 ± 6 y)	Double-blind, randomised control trial	28 days' supplementation 70 ml·day^{-1} BR (6.6 mmol·day^{-1} NO_3^-) (Note PL used was prune juice)	NT	↑ Plasma [NO_3^-] – day 14	↓ SBP - day 14 ↓ DBP - day 14 ↑ Large-vessel endothelial function - days 14 and 28 ↔ Microvascular function	NT	NT
Kapil et al. (2015)	PL: 10 male and 22 female, BR: 16 male and 16 female hypertensive patients	Double-blind, randomised control trial	28 days' supplementation 250 ml·day^{-1} BR (6.4 mmol·day^{-1} NO_3^-)	NT	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	↓ SBP ↓ DBP ↑ Endothelial function (~20%)	NT	NT

	(PL: age 56.3 ± 16.4, BR: age 57.6 ± 13.9 y)					↓ Arterial Stiffness		
Kelly et al. (2013)	6 male and 6 female older adults (age 62 ± 2; 64 ± 3 y)	Randomised, double-blind crossover	3 days' supplementation 140 ml·day^{-1} BR (9.6 mmol·day^{-1} NO_3^-)	Walking treadmill test and leg ergometer. Cognitive function tests	↑ Plasma [NO_2^-]	↓ SBP ↓ DBP	↔ Cognitive tests	↓ $\dot{V}O_2$ response time from rest to walking
Kenjale et al. (2011)	4 male and 4 female PAD patients (age 67 ± 13 y)	Randomised, open-label, crossover	Acute supplementation 3 h prior to exercise 500 ml BR (9 mmol NO_3^-) (Note PL used was orange juice)	Walking maximal cardiopulmonary exercise (CPX) test	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	↑ Tissue blood flow (NIRS) ↓ DBP ↓ SBP and HR during recovery	NT	↑ Walking duration longer before onset of pain (18%). ↑ Maximum walking time (17%).
Lansley et al. (2011a)	9 competitive male cyclists (club level) (age 21 ± 4 y)	Randomised, crossover	Acute supplementation 2 – 2.5 h prior to exercise 500 ml BR (6.4 mmol NO_3^-)	4 and 16.1 km TT on cycle ergometer	↑ Plasma [NO_2^-]	↓ SBP	NT	↑ 4 km TT performance. (2.8%) ↑ 16.1 km TT performance. (2.7%)
Lansley et al. (2011b)	9 physically active men (age 22 ± 4 y)	Randomised, double-blind crossover	6 days' supplementation 140 ml·day^{-1} BR (6.4 mmol·day^{-1} NO_3^-)	Walking, 4 Mod-I and 2 High-I running sessions on the treadmill (on days 4 – 6 of supplementation)	↑ Plasma [NO_2^-]	↓ SBP (4%)	NT	↓ $\dot{V}O_2$ during walking, MI, and High-I running ↑ High-I running time to exhaustion
Larson et al. (2007)	9 well trained, male cyclists or triathletes (age 28 ± 6 y)	Randomised, double-blind crossover	3 days' supplementation sodium nitrate (0.1 mmol·kg^{-1}·day^{-1})	Submaximal and maximal cycling for 5 min at 45, 60, 70, 80, 85, and 100% $\dot{V}O_{2max}$	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	↓ SBP ↓ DBP	NT	↓ $\dot{V}O_2$ for 4 lowest work rates ↑ Gross efficiency
Larson et al. (2011)	14 recreationally active males (n = 11) and females (n = 3) (age 25 ± 1 y)	Randomised, double-blind crossover	3 days' supplementation sodium nitrate (0.1 mmol·kg^{-1}·day^{-1})	Low-I cycling on ergometer	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	NT	NT	↑ Mitochondrial P/O ratio
Presley et al. (2011)	16 older adults (age 74.7 ± 6.9 y)	Placebo-controlled, crossover	High NO_3^- diet with BR (-12.4 mmol NO_3^-) vs low NO_3^- diet (-0.089 mmol NO_3^-)	NT	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	↑ Cerebral Perfusion (MRI)	NT	NT
Rogerson et al. (2022)	Healthy younger (age 24.2 ± 3.5 y; n = 17) and older (age 68.7 ± 10.3 y; n = 7) adults	Randomised, double-blind crossover	Acute supplementation 70 ml BR (6.4 mmol NO_3^-)	BP measured every 20 min for 3 h	↑ Urinary [NO_3^-] – younger and older	↔ SBP and DBP ↔ Microcirculatory endothelial function.	NT	NT
Thompson et al. (2014)	16 recreationally active males (age 24 ± 4 y)	Randomised, double-blind crossover	Acute supplementation 90 min prior to cognitive tests 500 ml BR (5 mmol NO_3^-)	Continuous cycling test (20 min at 50% and 70%, followed by 90% until voluntary exhaustion and cognitive tests	↑ Plasma [NO_2^-]	↓ SBP ↑ Cerebral oxygenation (NIRS)	↔ Cognitive tests	↓ $\dot{V}O_2$ at 50% $\dot{V}O_{2\,Max}$ ↑ Exercise tolerance

Thompson et al. (2015)	16 male team-sports players (age 24 ± 5 y)	Randomised, double-blind crossover	7 days' supplementation 140 ml·day^{-1} BR (12.8 mmol·day^{-1} NO_3^-)	Prolonged intermittent sprint test and cognitive function tests	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	NT	↑ Reaction time in second half of sprint test	↑ Total work done during sprint test
Wightman et al. (2015)	40 healthy adults (aged 18 – 27 y)	Randomised, double-blind, placebo-controlled	Acute supplementation 90 min prior to cognitive tests 450 ml BR (5.5 mmol NO_3^-)	3 cognitive tests using COMPASS and mood test	↑ Plasma [NO_2^-]	↑ Cerebral oxygenation (NIRS) / CBF ↔ SBP and DBP	↑ Serial 3s subtraction performance	NT

↑, significant increase; ↓, significant decrease; ↔, no significant difference; NT, not tested; [NO_2^-], nitrite concentration; [NO_3^-], nitrate concentration; BR, beetroot juice; PL, placebo; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; $\dot{V}O_2$, oxygen uptake; ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate; PCr, creatine phosphate; P/O ratio, phosphate/oxygen ratio; TT, time trial; Low-I, low-intensity; Mod-I, moderate-intensity; High-I, high-intensity; CBF, cerebral blood flow; NIRS, near infrared spectroscopy; MRI, magnetic resonance imaging; [HHb], deoxyhemoglobin concentration; PAD, peripheral arterial disease.

2.4.1 Cardiovascular Health

Over the past decade, a significant amount of research has shown improvements in blood pressure following supplementation with dietary nitrate (Kapil et al., 2015, Kelly et al., 2013, Larsen et al., 2006, Wylie et al., 2013), however, some studies have shown no beneficial effect on blood pressure (Cermak et al., 2012, Gilchrist et al., 2013; Table 2.2). Specifically, Larsen and colleagues (2006) were the first to investigate the effects of oral NO_3^- supplementation (3 days of 0.1 mmol·kg^{-1}·day^{-1} sodium NO_3^-) on blood pressure and found a significant reduction in mean DBP (3.7 mmHg) and mean arterial pressure (MAP; 3.2 mmHg) with NO_3^- supplementation compared to placebo (sodium chloride); however, there was no difference in SBP between treatments. In support of these findings, investigation into the effects of supplementation with three dosages of NO_3^--rich beetroot juice (4.2, 8.4 and 16.8 mmol of NO_3^-) showed peak plasma [NO_2^-] occurred 2 – 2.5 hours after ingestion, corresponding with the highest reductions in blood pressure, thus further indicating the effect of NO_3^- supplementation on health outcomes via the reversal of characteristics of endothelial dysfunction (Wylie et al., 2013). These benefits have also been shown in older adults (60 – 70 y) where NO_3^--rich beetroot juice supplementation (9.6 mmol·day^{-1} NO_3^-) significantly reduced both SBP (115 ± 9 vs. 120 ± 6 mmHg) and DBP (70 ± 5 vs. 73 ± 5 mmHg) (Kelly et al., 2013). Furthermore, as proof of concept, Ghosh et al. (2013) investigated the effects of acute supplementation with dietary NO_3^- (3.5 mmol) on blood pressure in hypertensive rats and found a clinically significant reduction in SBP (~12 mmHg) and DBP. This was then further investigated in hypertensive human patients, which also showed a significant reduction in 'in clinic' measured blood pressure, 24 h ambulatory blood pressure, and 'at-home' blood pressure following 28 days of supplementation with nitrate-rich beetroot juice (~6.4 mmol·day^{-1}) (Kapil et al., 2015). These results suggest that NO_3^- supplementation as a NO stimulator can be an effective, low-cost alternative to hypertensive drug treatments. It also suggests the potential for greater health benefits in individuals already suffering from endothelial dysfunction from reduced NO bioavailability, thus effects could be more pronounced in the older population who may have suboptimal cardiovascular functioning capacity (Donato et al., 2018, Kelly et al., 2013, Stanaway et al., 2019).

In addition to reductions in blood pressure, dietary NO_3^- supplementation has also been shown to promote improvements in endothelial function alongside increased blood flow (Kapil et al., 2015, Kenjale et al., 2011). Acute inhibition of blood platelet aggregation, and prevention or reversal of diminished endothelial function has been seen from High NO_3^- containing foods (Webb et al., 2008), this particularly holds greater significance in the older population who are at a higher risk of reduced blood flow and endothelial dysfunction due to ageing (Donato et al., 2018, Presley et al., 2011). Therefore, if endogenous production of NO is diminished via reduced activity of NOS, then

supplementation of dietary NO_3^- may help provide an alternative pathway for NO production, thus helping maintain blood flow and circulation. (Webb et al., 2008). Previous studies in animals have shown NO_3^- supplementation can reduce smooth muscle cell proliferation, arterial stiffness, arterial membrane thickness and inflammation of the arteries; all of which led to an increase in blood flow and reduced arterial resistance, thus reducing the risk of cardiovascular disease, and improving cardiovascular health (reviewed in Clements et al., 2014). In further support of this, one study in humans investigated the effects of 28 days' supplementation with dietary NO_3^- (~6.4 $mmol \cdot day^{-1}$) on pulse wave velocity and flow mediated dilation and showed a significant reduction in arterial stiffness (0.59 $m \cdot s^{-1}$) and improvement in endothelial function (~20%) in adults (18-85 y) with hypertension (Kapil et al., 2015). In addition, Kenjale et al. (2011) used near infrared spectroscopy (NIRS) and found an increase in blood flow to the working muscles in older adults which resulted in an increase in walking time prior to the onset of pain from peripheral arterial disease (PAD). These results suggest the potential for dietary NO_3^- supplementation to improve endothelial function and increase blood flow, particularly in individuals at risk of endothelial dysfunction. It is important to note that studies investigating the effects of dietary NO_3^- supplementation on endothelial function are still limited and present mixed findings (Gilchrist et al., 2013, Lara et al., 2016). Therefore, further research is required in this area with particular attention on populations which may experience dysregulation of the endothelium, such as older adults.

2.4.2 Cerebrovascular Health

In contrast to the cardiovascular health benefits of NO_3^- supplementation, research into investigation of cerebrovascular health benefits is still limited. Neural diseases such as dementia and Parkinson's disease are now the leading cause of 'health loss' in New Zealand (Ministry of Health, 2016). Nitrate supplementation has the potential to attenuate age-related decreases in NO activity and loss of cerebral blood flow, resulting in improved cerebrovascular health (Kelly et al., 2013, Stanaway et al., 2017). Presley et al. (2011), using magnetic resonance imaging (MRI), showed increased perfusion in the pre-frontal cortex of the brain of older adults following dietary NO_3^- supplementation. This indicates that dietary NO_3^- supplementation may counteract the negative effects of ageing and have the potential to slow or even prevent development of cerebrovascular dysfunction. Furthermore, it has been shown that supplementation with NO_3^--rich beetroot juice increased cerebral blood flow (CBF) and cognitive performance 90 min post consumption, thus indicating that dietary NO_3^- may stimulate neural function and oppose cerebrovascular dysfunction resulting in increased cerebrovascular health (Wightman et al., 2015). Despite these results, to date no study has directly investigated the dose-response relationship or the long-term effects of dietary NO_3^- supplementation on the development of neurodegenerative diseases, thus cause and effect

cannot be confirmed. Therefore, further research is required to investigate the effects of dietary NO_3^- supplementation on specific diseases of the brain and determine its benefits to cerebrovascular health.

2.4.3 Potential Risks of Nitrate and Nitrite Consumption

Despite the benefits of dietary NO_3^- supplementation, some issues have been proposed with regards to the safety of regular consumption of dietary NO_3^-. Nitrate and nitrite have historically gained a reputation for negative health effects due to their ability to form the carcinogenic compounds N-nitrosamines in the human body (Clements et al., 2014), which have been implicated in certain cancers including gastric, stomach, pancreatic, and thyroid cancers (Bryan and Loscalzo, 2011). However, there is no evidence for carcinogenic effects of prolonged dietary NO_3^- supplementation with both epidemiological and experimental studies unable to present consistent evidence for even N-nitrosamine formation much less increased risk of cancer (Machha and Schechter, 2012). Furthermore, studies that have indicated carcinogenic effects of prolonged NO_3^- and NO_2^- supplementation have not controlled for variations in sources such as those from cured meats versus vegetables (Bryan and Loscalzo, 2011). This is important, as NO_3^- and NO_2^- in cured meats have been more commonly associated with cancer and negative health effects (Clements et al., 2014). However, vegetable sources such as beetroot juice have been shown to have positive health benefits, with increased vegetable intake being linked with a reduction in the risk of cancer (Gilchrist et al., 2010). In support of this, the International Agency for Research on Cancer stated that while there was no supporting evidence for the increased risk of cancer with dietary NO_3^- and NO_2^- supplementation, they did indicate that consumption of foods high in NO_2^- and low in vitamin C (for example processed meats) were the predominant combination associated with any potential increased risk (Clements et al., 2014). Vitamin C and other compounds such as polyphenols (which are both high in beetroot juice) can react with secondary amines to produce NO and water. Secondary amines also react with nitrous acid, formed from nitrite entering the acidic environment in the gut, to form nitrosamines which are mutagenic (Combet et al., 2007). Therefore, vegetables such as beetroot, which are high in vitamin C and polyphenols, have the potential to negate the possible negative effects of high nitrate levels and can be assumed safe and pose little or no health risk (Machha and Schechter, 2012). Collectively, research indicates that the negative health issues associated with dietary NO_3^- and NO_2^- supplementation are likely exaggerated and that currently there are no safety issues with their consumption from natural sources such as beetroot juice.

2.5 Nitrate Supplementation and Cognitive Performance

With increasing rates of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, due to the ageing population (as mentioned in Section 2.4), methods of slowing and/or preventing their onset have gained greater attention (Carter et al., 2020, Kelly et al., 2013). Dietary NO_3^- supplementation has become a topic of investigation in this area over the past decade, with the potential to improve cognitive performance (Stanaway et al., 2019, Thompson et al., 2015).

2.5.1 Cerebral Function

Studies examining the effects of nitrate supplementation on cognitive performance are few in number and the results are equivocal (Clifford et al., 2015, Stanaway et al., 2017). Some studies have shown significant improvements in cognitive performance, whereby 14 days of supplementation with NO_3^--rich beetroot juice (250 ml·day^{-1}, 7.5 mmol NO_3^-) in older adults (67.2 ± 4.9 years) with type 2 diabetes resulted in a significant improvement in simple reaction time compared to placebo (Gilchrist et al., 2014). However, there was no difference for the other cognitive tests including decision time, rapid processing, and spatial and shape memory. Thompson et al. (2015) supplemented team sport players (24 ± 5 y) with 140 ml of beetroot juice (12.8 mmol NO_3^-) or placebo (~0.08 mmol NO_3^-) daily for 7 days and investigated the effects on cognitive function during a prolonged intermittent sprint test. They found that beetroot juice consumption significantly improved reaction time between the first and second half of the sprint test (820 ± 96 vs. 817 ± 86 ms) compared to placebo (824 ± 114 vs. 847 ± 118 ms); however, there was no significant difference in response accuracy (Thompson et al., 2015).

Other studies have shown no effect of NO_3^- supplementation on cognitive performance. The effects of NO_3^--rich beetroot juice supplementation over 3 days (2 x 70 ml·day^{-1}, 9.6 mmol of NO_3^-) on cognitive performance in healthy, older adults (aged 60 – 70 y) showed no significant differences in memory, attention, and information processing capability between beetroot juice and placebo treatments (Kelly et al., 2013). Similarly, acute beetroot juice supplementation (500 ml, 5 mmol NO_3^-) in recreationally active males (24 ± 4 y) resulted in no differences in cognitive performance compared to placebo (blackcurrant drink) during a continuous cycling test despite there being a significant increase in cerebral oxygenation (via fNIRS) (Thompson et al., 2014). The differences in cognitive performance results seen between these four studies may be attributed to methodological variations, including the duration of supplementation and the cognitive tests used. For example, both Gilchrist et al. (2014) and Thompson et al. (2015) used longer supplementation periods (14 and 7 days, respectively) and included simple reaction tests; however, both Kelly et al. (2013) and Thompson et al. (2014) used shorter supplementation periods (3 days and acute supplementation,

respectively) and did not include a simple reaction test. Additionally, the dose of dietary NO_3^- used could have impacted the results as Thompson et al. (2015) used 12.8mmol of NO_3^- while Thompson et al. (2014) only used 5 mmol, thus as higher doses of dietary NO_3^- could result in a greater conversion to NO, larger effects may be expected. In contrast, Kelly et al. (2013) used 9.8mmol of NO_3^- while Gilchrist et al. (2014) used 7.5 mmol, however Kelly et al. (2013) had healthy older adult participants and Gilchrist et al. (2014) had diabetic participants which may have affected the results to a greater degree than the dose used.

2.5.2 Potential Mechanisms of Action

The potential reasons for improvements in cognition following supplementation with dietary NO_3^- include alteration to cerebral metabolism and increased cerebral blood flow (CBF) (Thompson et al., 2015). The most commonly used methods for investigating CBF following consumption of dietary NO_3^- are functional near infrared spectroscopy (fNIRS) and MRI. Functional NIRS and MRI are non-invasive tools used to provide measurements of brain function. Functional NIRS relies on the principle of neurovascular coupling (the relationship between cerebral blood flow and neural activity) to infer brain activity from changes in oxygenation (Vermeij et al., 2012), while MRI uses strong magnetic fields and radio waves with the body's natural magnetic components to render quality images of specific parts of the body to show activation (Berger, 2002). With the use of fNIRS, acute supplementation with NO_3^--rich beetroot juice (5 mmol) compared to PL has been shown to significantly reduce cerebral deoxyhemoglobin concentration during cognitive testing at rest, and during exercise (Thompson et al., 2014). This has been suggested to indicate a reduction in the amount of oxygen required for mental processing tasks, with the potential to counteract impaired cognitive performance and, with large enough increases in cerebral oxygenation, to possibly improve cognitive performance (Thompson et al., 2015, Wightman et al., 2015).

Changes in CBF have also been demonstrated using MRI where consumption of a high NO_3^- diet including beetroot juice (~12.4 mmol of NO_3^-) in older adults (74.7 ± 6.9 years), resulted in a significant increase in perfusion of the frontal cortex – an area of the brain involved in executive function, task management and memory which is critical for cognitive ability (Presley et al., 2011). Furthermore, acute supplementation with NO_3^--rich beetroot juice (500 ml, ~12.3 mmol of NO_3^-) versus placebo (orange juice) has been shown to reduce cerebrovascular resistance index at rest and during submaximal exercise, supporting the involvement of beetroot juice in increasing CBF (Bond et al., 2013). These results clearly indicate increased blood flow to the brain following dietary NO_3^- supplementation; however, the researchers did not concurrently measure cognitive performance in the same study, thus cause and effect cannot be directly related. This issue was later

elaborated on by Wightman et al. (2015) who investigated the effects of acute supplementation with NO_3^--rich beetroot juice (5.5 mmol NO_3^-) versus placebo (blackcurrant mix) on cognitive performance and CBF of the prefrontal cortex in healthy younger adults (18-27 y). The authors found a significant increase in CBF following initiation of cognitive tests and a concurrent improvement in the serial 3-s subtraction task (measure of mental tracking capacity and prefrontal cortex activation) with consumption of beetroot juice (Wightman et al., 2015). These results further suggest that NO_3^- mediated cognitive performance benefits could be due to increases in vasodilation resulting in greater blood flow to the brain.

Collectively, these results suggest that changes in cerebral metabolism that are sufficient to improve cognitive performance may be more significant following prolonged (potentially at least 7 days) dietary NO_3^- supplementation and may be more pronounced in testing measuring reaction time (Gilchrist et al., 2014, Thompson et al., 2015). Additionally, changes in cerebral oxygenation and increases in CBF, following supplementation with dietary NO_3^-, both play an important role for improvements in cognition (Thompson et al., 2015). Whilst results in this area are still conflicting, there is evidence for potential benefits which warrants further research in the field of dietary NO_3^- supplementation and cognitive performance.

2.6 Nitrate Supplementation and Physiological Performance

In comparison to cognitive performance, physiological performance benefits from dietary NO_3^- supplementation have been researched in more depth. Given the nature of competitive sports with athletes and coaches constantly seeking new, safe, and convenient methods of improving physiological performance, dietary NO_3^- has become a big area of interest (Clements et al., 2014). A meta-analysis by Hoon et al. (2013) examined 17 studies that investigated the physiological performance benefits of NO_3^- and found that NO_3^- supplementation (either acute or prolonged) resulted in a 0.9% improvement in time trial performance. Whilst this could be considered a small effect, when put in context, a 0.9% improvement is more than sufficient to significantly impact an athlete's performance and potentially affect the outcome of a sporting event (Hoon et al., 2013). The most common reasons why dietary NO_3^- supplementation may be beneficial for exercise performance will be discussed in Sections 2.6.1 and 2.6.2, with a summary of the evidence presented in Table 2.2.

2.6.1 Aerobic Performance Benefits of Nitrate Consumption

The competitiveness of sports continues to grow at a rapid pace, where even minor performance improvements of as little as 0.3 – 0.6% have been shown to separate first and third place over a

variety of sports (Hoon et al., 2013). The benefits of inorganic NO_3^- supplementation on physiological performance have been attributed to alterations in aerobic energy pathways including improved blood flow to the working muscles (Larsen et al., 2007), improved oxygen uptake ($\dot{V}O_2$) response (Lansley et al., 2011b, Wylie et al., 2016), reduced muscle phosphocreatine (PCr) depletion, reduced adenosine triphosphate (ATP) cost (Bailey et al., 2010), and increased time to fatigue (Lansley et al., 2011a); therefore, the greatest improvements are seen in aerobic performance (Hoon et al., 2013).

Early work by Larsen et al. (2007) investigated the effects of NO_3^- supplementation for 3 days (sodium nitrate ~7 – 10 mmol·day^{-1}) on metabolic and circulatory factors during exercise. They found that NO_3^- supplementation significantly increased blood flow and lowered $\dot{V}O_2$ at a set submaximal cycling workload compared to placebo (2.82 ± 0.58 vs. 2.98 ± 0.57 L·min^{-1}, respectively). Furthermore, they stated that the amount of sodium nitrate used (0.1 mmol·kg^{-1}·day^{-1}) corresponds to approximately 150 – 250 g of nitrate-rich vegetables such as beetroot, which could easily be achieved through dietary means. The effects of NO_3^- supplementation on $\dot{V}O_2$ were further demonstrated by Lansley et al. (2011b) who found that 6 days of supplementation with beetroot juice (500 ml·day^{-1}: 6.2 mmol of NO_3^-) resulted in a significant reduction in $\dot{V}O_2$ during walking (0.70 ± 0.12 vs. 0.87 ± 0.10 L·min^{-1}), moderate-intensity running (2.10 ± 0.28 vs. 2.26 ± 0.27 L·min^{-1}) and high-intensity running (3.50 ± 0.62 vs. 3.77 ± 0.57 L·min^{-1}) compared to placebo (500 ml·day^{-1}: ~0.003 mmol of NO_3^-). Additionally, investigation into the effects of NO_3^-supplementation for 3 days (in the form of beetroot juice; 2 x 70 ml·day^{-1}, 9.6 mmol of NO_3^-) on physiological performance in older adults (aged 60–70 y) showed that beetroot supplementation 2.5 h prior to exercise significantly reduced $\dot{V}O_2$ response time (25 ± 7 s) compared to placebo (28 ± 7 s), allowing for faster delivery of oxygen during transition from rest to walking (Kelly et al., 2013).

Dietary NO_3^- supplementation has also been suggested to improve aerobic performance by reducing PCr depletion and consequently reducing the ATP cost of the working muscles, so that less ATP is consumed for a given effort (Jones, 2014). Examination of the effects of NO_3^--rich beetroot juice supplementation (5.1 mmol·day^{-1} NO_3^-) for 6 days on two-legged knee extensor exercise (6 low-intensity and 3 high-intensity), showed beetroot juice consumption resulted in a significant reduction of PCr in both low- (36%) and high-intensity (59%) bouts, when compared to placebo (Bailey et al., 2010); this was a key finding in helping to explain the potential mechanisms for improved aerobic performance. Furthermore, it was shown that the estimated total ATP turnover rate was significantly reduced with beetroot supplementation (192 ± 38 µM·s^{-1}) versus placebo (296 ± 58 µM·s^{-1}); with both adenosine diphosphate (ADP) and inorganic phosphate (P_i), the by-products of ATP hydrolysis, also being significantly reduced following the consumption of beetroot juice. This

indicates that less energy was needed to maintain the same workload, thus allowing for greater performance due to reduced energy depletion (Jones., 2014).

Previous studies have also shown improvements in aerobic performance following NO_3^- supplementation i.e., increasing time to fatigue and improving time trial performance for cycling, walking, and running exercise (Bailey et al., 2012, Kenjale et al., 2011, Lansley et al., 2011a, Lansley et al., 2011b). The effects of 6 days of beetroot juice supplementation (5.5 mmol·day^{-1} NO_3^-) on cycle exercise including four moderate-intensity and two severe-intensity cycling bouts performed on days 4 – 6 of supplementation showed that beetroot juice supplementation increased time to fatigue during severe-intensity exercise by 16% compared to placebo (blackcurrant cordial) (Bailey et al., 2009). Additionally, investigation into the effects of acute supplementation (3 hours prior to exercise) of beetroot juice (8 mmol NO_3^-) in older adults (67 ± 13 y) with PAD during a walking cardiopulmonary exercise test showed that supplementation with beetroot juice increased walking time before onset of PAD-related pain by 18% and increased maximal walking time by 17% compared to placebo (Kenjale et al., 2011). It is important to note however, that this study used an open-label design and the control drink for the trial was orange juice which could have resulted in a 'placebo' effect. Furthermore, the time-to-exhaustion tests used in these studies (Bailey et al., 2009, Kenjale et al., 2011) are not direct measures of exercise performance, but rather exercise capacity. While this is still valid for research into PAD, it lacks ecological validity for the majority of sporting events, making it difficult to relate these results to sporting competitions which involve completing a set distance in the shortest time possible (Jones, 2014). This issue was addressed by Lansley et al. (2011a) who investigated the effects of acute supplementation (2-2.5 hour prior to exercise) with NO_3^--rich beetroot juice (6.2 mmol NO_3^-) on time trial performance and found that beetroot juice significantly improved 4 and 16.1 km cycling time trial performance by 2.8% and 2.7%, respectively. Furthermore, 6 days of supplementation with beetroot juice (140 ml·day^{-1}: 8 mmol of NO_3^-) resulted in a 1.2% improvement in 10-km cycling time trial performance and a 2% increase in power output compared to placebo (NO_3^- depleted beetroot juice; Cermak et al., 2012).

2.6.2 Potential Mechanisms of Action

The exact mechanisms of how dietary NO_3^- affects physiological performance are still unclear, however, the most commonly suggested mechanisms are enhanced blood flow to the working muscle, improvements in mitochondrial efficiency, and/or improved muscle contractile function (Bailey et al., 2010). Supplementation with dietary NO_3^- results in a significant reduction in both systolic and diastolic blood pressure (Kelly et al., 2013, Lansley et al., 2011a) which is indicative of increased vasodilation and therefore enhanced blood flow. In support of this, participants

consuming beetroot juice (9 mmol NO_3^-) experienced a significant drop in DBP, SBP and HR indicating NO-mediated vasodilation and increased blood flow to the working muscles as well as the previously mentioned physiological performance benefits (Kenjale et al., 2011). Further support came via NIRS which was used to directly measure oxygenation of the gastrocnemius muscle during walking exercise, where an increase in muscle tissue oxygenation was shown (Kenjale et al., 2011). Thus, validating the conclusion that beetroot juice supplementation increases vasodilation and subsequent blood flow to the working muscle leading to improved physiological performance (Kenjale et al., 2011). Increased blood flow promotes greater transportation of oxygen to active muscles which could improve metabolic control and prolong time to fatigue at a given intensity, thus resulting in improved physiological performance (Jones, 2014).

Another suggested mechanism for the effects of NO_3^- supplementation on physiological performance is increased mitochondrial efficiency (Bailey et al., 2012, Jones et al., 2018). Muscle biopsies taken pre- and post-submaximal exercise following 3 days of sodium nitrate supplementation (0.1 mmol·kg^{-1}·day^{-1}: 6.8 – 7.2 mmol) showed reduced expression of the protein adenine nucleotide translocase (ANT), which is involved in the flow of mitochondrial protons, thus reducing proton leakage, and increasing oxidative phosphorylation and mitochondrial efficiency (Larsen et al., 2011). In addition, NO_3^- supplementation increased mitochondrial P/O (phosphate to oxygen) ratio (the amount of ATP produced from a given amount of oxygen reduced) by 19% which also strongly correlated (r^2 = -0.80) with the reduction in $\dot{V}O_2$ cost of submaximal exercise (Larsen et al., 2011). Therefore, the physiological performance-enhancing benefits, seen alongside a reduction in $\dot{V}O_2$ following NO_3^- supplementation, may be attributed to an increased mitochondrial efficiency. In contrast, direct application of NO_2^- to isolated mitochondria (*in vitro)* from human muscle biopsies had no effect on the mitochondrial P/O ratio, indicating the potential needed for prolonged (multiple doses over several days) rather than acute (single dose) NO_3^- supplementation to experience these changes in mitochondrial efficiency, likely due to the time required to downregulate ANT expression (Larsen et al., 2011, Jones, 2014). It is important to note however, this potential mechanism for increased mitochondrial efficiency following dietary NO_3^- supplementation is controversial with more recent research (Whitfield et al., 2016) showing conflicting results to that of Larsen and colleagues (2011).

Others have suggested the more dominant mechanism for NO_3^- effects on physiological performance occurs at the muscle contractile level (Hoon et al., 2013). Investigation into the energetics of the working muscles, using P-magnetic spectroscopy showed a reduction in PCr utilisation, and reduced ADP and P_i production following beetroot juice supplementation, suggesting a decrease in the rate of ATP utilisation for muscle force generation (Bailey et al., 2010). This effect

was seen alongside reductions in the $\dot{V}O_2$ cost of exercise, indicating that NO_3^- (via NO-mediated effects) may alter the ATP consumption rate during muscle contraction at the actomyosin-ATPase and Ca^{2+}-ATPase. Therefore, the oxygen cost required for contraction may be lowered due to reductions in ATP utilisation from these two ATPases (Bailey et al., 2012). This is supported by research performed in mice, which showed increased myoplasmic calcium concentrations and calcium-handling proteins following 7 days of NO_3^- supplementation (Hernandez et al., 2012), indicating increased contractile efficiency which resulted in an increase in fast-twitch muscle fibre force production.

2.7 Potential Benefits of Nitrate in the Older Population

Research into dietary NO_3^- supplementation indicates it may have the potential to slow the effects of ageing and allow the opportunity for a more active lifestyle with lower rate of disease (Jackson, 2011, Kelly et al., 2013, Stanaway et al., 2019). Currently the limited research available investigating dietary NO_3^- supplementation in the older population (Stanaway et al., 2017) has shown potential for improvements including: reduced blood pressure (Kelly et al., 2013, Kemmner et al., 2017, Stanaway et al., 2019), improved $\dot{V}O_2$ kinetics (Kelly et al., 2013), increased blood flow to the brain (Presley et al., 2011), and improved reaction time (Gilchrist et al., 2014, Stanaway et al., 2019). As older adults have age-related diminishments in functional epithelial vasodilation capacity (reducing blood flow), cardiovascular function, cognitive function, and mood, there is greater potential for improvements in these parameters following NO_3^- supplementation relative to younger adults who generally do not possess these diminishments (Kenjale et al., 2011, Clifford et al., 2015).

Furthermore, to the authors knowledge, only two studies have directly compared the effects of dietary NO_3^- supplementation on cardiovascular response in younger and older adults (Hughes et al., 2016, Rogerson et al., 2022), with no studies examining the effects on cognitive function. Hughes and colleagues (2016) investigated the effects of acute NO_3^--rich beetroot juice supplementation (9.4 mmol of NO_3^-) in younger (25 ± 4 y) and older (64 ± 5 y), adults on aortic blood pressure and wave reflection (a measure of vascular changes) and found a significant reduction in aortic blood pressure in both younger and older age groups but no difference in reductions between age groups. However, this trial lacked a placebo control, and was not double blind or crossover designed, which could have impacted results due to the 'placebo effect'. More recently, acute supplementation with NO_3^--rich beetroot juice (6.4 mmol of NO_3^-) was found to have no benefit on blood pressure or microcirculatory endothelial function in younger (24.2 ± 3.5 y) or older (68.7 ± 10.3 y) adults with no difference shown between the age groups (Rogerson et al., 2022). This study was a pilot trial, and the sample sizes between the groups were not balanced with older adults (n = 7) having notably

lower numbers compared to younger adults (n = 18). Additionally, neither study examined differences in physiological or cognitive performance measures between the two age-groups, thus only one aspect of the potential benefits of dietary NO_3^- supplementation was investigated. Therefore, further research with a more robust study design and larger sample size is required to compare the potential benefits of dietary NO_3^- supplementation on health, physiological performance, and cognitive performance in younger versus older adults.

2.8 Summary and Future Directions

Dietary NO_3^- supplementation can have many effects in the body following reduction to NO, with the most significant being improved health parameters (reduced blood pressure, and increased blood flow), improved cognitive performance, improved physiological performance (reduced oxygen cost and reduced ATP utilisation), and improved exercise performance (increased time to fatigue). The exact mechanisms underpinning these effects remain unclear. It is also evident that these effects can be present in both younger and older populations, with the potential for greater benefits in older adults. However, there is still limited research on the effects of dietary NO_3^- supplementation in the older population and only two studies have directly examined its effects on cardiovascular health with no studies examining potential cognitive differences between healthy younger and older adults. Additionally, there has been no investigation into the optimal supplementation dose of dietary NO_3^- which may be needed to elicit maximal benefits for cardiovascular responses and cognition in older adults. Therefore, further research is required in all these areas

Chapter 3
Performance and Health Benefits of Dietary Nitrate Supplementation in Older Adults: A Systematic Review

This chapter contains a reformatted version of the article "Performance and Health Benefits of Dietary Nitrate Supplementation in Older Adults: A Systematic Review", authored by Luke Stanaway, Kay Rutherfurd-Markwick, Rachel Page and Ajmol Ali; published in *Nutrients* on October 27th, 2017. The published version and corresponding DRC 16 form can be found in Appendix 2.

This chapter addresses Research Question 1 (Section 1.3.1) and is a systematic review of the literature examining the potential benefits of dietary nitrate supplementation on health and performance in older adults. A wide variety of health and performance parameters have been examined in the previous literature (as presented in Chapter 2), however, most of this research has been conducted in younger adults. Therefore, this manuscript aimed to specifically review the efficacy of dietary nitrate supplementation on health and performance parameters in older adults, and to highlight areas where further research was required to explore these effects.

Abstract

Supplementation with nitrate (NO_3^-)-rich beetroot juice has been shown to improve exercise performance and cardiovascular (CV) responses, due to an increased nitric oxide (NO) availability. However, it is unclear whether these benefits are greater in older adults who have an age-related decrease in NO and higher risk of disease. This systematic review examines 12 randomised, crossover, control trials, investigating food-based NO_3^- supplementation in older adults and its potential benefits on physiological and cognitive performances, and CV, cerebrovascular and metabolic health. Four studies found improvements in physiological performance (time to exhaustion) following dietary NO_3^- supplementation in older adults. Benefits on cognitive performance were unclear. Six studies reported improvements in CV health (blood pressure and blood flow), while four found no improvement. One study showed improvements in cerebrovascular health and two found no improvement in metabolic health. The current literature indicates positive effects of dietary NO_3^- supplementation in older adults on physiological performance, with some evidence indicating benefits on cardiovascular and cerebrovascular health. Effects on cognitive performance were mixed and studies on metabolic health indicated no benefit. However, there has been limited research conducted on the effects of dietary NO_3^- supplementation in older adults, thus, further study, utilising a randomised, double-blind, control trial design, is warranted.

Keywords: nitric oxide; beetroot juice; older adults; cognition; cardiovascular; blood pressure

3.1. Introduction

Over the past decade, there has been a decrease in levels of functional activity (physiological performance) and an increase in frailty, in the ageing population, alongside increased rates of disease and age-related dysfunction (Clements et al., 2014, Hutton et al., 2009). An individual's performance and health in their later years of life can be influenced by many lifestyle factors; some are more controllable and have greater impact on performance and health-related outcomes than others (Clements et al., 2014). For example, dietary habits have been indicated as a dominant influencer of healthy ageing and age-related dysfunction, which can be easily improved to promote beneficial outcomes (Seeram, 2008). Due to this significant influence of diet, recent research has begun to focus on food-based supplements, which contain key bioactive compounds, proposed to promote performance and health benefits (Clifford et al., 2015).

One key nutritional ingredient that has gained recent attention is inorganic nitrate (NO_3^-). Nitrate is found in high concentrations in green leafy vegetables, such as spinach and rocket, and root vegetables such as beetroot (Clements et al., 2014). Additionally, nitrate is a precursor for nitric oxide (NO)—a bioactive molecule—which has many functions in the human body ranging from regulation of neurotransmission, immunity, and blood flow to alterations in oxygen consumption, and has been shown to have many performance (both physiological and cognitive) (Berry et al., 2015, Gilchrist et al., 2014) and health benefits (Kemmner et al., 2017). It is important to note that an acceptable daily intake of nitrate has been set at 3.7 $mg \cdot kg^{-1}$, as a small percentage of nitrate is also converted to N-nitrosamines in the human body, which have been implicated in certain cancers, such as gastric, pancreatic and thyroid cancers (Clifford et al., 2015). Negative side effects are commonly associated with consumption of nitrate salts, such as those found in cured meats (Clements et al., 2014, Clifford et al., 2015). Despite this, there is currently no evidence to indicate negative effects of prolonged nitrate consumption from vegetable sources, such as beetroot (Clements et al., 2014, Clifford et al., 2015).

There are two main pathways for NO production in the human body. The first is the endogenous pathway, where L-arginine is converted to NO by nitric oxide synthases (NOS), which until recently was believed to be the sole dominant pathway (Clifford et al., 2015, Kelly et al., 2013). The second pathway is the exogenous pathway. Following consumption of dietary NO_3^-, it is rapidly absorbed from the stomach and duodenum of the small intestine and enters the blood stream (Gilchrist et al., 2014). About 20–25% of the NO_3^- is absorbed from the circulation and concentrated (10 to 20-fold)

in the salivary glands (Bedale et al., 2016). Facultative anaerobes, located in the crypts on the dorsal surface of the tongue, reduce NO_3^- to the bioactive nitrite (NO_2^-), which is swallowed again (Clifford et al., 2015). Small amounts of this NO_2^- will be reduced to NO in the acidic environment of the stomach, while most re-enters the systemic circulation and is transported to specific locations in the body, where it can be reduced to NO, via one or more potential non-enzymatic or enzymatic pathways (Kapil et al., 2013). The exact mechanism behind how NO_2^- enters the circulation and is reduced is still unclear; however, this reduction tends to occur in hypoxic tissue and conditions of low pH (Clifford et al., 2015, Kapil et al., 2013). This reduction occurs predominately in the vasculature (Hord et al., 2009). It is also important to note that through this NO^3–NO^2–NO pathway, endogenous NO^{3-} can be recycled by oral bacteria to act as a reservoir of precursors for NO production and that interruption of this recycling (such as from use of antiseptic mouthwash) has been shown to decrease plasma nitrite levels and raise blood pressure (BP) in individuals in the absence of nitrate intake (Clifford et al., 2015, Kapil et al., 2013).

The ability for consumption of dietary NO_3^- to result in an increased production of NO, via the aforementioned exogenous pathway, has led to greater interest in dietary NO_3^- (particularly in the form of beetroot juice), as a food-based supplement with the potential to improve performance and health (Clements et al., 2014). Furthermore, it has been shown that NO production in the body decreases with age and is believed to be a contributor to age-related dysfunction and increased risk of disease (Clifford et al., 2015). Therefore, more recently, specific interest has been directed at the benefits of NO_3^- supplementation in the older population.

Previous studies have shown that both acute and prolonged supplementation with dietary NO_3^- in older adults can significantly improve oxygen uptake ($\dot{V}O_2$) responses during exercise (Kelly et al., 2013), and increase time to fatigue (Berry et al., 2015, Eggebeen et al., 2016, Kenjale et al., 2011), thus promoting improved exercise performance. Furthermore, it has also been shown that NO_3^- supplementation can significantly improve cognitive performance, as shown by enhanced reaction times (Gilchrist et al., 2014) in older adults. However, the investigation into cognitive performance benefits with dietary NO_3^- supplementation is scarce, with equivocal findings (Kelly et al., 2013). In addition to the potential performance benefits, studies have shown positive health-related outcomes in older adults, with acute and prolonged dietary NO_3^- supplementation, including reduced blood pressure (Eggebeen et al., 2016, Kemmner et al., 2017) and improved blood flow to the muscle and brain (Kenjale et al., 2011, Presley et al., 2011) indicative of potential cardiovascular and cerebrovascular benefits, which may result in a lower risk of certain diseases. As older adults

have age-related diminishments in functional capacity of epithelial vasodilation (reducing blood flow), cardiovascular function, cognitive function, and mood, there is greater potential for improvements in these areas with NO_3^- supplementation, relative to younger adults who generally possess optimal functional capabilities (Kenjale et al., 2011).

Therefore, the purpose of this systematic review was to critically evaluate the effects of dietary NO_3^- supplementation on performance (physiological and cognitive) and health factors (specifically, cardiovascular, cerebrovascular, and metabolic health) in older adults. This is the first systematic review of its kind, evaluating potential benefits of dietary NO_3^- supplementation, specifically in older adults.

3.2. Materials and Methods

A systematic literature search was conducted by one researcher (L.S.) to identify primary research in the area of inorganic dietary NO_3^- supplementation in older adults, with the aim of examining performance and health benefits associated with dietary NO_3^- consumption.

3.2.1. Types of Outcome Measures

The primary focuses of this systematic review were any performance and health parameters affected by dietary NO_3^- supplementation in older adults. This included changes in physiological and cognitive performance, as well as cardiovascular, cerebrovascular, metabolic, and bone health.

3.2.2. Search Method

The following online databases were systematically searched: PubMed, Ovid, Science Direct and Web of Science, from inception to May 2017. The keywords chosen for the search included: nitrate OR nitrates OR beetroot AND older adults OR elderly. This search was enhanced via manual examination of the reference lists of all retrieved papers in attempts to detect any other potentially eligible studies.

3.2.3. Inclusion and Exclusion Criteria

The inclusion and exclusion criteria for all retrieved papers were established by the researchers (L.S., A.A., K.R. and R.P.). This systematic review only included primary research published in peer-reviewed journals, in English, using a randomised, crossover, placebo-controlled design. In addition, studies had to meet a set criteria including: (1) participants had to be older adults (aged 50+) or involve older adults in the study as a separate age group, i.e., those comparing younger with older

adults; (2) participants could be healthy or possess one or more morbidities (e.g., hypertension, diabetes, peripheral arterial diseases); (3) studies had to investigate the effects of inorganic dietary NO_3^- supplementation (such as beetroot juice or high nitrate diet interventions) on performance and/or health parameters; (4) studies using multiple supplementation protocols, involving other supplements in addition to nitrate, had to show a clear separation in the effects of NO_3^- and the other supplements.

3.2.4. Study Selection

Following the removal of duplicate studies from the different search engines, independent screening of the title and abstract of the remaining articles was undertaken to determine eligibility. Selected papers were then read in full to finalise eligibility. A summary of this process is outlined in Figure 3.1.

3.2.5. Quality Assessment

Risk of bias was assessed informally, using a bias hierarchy checklist, as described by Wright et al. (2007), including the following assessment of key areas of bias: selection, performance, detection, and attrition bias. Each article was individually assessed by the reviewer, LS.

3.2.6. Data Extraction

Data were extracted independently by one of the researchers (L.S.) and reviewed by the other researchers (A.A., K.R. and R.P.), following a systematic selection list. This included: (1) participant data (number of participants, sex distribution, health stasis (i.e., healthy or diagnosed patients), and age); (2) study design; (3) supplementation protocol; (4) testing protocol used; and (5) primary outcome measures (physiological performance, cognitive function, and health-based parameter). Mean values were extracted for all data, where appropriate.

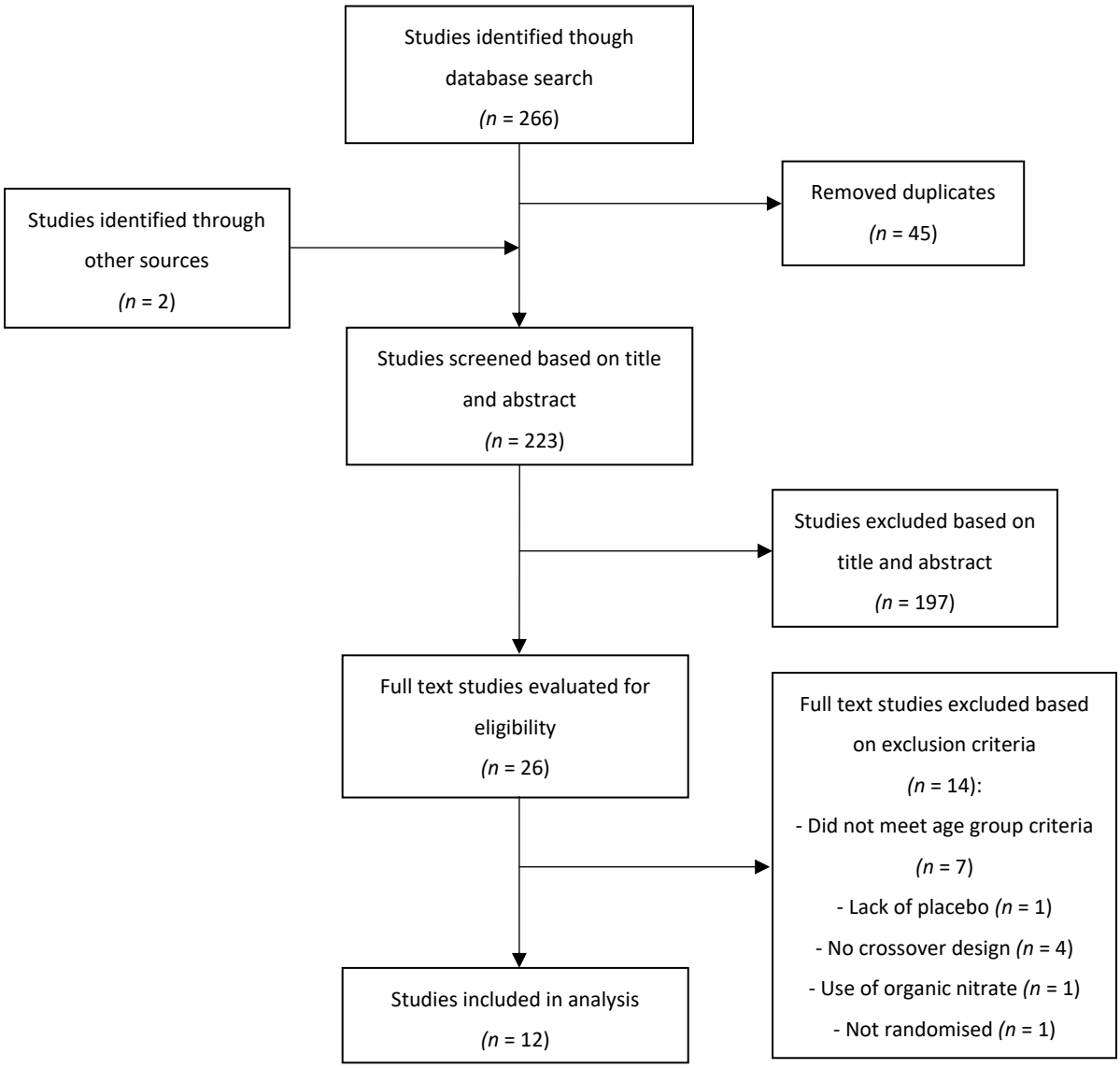

Figure 3.1. Flowchart showing study selection procedure and results

3.3. Results

Twelve studies (Berry et al., 2015, De Oliveira et al., 2016, Eggebeen et al., 2016, Gilchrist et al., 2013, Gilchrist et al., 2014, Kelly et al., 2013, Kemmner et al., 2017, Kenjale et al., 2011, Miller et al., 2012, Presley et al., 2011, Shepherd et al., 2016, Siervo et al., 2016) out of a total of 266 studies identified from preliminary research of online databases and other sources after reference list screening, were included in this systematic review (Figure 3.1).

3.3.1. Participant Characteristics

A summary of the characteristics of the studies is outlined in Table 3.1. From the 12 included studies, there were a total of 202 participants (95 males, 93 females, and 14 not stated). The number of participants in each trial ranged from 8 (Kenjale et al., 2011, Miller et al., 2012) to 27 (Gilchrist et al., 2013, Gilchrist et al., 2014), with mean ages ranging from 59.2 (Shepherd et al., 2016) to 74.7 years (Presley et al., 2011). These 12 studies targeted older adult participants of varying health status. Five studies recruited healthy older adults (Kelly et al., 2013, Presley et al., 2011, Miller et al., 2012, Shepherd et al., 2016, Siervo et al., 2016) two studies recruited older adults with type 2 diabetes (Gilchrist et al., 2013, Gilchrist et al., 2014), one study recruited participants with chronic obstructive pulmonary disease (COPD) (Berry et al., 2015), one recruited participants with heart failure with preserved ejection function (HFpEF) (Eggebeen et al., 2016), one with peripheral arterial disease (PAD) (Kenjale et al., 2011), one with chronic kidney disease (CKD) (Kemmner et al., 2017) and one with risk factors for cardiovascular disease (De Oliveira et al., 2016).

Table 3.1. Summary of studies investigating the effects of dietary nitrate supplementation on physical performance, cognitive performance, and health-based outcomes in older adults.

Reference	Participants	Study Design	Supplementation Protocol	Testing Protocols	Blood Measures (NO Indices)	Physiological Performance Outcomes	Cognitive Performance Outcomes	Health-Based Outcomes
Berry et al. 2015	15 COPD patients (m/f 12/3) (age 69.6 ± 8.5)	Randomised, single-blind crossover	Acute supplementation, 2.5 h prior to exercise, 140 mL BR (7.58 mmol NO_3]) (Note PL used was prune juice)	Constant work rate test at 75% max on cycle ergometer	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	↑ Time to exhaustion during submaximal cycle exercise	NT	↓ SBP ↓ DBP
Eggebeen et al. 2016	20 HFpEF patients (m/f 3/17) (age 69 ± 7)	Randomised, double-blind crossover	Phase 1: Acute supplementation, 1.5–2 h prior to exercise, 70 mL BR (6.1 mmol NO_3^-) Phase 2: 7 days supplementation, 70 mL·day^{-1} BR (6.1 mmol·day^{-1} NO_3^-)	Constant work rate test at 75% max on cycle ergometer	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	↑ Time to exhaustion during submaximal cycle exercise	NT	↓ SBP
Kenjale et al. 2011	4 male and 4 female PAD patients (age 67 ± 13)	Randomised, open-label, crossover	Acute supplementation, 3 h prior to exercise, 500 mL BR (9 mmol NO_3^-) (Note: orange juice was used as PL)	Walking maximal cardiopulmonary exercise (CPX) test	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	↑ Walking duration longer before onset of pain (18%) ↑ Maximum walking time (17%)	NT	↑ Tissue blood flow (NIRS) ↓ DBP ↓ SBP and HR during recovery
Siervo et al. 2016	19 healthy older adults (m/f 9/10) (age 64.7 ± 3)	Randomised, double-blind crossover	7 days supplementation, 2 × 70 mL·day^{-1} BR (12 mmol·day^{-1} NO_3^-)	Incremental cycle test to voluntary exhaustion Hand-grip strength test Time up-and-go test Repeated chair raising test 10-m walking test	↑ Plasma [NO_3^-] ↑ Urinary [NO_3^-]	↔ O_2 consumption ↔ Hand-grip strength ↔ Up-and-go test ↔ Repeated chair raising test ↔ 10-m walking test	NT	↔ BP
Kelly et al. 2013	6 male and 6 female older adults (age 64 ± 4; 63 ± 2)	Randomised, double-blind crossover	3 days supplementation, 140 mL·day^{-1} BR (9.6 mmol·day^{-1} NO_3^-)	Walking treadmill test and leg ergometer. Cognitive function tests	↑ Plasma [NO_2^-]	↓ $\dot{V}O_2$ response time from rest to walking ↔ 6 m walking test	↔ Cognitive tests	↓ SBP ↓ DBP
Gilchrist et al., 2014	27 older adults with type 2 diabetes (m/f 18/9) (age 67.2 ± 4.9)	Randomised, double-blind crossover	14 days supplementation, 250 mL·day^{-1} BR (7.5 mmol NO_3^-)	5 cognitive tests on E-Studio software	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	NT	↓ Simple reaction time (improvement)	NT

Presley et al. 2011	14 healthy older adults (sex not stated) (age 74.7 ± 6.9)	Placebo-controlled, crossover	2 days high NO_3^- diet with BR (~12.4 mmol NO_3^-) Low NO_3^- diet (~0.089 mmol NO_3^-)	NT	↑ Plasma $[NO_2^-]$ ↑ Plasma $[NO_3^-]$	NT	NT	↑ Cerebral perfusion (MRI)
de Oliveira et al. 2016	20 older adults with risk factors for cardiovascular disease (m/f 7/13) (age 70.5 ± 5.6)	Randomised, double-blind crossover	Acute supplementation, 2 h prior to measurements, 100 g BG (12.2 mmol NO_3^-) (PL used was NO_3^- depleted gel)	NT	↑ Urinary $[NO_2^-]$ ↑ Urinary $[NO_3^-]$	NT	NT	↑ Endothelial function ↔ BP ↔ Arterial stiffness
Gilchrist et al. 2013	27 older adults with type 2 diabetes (m/f 18/9) (age 67.2 ± 4.9)	Randomised, double-blind crossover	14 days supplementation, 250 mL·day^{-1} BR (7.5 mmol NO_3^-)	NT	↑ Plasma $[NO_2^-]$ ↑ Plasma $[NO_3^-]$	NT	NT	↔ BP ↔ MAP ↔ Endothelial function ↔ Insulin sensitivity
Kemmner et al. 2017	17 older adults with CKD (m/f 7/10) (age 72 ± 6)	Randomised, open-label crossover	Acute supplementation, 4 h prior to repeat measures, 200 mL BR (300 mg NO_3^-) (Note PL used was water)	Hemodynamic parameters and renal resistance index measure	↑ Plasma $[NO_3^-]$	NT	NT	↓ SBP ↓ DBP ↓ MAP ↓ RRI
Miller et al. 2012	8 normotensive older adults (m/f 3/5) (age 72.5 ± 4.7)	4-arm, randomised, crossover	3 days low NO_3^- diet (0.70 mmol NO_3^-) Low NO_3^- diet + supplementation, 500 mL BR (8.5 mmol NO_3^-) High NO_3^- diet (2.50 mmol NO_3^-) High NO_3^- diet + supplementation 500 mL BR	NT	↑ Plasma $[NO_2^-]$ ↑ Plasma $[NO_3^-]$	NT	NT	↔ BP
Shepherd et al. 2016	15 healthy older adults (m/f 8/7) (age 59.2 ± 6)	Randomised, double-blind crossover	Acute supplementation, 140 mL BR (11.91 mmol $[NO_3^-]$)	NT	↑ Plasma $[NO_2^-]$ ↑ Plasma $[NO_3^-]$	NT	NT	↔ BP ↔ Hepatic blood flow (MRI) ↔ Plasma glucose ↔ C-peptide ↔ [Incretin]

All values are mean ± SD. ↑ significant increase;↓ significant decrease; ↔ no significant difference; m/f, males/females; NT, not tested; $[NO_2^-]$, nitrite concentration; $[NO_3^-]$, nitrate concentration; BR, beetroot juice; PL, placebo; BG, beetroot gel; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; RRI, renal resistive index; $\dot{V}O_2$, oxygen uptake; HR, heart rate; NIRS, near infrared spectroscopy; MRI, magnetic resonance imaging; COPD, chronic obstructive pulmonary disease; HFpEF, heart failure with preserved ejection function; CKD, chronic kidney disease; PAD, peripheral arterial disease.

3.3.2. Study Characteristics

All studies implemented a randomised, crossover design, involving a placebo treatment and at least one NO_3^- treatment. Of the 12 studies, six used a NO_3^- depleted beetroot juice as the placebo (Eggebeen et al., 2016, Gilchrist et al., 2013, Gilchrist et al., 2014, Kelly et al., 2013, Shepherd et al., 2016, Siervo et al., 2016), two used a low NO_3^- diet (Miller et al., 2012, Presley et al., 2011) and single studies used NO_3^- depleted gel (De Oliveira et al., 2016), water (Kemmner et al., 2017), prune juice (Berry et al., 2015), or orange juice (Kenjale et al., 2011). Seven studies were double-blinded (De Oliveira et al., 2016, Eggebeen et al., 2016, Gilchrist et al., 2013, Gilchrist et al., 2014, Kelly et al., 2013, Shepherd et al., 2016, Siervo et al., 2016), one was single-blinded (Berry et al., 2015), and four were open-label trials (Kemmner et al., 2017, Kenjale et al., 2011, Miller et al., 2012, Presley et al., 2011).

3.3.3. Study Quality

Based on an informal assessment of bias, the studies included in this systemic review were considered to be good to excellent, based on the above-mentioned study characteristics. All studies were placebo controlled, randomised crossover experiments (low selection/detection bias), with the majority being double- or single-blinded (low performance/detection bias).

3.3.4. Nitrate Supplementation

The majority of the studies (n = 10) utilised supplemented inorganic NO_3^- in the form of beetroot juice, while the remaining two studies used beetroot gel and a high NO_3^- diet, where beetroot juice was included in the diet. The difference in the NO_3^- dose used between studies ranged from 6.1 to 12.4 mmol·day^{-1} (approx. 350–700 mg·day^{-1}). In addition, the supplementation period varied between studies: five studies investigated acute supplementation (consumption 2–4 h prior to testing (Berry et al., 2015, De Oliveira et al., 2016, Kemmner et al., 2017, Kenjale et al., 2011, Shepherd et al., 2016), six investigated chronic supplementation (2.5–14 days of NO_3^- loading) (Gilchrist et al., 2013, Gilchrist et al., 2014, Kelly et al., 2013, Miller et al., 2012, Presley et al., 2011, Siervo et al., 2016), and one study investigated both acute (1.5–2 h prior) and chronic (7 days) supplementation (Eggebeen et al., 2016).

3.3.5. Adverse Effects of Supplementation

Five of the 12 studies (De Oliveira et al., 2016, Gilchrist et al., 2013, Miller et al., 2012, Presley et al., 2011, Siervo et al., 2016) reported minor adverse effects from dietary NO_3^- supplementation; two studies reported beeturia (red urine) (De Oliveira et al., 2016, Siervo et al., 2016), two studies

reported both beeturia and red stools (Gilchrist et al., 2013, Presley et al., 2011), and one study reported beeturia, gastrointestinal discomfort and headaches (Miller et al., 2012). No chronic adverse effects from NO_3^- supplementation were stated in any of the 12 studies.

3.3.6. Characteristics of Nitric Oxide Measures

The 12 studies incorporated various measures to investigate the potential changes in NO concentrations in the body following supplementation with dietary NO_3^-, including plasma [NO_3^-] and [NO_2^-], and urinary [NO_3^-] and [NO_2^-] measures. Eight studies reported both plasma [NO_3^-] and [NO_2^-] (Berry et al., 2015, Eggebeen et al., 2016, Gilchrist et al., 2013, Gilchrist et al., 2014, Kenjale et al., 2011, Miller et al., 2012, Presley et al., 2011, Shepherd et al., 2016), one measured solely plasma [NO_2^-] (Kelly et al., 2013), one measured solely plasma [NO_3^-] (Kemmner et al., 2017), one measured both plasma and urinary [NO_3^-] (Siervo et al., 2016), and one measured both urinary [NO_3^-] and [NO_2^-] (De Oliveira et al., 2016). Similarly, various methods were used to determine the aforementioned values. Five studies used a Sievers NO analyser (chemiluminescence-based) (Gilchrist et al., 2013, Gilchrist et al., 2014, Kenjale et al., 2011, Presley et al., 2011, Shepherd et al., 2016); three used an ENO-20 NO analyser (based on colorimetric Griess assay) (Berry et al., 2015, Eggebeen et al., 2016, Miller et al., 2012); one used high-performance liquid chromatography (HPLC) (De Oliveira et al., 2016); one used gas chromatography-mass spectroscopy (GC-MS) (Siervo et al., 2016); one used an enzyme-linked immunosorbent assay (Kemmner et al., 2017); and one used a modified chemiluminescence method (Kelly et al., 2013).

3.3.7. Outcomes

3.3.7.1. Nitric Oxide Indices

Despite the different NO indices used between studies, dietary NO_3^- supplementation resulted in a significant increase in all NO indices (compared to placebo) in all 12 studies. Specifically, increases in plasma [NO_3^-] ranged from 150% (Siervo et al., 2016) to 938% (Berry et al., 2015), while increases in plasma [NO_2^-] ranged from 168% (Gilchrist et al., 2013) to 503% (Kelly et al., 2013) and increases in urinary [NO_3^-] were 283% (De Oliveira et al., 2016) and 979% (Siervo et al., 2016), while the increase in urinary [NO_2^-] was 214% (De Oliveira et al., 2016), at pre- versus post-supplementation, with dietary NO_3^-.

3.3.7.2. Performance Outcomes

Physiological Performance

Five of the 12 studies investigated the effects of dietary NO_3^- supplementation on physiological performance in older adults (Berry et al., 2015, Eggebeen et al., 2016, Kelly et al., 2013, Kenjale et al., 2011, Siervo et al., 2016). Acute supplementation with dietary NO_3^- (7.58 mmol/400 mg NO_3^-), 2.5 h prior to exercise, significantly improved time to exhaustion during submaximal cycling (p = 0.031) (Berry et al., 2015). Likewise, 7 days of supplementation with NO_3^- rich beetroot juice (6.1 mmol·day^{-1}/350 mg NO_3^-) also improved time to exhaustion during submaximal cycling exercise (p = 0.02) (Eggebeen et al., 2016); however, this improvement was not seen during the acute supplementation protocol in the study. Additionally, acute supplementation with NO_3^- rich beetroot juice (9 mmol/500 mg NO_3^-) improved walking duration prior to onset of pain by 18%, and maximal walking time by 17% in older adults with chronic obstructive pulmonary disease (PAD) (Kenjale et al., 2011). Similarly, an increase in the $\dot{V}O_2$ response time from rest to moderate intensity walking ($p < 0.05$) was shown, following 3 days of supplementation with beetroot juice (9.6 mmol·day^{-1}/550 mg NO_3^-) (Kelly et al., 2013). Despite this, the same study found no change in a 6-min walking test (Kelly et al., 2013). Additionally, it was reported that 7 days of supplementation with NO_3^- rich beetroot juice (12 mmol·day^{-1}/700 mg NO_3^-) had no effect on O_2 consumption, a 10-m walking test, a hand-grip strength test, an up-and-go test, or a repeated chair raising test, in healthy older adults (Siervo et al., 2016).

Cognitive Performance

Of the 12 studies, only three investigated the effects of dietary NO_3^- supplementation on cognitive performance in older adults (Gilchrist et al., 2014, Kelly et al., 2013, Presley et al., 2011), with only two directly measuring cognitive function (Gilchrist et al., 2014, Kelly et al., 2013). The potential for dietary NO_3^- as a cognitive performance aid in older adults consuming a high NO_3^- diet, including beetroot juice (total NO_3^- ~12.4 mmol/700 mg), for 2 days, was shown by an increase in cerebral blood flow to the white matter of the frontal lobe ($p < 0.005$) (Presley et al., 2011). Additionally, 14 days of supplementation with NO_3^- rich beetroot juice (250 mL·day^{-1}, 7.5 mmol/~400 mg NO_3^-) in older adults (67.2 ± 4.9 years) with type 2 diabetes, resulted in a significant improvement in simple reaction time, compared to a placebo (Gilchrist et al., 2014). Despite this, there was no difference between treatments for other cognitive tests, including decision time, rapid processing, and spatial and shape memory (Gilchrist et al., 2014). Investigation of the effects of NO_3^- rich beetroot juice supplementation over 3 days (2 × 70 mL·day^{-1}, 9.6 mmol/~550 mg of NO_3^-) on cognitive performance, in healthy, older adults (aged 60–70), showed no significant differences in memory,

attention and information processing capabilities between the beetroot juice and placebo treatments (Kelly et al., 2013).

3.3.7.3. Health Outcomes

Cardiovascular Health

Ten studies investigated the potential benefits of dietary NO_3^- supplementation on BP in older adults. Five of these studies showed that dietary NO_3^- supplementation resulted in a significant decrease in systolic blood pressure (SBP) (Berry et al., 2015, Eggebeen et al., 2016, Kelly et al., 2013, Kemmner et al., 2017, Kenjale et al., 2011), with four of the five studies also showing a significant decrease in diastolic blood pressure (DBP) following NO_3^- supplementation, compared to a placebo (Berry et al., 2015, Kelly et al., 2013, Kemmner et al., 2017, Kenjale et al., 2011). In contrast, five studies found no change in the BP of older adults after supplementation with dietary NO_3^- (De Oliveira et al., 2016, Gilchrist et al., 2013, Miller et al., 2012, Shepherd et al., 2016, Siervo et al., 2016). There were two studies that did not measure changes in BP (Gilchrist et al., 2014, Presley et al., 2011). Furthermore, two studies also investigated mean arterial pressure (MAP) (the average pressure in arteries from one cardiac cycle) following beetroot juice supplementation. Gilchrist et al. (2013) found no difference between treatments (BR, 7.5 mmol/400 mg NO_3^- vs. nitrate-depleted PL) in older adults with type 2 diabetes, whereas Kemmner et al. (2017) showed a decrease in MAP in older adults with CKD, following acute supplementation with NO_3^- rich beetroot juice (200 ml, 6 mmol/350 mg NO_3^-) (p = 0.012). Three studies investigated endothelial function and blood flow in older adults in response to dietary NO_3^- supplementation (De Oliveira et al., 2016, Gilchrist et al., 2013, Kenjale et al., 2011). Acute beetroot juice supplementation in older adults (67 ± 13 years) with PAD was found to increase blood flow to the working muscles, using near infrared spectroscopy (NIRS) (Kenjale et al., 2011). Acute supplementation with 100 g of NO_3^- rich beetroot gel (12.2 mmol/700 mg NO_3^-) in older adults with risk factors of cardiovascular disease has also been reported to significantly improve brachial flow-mediated dilation (FMD) (77%), blood flow velocity (BFV) (31%), and reactive hyperaemia (RH) (18%), thus improving endothelial function (De Oliveira et al., 2016). However, 14 days of supplementation with NO_3^- rich beetroot juice in older adults with type 2 diabetes had no significant effect on FMD or Doppler perfusion, and thus, did not improve endothelial function (Gilchrist et al., 2013). The study by Kemmner et al. (2017) also reported a decrease in the renal resistance index (RRI) (p = 0.017) following acute supplementation with NO_3^- rich beetroot juice in older adults with CKD.

Cerebrovascular Health

Only one study has investigated the potential cerebrovascular health benefits of dietary NO_3^- supplementation in older adults (Presley et al., 2011). This study used magnetic resonance imagining (MRI) to show increased perfusion in the frontal cortex of the brain ($p < 0.005$) of older adults following 2 days of a high NO_3^- diet (including beetroot juice), compared to a diet low in NO_3^- (Presley et al., 2011).

Metabolic Health

Of the 12 studies, two investigated various metabolic health parameters following dietary NO_3^- supplementation in older adults (Gilchrist et al., 2013, Shepherd et al., 2016). Chronic supplementation with NO_3^- rich beetroot juice in older adults with type 2 diabetes had no significant effect on insulin sensitivity, measured via the hyperinsulinemic isoglycemic clamp technique (Gilchrist et al., 2013). Furthermore, acute supplementation with NO_3^- rich beetroot juice was also shown to have no significant effect on acute plasma glucose, hepatic blood flow, C-peptide concentration, or incretin concentration (glucagon-like peptide-1 (GLP-1)), in older adults with type 2 diabetes (Shepherd et al., 2016).

3.4. Discussion

This systematic review investigated the effects of NO_3^- supplementation on performance and health-related outcomes in older adults. The current literature indicates positive effects of dietary NO_3^- supplementation in older adults on physiological performance, with some evidence indicating benefits on cardiovascular and cerebrovascular health. Effects on cognitive performance were mixed and studies on metabolic health indicated no benefit.

3.4.1. Nitrate Supplementation and Physiological Performance

Dietary NO_3^- supplementation has the potential to improve physiological performance, by prolonging time to exhaustion and increasing the $\dot{V}O_2$ response time in older adults. Four of the five studies that investigated physiological performance in older adults found positive outcomes in time to exhaustion during submaximal exercise (Berry et al., 2015, Eggebeen et al., 2016, Kenjale et al., 2011), and in $\dot{V}O_2$ response time (Kelly et al., 2013), while only one study showed no significant effects on physiological performance parameters (Siervo et al., 2016). This is a similar finding to the systematic review and meta-analysis conducted by Hoon et al. (2013) on the effects of dietary NO_3^- supplementation in exercise performance of healthy, young individuals, who reported a pooled effect size of 0.79 for time to exhaustion trials, with favourable benefits from beetroot juice supplementation.

Two studies reported an increase in total exercise capacity following acute supplementation with NO_3^- rich beetroot juice, during submaximal cycling and walking exercise in older adults with COPD and PAD, respectively (Berry et al., 2015, Kenjale et al., 2011). In contrast, another study found no significant effect of acute beetroot juice supplementation on time to exhaustion during submaximal cycling exercise in older adults with HFpEF (Eggebeen et al., 2016). The disparity in findings between these studies may have been due to variances in study design. The two studies which reported improvements in time to exhaustion used an open-label design, where the drinks for the control trials were prune juice (Berry et al., 2015) and orange juice (Kenjale et al., 2011), which could have produced a 'placebo' effect; however, Eggebeen et al. (2016) used a double-blind design with NO_3^- depleted beetroot juice as the control. Moreover, Berry et al. (2015) and Kenjale et al. (2011) also utilised higher dosages of NO_3^- and longer absorption periods (7.58 mmol NO_3^-, 2.5 h prior, and 9 mmol NO_3^-, 3 h prior, respectively), while Eggebeen et al. (2016) had participants consume 6.1 mmol NO_3^-, 1.5–2 h prior to exercise. These reductions in dosage and absorption times could have prevented adequate concentration and/or time for the supplementation to elicit an effect, especially when, in the same study by Eggebeen et al. (2016), while no acute effect was seen, they did show an increased time to exhaustion, after 7 days of dietary NO_3^- supplementation. Nevertheless, another study showed no effect of 7 days supplementation (12 mmol·day^{-1} NO_3^-) on time to exhaustion during an incremental cycle test (Siervo et al., 2016); thus, the discrepancies between the studies could be due to other factors.

Siervo et al. (2016) also found no significant improvements in O_2 consumption, grip strength, up-and-go test, repeated chair raises, or 10-m walking time. However, upon closer examination of the results there was a trend (p = 0.10) for improved time to exhaustion and an improvement in all tests, following beetroot versus placebo treatment (Siervo et al., 2016). This may be a result of insufficient sample sizes to portray the full effects (n = 19). Additionally, the absence of significant differences in this study may have been due to the use of healthy older adults, where the aforementioned studies who showed improvements utilised COPD (Berry et al., 2015), PAD (Kenjale et al., 2011), and HFpEF (Eggebeen et al., 2016) patients who already had diminished physiological functions, and thus had a greater potential to benefit from dietary NO_3^- supplementation (Clifford et al., 2015).

The aforementioned improvements in time to fatigue following NO_3^- supplementation may be due to enhanced blood flow to the working muscle, improvements in mitochondrial efficiency, and/or improved muscle contractile function (Jones, 2014). There is strong evidence to suggest that supplementation with dietary NO_3^- results in a significant reduction in both systolic and diastolic blood pressure in older adults, which is indicative of increased vasodilation and therefore enhanced

blood flow (Kelly et al., 2013, Kemmner et al., 2017). Kenjale et al. (2011) showed that, alongside the physiological performance benefits of beetroot juice supplementation, participants also experienced significant drops in DBP, SBP and heart rate (HR), indicating NO-mediated vasodilation and increased blood flow to the working muscles. Near infrared spectroscopy (NIRS) also showed increased muscle tissue oxygenation following supplementation with NO_3^- rich beetroot juice (Kenjale et al., 2011). Increased blood flow to the working muscle allows for greater transport of oxygen, which can result in more efficient metabolic control and prolonged time to fatigue at a given workload, thus improving physiological performance (Jones, 2014).

Furthermore, NO_3^- rich beetroot juice has been shown to improve mitochondrial efficiency via up-regulation of the protein, adenine nucleotide translocase (ANT), which is involved in the control of flow of mitochondrial protons, resulting in a reduction in proton leakage and increased efficiency of oxidative phosphorylation (Larsen et al., 2011). In addition, dietary NO_3^- supplementation increased the mitochondrial phosphate to oxygen (P/O) ratio (the amount of ATP produced from a given amount of oxygen reduced), thus reducing the amount of O_2 required to maintain a given workload and prolonging time to exhaustion (Larsen et al., 2011).

Bailey et al. (2010) used ^{31}P-magnetic resonance spectroscopy to show a decrease in utilisation of skeletal muscle phosphocreatine (PCr), along with a decrease in production of adenosine diphosphate (ADP) and inorganic phosphate (P_i), indicating a reduction in the rate of ATP consumption following consumption of NO_3^- rich beetroot juice. This effect indicated that supplementation with dietary NO_3^- (via NO mediated effects) may alter the ATP consumption rates of actomyosin-ATPase and Ca^{2+}-ATPase during muscle contraction (Bailey et al., 2010). Therefore, the reductions in ATP utilisation from these two ATPases during contraction would result in the ability to perform a given work rate for longer, thus prolonging time to exhaustion (Bailey et al., 2012).

Collectively, the results indicate that dietary NO_3^- supplementation can result in improved blood flow and mitochondrial efficiency, and a reduced rate of ATP utilisation, leading to a prolonged time to exhaustion during submaximal exercise (Jones, 2014). It is important to note that some of the results were based on studies using younger adults (Bailey et al., 2010, Bailey et al., 2012, Larsen et al., 2011); however, it has been suggested that the same underlying mechanisms would be present in the older population (Clements et al., 2014). Additionally, this emphasises the need for further research into the effects on dietary NO_3^- supplementation in older adults.

3.4.2. Nitrate Supplementation and Cognitive Performance

Older adults show reductions in cerebral blood flow (CBF), which may be due to an age-related decrease in the production of NO (Clifford et al., 2015). This reduction in blood flow to the brain has been indicated as a major risk factor for the impairment of cognitive function and development of neurodegenerative diseases, such as dementia (Kelly et al., 2013). Therefore, supplementation with NO_3^- rich beetroot juice could have greater benefits on cognitive function in older adults, via its NO mediated increase in CBF (Presley et al., 2011). However, in comparison to the effects of dietary NO_3^- supplementation on physiological performance, the effects on cognitive performance in older adults have only just begun to be investigated, thus the effects are less clear (Clifford et al., 2015).

Only two of the 12 studies directly measured the effects of dietary NO_3^- supplementation on cognitive performance in older adults (Gilchrist et al., 2014, Kelly et al., 2013). One study showed an improvement in cognitive performance, via a reduction in simple reaction time, with beetroot juice consumption compared to placebo, following a chronic dosing protocol (14 days of NO_3^- rich beetroot juice, 250 mL·day^{-1}, 7.5 mmol NO_3^-), in type 2 diabetic, older adults (67.2 ± 4.9 years) (Gilchrist et al., 2014). However, they found that performances in decision time, rapid processing, spatial and shape memory tests were not significantly different. The earlier study by Kelly et al. (2013) found no effect of dietary NO_3^- supplementation on cognitive performance following 3 days of supplementation with beetroot juice (2 × 70 mL·day^{-1}, 9.6 mmol of NO_3^-) in healthy, older adults (males 64 ± 4 and females 63 ± 2 years). Specifically, they reported no significant differences in memory, attention and information processing capabilities between beetroot juice and placebo treatments.

The differences in cognitive performance results seen between these two studies could be attributed to methodological variations, including the duration of supplementation and the cognitive tests used. Gilchrist et al. (2014) used a longer supplementation period (14 days) and included a simple reaction test; however, Kelly et al. (2013) used a much shorter supplementation period (3 days) and did not include a simple reaction test. Additionally, participant selection may have impacted results as one study used older adults with type 2 diabetes (Gilchrist et al., 2014), while the other used healthy older adults (Kelly et al., 2013). Therefore, the findings of this systematic review suggest that there may be some potential cognitive performance benefits for older adults following supplementation with dietary NO_3^-; however, these benefits may be specific to certain brain functions, and may be affected by the duration of supplementation as well as participant health status. Nevertheless, with only two studies to draw conclusions from, which provide conflicting results, more research is required to investigate these potential effects.

3.4.3. Nitrate Supplementation and Health

3.4.3.1. Cardiovascular

Investigations into the possible cardiovascular health benefits of NO_3^- supplementation in older adults has grown over the past 5 years, with a significant amount of research examining the effects on BP (Clements et al., 2014); more recently, the effects on MAP (Kemmner et al., 2017), changes in blood flow (Kenjale et al., 2011), and endothelial function (De Oliveira et al., 2016) have become additional areas of examination.

Due to age-related losses in NO production from decreased activity of NOS, older adults are at a higher risk for endothelial dysfunction and cardiovascular disease, of which, high BP and MAP are major risk factors (Clements et al., 2014). Supplementation with dietary NO_3^- increases NO production, which, in turn, has been shown to activate the enzyme, guanylate cyclase, which catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) (Bailey et al., 2010). Cyclic guanosine monophosphate acts on smooth muscle, causing relaxation, which results in vasodilation of arteries and veins, thus decreasing BP and reducing the risk of cardiovascular disease (Kelly et al., 2013). Despite this, the findings of this review indicate mixed results for the effects of acute and chronic dietary NO_3^- supplementation on BP and MAP in older adults. Whilst five of the reviewed studies reported a reduction in SBP (Berry et al., 2015, Eggebeen et al., 2016, Kelly et al., 2013, Kemmner et al., 2017, Kenjale et al., 2011), with four of these also showing a reduction in DBP (Berry et al., 2015, Kelly et al., 2013, Kemmner et al., 2017, Kenjale et al., 2011), a further five studies found no benefits (De Oliveira et al., 2016, Gilchrist et al., 2013, Miller et al., 2012, Shepherd et al., 2016, Siervo et al., 2016). Additionally, a more recent study found a reduction in MAP (Kemmner et al., 2017); however, an earlier study showed no significant change (Gilchrist et al., 2013).

The reasons for these conflicting results for BP may be due to differences in the health statuses of the participants. Of the five studies that reported a reduction in BP, only one used healthy older adults (Kelly et al., 2013), while the others used older adults with COPD (Berry et al., 2015), CKD (Kemmner et al., 2017), HFpEF (Eggebeen et al., 2016), or PAD (Kenjale et al., 2011). Comparatively, three of the five studies which showed no significant change in BP, used healthy older adults (Miller et al., 2012, Shepherd et al., 2016, Siervo et al., 2016), while one (De Oliveira et al., 2016) used participants with risk factors for cardiovascular disease (not yet morbid), and one (Gilchrist et al., 2013) used type 2 diabetic older adults. These differences could suggest that supplementation with dietary NO_3^- may be more beneficial for reducing BP in older adults with morbidities closely related

to an already reduced endothelial function, beyond that of age-related changes. Furthermore, the study by de Oliveira et al. (2016) used a shorter absorption time of 2 h compared to the more common 2.5–4 h, used by studies which have shown significant reductions in BP (Berry et al., 2015, Kemmner et al., 2017). This may not have provided sufficient time to allow for significant reductions in BP. In addition, Gilchrist et al. (2013) investigated effects on BP while participants were taking antihypertensive and hypoglycaemic medication, which is likely to have impacted the results, as the participants' baseline BPs would have already been lowered, due to the antihypertensive medication. However, one study has shown a decrease in BP in hypertensive patients, aged 18–85, following supplementation with NO_3^--rich beetroot juice, while still adhering to their antihypertensive medication protocol, suggesting improvements may still be possible with supplementation in conjunction with drug protocols (Kapil et al., 2015).

Alternatively, the conflicting results seen between these studies may have been due to differences in study design. Three of the studies which reported beneficial outcomes on BP in older adults following dietary NO_3^- supplementation used prune juice (Berry et al., 2015), water (Kemmner et al., 2017) and orange juice (Kenjale et al., 2011) as the placebo controls, while all other studies (De Oliveira et al., 2016, Eggebeen et al., 2016, Kelly et al., 2013, Gilchrist et al., 2013, Miller et al., 2012, Shepherd et al., 2016, Siervo et al., 2016), utilised a NO_3^- depleted beetroot control, so there was potential for a 'placebo' effect. Also, as these three studies (Berry et al., 2015, Kemmner et al., 2017, Kenjale et al., 2011) utilised beetroot juice as the NO_3^- source but did not utilise a NO_3^- depleted beetroot control, it is possible that other bioactive compounds found in beetroot juice (e.g., antioxidants and polyphenols) may have contributed to the reduction in BP (Lansley et al., 2011a). However, other studies using a NO_3^- rich beverage and NO_3^- depleted beetroot juice (placebo) crossover design have still shown reductions in BP of older adults (Kelly et al., 2013). Furthermore, an investigation into the interruption of the NO_3^-–NO_2^-–NO pathway, via the use of mouthwash or spitting of saliva following beetroot juice consumption, has been shown to negate the reductive effects on BP, indicating high concentrations of NO_3^- as the major contributor to a decreased BP (Clifford et al., 2015). The reasons for mixed results in MAP are likely similar to those mentioned above; including the continued use of participants' antihypertension medications (Gilchrist et al., 2013), or the use of an inappropriate study design (open-labelled with the placebo being water (Kemmner et al., 2017).

In addition to reductions in BP and MAP, supplementation with dietary NO_3^- in older adults has also been proposed to benefit cardiovascular health of older adults through its effects on blood flow and endothelial function (Eggebeen et al., 2016). Two studies found that acute supplementation with

NO_3^- rich beetroot gel (12.2 mmol NO_3^-, 2 h prior) (De Oliveira et al., 2016) or juice, (9 mmol NO_3^-, 3 h prior) (Kenjale et al., 2011) improved endothelial function and blood flow in older adults. This was expected, as foods high in NO_3^- have been shown to inhibit blood platelet aggregation and reverse or prevent further degradation in endothelial function (Webb et al., 2008), which is particularly important in older adults, as ageing is associated with reduced blood flow and endothelial dysfunction (Presley et al., 2011). Therefore, NO_3^- supplementation plays an important role in maintaining NO homeostasis when endogenous production may be diminished, thus maintaining adequate blood flow and distribution (Webb et al., 2008). Despite these results, Gilchrist et al. (2013) found no significant differences in endothelial function following 14 days of NO_3^- supplementation in older adults with type 2 diabetes, contradicting the aforementioned findings. As mentioned above, a major limitation in the study by Gilchrist et al. (2013) was the continued use of participants' antihypertensive medications, which are designed to promote vasodilation and subsequently increase blood flow, (Clements et al., 2014, Clifford et al., 2015). Therefore, it is unlikely that supplementation with dietary NO_3^- would promote further benefits to result in improved endothelial function. Based on this, the lack of improvements in blood flow and endothelial function seen by Gilchrist et al. (2013) are as expected.

Kemmner et al. (2017) reported a reduced RRI in older adults with CKD following supplementation with 200 mL of NO_3^- rich beetroot juice (6 mmol/~300 mg NO_3^-). Chronic kidney disease is another major risk factor for CVD, where hypertension can both result from, or be a large contributor to, the development of the disease (Kemmner et al., 2017). Furthermore, a high RRI is associated with an increased risk of cardiovascular dysfunction (Kemmner et al., 2017). Therefore, as older adults are at a greater risk of kidney dysfunction, due to age-related changes, a reduced RRI following supplementation with dietary NO_3^- is likely to result in reduced risk of a cardiovascular event, thus improving cardiovascular health (Kemmner et al., 2017). It is also important to note that CKD and high RRI can impact type 2 diabetes, potentially affecting metabolic health as well.

Despite the mixed results reported for changes in the BP and MAP of older adults following dietary NO_3^- supplementation, the review of the published data indicates that there is potential for beneficial effects which can result in improved cardiovascular health. Furthermore, some evidence from the reviewed studies also indicates potential benefits to cardiovascular health of older adults, via improved blood flow, endothelial function and/or RRI post-supplementation with dietary NO_3^-. Therefore, the conflicting results warrant further research, to fully evaluate the extent to which dietary NO_3^- supplementation impacts cardiovascular health in older adults. Additionally, specific

attention should be drawn to participant health status and the use of a double-blind study design and NO_3^- depleted control.

3.4.3.2. Cerebrovascular

Only one study (Presley et al., 2011) met the inclusion criteria for examining the relationship between dietary NO_3^- consumption and cerebrovascular health. Presley et al. (2011) showed increased perfusion in the frontal cortex of the brain (using MRI), of older adults following a diet high in NO_3^- with additional beetroot juice supplementation. This suggests that dietary NO_3^- supplementation may attenuate the age-related drop in NO activity and loss of cerebral blood flow (a major contributor to neurogenerative diseases) (Berry et al., 2015), thus potentially slowing or even preventing the development of cerebrovascular dysfunction. However, with only one study to draw conclusions from, cause and effect cannot be confirmed. Additionally, no study to date has directly investigated the long-term effects of dietary NO_3^- supplementation on the development of neurodegenerative diseases. Therefore, further research is required to investigate the effects of dietary NO_3^- supplementation on specific diseases of the brain and determine its possible benefits to cerebrovascular health in older adults.

3.4.3.3. Metabolic

The current systematic review found two studies which examined the relationship between metabolic health and dietary NO_3^- supplementation in older adults, both focusing predominately on insulin sensitivity and changes in plasma glucose levels (Gilchrist et al., 2013, Shepherd et al., 2016). Nitric oxide helps shuttle glucose absorbed in the small intestine into skeletal muscle (Kingwell et al., 2002). A reduction in NO bioavailability has been shown to result in a decrease in insulin secretion and reduced activation of GLUT4 transporter proteins, and thus a subsequent drop in glucose uptake (Shepherd et al., 2016, Lansley et al., 2011a). Therefore, older adults may have an age-related drop in insulin sensitivity, due to a reduction in NO production (Gilchrist et al., 2013). Consequently, supplementation with dietary NO_3^- in older adults may improve insulin sensitivity, improving metabolic health. However, the results from the studies included in this review do not support this suggestion. Neither acute supplementation with NO_3^- rich beetroot juice (140 mL, 11.91 mmol/~600 mg NO_3^-) in healthy older adults (Shepherd et al., 2016) nor chronic supplementation (250 mL·day^{-1}, 7.5 mmol/~400 mg NO_3^-) in type 2 diabetic older adults (Gilchrist et al., 2013) resulted in significant differences in plasma glucose levels or insulin sensitivity. These results suggest that dietary NO_3^- supplementation in older adults does not affect metabolic health via improvements in insulin sensitivity. However, it is important to note that there were some limitations to these studies. Gilchrist et al. (2013) had participants continue their prescribed hypoglycaemic medications, which

are designed to increase insulin secretion or improve insulin action (Zammitt and Frier, 2005), thereby potentially masking any potential metabolic effects of dietary NO_3^- supplementation. Additionally, Shepherd et al. (2016) used healthy older adults with a younger mean age (59.2 ± 6 years) compared to other studies, which may have impacted results, as age-related NO dysfunction may not become an issue until individuals are in their mid-60's (Clifford et al., 2015), thus, the potential benefits would not be as great in the early stages of older adulthood. With only two studies examining the metabolic health benefits of dietary NO_3^- supplementation in older adults, further investigation is required to provide a stronger evidence base, to allow more in-depth analyses and conclusions to be made.

Certain limitations for this systematic review should be noted. Firstly, there was incomplete retrieval of identified research, as only English articles were included in this systematic review. Secondly, studies were generally characterised by small sample sizes and there were few studies investigating some outcome variables, which may have affected the reliability of the conclusions drawn in this systematic review. A third limitation to our analysis was the lack of a meta-analysis. Due to the small number of studies for each outcome variable, completing a meta-analysis would have resulted in weak representation of the data, thus, it is suggested that a meta-analysis be conducted in the future, once more studies have been published. This would be beneficial because it would provide greater statistical evidence on the effects of dietary NO_3^- supplementation in older adults.

3.5. Conclusions

In summary, the findings of this systematic review provide convincing evidence for improved physiological performance (increased time to fatigue), in older adults following dietary NO_3^- supplementation. Some evidence suggests positive outcomes of dietary NO_3^- supplementation on cardiovascular health (reduced BP and MAP, increased blood flow and improved endothelial function, and reduced RRI) and cerebrovascular health (increased CBF), while results are mixed as to whether dietary NO_3^- supplementation has benefits on cognitive performance. Metabolic health in older adults does not seem to be improved via changes in insulin sensitivity or plasma glucose levels following dietary NO_3^- supplementation; however, more research is required before firm conclusions can be made. Furthermore, it is evident that these effects may be influenced by the dosage of NO_3^-, duration of supplementation, blinding/placebo-control, and health status of participants. Therefore, further research is required in this area, with a focus on randomised, double-blind, NO_3^- depleted control-based studies, with particular attention to participant health status.

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Chapter 4

Dose Response Relationship of Dietary Nitrate Consumption on Cardiovascular and Cognitive Responses in Older Adults

This chapter contains the article "Dose Response Relationship of Dietary Nitrate Consumption on Cardiovascular and Cognitive Responses in Older Adults", authored by Luke Stanaway, Kay Rutherfurd-Markwick, Rachel Page, Marie Wong, Ben Jones and Ajmol Ali. This article will be submitted to *Nutrition, Metabolism & Cardiovascular Diseases*. Other relevant documents for this chapter, such as the ethics approval and participant info sheet, can be found in Appendices 4.1-4.8.

This study addresses Research Question 2 (Section 1.3.2). The literature review (Chapter 2) and systematic review (Chapter 3) identified three key areas where further research was required: 1) the effects of dietary NO_3^- supplementation on cognitive function and performance, especially in older adults; 2) the mechanisms behind the shown improvements in cardiovascular responses following consumption of NO_3^--rich beetroot juice; 3) the dose response relationship of dietary NO_3^- for cardiovascular and cognitive responses in older adults with an indication of an optimal dose.

This study was designed to investigate the dose response relationship of dietary NO_3^- on physiological responses in older adults. The primary objective was to find the optimal dose for beneficial effects on cardiovascular and cognitive function in older adults and secondly to explore certain potential mechanisms of action via systemic vascular resistance measured with the USCOM.

Abstract

Nitrate-rich beetroot juice (BR) supplementation has been shown to improve cardiovascular and cognitive responses in older adults following consumption of varying doses of nitrate. However, the dose-response relationship between the concentration of dietary nitrate consumed and the effects on cardiovascular responses and cognition in older adults has not been examined. We hypothesised that acute supplementation with varying doses of nitrate-rich beetroot juice would increase plasma nitrate and nitrite and improve cardiovascular responses and cognitive performance dose-dependently, with the greatest improvements being in the 9.8 mmol treatment. Eleven older (50–80 years) adults consumed three 250 mL doses of nitrate-rich beetroot juice (9.8, 4.9, and 2.5 mmol nitrate) and placebo (PL; 0.15 mmol nitrate) in a randomised, double-blind, crossover design. Plasma nitrate and nitrite concentrations, blood pressure (BP), resting metabolic rate, heart rate, and cognitive function were measured pre- and 2.25 h post-supplementation. BR consumption increased plasma nitrate and nitrite concentrations dose-dependently. Systolic BP, diastolic BP, and mean arterial pressure were reduced following consumption of 9.8 mmol nitrate compared to PL, 2.5, and 4.9 mmol treatments ($p < 0.05$). Corsi span was improved in the Corsi block tapering test (CBT) following 9.8 mmol nitrate supplementation compared to PL ($p = 0.013$) and 2.5 mmol ($p = 0.004$) treatments. Acute BR supplementation increased plasma nitrate and nitrite concentrations dose-dependently. However, BP and cognitive performance were only significantly improved following supplementation with 9.8 mmol of nitrate, suggesting higher doses of nitrate-rich BR may promote greater improvements in cardiovascular health and cognition.

Keywords: nitric oxide; nitrate; blood pressure; cognition; older adults; dose-response

4.1. Introduction

Due to advances in health systems around the world, people are living longer which is driving an increase in the aging population, however, within this cohort there is a high proportion who live with multiple morbidities (Ministry of Health, 2016). In New Zealand, older adults account for one-third of all health loss (the loss of healthy life due to disability, illness, or death), while only making up one-eighth of the population (Ministry of Health, 2016). The majority of this health loss and many of the morbidities in older adults can be attributed to cardiovascular disease (CVD; such as hypertension) and neurological disorders (such as dementia) (Ministry of Health, 2016). This high portion of morbidities in older adults creates dependence and substantially increases healthcare costs (Jackson, 2011).

A proposed major risk factor for both CVD and neurological disorders is a reduction in nitric oxide (NO) availability (Clifford et al., 2019, Sindler et al., 2011). Nitric oxide has important functions in the human body with many critical to cardiovascular and cerebrovascular health including increasing vasodilation, neurotransmission, and regulation of endothelial function (Clifford et al., 2019, Lara et al., 2016). Most of the production of NO in the human body occurs via the L-arginine/NOS pathway, where L-arginine is reduced by nitric oxide synthase (NOS) to NO (Bailey et al., 2012). It has been proposed that as individuals age, production of NO is reduced due to potential dysfunction in the L-arginine/NOS pathway, resulting in a decrease in endothelial function, cerebral blood flow (CBF), and cognitive function; leading to increased risk of CVD and neurological disorders (Clifford et al., 2019, Sindler et al., 2011, Stanaway et al., 2019). However, in the past two decades another major pathway for NO production has been investigated: an exogenous pathway where consumption of dietary nitrate (NO_3^-) is converted to nitrite (NO_2^-) by anaerobic bacteria in the mouth and then to NO in the stomach and various locations throughout the body (Bailey et al., 2012). Interest in this pathway has led to further research into the potential health benefits of supplementation with NO_3^--rich beetroot juice in older adults.

Acute supplementation with dietary NO_3^- can improve cardiovascular responses in older adults with reductions in blood pressure (BP) (Berry et al., 2015, Kemmner et al., 2017, Stanaway et al., 2019) and improvements in endothelial function (de Oliveira et al., 2016). Acute supplementation with 4.9 mmol of dietary NO_3^- was shown to significantly reduce systolic blood pressure (SBP; -8.2 mmHg), diastolic blood pressure (DBP; -8.5 mmHg) and mean arterial pressure (MAP; -8.2 mmHg) in older adults with chronic kidney disease (CKD) (Kemmner et al., 2017). Similarly, consumption of NO_3^--rich beetroot juice (10.5 mmol NO_3^-) reduced SBP (-7.4 mmHg) and DBP (-5.7 mmHg) 2.25 h post-supplementation in healthy older adults (Stanaway et al., 2019). These reductions in BP can be

deemed clinically significant for potential use in a medical setting as reductions as little as 2 mmHg (in SBP and/or DBP) can greatly reduce the risk of CVD-related incidents (Collaboration, 2003). However, one study in healthy older adults showed acute supplementation with 11.9 mmol dietary NO_3^- had no effect on BP (Shepherd et al., 2016). While many studies have shown a significant decrease in BP following various doses of dietary NO_3^- consumption in older adults, there are equivocal results despite similar doses being used. This is likely due to other methodological differences in participant health status and the type of placebo treatment utilised.

Supplementation with dietary NO_3^- has also been shown to enhance cognitive performance in older adults (Gilchrest et al., 2014, Stanaway et al., 2019). An improvement in simple reaction time was shown following 14 days of chronic supplementation with 7.5 mmol·day^{-1} dietary NO_3^- in older adults (Gilchrest et al., 2014). Similarly, Stanaway et al. (2019) showed a decrease in reaction time in older adults during the Stroop test 2.5 h after acute supplementation with 10.5 mmol of dietary NO_3^-. In further support of cognitive benefits, two days' supplementation with NO_3^--rich beetroot juice, in conjunction with a diet high in NO_3^--containing foods (a total of 12.4 mmol NO_3^-), increased cerebral perfusion to the prefrontal cortex in older adults (Presley et al., 2011). Thus, as reduced cognitive function and CBF are major risk factors for neurological disorders, results from several studies indicate that supplementation with dietary NO_3^- may be able to attenuate age-related decline in cognitive function (Stanaway et al., 2017). However, another study investigating cognitive function in older adults showed no improvement following three days of supplementation with 9.6 mmol of NO_3^--rich beetroot juice (Kelly et al., 2013). These conflicting results, despite using similar doses of dietary NO_3^-, indicate the need for more research into the effects of various doses of beetroot juice using similar methodological controls to further explore the potential benefits of dietary NO_3^- supplementation on cognitive function in older adults.

The dose of dietary NO_3^- used may be one of the key factors in determining whether cardiovascular and cognitive benefits exist. While studies have shown positive health benefits in older adults from supplementation with as low as 4.9 mmol (Kemmner et al., 2017) to a high of 12.4 mmol (Presley et al., 2011) of dietary NO_3^-, no optimal dose has been established in older adults. Only one study has examined the effect of varying doses of dietary NO_3^- supplementation in the older cohort, which showed that supplementation with 100 g (~4.5 mmol NO_3^-), 200 g (~8.9 mmol NO_3^-), and 300 g (~13.4 mmol NO_3^-) of cooked beetroot increased plasma, urinary, and salivary NO_3^- dose-dependently in younger and older adults (Capper et al., 2022). Similarly, another study investigated the dose-response relationship of dietary NO_3^- supplementation, using treatments of 0 (placebo), 4.2, 8.4, and 16.8 mmol NO_3^- and found a dose-dependent increase in plasma [NO_3^-] and [NO_2^-], reduction in

SBP and MAP up to the 8.4 mmol dose, and reduction in the oxygen cost of moderate exercise (Wylie et al., 2013). However, Capper et al. (2022) did not examine any cardiovascular or cognitive measures and did not use a double-blind, placebo-controlled study, whilst Wylie and colleagues only conducted their study on younger adults (22 ± 5 y) and did not investigate cognitive function. Thus, further research is required to help establish an optimal dose of dietary NO_3^- for use in older adults for potential cardiovascular and cognitive benefits.

The purpose of this study was therefore to examine the dose-response relationship between supplementation with NO_3^--rich beetroot juice and cardiovascular responses and cognition in older adults. To the authors' knowledge, this is the first study to investigate the dose-response relationship of dietary NO_3^- supplementation on physiological responses specifically in older adults.

4.2. Materials and Methods

4.2.1. Sample Size

A power analysis was conducted (G-Power 3.1) to calculate the sample size based on the primary outcome measure of blood pressure (Faul et al., 2009). Previous literature in healthy older adults have shown a mean change in BP of 10 mmHg between NO_3^--rich beetroot juice (9.6 mmol nitrate) and control (Kelly et al., 2013). Based on this, the required sample size was 11, using a SD of 9, with a statistical power of 0.82 and α-level set at 0.05.

4.2.2. Participants

Eleven healthy recreationally active older adults (50-80 years) volunteered for this study. Participants completed a health-screening questionnaire to ensure suitability. Exclusion criteria included smokers, users of nitrate-based dietary supplements and well-trained/elite athletes (defined as those completing >4 training sessions per week, or >6 h of intense exercise per week, or having played/playing for a national or age-equivalent sports team). Those with known diseases (e.g., dementia and cardiovascular disease) were also excluded. Prior to testing, all participants were informed of the procedures, associated risks, and the possible benefits of participation, after which written informed consent was obtained. The study was approved by the Massey University Human Ethics Committee (SOA 18/30) and registered with the Australia New Zealand Clinical Trials Registry (ACTRN12620000005954).

4.2.3. Study Design

4.2.3.1. Study Summary

Participants were required to visit the laboratory on five separate occasions, an initial familiarisation visit followed by four main trials. Each participant was randomly allocated (using a random number generator and Latin square method) the order of consumption of the four beverages in a double-blind, crossover design.

4.2.3.2 Experimental Procedures

During the first visit, participants were familiarised with the testing procedures and equipment. Tests included cognitive function tests, body weight (kg) and body composition (measured using bioelectrical impedance analysis; BIA, Inbody 230, Biospace Corp., Seoul, Korea), and height (m) via a stadiometer. Resting metabolic rate (RMR) measures were also collected using online gas analysis to familiarise them with the procedure.

Participants were instructed to complete a food diary for two days prior to the second study visit and to replicate their diet and lifestyle factors for the third, fourth and fifth study visits. Dietary nitrate was not restricted to investigate additional benefits of BR in conjunction with normal daily intake; the food diary was used to evaluate intake of dietary nitrate. Participants were asked to arrive at the laboratory in a well-rested, fasted state and to refrain from excessive exercise (anything more than a walk or daily activities like gardening) and alcohol consumption 24 h prior and caffeine 12 h prior to each study visit. All trials were performed in the morning at approximately the same time of day (±2 h).

For the four supplementation trials participants consumed 250 ml of a high dose (High-NO_3^-; 9.8 mmol / 600 mg NO_3^-), medium dose (Mod-NO_3^-; 4.9 mmol / 300 mg NO_3^-), low dose (Low-NO_3^-; 2.5 mmol / 150mg NO_3^-) or placebo (PL; 0.15 mmol / 9 mg NO_3^-) beetroot drink. Drinks were consumed with a standardised, isocaloric breakfast (cereal or toast) with the same macronutrient breakdown (15 g protein, 30 g carbohydrate and 10 g fat). Participants consumed the same breakfast type (cereal or toast) for all supplementation trials. Visits 2-5 were separated by a one-week washout period providing sufficient time for normalisation of NO_3^- and NO_2^- levels in the body (Lansley et al., 2011a).

The protocol for visits 2-5 is illustrated in Figure 4.1. Prior to consumption of the beverage, baseline measures of blood pressure (mean value of three measurements taken from the left arm: deluxe HEM-7130; OMRON Healthcare CO. Ltd.; Kyoto, Japan), heart rate (A1 Polar chest transducer; Polar

Electro Oy, Kempele, Finland), systemic vascular resistance (SVR) and MAP (USCOM 1A; USCOM Ltd, Sydney, Australia), cognitive function, and RMR (COSMED, Quark cardiopulmonary exercise testing (CPET)) were completed followed by collection of a resting blood sample. Participants then consumed the allocated drink with breakfast, within a 10-min period. Participants were asked to remain in the laboratory for a 2.25-h absorption period while a home development show was aired for entertainment purposes (has minimal effect on cognitive stimulation or arousal levels (Kelly et al., 2013)), or participants could complete computer work or study, whichever the participant chose was replicated for each trial. The 2.25-h absorption period was chosen as previous research has shown that peak plasma nitrite concentrations occur 2–3 h after beetroot juice consumption (Wylie et al., 2013). After 2.25 h, the aforementioned measurements were repeated.

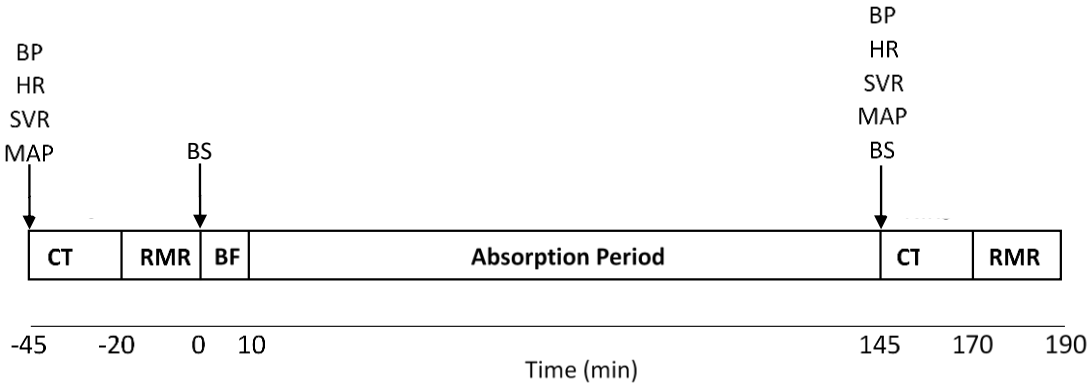

Figure 4.1. Schematic of dose-response study protocol. CT, cognitive tests; BF, breakfast; BP, blood pressure; BS, blood sample; HR, heart rate; MAP, mean arterial pressure; SVR, systemic vascular resistance, RMR, resting metabolic rate.

4.2.4. Intervention

The placebo drink was produced using beetroot juice concentrate and standardised to the same constant soluble solids concentration (11° Brix) but contained a low nitrate concentration (6 mg/250 mL). The BR drinks were produced using the placebo as a base then adding concentrated beetroot shots (Concentrated beetroot juice shot; Beet It Sport Nitrate 400, James White Drinks, Ipswich, UK) following the removal of the same amount of placebo to compensate for the added concentrate to make up the appropriate concentrations of 2.5 mmol, 4.9 mmol, and 9.8 mmol of nitrate per 250 ml. The nitrate concentrations in the placebo and concentrate were analysed prior to use, using high-performance liquid chromatography (HPLC) based on the method of Cheng and Tsang (1998). Briefly, isocratic separation was achieved using a Gracesmart C-18 column; mobile phase 0.01 M Octylammonium orthophosphate (pH 3–3.5) at a flow rate of 0.8 mL/min. Detection was carried out at 193 nm for nitrite and 213 nm for nitrate.

4.2.5. Blood Measurements

Plasma nitrite and nitrate were used as biomarkers for nitric oxide availability (Kleinbongard et al., 2003). Six millilitre venous blood samples were taken by venipuncture, from a vein within the antecubital area, and collected into heparinised tubes. Samples were mixed, centrifuged (MF–50 Hanil Science Industrial, Incheon, South Korea) at 3500 rpm (1330 g) for 10 min, and the collected plasma aliquoted into Eppendorf tubes (0.5 mL per tube) and stored at −80 °C for later analysis of nitrite and nitrate concentrations by HPLC. After filtration nitrite was analysed as per Li et al. (2000) and nitrate was analysed as per Cheng and Tsang (1998) described above.

4.2.6. USCOM Hemodynamics

Mean arterial pressure (via calculation) and SVR were measured using the USCOM-1a device. A 3.3-MHz transducer was placed on the suprasternal notch and Doppler waves provided a signal of the flow at the aortic valve. The signal was modified by the operator by moving the transducer until the clearest Doppler curve was obtained. An optimal Doppler curve is seen as a clear and well-defined peak, base, and flow curve with minimal interference, such as cord movement or participants talking (Beltramo et al., 2016). Systemic vascular resistance is calculated based on the equation SVR = MAP / cardiac output (CO) × 80, where MAP is calculated from SBP and DBP, and CO is measured by the USCOM 1a by determining heart rate and stroke volume.

4.2.7. Cognitive Measurements

Cognitive tests were completed in a quiet, isolated room using a computer placed at eye level. Participants were instructed to sit comfortably in a chair and maintain a calm and relaxed position. PsychoPy software, version 1.83.04 (Peirce, 2007) and PsyToolkit software, version 2.4.1 (Stoet, 2010, Stoet, 2017) were used to conduct the cognitive tests. The cognitive tasks included the Corsi block tapering test (CBT), rapid visual information processing (RVIP), and Stroop tests. Each task was performed a total of eight times per participant (two per supplementation trial; pre- and 2.25 h post-supplementation). These tests have been used to investigate the effects of nutritional supplementation on executive function, attention, and information processing speed (Kelly et al., 2013, Thompson et al., 2015).

4.2.7.1. Corsi Block Tapering (CBT)

The CBT task involves the display of nine blocks on the screen. The blocks light up in random order and the participant is required to remember and click which blocks lit up and the order they lit up in. The sequence starts at two blocks and goes up to nine blocks. If the participant gets the sequence

wrong, they are allowed another attempt on that level. When the participant gets the same level incorrect twice that is their final Corsi block span.

4.2.7.2. Rapid Visual Information Processing (RVIP)

The RVIP test requires participants to observe a chain of single numbers from 1 to 9, which appear in a pseudo-random order at a rate of 100 per minute. The aim of the test is to correctly identify target sequences (2–4–6, 3–5–7, and 4–6–8) as quickly as possible. The test lasts an average of 4 min with a total of 32 sequences to identify.

4.2.7.3. Stroop Test

The Stroop test displays different colour names ('RED', 'GREEN', and 'BLUE') which appear on the screen, one at a time in different-coloured fonts (red, green, or blue). The aim of this test is to press the key corresponding to the colour of the font that the present word was displayed in rather than the word itself.

4.2.8. Statistical Analysis

Statistical analyses were completed using IBM SPSS (Version 27.0). All data except for physical characteristics and baseline measurements were analysed using one-way analysis of variance (ANOVA) with treatment as the within subject factor. Sphericity was tested using the Mauchly's test to ensure the assumption of sphericity was not violated, and multivariate models were applied if these assumptions were not met. Where significant differences were found, Tukey post-hoc tests were undertaken to assess multiple comparisons. Outliers were excluded based on the Tukey test. Data are presented as mean ± standard deviation. Statistical significance was set at $p < 0.05$.

4.3. Results

4.3.1. Participants

The physical characteristics of the participants are presented in Table 4.1. All 11 participants completed the blood pressure, USCOM, cognitive tests, NIRS, and RMR. Of the 11, one participant's blood samples were not collected due to difficulties in obtaining blood. In addition, one participant was excluded from the change in blood pressure and MAP due to a high initial blood pressure reading as their result was determined to be an outlier falling outside the interquartile range.

Table 4.1. Physical characteristics and baseline plasma nitrite and nitrate measurements of dose-response study participants.

	Mean	**SD**		
Sex (m/f)	6 M:5 F			
Age (years)	69.2	5.4		
Height (cm)	170.0	8.3		
Body Mass (kg)	76.2	13.7		
SMM (kg)	29.6	4.9		
	Placebo	**Low-NO_3^-**	**Mod-NO_3^-**	**High-NO_3^-**
Plasma NO_2^- (nM)	311.6 ± 158.2	278.1 ± 215.9	234.9 ± 118.7	284.0 ± 167.2
Plasma NO_3^- (µM)	57.0 ± 57.5	65.2 ± 49.2	51.6 ± 27.9	54.9 ± 32.3

m, male; f, female; SMM, skeletal muscle mass; Low-NO_3^-, low dose; Mod-NO_3^-, moderate dose; High-NO_3^-, high dose; NO_2^-, nitrite; NO_3^-, nitrate.

4.3.2. Plasma Nitrite and Nitrate

Plasma [NO_3^-] was elevated above PL following consumption of High-NO_3^-, Mod-$NO_3^-$and Low-NO_3^- treatments (all p = <0.001 Figure 4.2A). Plasma [NO_3^-] increased dose-dependently following supplementation with NO_3^--rich beetroot juice (p <0.001 Figure 4.2A). Plasma [NO_2^-] was elevated post-supplementation with High-NO_3^- compared to PL (p = 0.004, Figure 4.2B). Additionally, there was a trend for an increase in plasma [NO_2^-] following supplementation with High-NO_3^- compared to Low-NO_3^- treatment (p = 0.058 Figure 4.2B). However, there were no other effects of treatment on plasma [NO_2^-] (p > 0.05).

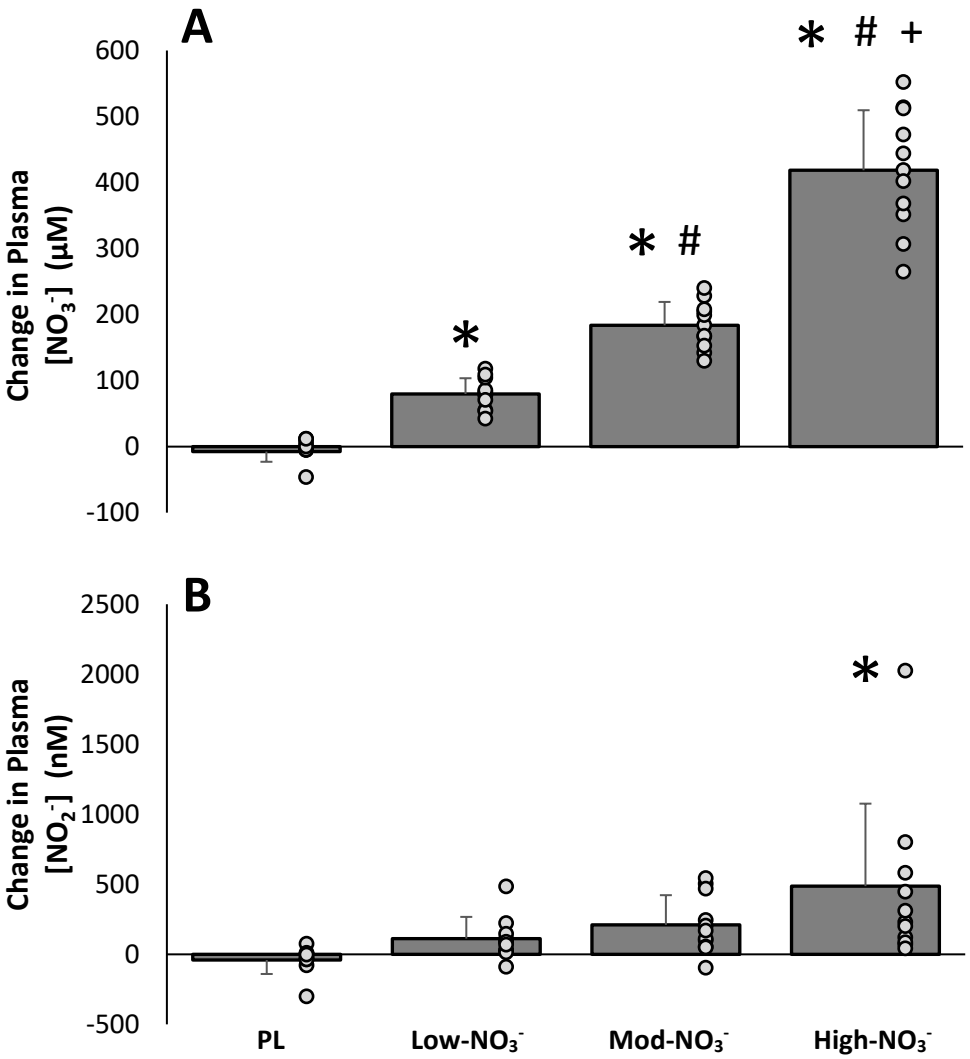

Figure 4.2. Changes in plasma nitrate and nitrite concentration in the dose-response study. (A), Change in plasma nitrate concentration ([NO_3^-]) (µM) pre- versus post-supplementation with high dose (High-NO_3^-) beetroot juice (9.8 mmol), moderate dose (Mod-NO_3^-) beetroot juice (4.9 mmol), low dose (Low-NO_3^-) beetroot juice (2.5 mmol), and placebo (PL). **(B)**, Change in plasma nitrite concentration ([NO_2^-]) (nM) pre- versus post-supplementation with High-NO_3^- beetroot juice (9.8 mmol), Mod-NO_3^- beetroot juice (4.9 mmol), Low-NO_3^- beetroot juice (2.5 mmol), and placebo.
Values of bars are expressed as means ± SD. Open circles represent individual data.

* Statistically significant mean difference from PL ($p < 0.05$). # Statistically significant mean difference from Low-NO_3^- ($p < 0.01$). + Statistically significant mean difference from Mod-NO_3^- ($p < 0.01$).

4.3.3. Blood Pressure and Hemodynamics

Mean arterial pressure was lower post-supplementation in High-NO_3^- relative to PL, Low-NO_3^- and Mod-NO_3^- treatments (all $p < 0.05$, Figure 4.3A). However, there was no reduction in MAP from Mod-NO_3^- or Low-NO_3^- treatments relative to PL ($p > 0.05$). Systolic BP was reduced following consumption of the High-NO_3^- relative to PL, Low-NO_3^- and Mod-NO_3^- ($p < 0.05$, Figure 4.3B). However, there was no effect on SBP from the other two treatments (Mod-NO_3^- and Low-NO_3^-) when compared to the PL ($p > 0.05$). There was also a reduction in change in DBP (post- versus pre-supplementation) following consumption of High-NO_3^- versus PL, Low-NO_3^-, and Mod-NO_3^- treatments ($p < 0.05$, Figure 4.3C). Change in DBP was not different following Low-NO_3^- and Mod-NO_3^- consumption compared to PL treatment ($p > 0.05$). There were no treatment effects on SVR ($p > 0.05$). However, there was a trend for a decrease in SVR following supplementation with High-NO_3^- versus Low-NO_3^- and Mod-NO_3^- treatments ($p = 0.096$ and 0.075, respectively; Figure 4.3D).

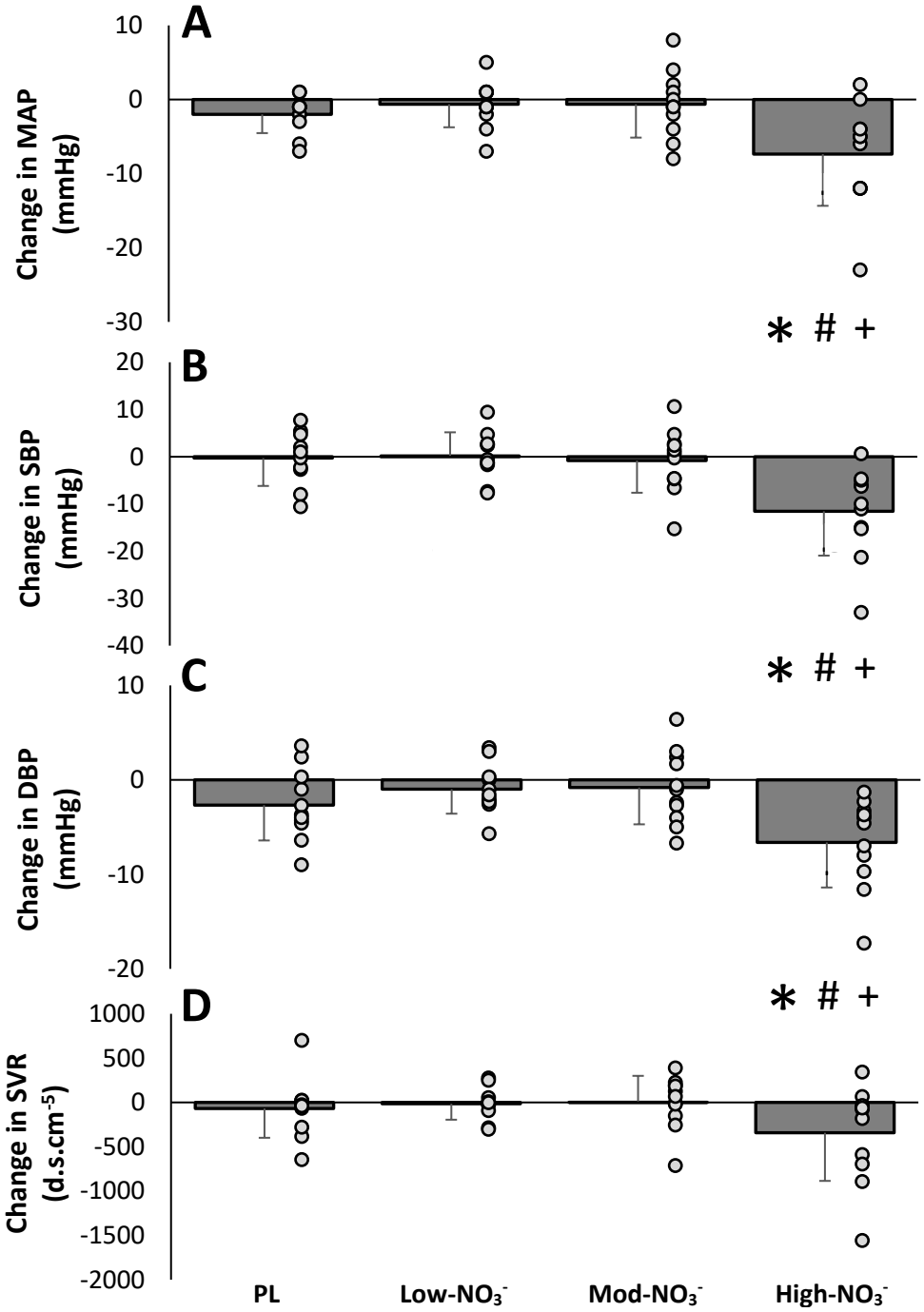

Figure 4.3. Changes in blood pressure and hemodynamic response in the dose-response study. (A), Change in mean arterial pressure (MAP) (mmHg) pre- versus post-supplementation with high dose (High-NO_3^-) beetroot juice (9.8 mmol), moderate dose (Mod-NO_3^-) beetroot juice (4.9 mmol), low dose (Low-NO_3^-) beetroot juice (2.5 mmol), and placebo (PL). **(B)**, Change in systolic blood pressure (SBP) (mmHg) pre- versus post-supplementation with High-NO_3^-beetroot juice (9.8 mmol), Mod-NO_3^- beetroot juice (4.9 mmol), Low-NO_3^- beetroot juice (2.5 mmol), and placebo. **(C)**, Change in diastolic blood pressure (DBP) (mmHg) pre- versus post-supplementation with High-NO_3^- beetroot juice (9.8 mmol), Mod-NO_3^- beetroot juice (4.9 mmol), Low-NO_3^- beetroot juice (2.5 mmol), and placebo. **(D)**, Change in systemic vascular resistance (SVR) (d.s.cm^5) pre- versus post-supplementation with High-NO_3^- beetroot juice (9.8 mmol), Mod-NO_3^- beetroot juice (4.9 mmol), Low-NO_3^- beetroot juice (2.5 mmol), and placebo.

Values are expressed as means ± SD. Open circles represent individual data.

* Statistically significant mean difference from PL ($p < 0.05$). # Statistically significant mean difference from Low-NO_3^- ($p < 0.05$). + Statistically significant mean difference from Mod-NO_3^- ($p < 0.05$).

4.3.4. Resting Metabollic Rate and Heart Rate

There was no effect of treatment (pre- and post-supplementation) on average HR (p > 0.05). Similarly, change in RMR was not affected by dose (p > 0.05).

4.3.5. Cognitive Performance

Supplementation with High-NO_3^- improved Corsi span during CBT from pre- to post-supplementation compared to PL and Low-NO_3^- treatments (p = 0.013 and 0.004, respectively, Table 4.2). There was also a trend for improved Corsi span during the CBT following consumption of High-NO_3^- compared to Mod-NO_3^- (p = 0.092). There was no effect on Corsi span from the other two treatments (Mod-NO_3^- and Low-NO_3^-) when compared to the PL (p > 0.05). There was no effect of treatment for change in RVIP percentage correct responses, percentage of errors, or reaction time (p > 0.05). Similarly, change in Stroop test percentage correct responses and reaction times were not affected by treatment (p > 0.05).

Table 4.2. Change in cognitive performance tests following supplementation with Placebo, Low-NO_3^- (2.5 mmol), Mod-NO_3^- (4.9 mmol), and High-NO_3^- (9.8 mmol) beetroot juice.

	Placebo	**Low-NO_3^-**	**Mod-NO_3^-**	**High-NO_3^-**
		Corsi Block Tapering Test		
Δ Corsi Block Span	-0.18 ± 0.75	-0.36 ± 1.4	0.18 ± 1.2	1.2 ± 0.75*#
		Rapid Visual Image Processing		
Δ Correct Response (%)	2.4 ± 14	2.6 ± 9.7	1.7 ± 6.2	6.0 ± 27
Δ Errors	0.73 ± 2.7	0.64 ± 1.9	0.64 ± 1.9	-0.30 ± 2.8
Δ Reaction Time (ms)	-0.46 ± 28	1.4 ± 40	-2.5 ± 39	-21 ± 20
		Stroop Test		
Δ Correct Response (%)	-0.68 ± 1.6	-0.03 ± 1.4	-1.0 ± 2.7	6.8 ± 19
Δ Reaction Time (ms)	-17 ± 29	-39 ± 60	-21 ± 79	31 ± 171

Values are expressed as means ± SD.
* Statistically significant mean difference between change in High-NO_3^- and PL (p < 0.05).
# Statistically significant mean difference between change in High-$NO_3^-$and Low-NO_3^- (p < 0.05).
Low-NO_3^-, low dose; Mod-NO_3^-, moderate dose; High-NO_3^-, high dose.

4.4. Discussion

We examined the effect of different doses of dietary NO_3^- intake on cardiovascular and cognitive responses in older adults. The main findings of this study demonstrated that (1) plasma [NO_3^-] and [NO_2^-] increased dose dependently; (2) supplementation with High-NO_3^- significantly reduced MAP, SBP, and DBP; (3) consumption of High-NO_3^- significantly improved Corsi span score.

4.4.1. Plasma [NO_3^-] and [NO_2^-]

Consumption of NO_3^--rich beetroot juice increased both plasma [NO_3^-] and [NO_2^-] dose-dependently. A recent study investigating the effects of acute consumption of ~4.5, ~8.9, and ~13.4 mmol of dietary NO_3^-, in the form of cooked beetroot, on varying measures of NO_3^- and NO_2^- concentrations, found that plasma, urinary, and salivary concentration of NO_3^- also increased dose-dependently in both younger and older adults (Capper et al., 2022). Another study conducted in younger adults showed a similar dose-dependent increase in plasma [NO_3^-] and [NO_2^-] following acute supplementation of 4.2, 8.4, and 16.8 mmol of NO_3^--rich beetroot juice (Wylie et al., 2013). While these findings are in a younger cohort it does show a similar trend to the results of the current study. However, while plasma [NO_3^-] showed a clear increase between treatments in the current study, plasma [NO_2^-] was only significantly increased following consumption of the High-NO_3^- compared to PL and showed a trend for increase when compared to Low-NO_3^- (Figure 4.2). This was most likely due to the inter-subject variability and potentially their variation in ability to convert NO_3^- to NO_2^- which can be affected by oral bacteria composition, saliva production, and possibly digestive function of older adults (Capper et al., 2022, Kenjale et al., 2011). Additionally, as the half-life of NO_2^- is much shorter than NO_3^- (20-45 min compared to 5-8 h) (Bondonno et al., 2015) any small delays in freezing or analysis could potentially affect NO_2^- levels more than NO_3^-. As only one other study (Capper et al., 2022) has examined varying doses of dietary NO_3^- on changes in plasma [NO_3^-] and [NO_2^-] in older adults, more work is required in the older cohort with larger sample sizes to expand on the current findings.

4.4.2. Blood Pressure and Hemodynamics

This is the first study investigating the dose-response relationship of supplementation with NO_3^--rich beetroot juice on BP and hemodynamics in older adults. Previous studies have shown that supplementation with varying doses (4.9 to 10.5 mmol) of dietary NO_3^- can reduce MAP, SBP, and DBP in older adults (Kelly et al., 2013, Kemmner et al., 2017, Stanaway et al., 2019). Therefore, with dose-dependent increases in plasma [NO_3^-] and [NO_2^-] and thus NO, following supplementation with the four NO_3^- doses (PL, 2.5, 4.9, and 9.8 mmol) it might be expected that a dose-dependent

decrease in BP would occur in this study. However, no dose-dependent decrease was observed for change in MAP, SBP, or DBP. This contrasts with a study in younger adults which showed a dose-dependent decrease in MAP and SBP following acute supplementation with PL, 4.2 and 8.4 mmol dietary NO_3^- (Wylie et al., 2013). Aging can impact the vasodilatory effect of NO due to reduced sensitivity, and therefore older adults may require higher doses of dietary NO_3^- to achieve significant reductions in BP and improvements in endothelial function (Lara et al., 2016). Thus, with no other studies in this area carried out in older adults, more research is required to further validate these BP and MAP findings.

MAP, SBP, and DBP were all reduced following supplementation with High-NO_3^- when compared with the other treatments. This was expected as supplementation with dietary NO_3^- leads to an increase in production of NO which activates guanylate cyclase, an enzyme involved in the formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) (Kapil et al., 2015, Kemmner et al., 2017). One of the functions of cGMP is to vasodilate the vasculature via relaxation of smooth muscle and thus decreasing BP (Stanaway et al., 2017). However, Mod-NO_3^- and Low-NO_3^- treatments did not significantly reduce MAP, SBP, and DBP compared to PL. While it was expected that the low dose (2.5 mmol) may not reduce BP due to an insufficient amount of dietary NO_3^- to provide significant benefits, one study using 4.9 mmol of NO_3^- showed reductions in MAP, SBP, and DBP in older adults (age 72 ± 6 y), following acute supplementation (Kemmner et al., 2017). However, Kemmner et al. (2017) used water as the placebo and the participants had CKD which could have affected the results as beetroot juice contains other bioactive compounds including polyphenols and betalains which can impact BP (Haswell et al., 2021), and benefits from dietary NO_3^- supplementation may be greater in morbid populations due to an already diminished vasculature function (Lara et al., 2016). The findings for change in MAP, SBP, and DBP in the current study indicate that there may be a minimum dose of dietary NO_3^- required to stimulate clinically significant BP reducing effects, and that the optimal dose used in this study was 9.8 mmol of dietary NO_3^-.

Previous studies investigating the effects of dietary NO_3^- consumption on endothelial function in older adults have shown mixed results, yet studies indicate the potential for beneficial effects due to changes in the vasculature, including increased vasodilation and reduced arterial stiffness (de Oliveira et al., 2016, Kapil et al., 2015). Endothelial function is most commonly measured via flow mediated dilation (FMD) and pulse wave velocity (PWV), which explore changes in arterial dilation and arterial stiffness (Lara et al., 2016). However, another important parameter involved in endothelial function is SVR (Woessner et al., 2020). With age-related decreases in endothelial function, increases in SVR could be expected and thus supplementation with NO_3^--rich beetroot juice

may counteract these effects (Woessner et al., 2020). However, the current study found there was no dose-response effect of supplementation with NO_3^--rich beetroot juice on SVR, although, the High-NO_3^- treatment did show a trend for decrease in SVR when compared to Low-NO_3^- (p = 0.096) and Mod-NO_3^- (p = 0.075) treatments. In comparison, one study showed a significant reduction in total vascular resistance at rest, following acute supplementation with ~12.3 mmol NO_3^--rich beetroot juice (Bond et al., 2013), indicating the potential for dietary NO_3^- to promote improvements in SVR. However, this study was conducted in younger adult females and used orange juice as the PL, thus is not directly equivalent to the current study. The large variability between subjects (Figure 4.2) may have contributed to the lack of significance seen for reduction in SVR in the current study, as the High-NO_3^- treatment considerably reduced SVR yet this was not statistically significant. This inter-subject variability could also explain why there was no trend between High-NO_3^- relative to the PL. This variability indicates the need for a larger sample size to further investigate these results. Additionally, as SVR can show changes in systemic vasculature it could be suggested that structural changes may require a longer duration of supplementation to occur before promoting significant effects on arterial dilation and stiffness.

4.4.3. Cognitive Performance

In the present study, there was no dose-dependent improvement in cognitive performance following supplementation with the four doses of dietary NO_3^-. These are important findings as this study is the first of its kind to investigate the dose-response relationship of dietary NO_3^- supplementation on cognitive function in older adults. Previous studies which have shown benefits to cognitive function and CBF following supplementation with dietary NO_3^- all used doses ranging from 7.5 to 12.4 mmol NO_3^- (Gilchrist et al., 2014, Presley et al., 2011, Stanaway et al., 2019). Therefore, there may be a threshold effect at which a certain intake of dietary NO_3^- is needed to elicit beneficial cognitive effects, indicating that the Low-NO_3^- and Mod-NO_3^- treatments used in this study may be below this threshold thus, resulting in no benefit.

Nevertheless, a significant improvement in cognitive function was seen in the Corsi block tapering test following supplementation with High-NO_3^- versus PL and Low-NO_3^- treatments. Other studies have also shown improvements in aspects of cognitive performance in older adults following supplementation with similar doses of dietary NO_3^- (Gilchrist et al., 2014, Stanaway et al., 2019). Specifically, Gilchrist et al. (2014) showed a reduction in simple reaction time in older adults (67.7 ± 4.9 y) following 14 days of daily supplementation with 7.5 mmol dietary NO_3^-. Additionally, acute supplementation with 10.5 mmol of NO_3^--rich beetroot juice improved reaction time in older adults (56 ± 6 y) during the Stroop test (Stanaway et al., 2019). The CBT is commonly used to measure

visuospatial working memory and can be commonly used in combination with other tests to identify early detection of Alzheimer's (Brunetti et al., 2014). Given the nature of the CBT test, common real work effects could include tasks that involve navigation or orientation such as driving and even shopping in a supermarket. The improvements in cognitive performance during the CBT following supplementation with the High-NO_3^- treatment may have been due to an increase in CBF and potential changes in cerebral metabolism from increased NO production and bioavailability (as indicated by increases in plasma NO_2^- and NO_3^- concentrations, Figure 4.2). Further supporting this, one study in younger adults (18-27 y) showed an increase in CBF alongside initiation of the serial 3-s subtraction cognitive task following acute supplementation with 5.5 mmol dietary NO_3^- (Wightman et al., 2015). Others have shown similar increases in cerebral perfusion in older adults, predominately at the prefrontal cortex, following 2 days' supplementation with 12.4 mmol of dietary NO_3^- using fMRI, indicating increased blood flow as a potential mechanism for improved cognitive performance (Presley et al., 2011). These promising results indicate the need for further research in this area, pairing measures of cerebral oxygenation or blood flow with measures of cognitive performance in older adults to further investigate these findings.

Interestingly, there were no other cognitive performance benefits observed following acute supplementation with NO_3^--rich beetroot juice for the two other cognitive tests, which could be due to the learning aspects of the RVIP and Stroop tests used. While the study by Stanaway et al. (2019) supports the potential of supplementation with dietary NO_3^- to have cognition boosting benefits, those results contrast with aspects of the current study which did not show an improvement in Stroop test reaction time. The lack of a significant difference in the Stroop test in the current study could have been due to a learning effect. Although the order of trials was balanced in both studies, in the current study, each participant had four supplementation trials versus two trials in the study by Stanaway et al. (2019). This could have resulted in a greater learning effect during the four trials and thus less improvement would be apparent. Additionally, the Stroop test and RVIP by nature have a faster learning curve, meaning participants could become proficient in the tests more quickly, as certain cognitive tests have been shown to have a greater practice effect (Basner et al., 2020). Therefore, further research is required with a more specific selection of the cognitive tests used to maximise cognitive demand and reduce chances of a learning effect and boredom.

4.4.4. Future Directions

The current findings clearly show that of the doses used, the High-NO_3^- treatment was the optimal dose for promoting improvements in cardiovascular and cognitive responses in healthy older adults. However, as this was the highest dose used in this study further research is needed to investigate

the dose-response relationship for higher doses of dietary NO_3^- and their potential to elicit greater effects in reducing BP and improving cognition. It has been suggested that older adults could have a decreased ability for conversion of NO_3^- /NO_2^- to NO and/or decreased sensitivity to the NO signalling pathways indicating the need for higher doses of dietary NO_3^- (Lara et al., 2016). The current findings also highlight the need for further research with larger sample sizes for measures of endothelial function to further investigate the potential benefits in these areas from dietary NO_3^- supplementation in older adults. Additionally, with the reductions in BP and improvements in cognitive performance in healthy older adults following consumption of NO_3^--rich beetroot juice, it could be suggested that greater benefits could be shown in morbid populations such as those with hypertension or neurological disorders for whom high BP and reduced cognition are major risk factors (Stanaway et al., 2017). Therefore, future research should investigate optimal doses of dietary NO_3^- for populations with various morbidities.

4.5. Conclusion

Acute supplementation with NO_3^--rich beetroot juice increased plasma [NO_3^-] and [NO_2^-] dose-dependently. Supplementation with High-NO_3^- reduced MAP, SBP, and DBP when compared to the other treatments and improved cognitive performance in the Corsi block tapering test compared to the PL and Low-NO_3^- treatments. Collectively, these results indicate that acute supplementation with dietary NO_3^- can reduce BP and improve cognitive performance in older adults and that the dose with the most beneficial effects seen in this study for improvements in cardiovascular and cognitive function was 9.8 mmol of dietary NO_3^-. Furthermore, the changes in SBP and DBP showed clinically significant reductions, indicating the potential for use of dietary NO_3^- in a clinical setting.

Chapter 5

Acute Supplementation with Nitrate-Rich Beetroot Juice Causes a Greater Increase in Plasma Nitrite and Reduction in Blood Pressure of Older Compared to Younger Adults

This chapter contains a reformatted version of the article "Acute Supplementation with Nitrate-Rich Beetroot Juice Causes a Greater Increase in Plasma Nitrite and Reduction in Blood Pressure of Older Compared to Younger Adults", co-authored by Kay Rutherfurd-Markwick, Rachel Page, Marie Wong, Wannita Jirangrat, Koon Hoong The, and Ajmol Ali. This article was published in *Nutrients* on July 18th, 2019. The published version and corresponding DRC 16 form can be found in Appendix 3, and other relevant documents for this chapter, such as the ethics approval and participant info sheet, can be found in Appendices 5.1-5.7.

This chapter addresses Research Question 3 (Section 1.3.3). From the research reviewed in Chapters 2 and 3, older adults may experience greater benefits from dietary nitrate supplementation due to the degenerative processes of aging. However, limited studies have compared the effects of dietary nitrate supplementation in younger versus older adults and there has been no investigation into this comparison with regards to cognition. Therefore, this study was designed to test the acute effects of consumption of a high dose of dietary nitrate on physiological responses in older versus younger adults.

Abstract

Nitrate-rich beetroot juice supplementation has been shown to improve cardiovascular and cognitive function in younger and older adults via increased nitric oxide production. However, it is unclear whether the level of effects differs between the two groups. We hypothesised that acute supplementation with nitrate-rich beetroot juice would improve cardiovascular and cognitive function in older and younger adults, with the potential for greater improvements in older adults. Thirteen younger (18–30 years) and 11 older (50–70 years) adults consumed either 150 mL of nitrate-rich beetroot juice (BR; 10.5 mmol nitrate) or placebo (PL; 1 mmol nitrate) in a double-blind, crossover design. Plasma nitrate and nitrite concentrations, blood pressure (BP), heart rate (HR), cognitive function, mood and perceptual tests were performed throughout the trial. BR consumption significantly increased plasma nitrate ($p < 0.001$) and nitrite ($p = 0.003$) concentrations and reduced systolic BP ($p < 0.001$) in both age groups and reduced diastolic BP ($p = 0.013$) in older adults. Older adults showed a greater elevation in plasma nitrite ($p = 0.038$) and a greater reduction in diastolic BP ($p = 0.005$) following BR consumption than younger adults. Reaction time was improved in the Stroop test following BR supplementation for both groups ($p = 0.045$). Acute BR supplementation increased plasma nitrite concentrations and reduced diastolic BP to a greater degree in older adults; whilst systolic BP was reduced in both older and younger adults, suggesting nitrate-rich BR may improve cardiovascular health, particularly in older adults due to the greater benefits from reductions in diastolic BP.

Keywords: nitric oxide; beetroot juice; blood pressure; cognition; aging

5.1. Introduction

The world's population is aging, with the number of people aged over 60 years expected to increase from 901 million to 1.4 billion by 2030 (United Nations, 2015). Rates of disease and age-related dysfunction are increasing rapidly (Clements et al., 2014) and are expected to cause a dramatic increase in costs in the health and disability services areas (United Nations, 2015). This has led to an increased interest in the use of food-based supplements and bioactive compounds to improve or maintain health and body functions (Velmurugan et al., 2016).

Beetroot juice contains high levels of nitrate (NO_3^-) which can be converted into the bioactive molecule, nitric oxide (NO) (Kelly et al., 2013). Nitric oxide plays a major role in many signaling pathways and biological processes, including improving neurotransmission and blood flow, alterations in mitochondrial oxygen consumption, promotion of cognitive benefits, mood, and cardiovascular function (Clements et al., 2014, Kelly et al., 2013). Currently, two major pathways are known to result in the generation of NO in humans. First, through the conversion of L-arginine to NO by the enzyme nitric oxide synthase (NOS), which is considered the dominant pathway. Secondly, via the reduction of dietary NO_3^- to NO_2^- (nitrite), which occurs in the mouth by lingual anaerobic bacteria (Kelly et al., 2013). Nitrite is then swallowed and ultimately enters the bloodstream where it can be reduced further to NO (Baily et al., 2012). It is through this second, exogenous pathway of NO production that nitrate-based food supplements such as BR are likely to exert their potential health benefits (Kelly et al., 2013, Thompson et al., 2014).

Acute supplementation with BR can improve physiological responses such as cardiovascular function in younger adults (Breese et al., 2013, Lansley et al., 2011a, Lansley et al., 2011b). Specifically, consumption of inorganic NO_3^-, either in the form of sodium nitrate or BR, has been shown to result in a significant increase in plasma NO_2^- levels and reduction of blood pressure in both younger (8 and 6 mmHg; systolic and diastolic blood pressure; respectively) (Larsen et al., 2007) and older (Kelly et al., 2013) (5 and 3 mmHg; systolic and diastolic blood pressure; respectively) adults. This positive effect on blood pressure has led to the suggestion that beetroot could potentially be used in medical settings as an alternative to traditional blood pressure-lowering drugs (Kapil et al., 2015).

Acute supplementation with BR (7.5 mmol nitrate) has been shown to improve simple reaction times in older adults (Gilchrist et al., 2014). This effect may be due to an increase in blood flow to the brain which has been observed in older adults following consumption of dietary NO_3^- (Presley et al., 2011). Cognitive diseases have been linked to age-related reductions in cerebral blood flow (CBF), therefore nitrate supplementation may slow the process of age-related cognitive decline due

to increased perfusion to the brain (Presley et al., 2011, Stanaway et al., 2017). However, other studies have shown no improvement in cognitive function or mood with acute or 3 days of NO_3^- supplementation in older adults (Kelly et al., 2013, Thompson et al., 2014). The equivocal findings are likely due to methodological differences in the cognitive tests used, the duration of supplementation (from 2 h to 14 days), and amount of NO_3^- consumed (6–12 mmol NO_3^-).

Ageing leads to a decline in body processes and functions resulting in increased blood pressure, reduced blood flow, and reduced oxygen delivery to the muscle. All of these measures have been shown to be improved following BR supplementation in both younger and older adults, however, data presented for older adults is limited (Bailey et al., 2012, Kelly et al., 2013). There may be greater potential for improvement in cardiovascular and cognitive function, and mood following NO_3^- supplementation in older adults relative to younger adults due to age-related changes (Kenjale et al., 2011). Few studies have examined the health benefits of BR supplementation in older adults, and, to our knowledge, only one study directly compared the effects in younger versus older adults (Hughes et al., 2016), therefore further research in this area is required.

We hypothesised that acute supplementation with nitrate-rich beetroot juice would provide a greater improvement in cardiovascular responses, cognition, and mood and perception in older adults (50–70 years) compared to younger adults (18–30 years). To examine this hypothesis we conducted a randomised, double-blind crossover trial to investigate and compare the effects of acute supplementation with nitrate-rich beetroot juice on plasma nitrate and nitrite concentrations, blood pressure and cognitive performance measures in older and younger adults.

5.2. Materials and Methods

5.2.1. Sample Size

A power analysis was conducted (G-Power 3.1) to calculate the sample size based on the primary outcome measure of blood pressure (Faul et al., 2009). Previous literature in healthy adults has shown a mean change in BP of 10 mmHg (9 SD) between BR (9.6 mmol nitrate) and control (Kelly et al., 2013). Based on this, the required sample size was 11 per group, using a SD of 9, with a statistical power of 0.82 and α-level set at 0.05.

5.2.2. Participants

Twenty-four healthy, normotensive, recreationally active adults (13 younger, 18–30 years, and 11 older, 50–70 years) volunteered for this study. Participants completed a health-screening questionnaire to ensure suitability. We did not recruit tobacco smokers, users of nitrate-based

dietary supplements or well-trained/elite athletes (defined as those completing >4 training sessions per week, or >6 h of intense exercise per week, or having played/playing for a national or age-equivalent sports team). Those with known diseases (e.g., dementia and cardiovascular disease) were also excluded. Prior to testing, all participants were informed of the procedures, associated risks, and the possible benefits of participation, after which written informed consent was obtained. The study was approved by the Massey University Human Ethics Committee (SOA 16/27) and registered with ACTRN (ACTRN12618001466235).

5.2.3. Intervention

Beetroot juice, extracted from fresh washed beetroots (*Beta vulgaris* 'Pablo'), was blended with other fruit juices to provide a standardised beetroot juice drink with a constant soluble solid concentration (11° Brix) and nitrate concentration (10.5 mmol/150 mL). The placebo beetroot drink was produced using beetroot juice concentrate and standardised to the same constant soluble solids concentration (11° Brix) but contained a low nitrate concentration (1 mmol/150 mL). The BR and placebo juice drinks had previously been presented to a consumer sensory panel using a triangle test, with 25 participants, and were not perceived as significantly different ($p > 0.05$). The nitrate concentration was analysed using high-performance liquid chromatography (HPLC) based on the method of Cheng and Tsang (1998). Briefly, isocratic separation was achieved using a Gracesmart C-18 column; mobile phase 0.01 M Octylammonium orthophosphate (pH 3–3.5) at a flow rate of 0.8 mL/min. Detection was carried out at 193 nm for nitrite and 213 nm for nitrate.

5.2.4. Study Design and Procedures

Participants were required to visit the laboratory on three separate occasions. During the first visit, they were familiarised with the testing procedure and equipment, including cognitive, perceptual and mood tests, and body weight (kg) and height (m) were measured using digital scales and a stadiometer, respectively. Participants were instructed to complete a food diary for two days prior to the second study visit and to replicate their diet and lifestyle factors for the third study visit (dietary nitrate was not restricted, in an attempt to investigate additional benefits of BR in conjunction with normal daily intake; however, the food intake diary was used to evaluate intake of dietary nitrate and correlated to baseline plasma nitrate and nitrite concentrations). Participants were asked to arrive at the laboratory in a well-rested, fasted state and to refrain from excessive exercise and alcohol consumption 24 h prior and caffeine 6 h prior to each study visit. All trials were performed in the morning at approximately the same time of day (±2 h).

The second and third visits were the supplementation trials, with each participant randomly allocated (using a random number generator) the order of consumption of the two beverages in a double-blind, crossover design. For these two study visits, participants consumed 150 mL of beetroot juice (BR; containing 10.5 mmol NO_3^-) during one visit and 150 mL of a placebo (PL; containing 1 mmol NO_3^-), on the other visit. A 150-mL beverage was used to obtain approximately 10.5 mmol of nitrate as this amount has been shown to be a sufficient dose to elicit beneficial effects (Kelly et al., 2013). Drinks were consumed with a standardised, isocaloric breakfast (cereal or toast) with the same macronutrient breakdown (15 g protein, 30 g carbohydrates, and 10 g fat). Participants consumed the same breakfast type for both supplementation trials. Visits two and three were separated by a one-week washout period providing sufficient time for normalisation of NO_3^- and NO_2^- levels in the body (Lansley et al., 2011a).

The protocol for visits two and three is illustrated in Figure 5.1. Prior to consumption of the beverage, baseline measures of blood pressure (mean value of three measurements taken from the left arm: deluxe HEM-7130; OMRON Healthcare CO. Ltd.; Kyoto, Japan), heart rate (A1 polar chest transducer), cognitive function, perceptual and mood tests were completed followed by collection of a resting blood sample. Participants then consumed the allocated drink with breakfast, within a 10-min period. Participants were asked to remain in the laboratory for a 2.25 h absorption period while a home development show was aired for entertainment purposes (has minimal effect on cognitive stimulation or arousal levels (Kelly et al., 2013)), or participants could complete computer work or study. The 2.25 h absorption period was chosen as previous research has shown that peak plasma nitrite concentrations occur 2–3 h after beetroot juice consumption (Wylie et al., 2013). After 2.25 h, the aforementioned measurements were repeated. Blood samples were also taken again 3.25 h post-supplementation.

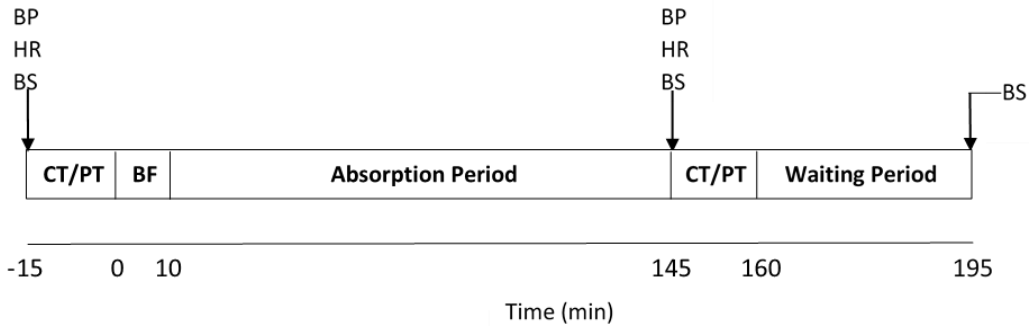

Figure 5.1. Schematic of the acute study protocol. CT, cognitive tests; PT, perceptual tests; BF, breakfast; BP, blood pressure; BS, blood sample; HR, heart rate.

5.2.5. Blood Measurements

Plasma nitrite and nitrate were used as biomarkers for nitric oxide availability (Kleinbongard et al., 2003). Six milliliter venous blood samples were taken by venepuncture, from a vein within the antecubital area, and collected into heparinised tubes. Samples were mixed, centrifuged (MF–50 Hanil Science Industrial, Incheon, South Korea) at 3500 rpm (1330 g) for 10 min, and the collected plasma aliquoted into Eppendorf tubes (0.5 mL per tube) and stored at −80 °C for later analysis of nitrite and nitrate concentrations by HPLC (Li et al., 2000).

5.2.6. Cognitive Measurements

Cognitive tests were completed in a quiet, isolated room using a computer placed at eye level (PsychoPy software, version 1.83.04 (Peirce, 2007)). The cognitive tasks included the choice reaction test (CRT), rapid visual information processing (RVIP), and Stroop tests. Each task was performed a total of four times per participant (two per trial; pre- and 2.5 h post-supplementation). These tests have been used to investigate the effects of nutritional supplementation on executive function, attention, and information processing speed (Kelly et al., 2013, Thompson et al., 2015).

5.2.6.1. Choice Reaction Test (CRT)

The CRT task involves participants pressing the left-hand key when the word ‘LEFT’ appears and the right-hand key when the word ‘RIGHT’ appears.

5.2.6.2. Rapid Visual Information Processing (RVIP)

The RVIP test involves participants following a chain of single numbers from 1 to 9, which appear in a pseudo-random order at the rate of 100 per minute. The aim of the test is to correctly identify target sequences (2–4–6, 4–6–8, and 3–5–7) as quickly as possible.

5.2.6.3. Stroop Test

The Stroop test involves the display of different colour names (‘RED’, ‘GREEN’, and ‘BLUE’) which appear on the screen, one at a time in different coloured fonts (red, green, or blue). The aim of this test is to press the key corresponding to the colour of the font that the present word was displayed in.

5.2.7. Mood and Perceptual Measurements

The feeling scale (FS) was used to measure the degree of displeasure or pleasure based on an 11-point scale ranging from −5 (very bad) to +5 (very good) (Hardy and Rejeski, 1989). The felt arousal scale (FAS) was used to measure the degree of arousal-activation based on a 6-point scale ranging

from 1 (low arousal) to 6 (high arousal) (Svebak et al., 1985). The profile of mood states (POMS) was used to evaluate mood based on ranking how one feels, on a 5-point Likert scale ranging from 0 (not at all) to 4 (extremely) for each word presented under the 7 mood states (fatigue, anger, vigor, tension, esteem, confusion, and depression) (McNair et al., 1971). The FS, FAS, and POMS measures were all completed prior to and 2.5 h post-supplementation.

5.2.8. Statistical Analysis

Statistical analyses were completed using IBM SPSS (Version 22.0). All data except for physical characteristics and baseline measurements were analysed using mixed-method repeated-measures analysis of variance (ANOVA) with treatment and time as within-subject factors, and age as the between-subject factor. Sphericity was tested using the Mauchly's test to ensure the assumption of sphericity was not violated, and multivariate models were applied if these assumptions were not met. Where significant differences were found, post-hoc tests with Holm-Bonferroni correction were undertaken to assess multiple comparisons. Outliers were excluded based on the Tukey test. Relative effect sizes were calculated using partial eta squared and defined as small (η^2_p = 0.01), medium (η^2_p = 0.06), or large (η^2_p = 0.14). Independent Student's *t*-tests were used to examine differences in physical characteristics (height, weight, age) between age groups. Data are presented as mean ± standard deviation. Statistical significance was set at $p < 0.05$.

5.3. Results

5.3.1. Participants

The physical characteristics and baseline nitrate, nitrite and blood pressure measurements for the younger and older group participants are reported in Table 5.1. All 24 participants completed the blood pressure, mood, perceptual, and cognitive tests. However, blood samples were only successfully obtained from 19 of the 24 (11 younger and 8 older) participants. One older participant was excluded from the Stroop test as their result was determined to be an outlier (using Tukey test) falling outside the interquartile range.

Table 5.1. Physical characteristics and baseline nitrate, nitrite, and blood pressure measurements of younger and older adult group participants in the acute study.

	Younger Adults (*n* = 13)		**Older Adults (*n* = 11)**		***p*-Value**	
Male	9		3			
Female	4		8			
Age (years)	25 ± 3		56 ± 6		<0.001 *	
Height (cm)	178.4 ± 10.1		169.8 ± 7.0		0.026 *	
Body Mass (kg)	75.3 ± 14.9		74.7 ± 7.9		0.90	
	Beetroot	**Placebo**	**Beetroot**	**Placebo**	**Beetroot (Between Groups)**	**Placebo (Between Groups)**
Plasma NO_2^- (nM)	193.9 ± 77.5	153.8 ± 57.7	145.4 ± 40.2	143.1 ± 60.3	0.19	0.86
Plasma NO_3^- (μM)	111.8 ± 112.5	116.0 ± 80.9	90.3 ± 62.1	127.0 ± 71.1	0.90	0.27
SBP (mmHg)	116.8 ± 8.1	114.9 ± 9.2	125.2 ± 9.6	120.9 ± 10.8	0.061	0.003 *
DBP (mmHg)	68.5 ± 6.0	68.8 ± 7.3	80.9 ± 6.9	77.9 ± 6.3	0.81	0.042 *

Values are means ± SD. * indicates statistically significant difference ($p < 0.05$). NO_2^-, nitrite; NO_3^-, nitrate; SBP, systolic blood pressure; DBP, diastolic blood pressure.

5.3.2. Plasma Nitrite and Nitrate

There was no significant difference ($p < 0.05$) between baseline plasma [NO_2^-] and [NO_3^-] between the groups (Table 5.1). Plasma [NO_2^-] was elevated 2.5 and 3.25 h post-supplementation following BR consumption compared to PL) in both groups ($p = 0.003$, $\eta^2_p = 0.41$, Figure 5.2A). Older adults had a greater increase in plasma [NO_2^-] 2.5 and 3.25 h post-supplementation compared to younger adults following BR supplementation versus PL ($p = 0.038$, $\eta^2_p = 0.23$). Plasma [NO_3^-] was also elevated 2.5 and 3.25 h post-supplementation with BR compared to PL in both groups ($p < 0.001$, $\eta^2_p = 0.67$, Figure 5.2B). There was a trend for a greater increase in plasma [NO_3^-] in older adults, 3.25 h post BR supplementation versus PL ($p = 0.064$, $\eta^2_p = 0.19$).

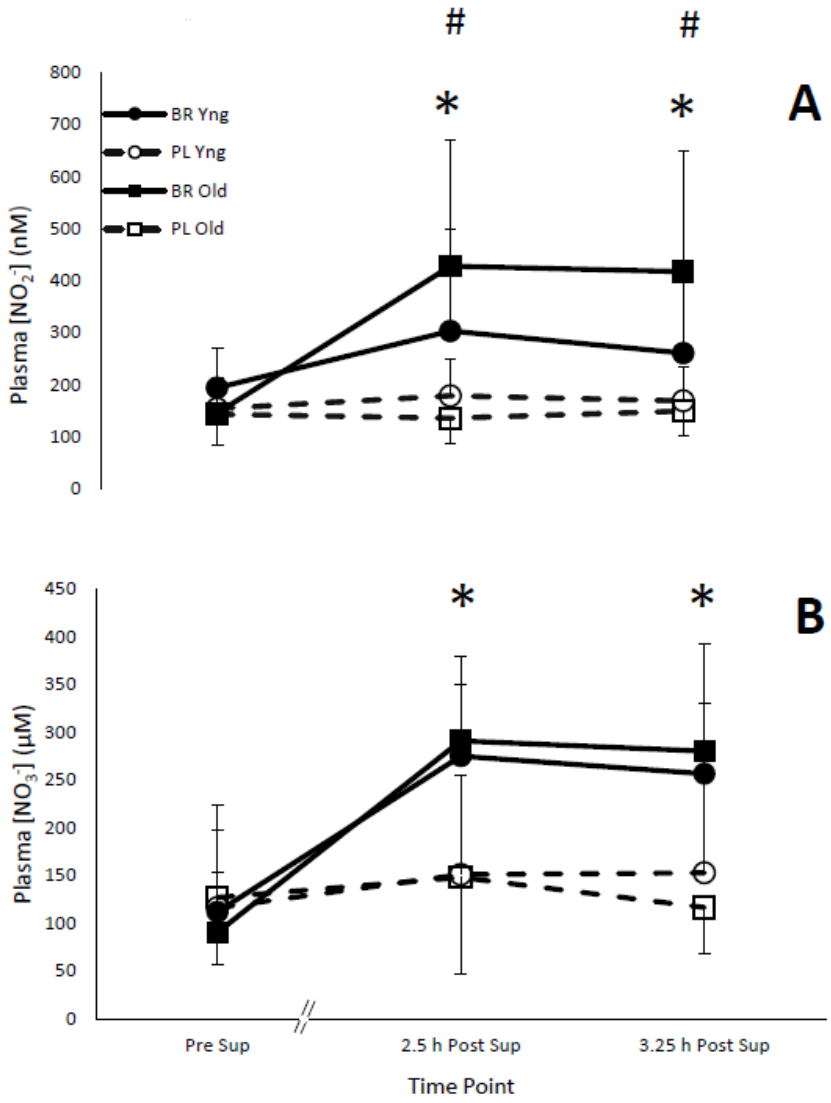

Figure 5.2. Changes in plasma nitrate and nitrite concentration in the acute study (**A**) Plasma nitrite concentration ($[NO_2^-]$) (nM) pre- (Pre Sup), 2.5 h post-supplementation (2.5 h Post Sup) and 3.25 h post-supplementation (3.25 h Post Sup) with beetroot juice (BR) and placebo (PL) in younger (Yng) (n = 13) and older (Old) adults (n = 11). (**B**) Plasma nitrate concentration ($[NO_3^-]$) (µM) pre- (Pre Sup), 2.5 h post-supplementation (2.5 h Post Sup) and 3.25 h post-supplementation (3.25 h Post Sup) with beetroot juice (BR) and placebo (PL) in younger (Yng) and older (Old) adults.
Values are expressed as means ± SD.
* Statistically significant mean difference between treatment (placebo vs. beetroot), and time (pre- vs. post-supplementation) ($p < 0.05$). # Statistically significant mean difference between treatment, time, and age ($p < 0.05$).

5.3.3. Blood Pressure

Systolic blood pressure (SBP) was reduced in both younger and older adults 2.25 h following consumption of BR ($p < 0.001$, $\eta^2_p = 0.45$, Figure 5.3A). However, there was no interaction effect for SBP of treatment*time*age ($p = 0.11$, $\eta^2_p = 0.11$). There was a reduction in DBP post-supplementation compared to pre-supplementation following consumption of BR versus PL for older but not younger adults ($p = 0.013$, $\eta^2_p = 0.25$, Figure 5.3B). Older adults had a greater reduction

in DBP post- versus pre-supplementation with BR versus PL, compared to younger adults (p = 0.005, η^2_p = 0.31, Figure 5.3B).

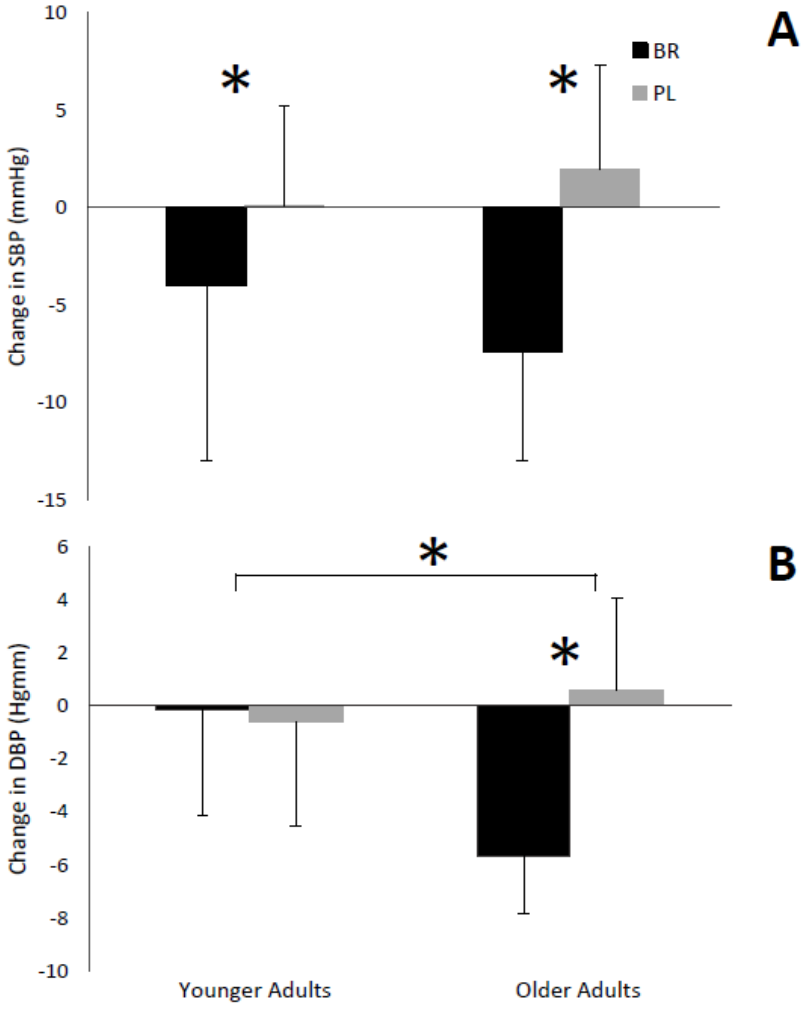

Figure 5.3. Changes in blood pressure following acute dietary nitrate supplementation (**A**) Change in systolic blood pressure (SBP) (mmHg) pre- versus post-supplementation with beetroot juice (BR) and placebo (PL) in younger (n = 13) and older adults (n = 11). (**B**) Change in diastolic blood pressure (DBP) (mmHg) pre- versus post-supplementation with beetroot juice (BR) and placebo (PL) in younger and older adults.
Values are expressed as means ± SD.
* Statistically significant mean difference between change in blood pressure (**A**, **B**) and change in blood pressure between older and younger adults (**B**) (p < 0.05).

5.3.4. Heart Rate

There was no interaction of treatment*time (pre- and post-supplementation) (p = 0.99, η^2_p < 0.001) or interaction of treatment*time*age (p = 0.62, η^2_p = 0.011) on average HR in either group.

5.3.5. Cognitive Performance

Supplementation with BR showed a trend for improved correct response percentage in the Stroop test from pre- to post-supplementation compared to PL (p = 0.075, η^2_p = 0.14). Beetroot juice improved reaction time during the Stroop test from pre- to post-supplementation compared to PL (p = 0.045, η^2_p = 0.18, Table 5.2). There was no interaction of treatment*time*age for correct responses (p = 0.99, η^2_p < 0.001) or reaction time (p = 0.38, η^2_p = 0.037) in the Stroop test. Placebo improved correct response percentage for CRT from pre- to post-supplementation compared to BR (p = 0.008, η^2_p = 0.28). There was no effect of treatment*time for reaction time in the CRT (p = 0.14, η^2_p = 0.094) or interaction effect of treatment*time and age for percentage correct responses (p = 0.96, η^2_p < 0.001) or reaction time (p = 0.076, η^2_p = 0.14) in the CRT (p = 0.96, η^2_p < 0.001). There were no interaction effects of treatment*time or treatment*time*age for RVIP percentage correct responses (p = 0.33 and 0.89, η^2_p = 0.043 and 0.001, respectively), percentage of errors (p = 0.92 and 0.65, η^2_p < 0.001 and 0.010, respectively), and reaction time (p = 0.31 and 0.99, η^2_p = 0.047 and <0.001, respectively).

Table 5.2. Cognitive performance tests and mood analysis pre- and post-supplementation with beetroot juice and placebo in younger and older adults.

	Younger Adults				**Older Adults**			
	Placebo		**Beetroot**		**Placebo**		**Beetroot**	
	Pre	***Post***	***Pre***	***Post***	***Pre***	***Post***	***Pre***	***Post***
Choice Reaction Test								
Correct Response (%)	96.2 ± 2.3	97.5 ± 1.7 *	96.2 ± 2.1	95.6 ± 2.6	98.6 ± 2.0	99.2 ± 0.4 *	98.7 ± 1.7	97.4 ± 2.2
Reaction Time (ms)	433 ± 48	426 ± 34	435 ± 46	421 ± 45	637 ± 125	588 ± 59	596 ± 96	615 ± 134
Rapid Visual Information Processing								
Correct Response (%)	87.2 ± 9.5	90.1 ± 8.8	81.1 ± 16.7	89.5 ± 7.7	57.8 ± 20.3	63.1 ± 21.4	55.9 ± 20.6	65.4 ± 16.9
Errors	1.2 ± 1.2	1.2 ± 1.1	1.4 ± 1.3	1.1 ± 1.1	2.4 ± 3.4	1.3 ± 1.7	2.6 ± 2.0	1.8 ± 1.6
Reaction Time (ms)	422 ± 34	402 ± 32	445 ± 67	409 ± 49	481 ± 65	495 ± 61	498 ± 68	496 ± 76
Stroop Test								
Correct Response (%)	96.5 ± 2.6	95.8 ± 3.6	95.6 ± 3.0	96.3 ± 2.8	98.0 ± 2.2	98.6 ± 1.1	96.7 ± 3.0	98.7 ± 1.6
Reaction Time (ms)	644 ± 112	607 ± 102	690 ± 204	616 ± 117 *	965 ± 194	970 ± 167	978 ± 189	891 ± 138 *
Mood Measures								
FS	1.9 ± 1.1	2.2 ± 1.1	2.6 ± 1.3	2.9 ± 1.3	2.7 ± 1.2	2.8 ± 1.5	2.2 ± 1.8	2.4 ± 1.3
FAS	1.9 ± 0.8	2.4 ± 1.0	2.2 ± 0.6	2.3 ± 1.0	1.9 ± 0.9	2.0 ± 1.1	2.1 ± 1.2	1.9 ± 1.3
POMS	22 ± 11	17 ± 7	22 ± 9	19 ± 8	18 ± 8	16 ± 7	25 ± 10	20 ± 10

Values are expressed as means ± SD. Younger (n = 13), Older [choice reaction, rapid visual information processing and mood tests (n = 11) Stroop test (n = 10). * Statistically significant mean difference between treatment (placebo vs. beetroot), and time (pre- vs. post-supplementation) ($p < 0.05$). FS, feeling scale; FAS, felt arousal scale; POMS, profile of mood states.

5.3.6. Perceptual Responses and Mood

There was no treatment or treatment*age effects between pre- and post-supplementation for ratings of pleasure-displeasure (FS) (p = 0.93 and 0.73, η^2_p < 0.001 and 0.006, respectively; Table 5.2) and perceived activation (FAS) (p = 0.12 and 0.78, η^2_p = 0.11 and 0.003 respectively; Table 5.2). There was no treatment or treatment*age effects for individual categories (p > 0.05) or total POMS score (p = 0.62 and 0.077, η^2_p = 0.012 and 0.14, respectively) between pre- and post-supplementation.

5.4. Discussion

We examined the effects of acute supplementation with NO_3^- rich beetroot juice (BR) on cardiovascular responses, cognition, and mood and perceptual responses in younger and older adults. The main findings of this study demonstrate that consumption of NO_3^- rich BR; (1) significantly elevated plasma NO_2^- and NO_3^- concentrations and reduced BP for both younger and older adults, (2) improved reaction time during the Stroop test pre- versus post-supplementation, and (3) elevated plasma NO_2^- to a greater degree and resulted in a greater reduction in DBP in older adults.

5.4.1. Plasma [NO_2^-] and [NO_3^-]

This is the first study to directly compare plasma [NO_2^-] and [NO_3^-] in younger and older adults following BR supplementation, with consumption of BR significantly elevating both plasma [NO_2^-] and [NO_3^-] in both age groups. Previous studies have found similar increases in plasma [NO_2^-] and [NO_3^-] following dietary NO_3^- supplementation in older (Kenjale et al., 2011) and younger adults (Larsen et al., 2011). Supplementation with BR resulted in a greater increase in plasma [NO_2^-] and showed a trend for a greater increase in plasma [NO_3^-] in older compared to younger adults (Figure 5.2). This finding was expected as older adults (60–70 years) have been suggested to have an age-related decrease in NO availability, suggesting that NO_3^- supplementation may have a greater impact on the older population, resulting in larger increases in plasma [NO_2^-] and [NO_3^-] (Kelly et al., 2013). Due to the novelty of this finding, further research is required directly comparing plasma [NO_2^-] and [NO_3^-] in younger and older adults following BR supplementation.

5.4.2. Blood Pressure

Systolic blood pressure was reduced in both age groups following BR supplementation, while DBP was reduced only in the older adults. Studies have shown that reductions in SBP and DBP of as little as 2 mmHg can greatly reduce the incidence of CVD in both hypertensive and normotensive individuals (Hess et al., 2014). Consequently, reductions such as those observed in this study are considered clinically meaningful (Wong and Wright, 2014).

The reductions in BP observed in this study were likely due to an increase in NO production (Lansley et al., 2011a), which has been shown to activate the enzyme guanylate cyclase which catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) (Bailey et al., 2010, Lansley et al., 2011a). cGMP acts on smooth muscle causing relaxation, resulting in vasodilation of arteries and veins, thus decreasing BP (Kelly et al., 2013). It would be expected that NO_3^- supplementation would also reduce DBP via this same mechanism; however, this only occurred in older adults. Previous studies have indicated that a reduction in DBP in younger adults following BR consumption is less common compared to a reduction in SBP (Bailey et al., 2009, Lansley et al., 2011a), while studies in older adults more commonly show reductions in DBP following BR supplementation (Kelly et al., 2013, Kenjale et al., 2011). Although it has been suggested that the BP-lowering effects of BR may be related to other bioactive compounds or micronutrients found in beetroot juice; studies using a nitrate depleted placebo (by ion-exchange chromatography), and thus containing all other compounds, have still shown similar reductions in BP, indicating the nitrate content is the determining factor (Lansley et al., 2011a, Lansley et al., 2011b).

Interestingly, older adults were found to have a larger reduction in DBP following supplementation with BR compared to younger adults; however, this was not seen for SBP. The interaction effect on DBP is a relevant finding as older adults are at a higher risk of endothelial dysfunction and cardiovascular disease, of which high blood pressure is a major risk factor, due to age-related reductions in NO production (Clements et al., 2014). This finding also supports the theory that older adults have a lower initial level of NO and reduced endothelial functioning capacity, thus NO_3^- supplementation may have a greater potential for reducing BP in older versus younger adults (Hughes et al., 2016, Kelly et al., 2013). In contrast to our findings, one study comparing the effects of dietary NO_3^- supplementation in younger versus older adults found no significant difference in SBP or DBP between the two age groups (Hughes et al., 2016), however, they did not use a placebo-controlled, crossover design which could have impacted the results.

5.4.3. Cognitive Performance

Current findings suggest only certain aspects of cognitive performance (Stroop but not CRT, RVIP) were improved following BR supplementation. This is consistent with the findings of others (Thompson et al., 2015), which showed significant improvements in Stroop test mean reaction time post BR (12.8 mmol of NO_3^-) consumption compared to PL (0.08 mmol of NO_3^-), but no changes in other cognitive performance tests. Supplementation with NO_3^- rich BR has been shown to significantly increase cerebral perfusion, specifically to the prefrontal cortex (Presley et al., 2011), therefore, improvements in reaction time in the Stoop test (a task that specifically targets the

prefrontal cortex) could be expected. This may be due to increases in cerebral oxygenation allowing for a decrease in the O_2 cost of mental processing (Wightman et al., 2015). The variation in results from the different cognitive tests may be due to differences in the cognitive demands of the tests, with the Stroop test placing greater strain on an individual's mental processing capacity compared to simple reaction and number recall tests. Thus, BR supplementation may only improve cognitive performance when a large degree of cognitive difficulty is imposed (Thompson et al., 2015). It is also important to note that placebo supplementation improved the correct response percentage for the CRT, however, due to the extremely high percentages of correct responses and small standard deviations, it is possible this result lacks biological significance and indicates the need for further investigation.

While it has been proposed that supplementation with NO_3^- rich BR could have greater benefits on cognitive function in older adults due to its ability to compensate for age-related decreases in NO production and CBF (Presley et al., 2011), our findings did not support this. The lack of difference between age groups may have been due to the small sample size used and slightly younger mean age (56 years) of the older adults, as results from the CRT showed a trend for greater reduction in reaction time for older adults following BR supplementation ($p = 0.076$). Another major limitation in this area was the lack of a direct measure of CBF, thus it was not possible to determine whether or not BR supplementation increased blood flow to the brain and whether any increase was greater in the older adults.

5.4.4. Mood and Perception

Supplementation with NO_3^- rich BR did not improve any mood or perceptual measures (Table 5.2), supporting the findings of others (Wightman et al., 2015). Mood is affected by changes in blood flow to the brain and increases in neuro-excitation (Ishizaki et al., 2008), thus, supplementation with dietary NO_3^- has been suggested as a mood enhancer due to its effects on cerebral vasodilation and stimulation of NO production (Stanaway et al., 2017, Thompson et al., 2014). Studies have also shown that age-related depression and alterations in mood are related to a reduction in CBF, and changes in neurotransmitter concentrations (Ishizaki et al., 2008). As BR supplementation increases CBF and stimulates increased NO production, it is possible that older adults may experience greater improvements in mood following consumption (Kelly et al., 2013, Presley et al., 2011). The lack of change in mood in this study could be due to insufficient sample size. In addition, it has been shown that age-related depression tends to occur in the mid-to-late 60s, (Ishizaki et al., 2008) hence the lack of significance in mood and perception values between age groups seen here may have been due to the mean age of the older group being only 56 y.

5.4.5. Future Directions

The current findings indicate the potential for NO_3^- rich BR as an intervention for reducing BP in normotensive younger and older adults, with further research needed to assess its viability in a medical setting; for example, being used in conjunction with antihypertensive medication or to help delay the need for pharmaceutical treatment in populations with hypertension. The findings also highlight the need for further research, with specific attention to direct comparisons of measures in younger and older adults, larger sample size for cognitive and mood measures, and more sensitive and specific measures for potential cognitive and mood benefits from supplementation with NO_3^- rich BR (e.g., near-infrared spectroscopy (NIRS) of the brain to monitor oxygenation and CBF (Kenjale et al., 2011)). Additionally, as this study showed a benefit on BP following NO_3^- rich BR supplementation in the healthy population this may suggest greater benefits in hypertensive groups, which should be an area for future research. Moreover, further research is needed to examine the effects of long-term consumption (chronic) of NO_3^- rich BR supplementation on health benefits. To date, most longer-term studies, involve daily consumption for periods of between 6 to 14 days (Gilchrist et al., 2014, Lansley et al., 2011b), so future studies should focus on investigating longer supplementation periods of at least 28 days duration.

5.5. Conclusions

Acute supplementation with NO_3^- rich BR increased plasma [NO_2^-] and [NO_3^-] and reduced SBP in both age groups. The increase in plasma NO_2^- was greater in older adults, and DBP was reduced in older compared to younger adults. There was no difference in HR. Reaction time was improved in the Stroop test following BR supplementation; however, no difference was seen between age groups or for any other cognitive tests. Supplementation with BR did not improve perceptual or mood measures pre- versus post-supplementation. Collectively, these results indicate that acute supplementation with BR can reduce BP and improve aspects of cognitive performance; thus, having potential health benefits for both younger and older adults.

Author Contributions: L.S., A.A., K.R.-M. and R.P. designed the research study; L.S. conducted the research trials; A.A., K.R.-M., K.H.T., M.W. and W.J. produced, analysed, and stored the beetroot juice; L.S., K.S. and K.R.-M. performed blood analysis; L.S. performed data and statistical analysis; L.S. wrote the paper. All authors contributed to the discussion, edited, and revised the manuscript, and accepted the final manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Chapter 6
Acute and Chronic Nitrate-rich Beetroot Juice Supplementation Improves Blood Pressure and Cognition to a Greater Extent in Older Compared to Younger Adults

This chapter contains the article "Acute and Chronic Nitrate-rich Beetroot Juice Supplementation Improves Blood Pressure and Cognition to a Greater Extent in Older Compared to Younger Adults", co-authored by Kay Rutherfurd-Markwick, Rachel Page, Marie Wong, Ben Jones, Stephen Bailey and Ajmol Ali. This article will be submitted to *The American Journal of Clinical Nutrition*. Other relevant documents for this chapter, such as the ethics approval and participant information sheet, can be found in Appendices 6.1-6.7. Due to the relevant findings and size of this article, splitting the article into two separate manuscripts may be considered post-submission of this thesis.

This chapter contains the results of the Chronic study addressing research question 4 (Section 1.3.4). The study design for this study was developed following key findings from Chapters 2, 3, 4, and 5. Firstly, from Chapters 2 and 3 it was clear that there was limited research on the effects of dietary NO_3^- on cognitive health and performance, the mechanisms behind the physiological effects, the comparison between effects in older versus younger adults, and the effects of duration of supplementation. Secondly, it was evident that the high dose (600 mg / 9.8 mmol) was the optimal dosage of dietary NO_3^- based on the treatments investigated in the study (Chapter 4), therefore, a 640 mg dose was chosen for this Chronic study. Thirdly, the results from the study presented in Chapter 4 indicated potential mechanisms of action based on the findings showing a trend for decreased SVR. Finally, acute dietary NO_3^- supplementation elicited greater benefits in older compared to younger adults (Chapter 5). Therefore, this RCT was designed to investigate acute and chronic (14 and 28 days) supplementation with a high (640 mg) dose of NO_3^--rich BR on cardiovascular and cognitive health and performance in older and younger adults. Additionally, a secondary outcome measure of the potential mechanism of action, SVR via USCOM was included.

Abstract

Although supplementation with dietary nitrate can reduce blood pressure (BP) and improve cognition in younger and older adults, age-related decline in cardiovascular health and cognition suggests greater scope for beneficial effects in older cohorts. Additionally, it is unclear how the duration of supplementation may impact effects on cardiovascular responses and cognition, and whether such responses differ with age. It was hypothesised that acute supplementation with nitrate-rich beetroot juice would provide greater benefits on cardiovascular health, cognition, and physiological performance in older compared to younger adults, and that daily supplementation compared to a single dose would elicit greater improvements in these parameters. Twenty younger (18–30 y) and 22 older (50–80 y) adults consumed either 250 mL of nitrate-rich beetroot juice (BR; 10.5 mmol nitrate) or placebo (PL; 0.15 mmol nitrate) daily for 28 days in a double-blind, randomised control trial. On days 0, 14, and 28 plasma [nitrate] and [nitrite], BP, systemic vascular resistance (SVR), heart rate, and cognitive function were measured pre- and 2.25 h post-supplementation, with time trial performance measured 2.5 h post-supplementation. Acute BR consumption compared to PL significantly reduced systolic BP, diastolic BP, and mean arterial pressure (MAP) in older adults on day 0 ($p < 0.001$) and 28 ($p < 0.01$) and younger adults on day 28 ($p < 0.05$), with a reduction also shown in older adults on day 14 following chronic supplementation ($p < 0.05$). The reductions in BP following acute supplementation with BR on day 0 and chronic supplementation on day 14 were greater in older compared to younger adults ($p < 0.05$). Reaction time for older adults was improved in the Stroop test on days 0 ($p = 0.042$) and 28 ($p = 0.006$) following acute BR supplementation, with a greater improvement in older compared to the younger adults shown on both days ($p = 0.012$ and 0.032, respectively). Older adults also had a greater improvement in Stroop test reaction time following 14 ($p = 0.016$) and 28 ($p = 0.02$) days' chronic supplementation compared to younger adults. The results of the current study indicate that acute and chronic BR supplementation has the potential to improve cardiovascular and cognitive responses by a greater magnitude in older compared to younger adults. This suggests that daily supplementation with BR may have positive implications for maintaining cardiovascular and cognitive health in the ageing population.

Keywords: nitric oxide; nitrate; blood pressure; cognition; chronic supplementation; aging

6.1 Introduction

The importance of nitric oxide (NO) in the human body is well established due to its critical role in several key biological processes and signaling pathways including regulation of vasodilation and endothelial function (Kapil et al., 2015, Melikian et al., 2009), neurotransmission (Garthwaite, 2008), and muscle contractility (Bailey et al, 2010). Most commonly, NO is produced endogenously via oxidation of L-Arginine to NO by nitric oxide synthase (NOS) in combination with the use of specific cofactors (e.g., nicotinamide adenine dinucleotide phosphate, flavin adenine dinucleotide, flavin mononucleotide) and oxygen (Bailey et al., 2012). However, dysfunction in the L-Arginine/NOS pathway can occur due to aging and lack of bioavailability of the substrates and essential cofactors (Bailey et al., 2012, Sindler et al., 2011). This can result in a decrease in NO availability and increased risk of neurological and cardiovascular diseases (CVD), and reduced exercise tolerance due to a decrease in endothelial function, blood flow to the brain, neurotransmission, and mitochondrial function (Clements et al., 2014, Clifford et al., 2019, Lara et al., 2016). This possible dysfunction in the endogenous pathway increases the importance of the exogenous pathway, where consumption of dietary nitrate (NO_3^-) is converted to nitrite (NO_2^-) and then to NO (Kemmner et al., 2017). This alternative pathway provides a mechanism to maintain or improve normal NO homeostasis in individuals that may have a diminished NO production capability (Hughes et al., 2016).

Older adults represent a group who are at risk of reduced NO synthesis and bioavailability, indicating the need for higher concentrations of NO to promote the same physiological responses (Donato et al., 2018, Lara et al., 2016). Furthermore, the older adult population is increasing, and with associated medical conditions (such as CVD and dementia) also on the rise, there is greater demand on the healthcare system to support the aging populace (Jackson, 2011, Ministry of Health, 2016). With increased risk for CVD and neurological disorders being linked to a reduction in NO bioavailability (Donato et al., 2018, Presley et al., 2011, Stanaway et al., 2017), there has been an increase in research examining food-based products high in dietary NO_3^- (most commonly beetroot juice) with the potential to promote increases in NO production via the exogenous (NO_3^- - NO_2^- - NO) pathway (Hughes et al., 2016, Kelly et al., 2013).

Previous studies have shown that acute and daily supplementation with NO_3^--rich beetroot juice (BR) can reduce blood pressure (BP) and improve physiological performance in younger (Bailey et al., 2010, Breese et al., 2013, Lansley et al., 2011a, Wylie et al., 2013) and older adults (Berry et al., 2015, Kelly et al., 2013, Stanaway et al., 2019). Specifically, acute supplementation with 8.4 mmol NO_3^--rich BR, significantly reduced systolic (-10 mmHg) and diastolic (-3 mmHg) BP in younger adults (Wylie et al., 2013), while acute supplementation with BR (10.5 mmol) reduced systolic blood

pressure (SBP) by -7.4 mmHg and diastolic blood pressure (DBP) by -5.7 mmHg in older adults (Stanaway et al., 2019). Daily supplementation for 6 days with 5.1 mmol·day^{-1} dietary NO_3^- reduced SBP (-7 mmHg) and DBP (-7 mmHg) in younger adults (Bailey et al., 2010). Similarly, consumption of 9.6 mmol·day^{-1} NO_3^- for 3 days reduced SBP (-10 mmHg) and DBP (-4 mmHg) in older adults (Kelly et al., 2013). To put these results in context, the mean reduction in systolic and diastolic BP seen from a standard dose of anti-hypertensive medication is 9.1 and 5.5 mmHg respectively (Law et al., 2009), thus supplementation with dietary NO_3^- may have the potential to achieve similar results for reduction in risk of CVD without the potential adverse effects. However, while many studies have shown a reduction in BP following acute or chronic supplementation with BR, some have shown no improvement in either younger (Cermak et al., 2012) or older (Gilchrist et al., 2013, Shepherd et al., 2016) adults. These conflicting results are likely due to methodological differences, for example Gilchrist et al. (2013) investigated subjects who maintained their normal prescriptions for BP-lowering medication, thus supplementation with BR had no effect in addition to existing effects of the medication.

Acute and daily consumption of BR has also been shown to improve time to exhaustion and oxygen uptake ($\dot{V}O_2$) responses in younger (Bailey et al., 2009, Lansley et al., 2011b) and older adults (Kelly et al., 2013, Kenjale et al., 2011) potentially via increased delivery of oxygen to the working muscles, which may result in a more rapid transition to aerobic metabolism and less reliance on the anaerobic system. Lansley and colleagues (2011b) showed that the oxygen cost during submaximal treadmill walking (in younger adults) was lower following supplementation with BR (6.4 mmol·day^{-1}) compared to placebo. In addition, a significant speeding in $\dot{V}O_2$ kinetics in older adults during submaximal treadmill walking was shown following consumption of BR (9.6 mmol·day^{-1}), yet no improvement in walking performance was found (Kelly et al., 2013). However, there is limited research directly comparing the effects of BR consumption on physiological performance in older compared to younger populations particularly looking at longer durations of supplementation (>15 days).

Acute and chronic supplementation with BR has also been found to improve cognitive performance in younger (Thompson et al., 2015, Wightman et al., 2015) and older (Gilchrist et al., 2014, Stanaway et al., 2019) adults. In particular, acute supplementation with 5.5 mmol (Wightman et al., 2015) and 7 days' supplementation with 12.8 mmol·day^{-1} (Thompson et al., 2015) of NO_3^--rich BR, significantly improved performance during the serial 3-s subtraction test and reduced Stroop test reaction time, in younger adults. While in older adults, acute supplementation with BR (10.5 mmol NO_3^-) showed an improvement in reaction time during the Stroop test (Stanaway et al., 2019) and 14 days'

supplementation (7.5 mmol·day^{-1} NO_3^-) showed improvements in simple reaction time (Gilchrist et al., 2014). However, others have reported no benefits to cognitive function for younger and older adults following consumption of dietary NO_3^- (Kelly et al., 2013, Thompson et al., 2014). Nevertheless, the improvements that have been observed in cognitive function suggest the potential for utilisation of dietary NO_3^- to enhance or maintain normal cerebral function (Stanaway et al., 2019). This is particularly important in older adults who experience a decline in cognition with aging (Clifford et al., 2015). Indeed, there is currently a limited number of studies that have investigated the benefits of BR supplementation on cognition and with incongruent findings more research is required.

With older adults being at greater risk of diminished NO availability due to aging (Sindler et al., 2011), they may experience greater benefits in cardiovascular and cognitive outcomes from dietary NO_3^- supplementation compared to younger adults who are more likely to have normal NO production (Stanaway et al., 2017). This was demonstrated by our previous work (Chapter 5), where we showed older adults had a greater increase in plasma [NO_2^-] and reduction in DBP compared to younger adults following BR consumption (Stanaway et al., 2019). In addition, it has been proposed that multiple day supplementation compared to an acute single dose of NO_3^--rich BR may elicit greater benefits in cardiovascular and cognitive health and performance due to potential accumulating effects and structural changes in the vasculature and skeletal muscle (de Oliveira et al., 2016, Presley et al., 2011, Stanaway et al., 2019, Vanhatalo et al., 2010, Wylie et al., 2016). One study showed a reduction in steady state $\dot{V}O_2$ following 28- or 30-days supplementation with BR (6 mmol NO_3^-) 24 h after the last BR supplement was consumed and when plasma [nitrite] had returned to baseline, thus suggesting potential structural changes beyond an acute increase in plasma [NO_3^-] and [NO_2^-] (Wylie et al., 2016). Aside from the study by Wylie and colleagues (2016), few studies have investigated the independent chronic effects of BR consumption in the absence of an acute dose of dietary NO_3^-. Instead, many studies examining the effects of chronic supplementation with NO_3^--rich BR on cardiovascular and cognitive responses in both younger and older adults have used protocols with the final dose of NO_3^- being consumed 2-5 h prior to testing (Eggebeen et al., 2016, Kelly et al., 2013, Thompson et al., 2015, Vanhatalo et al., 2010). Consuming the final dose of dietary NO_3^- hours before testing incorporates a combined chronic plus acute effect, rather than an independent chronic effect (measurements taken the day following consumption of the last dose) and thus does not adequately isolate the effects of chronic supplementation in the absence of an acute dose and subsequent peak in plasma [NO_3^-] and [NO_2^-] (Wylie et al., 2016). Hence, further research is required to investigate the potential chronic effects of dietary NO_3^- supplementation on cardiovascular and cognitive health and performance.

Therefore, we hypothesised that acute supplementation with nitrate-rich beetroot juice would provide a greater improvement in cardiovascular health, cognition, and exercise performance in older adults (50–80 years) compared to younger adults (18–30 years), and that chronic supplementation would elicit greater improvements in these parameters. To examine this hypothesis we conducted a randomised, double-blind control trial to investigate and compare the effects of acute and chronic (14 and 28 days) supplementation with NO_3^--rich beetroot juice on plasma nitrate and nitrite concentrations, blood pressure, mean arterial pressure, systemic vascular resistance, cognitive performance, and physiological performance (work to completion time trial and $\dot{V}O_2$ response) measures in older and younger adults.

6.2 Materials and Methods

6.2.1. Sample Size

A power analysis was conducted (G-Power 3.1) to calculate the sample size based on the primary outcome measure of blood pressure (Faul et al., 2009). Previous literature in healthy adults has shown an effect size of partial η2 = 0.45 for reduction in blood pressure between nitrate-rich beetroot juice (10.5 mmol nitrate) and control (Stanaway et al., 2019). Based on this, the required sample size was 8 per treatment arm (16 per age group) with a statistical power of 0.82 and α-level set at 0.05.

6.2.2. Participants

Forty-two healthy, recreationally active adults (20 younger, 18–30 y, and 22 older, 50-80 y) volunteered for this study. Participants completed a health-screening questionnaire to ensure suitability. Exclusion criteria included tobacco smokers, users of nitrate-based dietary supplements, or well-trained/elite athletes (defined as those completing >4 training sessions per week, or >6 h of intense exercise per week, or having played/playing for a national or age-equivalent sports team). Those with known diseases (e.g., dementia and cardiovascular disease) were also excluded. Prior to testing, all participants were informed of the procedures, associated risks, and the possible benefits of participation, after which written informed consent was obtained. The study was approved by the Massey University Human Ethics Committee (SOA 17/36) and registered with Australia New Zealand Clinical Trials Registry (ACTRN12620000081910).

6.2.3. Study Design and Procedures

Participants were required to visit the laboratory on five separate occasions. During the first visit, they were familiarised with the testing procedure and equipment, including cognitive, perceptual

and mood tests, and body weight (kg) and height (m) measured using digital scales and a stadiometer, respectively. An incremental cycle test was carried out to determine the work to be completed for the cycle test during the next four visits. Younger adults warmed up at 75 W for 5-min and the test started at 95 W increasing by 35 W every 2-min until voluntary exhaustion. Older adults warmed up at 50 W for 5-min and the test started at 70 W increasing by 25 W every 2-min until voluntary exhaustion. This was used to calculate Wmax (estimated maximal power output) based on calculations by Hodgson et al. (2013):

$$Wmax = Wout+[(t/120) \times 35] \text{ (younger cohort)}$$
$$Wmax = Wout+[(t/120) \times 25] \text{ (older cohort)}$$

Where, Wout is the final power output that the participant achieved during the incremental cycle test, while t (s) is the time spent at the final power output before fatigue. Participants were instructed to complete a food diary for two days prior to the second study visit and to replicate their diet and lifestyle factors for all subsequent study visits. Dietary nitrate was not restricted to investigate additional benefits of BR in conjunction with normal daily intake; however, the food diary was used to evaluate intake of dietary nitrate in relation to potential abnormalities in baseline plasma nitrate and nitrite concentrations. Participants were asked to arrive at the laboratory in a well-rested, fasted state and to refrain from excessive exercise (anything more than a walk or daily activities like gardening or work) and alcohol consumption 24 h prior and caffeine 12 h prior to each study visit. All trials were performed in the morning at approximately the same time of day (±2 h).

The second visit was the baseline cycle trial where participants arrived in the lab having eaten breakfast. Baseline measures of blood pressure (mean value of three measurements taken from the left arm; deluxe HEM-7130; OMRON Healthcare CO. Ltd.; Kyoto, Japan), heart rate (A1 polar chest transducer; Polar Electro Oy, Kemple, Finland), systemic vascular resistance and mean arterial pressure (USCOM 1A; USCOM Ltd, Sydney, Australia), and cognitive function were completed. This was followed by the exercise test which was a work done time trial (completion of a predetermined amount of work as fast as possible) on a cycle ergometer (Velotron DynaFit Pro Racer-Mate, Seattle, WA). Work to completion was calculated based on each individual's estimated total work that could be completed during a 20-min (1200 s) cycle working at 75% of their Wmax (Jeukendrup et al., 1996):

$$\text{Total Work to completion (J)} = Wmax \times 0.75 \times 1200.$$

Wmax was established during the familiarisation trial. During the cycle test, expired air was sampled throughout the cycle, in 10-s intervals, using an online gas analysis system using a mixing chamber (COSMED, Quark CPET, Rome, Italy). Mean heart rate was recorded at 0, 20, 40, 60, 80, and 100%

of work completed. Following the completion of the cycle test, baseline measures of blood pressure and heart rate were repeated.

Visits 3-5 were the supplementation trials, with each participant randomly allocated (using a random number generator) to either 250 mL of beetroot juice (BR; 10.5 mmol / 640 mg NO_3^-) or 250 mL of a placebo (PL; 0.15 mmol / 9 mg NO_3^-) in a double-blind, randomised control trial design. Participants were asked to consume the drink daily (for 28 days) in the morning with breakfast. For the three supplementation visits (days 0, 14 and 28), participants were provided the drink in the lab. A 250-mL beverage was used to deliver 10.5 mmol of NO_3^- as this amount had previously been shown to be a sufficient dose to elicit beneficial effects on cardiovascular and cognitive measures (Stanaway et al., 2019). Drinks were consumed with a standardised, isocaloric breakfast (cereal or toast) with the same macronutrient content (15 g protein, 30 g carbohydrates and 10 g fat). Participants consumed the same breakfast type (cereal or toast) for all supplementation trials.

The protocol for visits 3-5 is illustrated in Figure 6.1. Prior to consumption of the beverage, baseline measures for blood pressure, heart rate, systemic vascular resistance and mean arterial pressure, and cognitive function tests were completed, followed by collection of a resting blood sample. Participants then consumed the allocated drink with breakfast, within a 10-min period. Participants were asked to remain in the laboratory for a 2.25 h absorption period while a home development show was aired for entertainment purposes (has minimal effect on cognitive stimulation or arousal levels (Kelly et al., 2013)), or participants could complete computer work or study. The 2.25-h absorption period was chosen as peak plasma nitrite concentrations occur 2–3 h after beetroot juice consumption (Wylie et al., 2013). After 2.25 h, the measurements were repeated prior to commencing the exercise protocol, approximately 2.5 h after beverage consumption. The exercise test was completed as outlined above, and following completion, baseline measures of blood pressure, heart rate, and blood sample were repeated. Effects from acute supplementation were measured pre- (baseline) versus post-supplementation (2.25 h) with NO_3^--rich beetroot juice on day 0; effects from chronic supplementation were measured from baseline on day 0 and pre-supplementation on days 14 and 28; effects from acute + chronic supplementation were measured pre- versus post-supplementation on days 14 and 28 following 14 and 28 days of daily supplementation; and effects from chronic + acute supplementation (physiological performance measures) were measured from baseline on day 0 versus post-supplementation on days 14 and 28 following 14 and 28 days of daily supplementation.

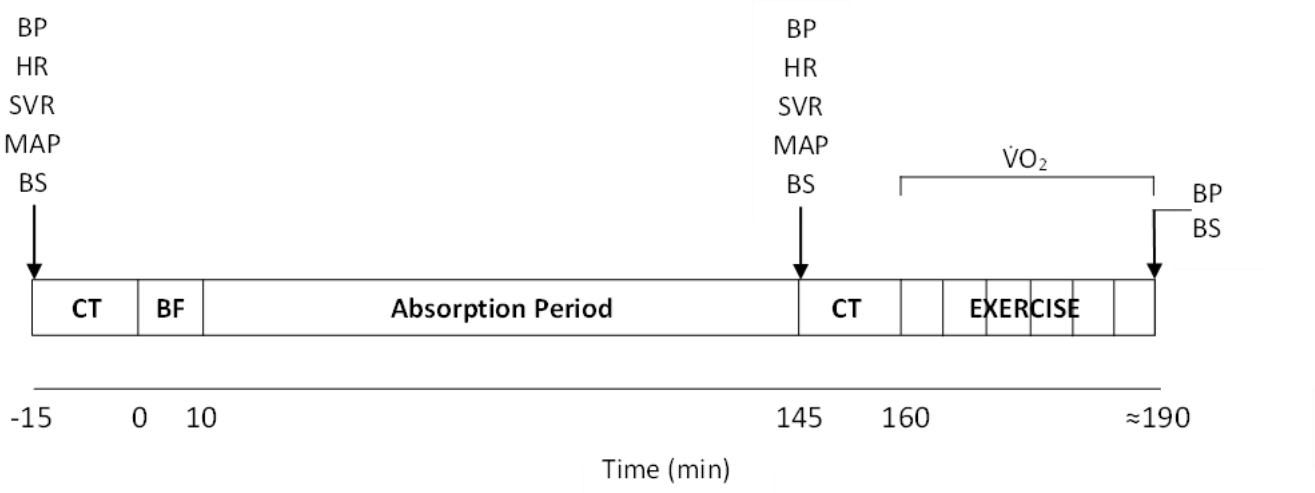

Figure 6.1. Schematic of the chronic study protocol. CT, cognitive tests; BF, breakfast; BP, blood pressure; BS, blood sample; HR, heart rate; MAP, mean arterial pressure; SVR, systemic vascular resistance; $\dot{V}O_2$, oxygen uptake.

6.2.4. Intervention

Beetroot juice extracted from fresh, washed beetroot *(Beta vulgaris*) was blended with other fruit juices to provide a standardised beetroot juice drink with a constant soluble solid concentration (11° Brix) and nitrate concentration (10.5 mmol/250 mL). The placebo beetroot drink was produced using beetroot juice concentrate and standardised to the same constant soluble solids concentration (11° Brix), but contained a low nitrate concentration (0.15 mmol/250 mL). The BR and placebo juice drinks had previously been presented to a consumer sensory panel using a triangle test (with 25 participants) and were not perceived as significantly different from each other ($p > 0.05$). The nitrate concentration was analysed using high-performance liquid chromatography (HPLC) based on the method of Cheng and Tsang (1998). Briefly, isocratic separation was achieved using a Gracesmart C-18 column; mobile phase 0.01 M Octylammonium orthophosphate (pH 3–3.5) at a flow rate of 0.8 mL/min. Detection was carried out at 193 nm for nitrite and 213 nm for nitrate.

6.2.5. Blood Measurements

Plasma nitrite and nitrate were used as biomarkers for nitric oxide availability (Kleinbongard et al., 2003). Six milliliter venous blood samples were taken by venepuncture, from a vein within the antecubital area, and collected into heparinised tubes. Samples were mixed, centrifuged (MF–50 Hanil Science Industrial, Incheon, South Korea) at 3500 rpm (1330 g) for 10 min, and the collected plasma aliquoted into Eppendorf tubes (0.5 mL per tube) and stored at −80 °C for later analysis of nitrite and nitrate concentrations by HPLC. After filtration, nitrite (Li et al., 2000) and nitrate (Cheng and Tsang 1998) were analysed using established methods.

6.2.6. USCOM Hemodynamics

Mean arterial pressure (via calculation) and systemic vascular resistance were measured using ultrasound (USCOM-1A). A 3.3-MHz transducer was placed on the suprasternal notch and Doppler waves provide a signal of the flow at the aortic valve. The signal was modified by the operator by moving the transducer until the clearest Doppler curve was obtained. Systemic vascular resistance is calculated using the equation SVR = MAP / cardiac output (CO) × 80, where MAP is calculated from SBP and DBP, and CO is measured by the USCOM 1A by determining heart rate and stroke volume (Cattermole et al., 2017).

6.2.7. Cognitive Measurements

Cognitive tests were completed in a quiet, isolated room using a computer placed at eye level (PsychoPy software, version 1.83.04 (Peirce, 2007) and PsyToolkit software, version 2.4.1 (Stoet, 2010, Stoet, 2017)). Based on previous studies (Ong et al., 2022, Thompson et al., 2015 Wightman et al., 2015) examining the effects of nutritional supplementation on executive function, attention, and information processing speed; this study included the Corsi block tapering test (CBT), rapid visual information processing (RVIP), and Stroop tests. Each task was performed a total of seven times per participant (once in visit 2; prior to the cycle and twice each for visits 3-5; pre- and 2.5 h post-supplementation).

6.2.7.1. Corsi Block Tapering (CBT)

The CBT task involves the display of nine blocks on the screen. The blocks light up in random order and the participant needs to remember and click which blocks lit up and the order they lit up in. The sequence starts at two blocks and goes up to nine. If the participant gets the sequence wrong, another attempt is allowed on that level. When the participant gets the same level wrong twice that is their final Corsi block span.

6.2.7.2. Rapid Visual Information Processing (RVIP)

The RVIP test involves participants following a chain of single numbers from 1 to 9, which appear in a pseudo-random order at the rate of 100 per minute. The aim of the test is to correctly identify target sequences (2–4–6, 4–6–8, and 3–5–7) as quickly as possible.

6.2.7.3. Stroop Test

The Stroop test involves the display of different colour names (‘RED’, ‘GREEN’, and ‘BLUE’) which appear on the screen, one at a time in different-coloured fonts (red, green, or blue). The aim of this

test is to press the key corresponding to the colour of the font that the present word was displayed in.

6.2.8. Statistical Analysis

Statistical analyses were completed using IBM SPSS (Version 27.0). One-way analysis of variance (ANOVA) was used to examine physical and baseline characteristics. All other data were analysed using two-way (treatment × age) analysis of variance (ANOVA) with a between subject's design. Sphericity was tested using the Mauchly's test to ensure the assumption of sphericity was not violated, and multivariate models were applied if these assumptions were not met. Where significant differences were found, post-hoc tests with Holm-Bonferroni correction were undertaken to assess multiple comparisons. Relative effect sizes were calculated using partial eta squared and defined as small (η^2_p = 0.01), medium (η^2_p = 0.06), or large (η^2_p = 0.14). Data are presented as mean ± standard deviation. Statistical significance was set at $p < 0.05$.

6.3 Results

6.3.1. Participants

The physical characteristics and baseline BP measurements for the younger and older group participants in both the BR and PL arms are reported in Table 6.1. Any missing data were assessed using Little's MCAR test to determine whether or not data were missing at random. With data shown to be missing randomly, expectation maximisation imputation was used to compute values for the missing data points. Data that is missing completely at random will show a non-significant result in Little's MCAR test, and expectation maximisation imputation can then be conducted to impute missing values in a set of data (Molenberghs and Verbeke, 2005). Expectation maximisation imputation is a technique used to calculate the maximum likelihood estimates from a data set with missing values (Ghomrawi et al., 2011).

Table 6.1. Physical characteristics and baseline blood pressure measurements of the younger and older adult participants in the chronic study.

	Younger BR (*n* = 12)	**Younger PL (*n* = 8)**	**Older BR (*n* = 10)**	**Older PL (*n* = 12)**
Male	5	4	4	4
Female	7	4	6	8
Age (y)	24 ± 3	23 ± 3	70 ± 4	61 ± 9
Height (cm)	177.1 ± 8.4	172.8 ± 8.7	158.3 ± 27.5	161.3 ± 23.3
Body mass (kg)	72.8 ± 10.8	70.5 ± 13.5	81.3 ± 34.1	75.8 ± 35.2
SBP (mmHg)	111.8 ± 11.2	114.0 ± 7.9	139.1 ± 23.1	124.8 ± 18.2
DBP (mmHg)	68.9 ± 7.5	69.9 ± 8.1	85.0 ± 13.3	79.7 ± 12.2
SVR (d.s.cm^{-5})	1239.0 ± 367.0	1316.5 ± 409.1	2232.8 ± 868.2	2064.3 ± 798.0

Values are means ± SD. BR, beetroot juice; PL, placebo; SBP, systolic blood pressure; DBP, diastolic blood pressure; SVR, systemic vascular resistance.

6.3.2. Plasma Nitrite and Nitrate

Due to COVID-19, access to the biochemical laboratory was restricted for processing of the plasma NO_3^- and NO_2^-. Plasma NO_3^- and NO_2^- analysis will be completed post-submission of this PhD study and added to the manuscript prior to submission for publication.

6.3.3. Effects of Acute Supplementation with BR on Blood Pressure and Hemodynamics

Systolic and diastolic BP were reduced on day 0 following acute consumption of BR (versus PL) for older (p = <0.001, η^2_p = 0.47 and 0.40, respectively; Figures 6.2A and B), but not younger adults (p = 0.61 and 0.54, η^2_p = 0.007 and 0.01, respectively). Older adults also showed a greater reduction in SBP (p = 0.005, η^2_p = 0.19, Figure 6.2A) and DBP (p = 0.005, η^2_p = 0.19, Figure 6.2B) following supplementation with BR when compared to younger adults. Similarly, MAP was reduced post-supplementation with BR compared to PL on day 0 in older (p = <0.001, η^2_p = 0.40, Figures 6.2C) but not younger (p = 0.46, η^2_p = 0.015) adults. There was also a greater reduction in MAP in older compared to younger adults following BR supplementation (p = 0.006, η^2_p = 0.19, Figure 6.2C). There was no main effect of treatment (BR versus PL) (p = 0.40, η^2_p = 0.018, Figure 6.2D) or interaction of treatment*age (p = 0.48, η^2_p = 0.013, Figure 6.2D) on SVR between groups on day 0.

6.3.4. Effects of Acute + Chronic Supplementation with BR on Blood Pressure and Hemodynamics

On day 14 there was no effect of treatment on SBP, DBP, or MAP in younger adults (p > 0.05), however, there were trends detected for a reduction in SBP (p = 0.081, η^2_p = 0.078, Figure 6.2A), DBP (p = 0.098, η^2_p = 0.07, Figure 6.2B), and MAP (p = 0.088, η^2_p = 0.075, Figure 6.2C) in older adults

following BR consumption compared to PL. There were no interaction effects (treatment*age) for SBP ($p = 0.66$, $\eta^2_p = 0.005$), DBP ($p = 0.72$, $\eta^2_p = 0.003$), or MAP ($p = 0.67$, $\eta^2_p = 0.005$) between groups. Systemic vascular resistance was reduced following acute + chronic consumption of BR for older adults when compared to placebo ($p = 0.032$, $\eta^2_p = 0.12$, Figure 6.2D) however, this was not seen in younger adults ($p = 0.20$, $\eta^2_p = 0.04$, Figure 6.2D). There was also a greater reduction in SVR in older compared to younger adults, post-supplementation with BR ($p = 0.015$, $\eta^2_p = 0.15$, Figure 6.2D).

On day 28, following supplementation with BR compared to PL, both younger and older adults showed an acute + chronic reduction in SBP ($p = 0.041$ and 0.005, $\eta^2_p = 0.11$ and 0.19, respectively; Figure 6.2A), DBP ($p = 0.031$ and 0.008, $\eta^2_p = 0.12$ and 0.17, respectively; Figure 6.2B), and MAP ($p = 0.019$ and 0.003, $\eta^2_p = 0.14$ and 0.21, respectively; Figure 6.2C). However, there were no interaction effects (treatment*age) for SBP ($p = 0.63$, $\eta^2_p = 0.006$), DBP ($p = 0.78$, $\eta^2_p = 0.002$), or MAP ($p = 0.71$, $\eta^2_p = 0.004$). Acute + chronic consumption of BR reduced SVR on day 28 in older ($p = 0.016$, $\eta^2_p = 0.14$, Figure 6.2D) but not younger ($p = 0.61$, $\eta^2_p = 0.007$, Figure 6.2D) adults. There was also a trend for a greater reduction in SVR in older compared to younger adults, post-consumption of BR versus PL ($p = 0.056$, $\eta^2_p = 0.093$, Figure 6. 2D).

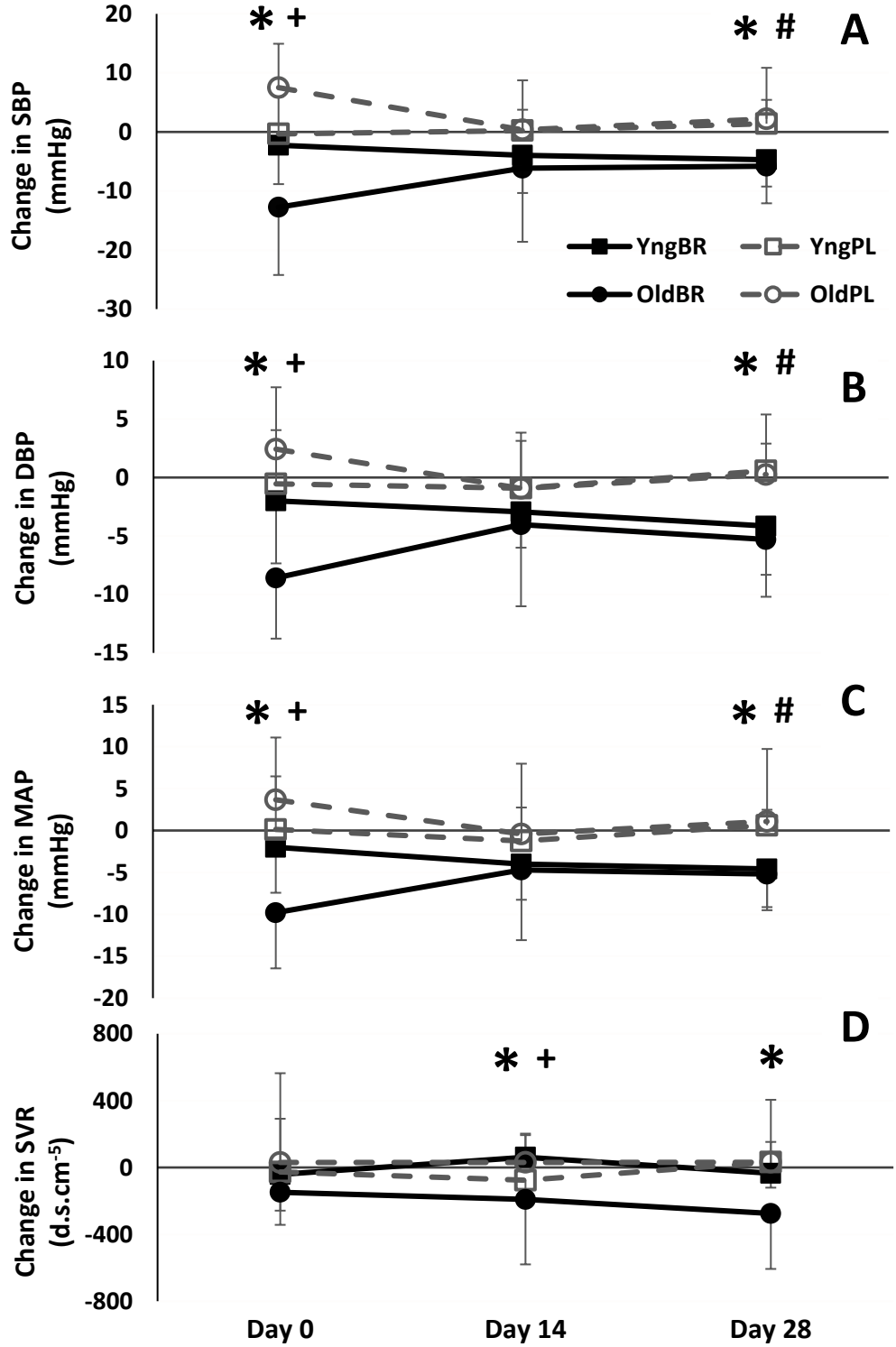

Figure 6.2. Acute changes in blood pressure and hemodynamic response in the context of the chronic study. (A), Acute and acute plus chronic changes in *s*ystolic blood pressure (SBP) (mmHg) pre- versus post-supplementation with beetroot juice (BR) and placebo (PL) in younger (Yng) and older (Old) adults, days 0, 14, and 28. **(B)**, Acute and acute plus chronic changes in diastolic blood pressure (DBP) (mmHg) pre- versus post-supplementation with beetroot juice (BR) and placebo (PL) in younger (Yng) and older (Old) adults, days 0, 14, and 28 **(C)**, Acute and acute plus chronic changes in mean arterial pressure (MAP) (mmHg) pre- versus post-supplementation with beetroot juice (BR) and placebo (PL) in younger (Yng) and older (Old) adults, days 0, 14, and 28. **(D)**, Acute and acute plus chronic changes in systemic vascular resistance (SVR) (d.s.cm^{-5}) with beetroot juice (BR) versus placebo (PL) in younger (Yng) and older (Old) adults, days 0, 14, and 28.

Values are expressed as means ± SD.

*Statistically significant mean difference from PL for older adults ($p < 0.05$). $^{\#}$Statistically significant mean difference from PL for younger adults ($p < 0.05$). $^{+}$Statistically significant mean difference between older and younger adults following BR versus PL supplementation ($p < 0.05$).

6.3.5. Effects of Chronic Supplementation with BR on Blood Pressure and Hemodynamics

Consumption of BR reduced SBP, DBP, and MAP from pre-supplementation on day 0 to pre-supplementation on day 14 in older (p = 0.019, 0.004, and 0.005, η^2_p = 0.14, 0.20, and 0.19, respectively; Figures 6.3A, B, and C) but not younger (p = 0.24, 0.91, and 0.85 η^2_p = 0.036, <0.001, and 0.001, respectively; Figures 6.3A, B, and C) adults. From day 0 to 14 older adults had a greater reduction in SBP (p = 0.018, η^2_p = 0.14, Figure 6.3A), DBP (p = 0.002, η^2_p = 0.22, Figure 6.3B), and MAP (p = 0.001, η^2_p = 0.25, Figure 6.3C) following chronic supplementation with BR when compared to younger adults. There was no effect of treatment (p = 0.20, η^2_p = 0.043, Figure 6.3D) or interaction of treatment*age (p = 0.78, η^2_p = 0.02, Figure 6.3D) on SVR from days 0 to 14.

There were no chronic treatment effects from day 0 to 28 for SBP (p = 0.36, η^2_p = 0.022, Figure 6.3A), DBP (p = 0.33, η^2_p = 0.025, Figure 6.3B), MAP (p = 0.29, η^2_p = 0.03, Figure 6.3C), or SVR (p = 0.73, η^2_p = 0.003, Figure 6.3D). There were also no interaction effects of treatment*age for SBP (p = 0.37, η^2_p = 0.021, Figure 6.3A), DBP (p = 0.59, η^2_p = 0.008, Figure 6.3B), MAP (p = 0.61, η^2_p = 0.007, Figure 6.3C), or SVR (p = 0.98, η^2_p = <0.001, Figure 6.3D) from day 0 to 28 of supplementation.

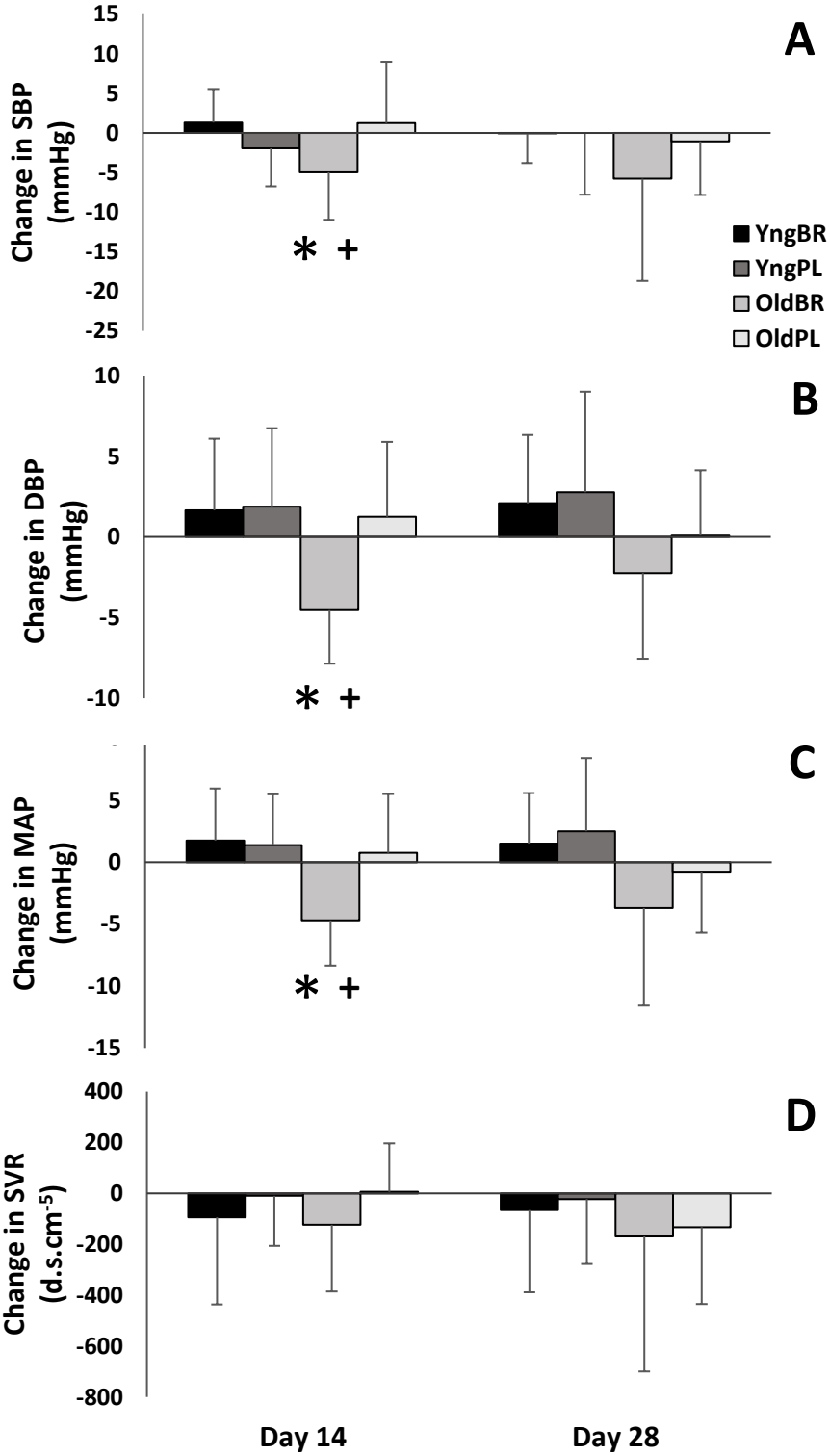

Figure 6.3. Chronic changes in blood pressure and hemodynamic response in the chronic study. (A), Chronic change in *s*ystolic blood pressure (SBP) (mmHg) from baseline to day 14 and 28 following supplementation with beetroot juice (BR) and placebo (PL) in younger (Yng) and older (Old) adults. **(B)**, Chronic change in diastolic blood pressure (DBP) (mmHg) from baseline to day 14 and 28 following supplementation with beetroot juice (BR) and placebo (PL) in younger (Yng) and older (Old) adults. **(C)**, Chronic change in mean arterial pressure (MAP) (mmHg) from baseline to day 14 and 28 following supplementation with beetroot juice (BR) and placebo (PL) in younger (Yng) and older (Old) adults. **(D)**, Chronic change in systemic vascular resistance (SVR) (d.s.cm^{-5}) from baseline to day 14 and 28 following supplementation with beetroot juice (BR) and placebo (PL) in younger (Yng) and older (Old) adults.

Values are expressed as means ± SD.

*Statistically significant mean difference from PL for older adults ($p < 0.05$). $^{+}$ Statistically significant mean difference between older and younger adults following BR versus PL supplementation ($p < 0.05$).

6.3.6. Effects of Acute and Acute + Chronic Supplementation with BR on Cognitive Performance

On day 0, beetroot juice consumption improved reaction time during the Stroop test following acute supplementation in older (p = 0.042, η^2_p = 0.10, Table 6.2) but not younger (p = 0.48, η^2_p = 0.013) adults. Older compared to younger adults also had a greater improvement in reaction time during the Stroop test following BR consumption on day 0 (p = 0.012, η^2_p = 0.16, Table 6.2). However, following acute + chronic supplementation with BR on day 14 there was no main effect of treatment (p = 0.41, η^2_p = 0.018, Table 6.2) or interaction effect of treatment*age (p = 0.14, η^2_p = 0.056, Table 6.2) for reaction time during the Stroop test. On day 28 acute + chronic supplementation with BR improved reaction time during the Stroop test in older adults (p = 0.006, η^2_p = 0.18, Table 6.2) and showed a trend for improved reaction time in younger adults (p = 0.066, η^2_p = 0.086). Older adults also had a greater improvement in reaction time compared to younger adults following BR consumption on day 28 (p = 0.032, η^2_p = 0.12, Table 6.2). There were no acute or acute + chronic effects of treatment or interaction effects of treatment*age for percentage correct responses in the Stoop test, Corsi span during the CBT, or percentage correct responses, percentage of errors, and reaction time during the RVIP test on days 0, 14, or 28 (p > 0.05, Table 6.2).

Table 6.2. Changes in cognitive performance following acute supplementation with beetroot juice versus placebo and younger compared to older adults in the context of a 28-day chronic trial.

	Younger Beetroot			Younger Placebo			Older Beetroot			Older Placebo		
	Day 0	**Day 14**	**Day 28**	**Day 0**	**Day 14**	**Day 28**	**Day 0**	**Day 14**	**Day 28**	**Day 0**	**Day 14**	**Day 28**
Corsi Block Tapering Test												
Δ Corsi Block Span	-0.33 ± 1.3	-0.25 ± 1.5	0.17 ± 0.84	-0.50 ± 1.2	0.50 ± 0.93	-0.50 ± 1.2	0.50 ± 0.97	-0.20 ± 1.8	1.3 ± 2.4	0.00 ± 3.1	-0.50 ± 1.2	0.42 ± 1.1
Rapid Visual Image Processing												
Δ Correct Response (%)	3.1 ± 9.6	1.1 ± 6.6	-0.63 ± 15	10 ± 10	0.38 ± 7.9	2.8 ± 6.8	1.6 ± 16	3.8 ± 8.6	3.8 ± 9.4	0.52 ± 9.0	0.78 ± 7.8	3.1 ± 4.4
Δ Errors	0.42 ± 1.8	-0.42 ± 1.4	-0.42 ± 1.2	0.00 ± 0.93	0.38 ± 1.4	0.25 ± 0.89	-1.2 ± 2.8	-0.20 ± 2.4	-1.2 ± 3.3	-0.33 ± 3.3	0.00 ± 2.0	-0.42 ± 2.0
Δ Reaction Time (ms)	-4.6 ± 40	11 ± 44	-20 ± 25	-4.4 ± 23	-5.8 ± 18	-11 ± 32	-8.5 ± 62	-29 ± 95	-11 ± 32	-17 ± 51	-11 ± 41	-0.39 ± 38
Stroop Test												
Δ Correct Response (%)	-1.1 ± 1.6	0.06 ± 3.2	-0.17 ± 2.3	0.03 ± 2.8	-0.84 ± 1.5	-1.6 ± 2.0	0.00 ± 2.0	-0.72 ± 1.5	0.14 ± 1.4	-0.37 ± 1.9	0.37 ± 1.7	-0.06 ± 1.1
Δ Reaction Time (ms)	-14 ± 40	-5.4 ± 81	-31 ± 43	-36 ± 58	-56 ± 68	-8.5 ± 12	-91 ± 112*+	-50 ± 79	-75 ± 77*+	-30 ± 45	-35 ± 47	-17 ± 23

Values are expressed as means ± SD.

* Statistically significant mean difference from PL for older adults ($p < 0.05$).

+ Statistically significant mean difference between older and younger adults following BR versus PL supplementation ($p < 0.05$).

6.3.7. Effects of Chronic Supplementation with BR on Cognitive Performance

There were no chronic effects of treatment for Corsi span during the CBT; percentage correct responses, percentage of errors, and reaction time during the RVIP test; or percentage correct responses and reaction time during the Stroop test at day 14 or 28 ($p > 0.05$, Table 6.3). Older compared to younger adults showed a greater improvement in reaction time during the Stroop test following 14 ($p = 0.016$, $\eta^2_p = 0.14$, Table 6.3) and 28 ($p = 0.020$, $\eta^2_p = 0.13$, Table 6.3) days' chronic supplementation with BR. Similarly, there was a trend for a greater decrease in reaction time during the RVIP test in older compared to younger adults at day 28 compared to baseline ($p = 0.072$, $\eta^2_p = 0.13$, Table 6.3). There were no other interaction effects of treatment*age for Corsi span during the CBT; percentage correct responses, percentage of errors, and reaction time during the RVIP test; or percentage correct responses during the Stroop test following 14 or 28 days chronic supplementation ($p > 0.05$, Table 6.3).

Table 6.3. Changes in cognitive performance following chronic supplementation for 14 and 28 days with beetroot juice versus placebo in younger compared to older adults.

	Younger Beetroot		Younger Placebo		Older Beetroot		Older Placebo	
	Day 14	**Day 28**	**Day 14**	**Day 28**	**Day 14**	**Day 28**	**Day 14**	**Day 28**
Corsi Block Tapering Test								
Δ Corsi Block Span	0.50 ± 1.1	0.33 ± 1.3	-0.38 ± 1.5	0.38 ± 1.1	-0.50 ± 1.2	-0.50 ± 1.3	0.50 ± 2.8	0.50 ± 2.2
Rapid Visual Image Processing								
Δ Correct Response (%)	4.4 ± 8.7	4.4 ± 8.9	5.5 ± 10	4.7 ± 10	7.5 ± 14	13 ± 12	7.5 ± 14	13 ± 13
Δ Errors	0.67 ± 2.0	-0.42 ± 1.2	-0.38 ± 1.3	0.25 ± 0.89	-2.8 ± 6.9	-1.2 ± 3.3	-0.33 ± 1.9	-0.42 ± 2.0
Δ Reaction Time (ms)	-2.1 ± 42	9.9 ± 27	-4.1 ± 28	2.5 ± 41	-31 ± 117	-43 ± 108	-6.3 ± 46	-19 ± 63
Stroop Test								
Δ Correct Response (%)	-1.1 ± 1.9	-1.3 ± 1.5	-0.64 ± 3.0	0.11 ± 2.9	0.29 ± 1.4	0.16 ± 1.3	-0.008 ± 2.6	0.23 ± 3.0
Δ Reaction Time (ms)	-5.0 ± 48	-12 ± 65	19 ± 77	-53 ± 68	-88 ± 142[+]	-112 ± 148[+]	-45 ± 57	-72 ± 82

Values are expressed as means ± SD.

[+] Statistically significant mean difference between older and younger adults following BR versus PL supplementation ($p < 0.05$).

6.3.8. Effects of Supplementation with BR on Exercise Performance and HR

There was no acute (on day 0) or chronic + acute (on day 14) main effect of treatment (p = 0.64 and 0.37, η^2_p = 0.006 and 0.021, respectively) or interaction of treatment*age (p = 0.74 and 0.29, η^2_p = 0.003 and 0.029, respectively) for work to completion time trial performance. On day 28, supplementation with PL compared to BR improved time trial performance in older (p = 0.007, η^2_p = 0.17) but not younger (p = 0.29, η^2_p = 0.029) adults. A trend for a greater improvement in time trial performance in older compared to younger adults following chronic + acute consumption of the PL treatment (p = 0.052, η^2_p = 0.096) was also observed on day 28. On days 0, 14, and 28 there were no significant differences between BR and PL supplementation for change in $\dot{V}O_2$ at the start, 20%, 40%, 60%, 80%, or 100% of work completion during the cycle time trial in younger or older adults (p > 0.05 , Figure 6.4A, B, and C), however, there was a trend for reduction in $\dot{V}O_2$ in older adults following chronic + acute supplementation with BR compared to PL on day 14 at 80% completion of the time trial (p = 0.087, η^2_p = 0.092, Figure 6.4B). Older adults showed a greater improvement in $\dot{V}O_2$ following acute supplementation with BR at 20% completion of the time trial on day 0 (p = 0.044, η^2_p = 0.11, Figure 6.4A). There were no other interaction effects of treatment*age at any other percentage of completion during the cycle trial on days 0, 14, or 28. There were no acute, acute + chronic, chronic + acute or chronic effects of treatment or interaction effects of treatment*age on average heart rate across any time points at rest or during exercise (p > 0.05).

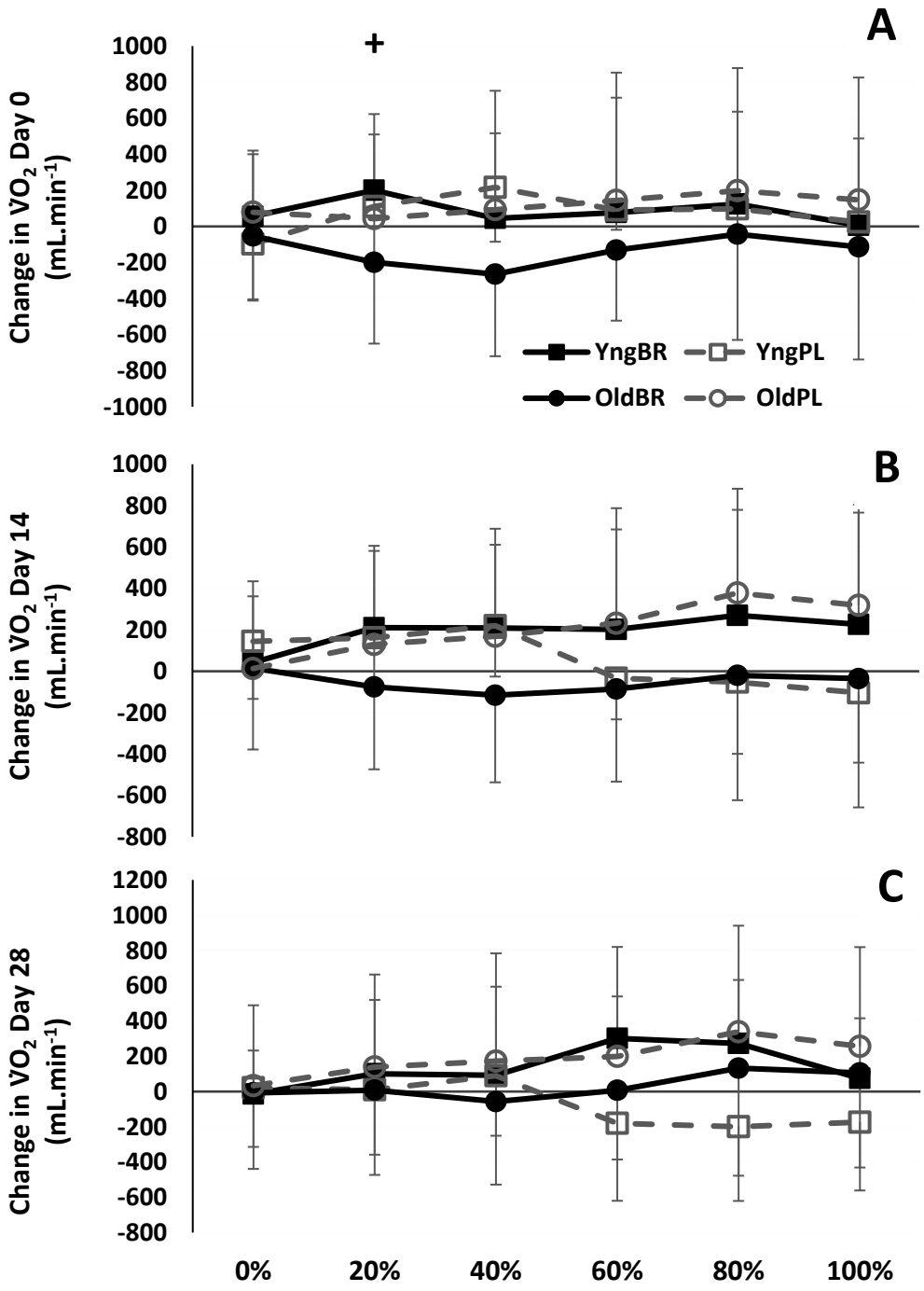

Figure 6.4. Changes in oxygen uptake in the chronic study. (A), Acute change in oxygen uptake ($\dot{V}O_2$) (mL/min) at each percentage completion of the cycle time trial pre- versus post-supplementation with beetroot juice (BR) and placebo (PL) in younger (Yng) and older (Old) adults on day 0. **(B)**, Chronic plus acute change in oxygen uptake ($\dot{V}O_2$) (mL/min) at each percentage completion of the cycle time trial from baseline day 0 to post-supplementation with beetroot juice (BR) and placebo (PL) in younger (Yng) and older (Old) adults on day 14. **(C)**, Chronic plus acute change in oxygen uptake ($\dot{V}O_2$) (mL/min) at each percentage completion of the cycle time trial from baseline day 0 to post-supplementation with beetroot juice (BR) and placebo (PL) in younger (Yng) and older (Old) adults on day 28.

Values are expressed as means ± SD.

[+] Statistically significant mean difference between older and younger adults following BR versus PL supplementation ($p < 0.05$).

6.4 Discussion

We examined the acute and chronic effects of supplementation with NO_3^--rich BR on cardiovascular responses and cognition in younger compared to older adults over 28 days. The main findings of this study demonstrated that consumption of dietary NO_3^- (1) significantly reduced SBP, DBP, and MAP following acute supplementation in older adults on day 0, and both older and younger adults following acute + chronic supplementation on day 28, with greater reductions seen in older compared to younger adults on day 0; (2) reduced SVR in older adults following acute + chronic supplementation on days 14 and 28, with a greater reduction seen in older compared to younger adults on day 14; (3) improved reaction time during the Stroop test in older adults following acute supplementation on days 0 and acute + chronic supplementation on day 28, while also showing a larger reduction in reaction time in older versus younger adults on both days; (4) significantly reduced SBP, DBP, and MAP following 14 days chronic supplementation in older adults with a larger reduction in older compared to younger adults.

6.4.1. Changes in Blood Pressure and Hemodynamics Following Acute Supplementation

Acute supplementation with BR on day 0 reduced SBP, DBP, and MAP in older adults. This reduction in BP was likely due to increases in plasma NO_2^-, and subsequent increases in NO bioavailability and cyclic guanosine monophosphate (cGMP) concentrations (a potent vasodilator which relaxes smooth muscle) which is produced from guanosine triphosphate (GTP) by the enzyme guanylate cyclase (Bailey et al., 2010, Kapil et al., 2015). However, this same increase in NO and cGMP would be expected in younger adults, hence a significant drop in SBP, DBP, and MAP post-supplementation with BR could also be expected in this group, yet this was not the case at day 0. This was unexpected as previous studies have shown a significant decrease in SBP following acute dietary NO_3^- supplementation in younger adults (Hughes et al., 2016, Thompson et al., 2014, Wylie et al., 2013), while reductions in DBP and MAP are not as definitive, with previous literature indicating no improvement following acute BR consumption (Lansley et al., 2011a, Stanaway et al., 2019). The lack of a significant change in SBP seen in younger adults may be due to the lower baseline SBP and DBP seen in the younger compared to older cohort (Table 6.1). Anti-hypertension medication is known to promote greater reductions in BP as baseline BP measures increase (Law et al., 2009). Therefore, assuming this was translatable to NO_3^- supplementation, the effects of increased NO from BR consumption on the vasculature may not be as strong or may be limited when resting BP is lower to prevent inducing hypotension. It is normal for older adults to have a higher resting BP because of aging; thus, older adults may experience greater benefits from dietary NO_3^- supplementation (Sindler et al., 2011).

Older adults had a greater reduction in SBP, DBP, and MAP compared to younger adults following BR consumption on day 0. We have previously shown similar results with a greater reduction in DBP in older compared to younger adults following acute supplementation with dietary NO_3^- (10.5 mmol) (Stanaway et al., 2019). The reason for this larger reduction in BP is likely due to the variation in NO bioavailability between older and younger adults, whereby older adults are suggested to have lower bioavailability and subsequently diminished endothelial and vascular function due to aging (Lara et al., 2016), thus may gain greater benefit from BR consumption compared to younger adults with normal NO production (Kelly et al., 2013, Sindler et al., 2011). Importantly, age and high BP are both major risk factors for CVD and endothelial dysfunction, therefore, supplementation with dietary NO_3^- may help promote normal NO homeostasis and reduce the risk of vascular dysfunction and disease in older adults (Sindler et al., 2011, Stanaway et al., 2019). In contrast, two studies comparing acute supplementation with BR (9.4 mmol and 6.4 mmol) in younger compared to older adults found no difference in change in SBP or DBP between age groups (Hughes et al., 2016, Rogerson et al., 2022). However, Hughes et al. (2016) did not use a placebo-control, crossover design and Rogerson et al. (2022) had unequal sample sizes with a considerably lower numbers of older adults; both these factors could have impacted the results observed in the respective studies.

6.4.2. Changes in Blood Pressure and Hemodynamics Following Acute + Chronic Supplementation

Following acute + chronic supplementation with NO_3^--rich BR on day 14 there was no change in SBP, DBP, or MAP for younger or older adults and no interaction effect of age and treatment. Considering the findings on day 0, this lack of effect was unexpected for older adults and may be attributable to the changes in pre-supplementation BP at day 14, as due to the chronic effects of supplementation with BR (discussed in detail below) pre-supplementation BP was significantly lower on day 14 compared to day 0. This could have resulted in a smaller effect from acute supplementation on day 14 due to the already present chronic effect of lowered BP and may also explain the lack of difference between the age groups. Nonetheless, a trend for a decrease in SBP (p = 0.081), DBP (p = 0.098) and MAP (p = 0.088) following acute + chronic consumption of BR on day 14 was seen for older adults, indicating the presence of potential acute effects in addition to chronic. Additionally, the lack of change in BP for younger adults could be due to their lower pre-supplementation BP as mentioned above. A previous study also showed no reduction in SBP or DBP for younger adults after 7 days' supplementation with BR (9.7 mmol·day^{-1} NO_3^-) followed by an acute dose 2.25 h prior to the final BP measurement (Haider and Folland, 2014). Although another study in younger males showed a reduction in SBP, DBP, and MAP following 15 days' supplementation with dietary NO_3^- (5.2 mmol·day^{-1}) with the final dose 2.5 -5 h prior to testing on day 15 (Vanhatalo et al., 2010), they used

a low-calorie cordial drink as the PL which could have impacted the results. However, it is important to note that these authors (Haider and Folland, 2014; Vanhatalo et al., 2010) compared changes in BP between baseline measures on day 1 and following an acute dose on days 7 and 15, respectively. This methodology equates to chronic + acute supplementation, as opposed to acute + chronic which was investigated in the current study. Therefore, further research is needed to elaborate on the effects of acute supplementation with dietary NO_3^- on BP in younger and older adults in combination with daily supplementation.

Similar to day 0, there was a reduction in SBP, DBP, and MAP for older adults on day 28 of supplementation from pre to 2.25 h after the final dose of BR (acute + chronic supplementation). This was likely due to the increases in NO production and subsequent activation of guanylate cyclase (Bailey et al., 2010) and possible accumulating chronic effects. There was also a decrease in SBP, DBP, and MAP in younger adults following acute + chronic supplementation with dietary NO_3^- on day 28, despite no improvements seen on days 0 and 14. Chronic supplementation may have led to structural changes of the vasculature (reduced arterial stiffness) and/or changes in the microbiome (potential increases in concentrations of NO_3^- reducing species which may have resulted in a greater percentage of NO_3^- conversion to NO_2^- thus promoting greater increases in plasma [NO_3^-] and [NO_2^-]) (de Oliveira et al., 2016, Velmurugan et al., 2016). These potential structural and microbiome changes may have increased sensitivity to or production of NO, hence making younger adults more responsive to acute supplementation, thus explaining the significant changes in BP observed on day 28 following an acute dose of dietary NO_3^-. Furthermore, on day 0, older but not younger adults showed a decrease in SBP, DBP, and MAP following acute supplementation with BR. However, on day 28 both age groups had similar decreases in SBP, DBP, and MAP (pre- to post-supplementation), and hence there was no effect of age at this time point (day 28). Based on these findings, more research is required to investigate potential age-related differences in the effects of acute + chronic BR supplementation on BP.

An important measure to investigate changes in vasodilation and endothelial function is SVR (Jones et al., 2019, Woessner et al., 2020). Previous studies have suggested that acute supplementation with dietary NO_3^- in younger and older adults may improve vascular resistance and thus improve endothelial function (Bond et al., 2013, Woessner et al., 2020). However, in the current study we showed no significant improvement in SVR in younger or older adults following acute supplementation with BR on day 0. This was similar to the authors previous work in older adults which showed no improvement in SVR following acute supplementation with 9.8 mmol of dietary NO_3^- (Chapter 4). In contrast, Bond et al. (2013) found a significant improvement in total vascular

resistance (TVR) 2 h post-supplementation with ~12.3 mmol of dietary NO_3^- in younger female adults; however, orange juice was used as the placebo beverage and as orange juice does not contain similar bioactive ingredients found in BR such as betalains or high levels of polyphenols, this may have impacted the findings of the study (Haswell et al., 2021).

Despite the lack of change in SVR on day 0, an acute dose of BR following 14 and 28 days supplementation (acute + chronic supplementation) reduced SVR in older but not younger adults. Additionally, older adults showed a greater reduction in SVR compared to younger adults on day 14 and a trend (p = 0.056) for greater reduction on day 28. Due to diminished bioavailability of NO and age-related increases in arterial stiffness, older adults tend to have a higher SVR than healthy younger adults with optimal vascular function (Donato et al., 2018, Woessner et al., 2020). Therefore, it could be expected that supplementation with dietary NO_3^- may compensate for this reduction in NO bioavailability by increasing exogenous NO production in older adults, thus promoting vasodilation with potential reductions in arterial stiffness (Hughes et al., 2016), resulting in a decrease in SVR in older but not younger adults, with a large enough difference in change in SVR to result in a significant difference between the age groups. It has been suggested that longer durations of BR supplementation may be required to promote greater changes in the arterial wall (de Oliveira et al., 2016), which would be a major contributor to improved SVR. One study in hypertensive adults (18 – 85 y) showed that 28 days supplementation with NO_3^--rich BR (~6.4 mmol) significantly reduced arterial stiffness by 0.59 $m \cdot s^{-1}$ (Kapil et al., 2015). Therefore, acute supplementation in combination with daily doses of BR may explain the improvements in SVR seen for older adults on days 14 and 28 when compared to the lack of change seen on day 0 from solely acute supplementation. In contrast, a previous systematic review and meta-analysis investigating the effects of dietary NO_3^- on endothelial function suggested that due to aging and increased arterial stiffness, older adults experienced reduced effects from dietary NO_3^- (Lara et al., 2016). However, this review only included five studies investigating endothelial function in older adults, of which three used fruit juice as the PL, which may have affected the strength of the meta-analysis. Furthermore, the findings in this study suggest the opposite, that older adults may have a greater improvement in vascular resistance and potential structural changes from accumulative effects of acute and chronic supplementation. While these results in SVR indicate benefits from supplementation with BR on vasodilation and improved endothelial function and suggest the potential for reductions in arterial stiffness in older adults, further research in this area is needed. The use of more direct measures of endothelial function such as pulse wave velocity (PWV) may provide more detail into the potential benefits of dietary NO_3^- supplementation as PWV is the gold standard measure of arterial stiffness which has a strong relationship to cardiovascular risk (Ben-

Shlomo et al., 2014). It is also important to note that the lack of significance observed between age groups on day 28, was likely due to the large inter-subject variability (Figure 6.3D) and may indicate larger sample sizes are needed to further investigate the benefits of dietary NO_3^- supplementation on SVR.

6.4.3. Changes in Blood Pressure and Hemodynamics Following Chronic Supplementation

Previous studies have indicated that daily supplementation with BR for longer durations may elicit greater beneficial effects in BP responses beyond the initial acute effects (de Oliveira et al., 2016, Vanhatalo et al., 2010). The current study supports this, with reductions in SBP, DBP, and MAP observed in older adults following 14 days' supplementation, in the absence of an acute dose of BR. This suggests chronic supplementation may maintain reductions in BP beyond the acute increase in plasma [NO_3^-], [NO_2^-] and potential for O_2 independent NO synthesis, and that alternative mechanisms for these improvements may be present. One potential mechanism for the maintained reduction in BP following 14 days' chronic supplementation with BR is that the increased NO bioavailability in the vasculature may be preserved beyond the initial peak in plasma [NO_2^-], inducing structural changes in the vasculature over time (de Oliveira et al., 2016, Lara et al., 2016, Wylie et al., 2016). Studies in mice have indicated that NO_2^- levels can accumulate in mammalian tissue including the heart (Bryan et al., 2005) which, if translatable to humans, could result in NO_2^- storage for use over time as a precursor to NO or as a direct signalling molecule itself (Hughes et al., 2016, Wylie et al., 2016). Although one study has shown no improvements in 24-h ambulatory BP following 14 days' supplementation in older adults, this study had participants who continued their BP medication alongside the BR supplementation which would likely have impacted the results (Gilchrist et al., 2013). For younger adults however, there was no reduction in SBP, DBP, or MAP in the current study following 14 days' chronic supplementation. This could again be explained by the lower baseline SBP and DBP values of younger compared to older adults (as discussed above), whereby smaller to no changes in BP may be expected due to already optimal BP. In support of this, one study in younger adults has shown that the decrease in SBP following dietary NO_3^- supplementation (9.7 mmol·day^{-1}) was negatively correlated with baseline SBP values, meaning that individuals with lower baseline SBP had smaller decreases in SBP following supplementation (Haider and Folland, 2014).

Despite the reduction in BP observed in older adults following 14 days' supplementation with BR, no improvements in SBP, DBP, or MAP were seen following 28 days' supplementation in younger or older adults. This is consistent with one study that showed no improvement in BP following 21 days' supplementation with BR (6 mmol·day^{-1}) in older adults (Jajja et al., 2014). In contrast, Kapil et al.

(2015) reported a significant reduction in BP following 28 days' supplementation with dietary NO_3^- (6.4 mmol·day^{-1}), however this study used hypertensive participants, thus a significant drop may have occurred due to higher baseline BP measures. Few studies have investigated the potential benefits of chronic BR supplementation in older and younger adults for durations as long as 28 days and further investigation is necessary. It is also important to note that despite the lack of change in BP from day 0 to day 28 for older adults there was also no significant difference in reductions in SBP, DBP, or MAP from day 14 to 28, indicating that the effects of chronic supplementation seen following 14 days supplementation may have been maintained at day 28. Therefore, the lack of significant change in SBP, DBP, and MAP following 28 days chronic supplementation with BR could be explained by the large standard deviation seen at day 28 (Figure 6.3). This may suggest that chronic adaptations peak before day 28 and that these adaptations may be maintained in older adults with regular daily doses of dietary NO_3^-.

Older adults showed a greater reduction in SBP, DBP, and MAP compared to younger adults after chronic supplementation with BR for 14 days. As the first study to investigate age related differences in cardiovascular measures following chronic supplementation with dietary NO_3^-, the results from this study provide novel findings, however the mechanisms for this larger improvement in BP for older adults are currently unclear. As discussed, this could be due to older adults having reduced NO bioavailability and impaired endothelial function due to aging, and thus they may benefit more from increased NO production following consumption of dietary NO_3^- compared to younger adults with normal NO bioavailability (Sindler et al., 2011, Stanaway et al., 2019). However, based on this rationale we would expect older adults to demonstrate a larger reduction in SBP, DBP, and MAP following 28 days' supplementation compared to younger adults, yet this was not observed in the current study. Therefore, it is important to further investigate the potential benefits of longer durations of supplementation with BR in older and younger adults.

Interestingly, there was no improvement in SVR for either older or younger adults, or differences between age groups following 14 or 28 days of chronic supplementation. In contrast, one study in younger adults showed a decrease in total peripheral resistance (TPR) following 15 days of supplementation with BR (6.4 mmol·day^{-1}) (Lee et al., 2015). Additionally, with the improvements in SVR in older adults following acute + chronic supplementation with BR on days 14 and 28, it could have been expected that chronic supplementation would improve SVR particularly in older adults due to their diminished NO bioavailability and endothelial function (de Oliveira et al., 2016, Stanaway et al., 2019), however, this was not seen in this study. One reason for the lack of statistically significant changes in SVR following chronic supplementation with BR could be due to

the large inter-subject variability seen in both older and younger adults (Figure 6.3D). Furthermore, the lack of statistical significance seen from chronic supplementation in the absence of an acute dose of BR prior to testing, may suggest greater benefits from acute supplementation with dietary NO_3^- in addition to daily consumption for 14 and 28 days.

6.4.4. Cognitive Performance Following Acute and Acute + Chronic Supplementation

Acute and acute + chronic supplementation with BR improved aspects of cognitive performance with a reduction in reaction time during the Stroop test in older adults on day 0 and 28. This is consistent with our previous findings which showed a similar reduction in reaction time in the Stroop test for older adults following consumption of BR, with no improvement seen in the other cognitive tests (Stanaway et al., 2019). The Stroop test activates the prefrontal cortex of the brain (Endo et al., 2018) and supplementation with dietary NO_3^- has resulted in improved cerebral perfusion to the prefrontal cortex in older adults (Presley et al., 2011). Therefore, improvements in cognitive performance during this test could be expected and may be attributed to increases in CBF allowing higher cerebral oxygenation for mental processing (Clifford et al., 2019, Presley et al., 2011). Notably, no improvements in reaction time were observed during the Stroop test on day 14 following acute + chronic dietary NO_3^- supplementation in older adults. Considering the improvements seen on day 0 and 28 this is unexpected; however, the magnitude of changes in plasma [NO_3^-] and [NO_2^-] (yet to be determined) that occurred on day 14 relative to day 0 and 28 may help explain this lack of significance.

There were no improvements in performance for any of the cognitive tests in younger adults following acute or acute + chronic supplementation with BR on days 0, 14, or 28. While some studies have shown improvements in cognitive function following supplementation with dietary NO_3^- in younger adults (Thompson et al., 2015, Wightman et al., 2015) others have shown no benefit (Shannon et al., 2017, Thompson et al., 2014). The equivocal results for change in cognitive performance indicate the need for more research in this area to clearly understand the effect of dietary NO_3^- supplementation on cognition in younger adults. A recent systematic review and meta-analysis investigating the potential benefits of dietary NO_3^- on cognitive function and CBF concluded that there was currently inconclusive evidence for benefits for cognition in younger adults (Clifford et al., 2019).

Older adults have reduced CBF due to the degenerative processes of aging, thus suggesting that they may experience greater benefits in cognitive performance following BR consumption compared to younger adults (Meyer et al., 1988, Presley et al 2011, Stanaway et al., 2017). In support of this,

the current study showed a greater reduction in reaction time during the Stroop test, in older relative to younger adults, following supplementation with BR on days 0 (acute) and 28 (acute + chronic). To the authors' knowledge this is the first study to show an age difference in improvements in cognitive function following dietary NO_3^- supplementation. These results add support to the potential for dietary NO_3^- to help mitigate negative cerebral health outcomes from aging, as reduced cognition is a major risk factor for neurological disorders such as dementia (Meyer et al., 1988). However, there was no difference between age groups on day 14 of supplementation following an acute dose of BR, which as mentioned above, may be explained by later analysis of plasma [NO_3^-] and [NO_2^-]. With limited research in this area, further studies are required to investigate age-related differences in change in cognition to elaborate on the findings of this study.

6.4.5. Cognitive Performance Following Chronic Supplementation

Few studies have examined the effects of chronic dietary NO_3^- supplementation on cognitive performance in older and younger adults, with, to the authors' knowledge, the longest duration of supplementation being 14 days (Gilchrist et al., 2014). The present study investigated 28 days' chronic supplementation with BR and found no improvements in cognitive performance in younger or older adults on days 14 or 28 pre-supplementation with an acute dose. While it has been speculated that potential changes in cerebral vasculature and metabolic efficiency may require a longer duration of supplementation, 28 days should be sufficient time to show this (Gilchrist et al., 2014), therefore, acute doses of dietary NO_3^- may be required to re-establish peaks in plasma [NO_3^-] and [NO_2^-] and thus NO production for acute vasodilation and increases in CBF. In support of this, a study in younger adults which explored 7 days' chronic supplementation with BR (12.8 mmol·day^{-1} NO_3^-) showed an improvement in reaction time (Thompson et al., 2015). However, the last dose of BR was taken 2.25 h prior to testing, thus stimulating an acute increase in plasma [NO_3^-] and [NO_2^-] in addition to the chronic effects from daily doses. Furthermore, an independent chronic effect (pre-supplementation with an acute dose) was not measured (Thompson et al., 2015). Another study found that 14 days' supplementation with dietary NO_3^- (7.5 mmol·day^{-1}) improved simple reaction time in older adults (Gilchrist et al., 2014). However, with few studies investigating longer durations of supplementation with BR on cognition more research is needed to investigate the conflicting results.

While there was no treatment effect for chronic supplementation with BR in older or younger adults, older adults did show a significantly greater reduction in reaction time during the Stroop test on days 14 and 28 and a trend for improved reaction time (p = 0.072) during the RVIP test on day 28. This is the first study to examine chronic effects of BR supplementation on cognition between older

and younger adults and further supports the acute findings that older adults may gain greater benefits in cognitive function with daily supplementation with BR. Additionally, this result suggests that chronic supplementation without an acute dose of dietary NO_3^- may still provide cognitive benefits, (albeit not statistically significant in this study) and that these slight improvements are still greater than those seen in younger adults, thus indicating the potential need for greater sample sizes to further evaluate any age-related differences in the effects of chronic supplementation with BR on cognition.

6.4.6. Physiological Performance

The present study showed no significant improvement in work to completion time trial performance or change in $\dot{V}O_2$ following acute (day 0) or chronic + acute (day 14 and 28) supplementation with NO_3^--rich BR in younger and older adults. In contrast, previous studies have shown that acute and chronic + acute supplementation with BR significantly improved exercise performance and reduced $\dot{V}O_2$ during exercise in both younger (Bailey et al., 2009, Wylie et al., 2016, Lansley et al., 2011a, Lansley et al., 2011b) and older (Berry et al., 2015, Eggebeen et al., 2016, Kenjale et al., 2011) adults. The mechanisms for improved exercise performance are still unclear but one study linked reductions in $\dot{V}O_2$ (following BR consumption) to a decrease in the rate of ATP consumption (Bailey et al., 2010). This effect was seen alongside reductions in the $\dot{V}O_2$ cost of exercise, indicating that supplementation with dietary NO_3^- (via NO mediated effects) may alter the ATP consumption rate of actomyosin-ATPase and Ca_2^+-ATPase during muscle contraction (Bailey et al., 2010). Therefore, the O_2 cost required for contraction may be lowered due to reductions in ATP utilisation from these two ATPases (Bailey et al., 2012). However, others have shown similar findings to the current study with no improvements in time trial performance or $\dot{V}O_2$ cost of exercise (Fowler et al., 2021, Kelly et al., 2013). In further support of the results in the current study, a systematic review and metanalysis concluded that supplementation with dietary NO_3^- is less likely to improve time trial performance, but more effective at improving endurance exercise capacity (McMahon et al., 2017).

Unexpectedly, older adults showed a significant improvement in time to completion during the cycle trial on day 28 of supplementation with the PL compared to the BR treatment. The reason for the observed improvement in time trial performance in older adults following the PL treatment could be explained by the difficulty in encouraging older adults to push themselves maximally to complete the cycle trial as quickly as possible. While this would normally be controlled for by the PL arm, as this was an RCT, if older adults in one arm of the study did not give it their maximal effort, then this would affect only that arm of the study. Another possible explanation could be the lack of adequate familiarisation as during the familiarisation trial participants only completed an incremental cycle

test, therefore did not have a direct practice of the work to completion time trial. It is also important to note that a trend for reduction in $\dot{V}O_2$ was observed (p = 0.087, Figure 6.4B) following chronic + acute supplementation with NO_3^--rich BR at 80% completion of the time trial on day 14, indicating the potential for improvements in $\dot{V}O_2$ in older adults following daily supplementation. However, the variability between individual responses may account for the lack of significance shown following BR supplementation, suggesting the need for further research, with the use of larger samples sizes, to explore this finding.

To the authors' knowledge this is the first study to directly compare time trial performance and $\dot{V}O_2$ response during exercise between younger and older adults. There was no effect of age on work to completion time trial performance following supplementation with NO_3^--rich BR on days 0, 14, or 28; however, $\dot{V}O_2$ was significantly reduced at 20% completion of the time trial in older compared to younger adults on day 0 following acute supplementation. Previous studies have suggested that older adults have an age-related decrease in NO production, reduced $\dot{V}O_{2\ peak}$, higher ATP cost of force production, and a reduction in mitochondrial efficiency (increased proton leakage) (Clements et al., 2014, Kelly et al., 2013, Layec et al., 2014). This suggests that older adults may have the potential for greater reductions in O_2 cost during exercise following NO_3^--rich BR consumption, as they have a lower base functional capacity and daily life tasks force them to work at a higher percentage of their maximal exercise ability (Stanaway et al., 2017). Therefore, the current findings provide support for greater beneficial effects of dietary NO_3^- supplementation on $\dot{V}O_2$ in older adults and indicate the need for more research in this area to elaborate on these findings.

6.4.7. Future Directions

The current findings show that both acute and chronic supplementation with NO_3^--rich beetroot juice reduces BP in older adults. However, as there is limited research investigating the chronic effects of dietary NO_3^- supplementation in older adults in the absence of an acute dose prior to testing, future studies are needed to further examine these independent chronic effects on cardiovascular responses. Additionally, the longest study investigating the effects of daily supplementation with dietary NO_3^- on BP and endothelial function is currently six weeks (Velmurugan et al., 2016), thus an important area for future research would be investigation into the effects of supplementation with NO_3^--rich beetroot juice in younger and older adults beyond six weeks. Older adults tend to have higher resting BP and reduced cognition due to age-related changes, suggesting that dietary NO_3^- supplementation may be more beneficial in populations with diminished cardiovascular responses and cognitive function such as hypertension and neurological disorders (Donato et al., 2018, Presley et al., 2011, Stanaway et al., 2017). Therefore, with the

improvements in cognition and clinically significant reductions in BP shown in older adults following acute and chronic supplementation with dietary NO_3^- in this study, future research should investigate the use of dietary NO_3^- supplementation in a clinical setting as potential preventative therapy for CVD or neurological disorders. The findings in this study also emphasise the need for future research investigating changes in $\dot{V}O_2$, SVR, and more direct measures of endothelial function such as PWV, with the use of larger sample sizes following daily supplementation with dietary NO_3^- in younger and older adults.

6.5 Conclusion

Acute supplementation with NO_3^--rich BR (10.5 mmol) reduced SBP, DBP, and MAP in older adults at day 0, while acute + chronic supplementation reduced SBP, DBP, and MAP in both older and younger adults on day 28. Systemic vascular resistance was significantly reduced in older adults on days 14 and 28 following acute + chronic supplementation with NO_3^--rich BR. Chronic supplementation with dietary NO_3^- reduced SBP, DBP, and MAP in older adults on day 14. The reductions in BP following acute supplementation on day 0 and chronic supplementation on day 14 were greater in older compared to younger adults, while reductions in SVR following acute + chronic supplementation on day 14 were also greater in the older cohort. Stroop test reaction time was improved on days 0 and 28 for older but not younger adults following acute and acute + chronic BR supplementation, with a greater improvement in reaction time for older compared to younger adults on both days. Older adults also had a greater reduction in $\dot{V}O_2$ following chronic + acute BR supplementation on day 14. Collectively, these results indicate that acute and chronic supplementation with BR can reduce BP and improve cognitive performance, particularly in older adults. Therefore, supplementation with BR may have greater cardiovascular and cognitive benefits in the older cohort, and with the aging population and increase in age-related diseases, daily consumption of BR may provide a potential means of preventative treatment. Furthermore, the reductions in BP were clinically significant, following both acute and chronic supplementation, indicating the potential for use of dietary NO_3^- in a clinical setting.

Chapter 7

General Discussion and Conclusions

This chapter provides a discussion of the main findings and presents the overall conclusions of this PhD study. It addresses the research from all chapters and summarises the results for each research question set out in Section 1.3. Additionally, it highlights the novel findings of this PhD project and outlines the limitations and potential future research directions.

7.1 Summary of Key Findings

A summary of the key findings, with their relevance to the four research questions (Chapter 1, Section 1.3) established for this PhD study are outlined below:

1. **Does supplementation with dietary NO_3^- improve cardiovascular, cerebrovascular, and metabolic health, and physiological and cognitive performance in older adults?**

   Based on the 12 studies examined in the systematic review (Chapter 3), strong evidence exists supporting improvements in physiological performance of older adults following consumption of NO_3^--rich beetroot juice, and some research also indicates the potential for beneficial outcomes to cardiovascular and cerebrovascular health. However, as there were only three studies (Gilchrist et al., 2014, Kelly et al., 2013, Presley et al., 2011) included in the systematic review that investigated cognitive function in older adults following supplementation with dietary NO_3^-, it was determined that more research was needed to further examine the potential benefits in this area.

2. **Does acute supplementation with different doses of dietary NO_3^- improve cardiovascular health and cognitive function in older adults (50 – 80 y) in a dose dependant manner?**

   The findings from the Dose-response study (Chapter 4) showed that plasma [NO_3^-] and [NO_2^-] increased dose dependently, with plasma [NO_3^-] significantly increasing between treatments (PL, 2.5, 4.9 and 9.8 mmol of NO_3^-) ($p < 0.001$). There was also a clinically significant reduction in SBP (11.6 mmHg), DBP (6.6 mmHg), and MAP (7.3 mmHg) following consumption of the 9.8 mmol dose when compared to the other three treatments. However, the lower doses showed no significant effects on BP. There was a trend for reduction in SVR following the 9.8 mmol treatment compared to the 2.5 mmol ($p = 0.096$) and 4.9 mmol ($p = 0.075$) treatments. The highest dose (9.8 mmol) also improved Corsi span (a measure of cognitive performance) compared to PL ($p = 0.013$) and 2.5 mmol ($p = 0.004$) treatments.

3. **Does acute supplementation with NO_3^--rich beetroot juice improve cardiovascular and cognitive health and performance in younger compared to older adults?**

   The findings from the Acute study (Chapter 5) showed that acute supplementation with 10.5 mmol dietary NO_3^- increased plasma [NO_3^-] and [NO_2^-] in both younger and older adults. Furthermore, the increase in plasma [NO_2^-] was greater in older when compared to younger adults. Similarly, SBP was reduced in both younger (4.0 mmHg) and older (7.4

mmHg) adults following acute supplementation with NO_3^--rich beetroot juice; however, DBP was only reduced in older adults (5.7 mmHg), hence there was a greater reduction in DBP in older compared to younger adults. Aspects of cognitive performance were improved for both younger and older adults following supplementation with dietary NO_3^-, with improvements in reaction time during the Stroop test. There was no improvement in mood and perceptual measures.

4. **Does chronic supplementation with NO_3^--rich beetroot juice improve cardiovascular health and performance, and cognition in younger compared to older adults?**

   The outcomes of the Chronic study (Chapter 6) showed that on day 0 following acute supplementation with 10.5 mmol of dietary NO_3^-, SBP, DBP, and MAP were clinically reduced in older (12.8, 8.6, and 9.8 mmHg, respectively) but not younger adults, thus resulting in a greater reduction in BP and MAP for older compared to younger adults. Similarly, 14 days of prolonged supplementation with dietary NO_3^- (in the absence of an acute dose) also resulted in a reduction in SBP (5.0 mmHg), DBP (4.5 mmHg), and MAP (4.7 mmHg) in the older cohort with a greater reduction shown in older when compared to the younger cohort. Comparing SVR pre- versus 2.25 h post-supplementation with dietary NO_3^- on days 14 and 28 in combination with chronic daily supplementation (acute + chronic supplementation) significantly reduced SVR in older but not younger adults, with older adults also having a greater reduction in SVR compared to younger adults on day 14. Improvements in reaction time during the Stroop test (a measure of cognitive performance) were also shown in older but not younger adults following acute supplementation with NO_3^--rich beetroot juice on day 0 and pre- versus post-supplementation with dietary NO_3^- on day 28 in combination with 28 days daily supplementation prior to the acute dose. The older cohort also showed a greater improvement in reaction time compared with the younger cohort following acute and acute + chronic supplementation on days 0 and 28. Furthermore, while neither younger nor older adults showed an improvement in cognitive performance following chronic supplementation with dietary NO_3^- on days 14 or 28, older adults showed greater reduction in reaction time during the Stroop test compared to the younger cohort. Finally, acute supplementation with NO_3^--rich BR on day 0 resulted in a greater reduction in $\dot{V}O_2$ in older compared to younger adults.

7.2 Discussion of Main Findings

The investigation into the effects of supplementation with NO_3^--rich beetroot juice on cardiovascular responses and cognition in younger and older adults throughout the dose-response, acute, and chronic studies demonstrated a common increase in plasma [NO_3^-] and [NO_2^-], along with decreases in BP and MAP, and improvements in aspects of cognition.

7.2.1. Plasma [NO_3^-] and [NO_2^-]

The findings in Chapters 4 and 5 support those shown by previous studies (Kenjale et al., 2011, Larsen et al., 2011, Wylie et al., 2013), whereby acute supplementation with NO_3^- significantly increased plasma [NO_3^-] and [NO_2^-] in both younger and older adults. Importantly, both Chapters 4 and 5 also presented novel findings. To the authors' best knowledge, the Dose-response study (Chapter 4) was the first to examine the effects of varying doses of dietary NO_3^- on cardiovascular and cerebrovascular responses in older adults and showed that plasma [NO_3^-] and [NO_2^-] increased in a dose dependant manner, with the highest dose providing the greatest increase. This result was similar to one study which examined changes in plasma, urinary, and salivary nitrate and nitrite concentrations in younger and older adults, where supplementation with ~4.5, ~8.9, and ~13.4 mmol of NO_3^- compared to PL also resulted in a dose-dependent increase in plasma, urinary, and salivary [NO_3^-] in both younger and older adults (Capper et al., 2022). Thus, higher doses of dietary NO_3^- may provide the greatest acute benefits due to higher levels of plasma NO_3^- and NO_2^- and consequently NO. Additionally, the Acute study (Chapter 5) showed that older compared to younger adults had a greater increase in plasma [NO_2^-] and a trend for increase in plasma [NO_3^-] (p = 0.064) following acute supplementation with dietary NO_3^-. The Acute study was the first study to show a difference in plasma [NO_3^-] and [NO_2^-] measures between younger and older adults following acute supplementation with NO_3^--rich beetroot juice, and as such provides evidence to support the hypothesis that older adults may experience greater benefits from dietary NO_3^- due to the potential compensation for age-related decline in NO bioavailability and reduced NO production via the L-Arginine/NOS pathway (Bailey et al., 2012, Sindler et al., 2011, Stanaway et al., 2017).

Due to COVID-19 restrictions, processing of the blood samples for analysis of plasma [NO_3^-] and [NO_2^-] from the Chronic study (Chapter 6) will be completed retrospectively to submission of this PhD Study.

7.2.2. Blood Pressure and Hemodynamics

Following acute supplementation with NO_3^--rich beetroot juice, results from all three experimental Chapters (4, 5, and 6) showed a significant reduction in SBP and DBP in older adults, with the Dose-response study and Chronic study (Chapters 4 and 6) also showing a reduction in MAP. These improvements in BP are consistent with previous findings (Berry et al., 2015, Kemmner et al., 2017, Kenjale et al., 2011), and are likely due to an increase in NO (via the exogenous pathway) with subsequent activation of the enzyme guanylate cyclase (Kapil et al., 2015, Kemmner et al., 2017). Guanylate cyclase acts in the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), with cGMP being a potent vasodilator which relaxes smooth muscle, resulting in a relaxation of vasculature and hence reduction in BP (Kapil et al., 2015, Kemmner et al., 2017, Stanaway et al., 2017). Therefore, the reductions in BP following acute supplementation with NO_3^--rich beetroot juice in older adults seen in the studies throughout this PhD project demonstrates a viable way for improving cardiovascular health in the older population.

Through the same mechanism discussed above, it could be assumed that younger adults would also experience a reduction in BP following acute supplementation with NO_3^--rich beetroot juice; however, the Acute study (Chapter 5) only showed a reduction in SBP but not DBP in young people, while the Chronic study (Chapter 6) showed no reduction in SBP, DBP, or MAP. The lack of reduction in DBP and MAP in these experiments (Chapter 5 and 6) may be expected as previous studies have shown that improvements in DBP and MAP following acute dietary NO_3^- supplementation are less common compared to improvements in SBP (Lansley et al., 2011a, Lansley et al., 2011b, Thompson et al., 2014). Despite this, the lack of reduction in SBP shown in the Chronic study contrasts with results from previous studies which have commonly shown reductions in SBP following acute supplementation with dietary NO_3^- (Lansley et al., 2011a, Thompson et al., 2014, Wylie et al., 2013). This conflicting result for change in SBP following acute dietary NO_3^- supplementation could be explained by the lower baseline SBP and DBP measures for younger compared to older adults. Therefore, it could be suggested that the increase in NO following supplementation with NO_3^--rich beetroot juice may not elicit as strong an effect on the vasculature when resting BP is lower or at a more optimal pressure, hence preventing the induction of hypotension. As we age it is normal to see an increase in BP (Sindler et al., 2011), thus it is expected that the older cohort would have a higher average resting BP and in turn may experience greater benefits from supplementation with NO_3^--rich beetroot juice.

Novel findings from Chapters 5 and 6 showed that older adults exhibited greater improvements in BP compared to younger adults following acute supplementation with NO_3^--rich beetroot juice. Specifically, the Acute study (Chapter 5) found that the older cohort showed a greater reduction in DBP, and the Chronic study (Chapter 6) found they had a greater reduction in SBP, DBP, and MAP compared to the younger cohort. In addition to older adults having a higher resting BP, the difference in change in BP seen between the two age groups may be due to variations in NO production (via the L-Arginine/NOS pathway) and utilisation, whereby aging has been suggested to lower NO production and bioavailability, and subsequently reduce vascular function (Lara et al., 2016, Sindler et al., 2011, Stanaway et al., 2017). Therefore, older adults may experience greater benefits from supplementation with NO_3^--rich beetroot juice compared to younger adults with optimal NO production and bioavailability (Kelly et al., 2013). However, two previous studies have shown opposing findings, with no difference in SBP or DBP seen between younger and older cohorts following acute supplementation with 9.4 and 6.4 mmol of NO_3^- (Hughes et al., 2016, Rogerson et al., 2022). These inconsistencies may be attributed to the study designs and sample sizes used. The study by Hughes et al. (2016) was not placebo-controlled and that of Rogerson et al. (2022) had unequal sample sizes with the numbers in the older cohort (n = 7) being considerably lower than that of the younger cohort (n = 18).

Acute supplementation with dietary NO_3^- on day 28 (pre- versus 2.25 h post-supplementation) in combination with 28 days of daily supplementation reduced SBP, DBP, and MAP in older adults. This was likely due to increases in NO levels resulting in activation of guanylate cyclase and thus increased vasodilation of the blood vessels (Kemmner et al., 2017, Stanaway et al., 2019). This reduction in BP at 28 days was also seen in younger adults despite the lack of change seen at day 0 and 14. It has been suggested that daily supplementation with dietary NO_3^- for longer durations may result in greater improvements in cardiovascular function beyond the effects seen from a single acute dose, which may be due to structural changes in the vasculature and increased NO bioavailability (de Oliveira et al., 2016, Vanhatalo et al., 2010). This means that an acute dose following daily supplementation with NO_3^--rich beetroot juice for 28 days may provide greater effects compared to that seen on day 0 and 14, resulting in a significant reduction in BP in younger adults on day 28.

In addition to benefits from acute supplementation, an important novel finding from this PhD project was that older adults exhibited a significant reduction in SBP, DBP, and MAP following 14

days' chronic supplementation with NO_3^--rich beetroot juice compared to PL in the absence of an acute dose, with this reduction being maintained following 28 days' supplementation. Older adults also had a significantly greater reduction in SBP, DBP, and MAP compared to younger adults following chronic supplementation with dietary NO_3^- on day 14. These results suggest that daily supplementation with dietary NO_3^- for 14 days may maintain reductions in the BP of older adults up to ~24 h beyond the last dose. These beneficial effects from chronic supplementation with dietary NO_3^- are maintained beyond the initial peak in plasma [NO_3^-], [NO_2^-] and thus NO, suggesting that other mechanisms may also be contributing to the observed decrease in BP. One study using mice has shown the potential for mammalian heart tissue to store NO_2^- (Bryan et al., 2005); assuming this was translatable to humans may mean supplementation with dietary NO_3^- could provide the precursor to a reservoir of NO_2^- for use over time as a direct signally molecule or for conversion to NO (Hughes et al., 2016, Wylie et al., 2016). Additionally, it has been suggested that increased NO bioavailability from dietary NO_3^- supplementation may be preserved in the vasculature, beyond initial peaks in plasma [NO_2^-] with daily doses of NO_3^- and this may stimulate structural changes in the vasculature over time (de Oliveira et al., 2016, Lara et al., 2016, Wylie et al., 2016). In support of the current findings, Jones et al. (2019) also showed a reduction in SBP and DBP of older adults following 14 days' chronic supplementation with NO_3^--rich beetroot juice (6.6 mmol) compared to baseline; however, there was no difference when compared to the PL treatment. Nonetheless, novel findings of this PhD project are the potential for chronic supplementation with NO_3^--rich beetroot juice to provide a sustained reduction in BP for older adults when compared to PL treatments and younger adults.

Putting the results of this PhD project into context with respect to changes in BP, the average 'acute' reduction in SBP and DBP for older adults based on the findings from the three experimental Chapters (3-6) were 9.4 and 6.6 mmHg respectively, while the average 'chronic' reduction, ~24 h after the last dose of dietary NO_3^-, in SBP and DBP were 5.0 and 4.5 mmHg, respectively. The average reduction in SBP and DBP from a standard dose of antihypertensive medication, given a pretreatment SBP of 150 mmHg and DBP of 90 mmHg, has been suggested to be approximately 8.7 and 4.7 mmHg, respectively, with the decrease in SBP and DBP being less if the initial pretreatment BP was lower (Law et al., 2009). Additionally, a meta-analysis of 147 studies showed that a reduction of 10 mmHg SBP or 5 mmHg DBP reduced risk of an adverse coronary heart disease event by 22% and risk of stroke by 41% (Law et al., 2009). Collectively, these results indicate that supplementation with NO_3^--rich beetroot juice may have the potential for use in a clinical setting for treatment or

preventative therapy against CVD and related health conditions in older adults. In particular, NO_3^--rich beetroot juice may be a suitable option for treatment in individuals who are averse to using certain drug interventions or those who may have unfavourable adverse reactions to standard antihypertensive medication.

For the first time, acute + chronic supplementation with NO_3^--rich beetroot juice has been shown to improve SVR in older adults on days 14 and 28, with a greater reduction in SVR seen in the older compared to younger cohort on day 14 and a trend (p = 0.056) for greater reduction on day 28 (Chapter 6). Older adults may have an age-related decrease in NO production and bioavailability (Lara et al., 2016, Sindler et al., 2011, Stanaway et al., 2017), while aging can also cause an increase in arterial stiffness and consequently SVR (Donato et al., 2018, Woessner et al., 2020). Therefore, the differences in the level of improvements in SVR seen in the different age groups may be due to an increase in NO production and utilisation from supplementation with dietary NO_3^-, thus compensating for the degenerative effects of aging in the older cohort, resulting in greater vasodilation and subsequent reduction in arterial stiffness for older compared to younger adults (Hughes et al., 2016).

It has been suggested that a longer duration of supplementation with dietary NO_3^- may promote structural changes in the arterial wall (de Oliveira et al., 2016), thus reducing arterial stiffness and therefore SVR. This could explain why improvements in SVR were only seen after days 14 and 28 of supplementation with NO_3^--rich beetroot juice and not acutely at day 0. Although one review examining the effects of dietary NO_3^- supplementation on endothelial function showed an association between aging and a reduction in the effectiveness of dietary NO_3^- consumption, only five studies examining endothelial function in the older population were reviewed with three using fruit juice as the PL, likely impacting the strength of the meta-analysis (Lara et al., 2016). The conflicting findings in relation to the results seen in this PhD study suggests the need for further research into the effects of dietary NO_3^- supplementation on vascular resistance and arterial stiffness in older adults with longer duration studies. Additionally, the use of more direct measures of arterial stiffness and endothelial function such as pulse wave velocity may provide more detail on potential mechanisms.

7.2.3. Cognitive Health and Performance

It has been suggested that supplementation with dietary NO_3^- may improve cognitive performance in younger and older adults due to increases in NO production leading to increased cerebral blood flow (CBF), resulting in greater oxygenation of the brain and potential reduction in the oxygen cost of mental processing tasks (Presley et al., 2011 Wightman et al., 2015). However, the results from this PhD study demonstrated mixed findings for younger adults, showing an improvement in aspects of cognition via a reduction in reaction time during the Stroop test in the Acute study (Chapter 5), but showed no improvements in cognition from supplementation with NO_3^--rich beetroot juice in the Chronic study (Chapter 6). Previous literature has also shown mixed findings on cognitive performance following supplementation with dietary NO_3^- in younger adults with some studies showing improvements in cognitive performance (Thompson et al., 2015, Wightman et al., 2015) and others showing no improvements (Shannon et al., 2017, Thompson et al., 2014). A recent systematic review and meta-analysis examining the effects of dietary NO_3^- supplementation on cognition and CBF suggested the current evidence was not strong enough to support the hypothesis that supplementation with dietary NO_3^- improves cognition in younger adults (Clifford et al., 2019). Therefore, the conflicting findings seen in this PhD study and previous literature suggest the need for further research in this area to adequately evaluate the effects of dietary NO_3^- supplementation on cognition in younger adults.

This PhD study has shown that acute supplementation with NO_3^--rich beetroot juice improved aspects of cognitive performance in older adults (Chapters 4, 5, and 6). An improvement in Corsi span during the Corsi block tapering test (CBT) was observed (Chapter 4) and a reduction in reaction time during the Stroop test occurred (Chapters 5 and 6). A reduction in reaction time during the Stroop test when comparing pre- versus 2.25 h post-supplementation with dietary NO_3^- on day 28, following 28 days of daily supplementation was shown in the Chronic study (Chapter 6). These improvements in cognition were likely due to the dietary NO_3^- supplementation increasing NO, and in turn CBF (Stanaway et al., 2019). In support of this one study showed increases in cerebral perfusion (via MRI) to the prefrontal cortex in older adults following 2 days' supplementation with 12.4 mmol dietary NO_3^- (Presley et al., 2011).

A novel finding highlighted in the Chronic study (Chapter 6) was that older adults exhibited a greater improvement in cognitive performance compared to younger adults following both acute and chronic supplementation with dietary NO_3^-. Specifically, reaction time during the Stroop test was

reduced on day 0 (acute) and day 28 (acute + chronic) following supplementation with NO_3^--rich beetroot juice. Furthermore, while there was no treatment effect following 14 and 28 days of chronic supplementation (in the absence of an acute dose of dietary NO_3^-) older adults showed a greater improvement in reaction time during the Stroop test compared to younger adults. This difference in results between the age groups could be explained by the degradative processes of aging which may cause reductions in CBF and cognition, thus supplementation with dietary NO_3^- may provide greater cognitive performance benefits in older compared to younger adults (Meyer et al., 1988, Presley et al., 2011, Stanaway et al., 2017). As reduced cognition is a major risk factors for neurological disorders such as dementia, this research indicates the potential for supplementation with NO_3^--rich beetroot juice to help delay the age-related decline in cognition.

Given that rates of dementia and other neurological diseases are increasing with the aging population (Ministry of Health, 2016) the potential benefits to cognition in combination with the anti-hypertensive effects from supplementation with NO_3^--rich beetroot juice indicates the potential for daily consumption to improve the quality of life as we age.

7.2.4. Physiological Performance

The findings from Chapter 6 also showed for the first time that acute supplementation with NO_3^--rich BR on day 0 resulted in a greater reduction in $\dot{V}O_2$ at 20% completion of the time trial in older compared to younger adults. While there was no corresponding effect of treatment at this timepoint, this finding still indicates the potential for greater beneficial effects of supplementation with dietary NO_3^- on $\dot{V}O_2$ response in older adults. This could be expected as older adults have been suggested to have reductions in NO bioavailability and production, higher ATP cost of force production, and reduced peak $\dot{V}O_2$ due to age-related changes (Clements et al., 2014, Hughes et al., 2016, Kelly et al., 2013, Layec et al., 2014). Therefore, greater reductions in $\dot{V}O_2$ during exercise following consumption of NO_3^--rich BR may be more likely in older adults with suboptimal exercise efficiency compared to younger adults with normal physiological functions (Stanaway et al., 2017). In further support of this, a trend (p = 0.087) was shown for reduction in $\dot{V}O_2$ response following chronic + acute supplementation with NO_3^--rich BR compared to PL in older but not younger adults at 80% completion of the time trial on day 14, thus suggesting the potential for beneficial effects of dietary NO_3^- supplementation in older adults. The lack of significance observed at this timepoint could be due to the large inter-subject variability (Figure 6.4), indicating the need for larger sample sizes. There were no other significant changes in $\dot{V}O_2$ between treatments or age groups at any

other percentage of work completed on days 0, 14, or 28. Therefore, further research with the use of larger sample sizes is required to expand on the current findings and implications of daily supplementation with NO_3^--rich BR on physiological performance in younger and older adults.

It is also important to note that chronic + acute supplementation with the PL treatment on day 28 improved work to completion time trial performance in older adults. This is in contrast to previous studies which have shown improvements in exercise performance following daily supplementation with BR treatments not PL (Berry et al., 2015, Eggebeen et al., 2016). One potential reason for this conflicting finding could be due to variations in the maximal effort performed by older adults as it is difficult for older adults to work at maximal exercise intensities and due to the nature of a RCT design if individuals in one arm of the study did not push themselves maximally for all trials, then this could affect just that arm of the study. Another explanation could be the lack of adequate familiarisation of the work to completion time trial as during the first visit (familiarisation trial) participants only completed an incremental cycle test with the second visit being the baseline cycle trial. Therefore, participants may not have had an adequate practice at the work to completion time trial.

7.3 Limitations

The limitations for the research conducted in this PhD project are discussed in specific detail in each associated chapter. The key limitations are as follows:

- The main limitation for the systematic review (Chapter 3) was the absence of a meta-analysis. With limited numbers of studies published investigating each outcome variable, particularly studies looking at cognitive health and performance, conducting a meta-analysis would have poorly represented the data. Therefore, as more research emerges on dietary NO_3^- supplementation in older adults, a meta-analysis would add valuable information.
- For Chapters 4 and 6, a common limitation was that the sample size was likely too small to adequately investigate the effects of dietary NO_3^- on the secondary outcome measure, changes in SVR. Whilst a prospective power analysis was completed, it was only conducted on the primary study outcomes, hence as a secondary outcome measure, SVR was not accounted for in this calculation. Based on the results found in Chapters 4 and 6, larger sample sizes may be required to appropriately evaluate the effects of supplementation with

NO_3^--rich beetroot juice on change in SVR. A retrospective power analysis was completed for SVR based on the data from this PhD study. The required sample size was calculated to be 26 per group (cf. 11 based on BP), using a partial η2 of 0.14, with a statistical power of 0.81 and α-level set at 0.05. Therefore, future research should use higher samples sizes greater than 26 participants per group to obtain statistically significant results related to SVR.

- Due to the COVID-19 pandemic, data collection for the dose-response (Chapter 4) and chronic (Chapter 6) studies were ended early. While both studies' sample sizes still met their minimum number of participants needed based on the power calculation for the primary outcome variables, this did mean that the intended sample sizes for each study were not achieved, and the studies were not as strongly powered as expected.
- Due to limited lab access throughout the pandemic, analysis of the blood samples for the Chronic study (Chapter 6) were not completed and will be conducted following submission of this thesis.
- Another challenge encountered in the Chronic study (Chapter 6) was the work to completion time trial performance for older adults. While the workloads were all set relative to the individuals' capabilities (based on the incremental cycle test) it was difficult to get some participants in the older cohort to push themselves during the cycle trials, thus impacting the cycle trial performance results. This would normally be balanced by the control (placebo) arm of the study; however, as this was an RCT, each participant only completed one treatment group of the study. Therefore, participants in the older group who did not push themselves maximally, would affect that arm of the study.

7.4 Future Directions

The results found in this PhD study have highlighted several areas of future research into the effects of supplementation with NO_3^--rich beetroot juice on cardiovascular and cognitive health and performance.

1. The findings of the Dose-response study (Chapter 4) showed that the highest dose (9.8 mmol) of dietary NO_3^- provided the greatest benefits to cardiovascular and cognitive function in older adults. However, it is unclear whether a higher dose (beyond the 9.8 mmol NO_3^- dose) may provide even greater benefits. In support of this, it has been suggested that older compared to younger adults may require higher doses of dietary NO_3^- due to impaired

processing of NO_3^- and bioavailability of NO (Lara et al., 2016). Therefore, future research could investigate the dose-response relationship of dietary NO_3^- supplementation in older adults using a higher range of doses to determine if this results in greater cardiovascular and cognitive benefits, and to establish whether there is an optimal dose for supplementation beyond which no further benefits are seen.

2. Future studies could explore potential mechanisms behind the maintained reduction in BP for older adults following chronic (14 days) supplementation with NO_3^--rich beetroot juice, as shown in Chapter 6. It has been suggested that structural changes in the vasculature may be one of the potential mechanisms behind this lasting reduction in BP following chronic consumption of NO_3^--rich beetroot juice (de Oliveira et al., 2016, Lara et al., 2016). Therefore, future research extending from the Chronic study could examine more specific measures of arterial stiffness in older adults, such as pulse wave velocity (PWV), following prolonged supplementation for at least 14 days in the absence of an acute dose of dietary NO_3^-. Pulse wave velocity has the most research supporting its relationship with cardiovascular risk and is seen as the criterion measure for arterial stiffness (Ben-Shlomo et al., 2014).
3. Currently, the longest supplementation period used when investigating the potential benefits of daily doses of dietary NO_3^- is 6 weeks (Velmurugan et al., 2016), hence it remains unclear whether the benefits from prolonged daily consumption would be maintained with continued supplementation. Additionally, the safety of long-term supplementation with high doses of dietary NO_3^- is still unknown. Therefore, longer duration human studies examining key outcome variables such as plasma [NO_3^-] and [NO_2^-], and BP, is warranted. Prolonged supplementation trials can be demanding on the participant due to the time commitment and the ethical cost (invasive measurements). Therefore, investigating only key outcome variables may also help reduce participant demand by reducing the number of variables measured and thus the time commitment required.
4. Future research could examine, in more depth, two key populations; those with elevated BP (pre-hypertensive and hypertensive) and those with early onset dementia to establish if supplementation with dietary NO_3^- may provide a clinical means for treatment or preventative therapy.
    - Research in the pre-hypertensive population with larger sample sizes will provide important information including whether daily supplementation with dietary NO_3^-

may lower BP to normotensive ranges thus significantly improving health outcomes. The next step would then be to investigate a hypertensive population, to establish whether NO_3^--rich beetroot juice may have the potential as a treatment in combination with or instead of anti-hypertensive medication. More research is required to elaborate on the promising findings of the chronic study (28 days) carried out in hypertensive participants (Kapil et al., 2015).

- Due to the complex nature of dementia, future studies could investigate the potential preventative effects of higher daily consumption of dietary NO_3^- in healthy adults as they age via a longitudinal study.

The effects of dietary NO_3^- supplementation on BP and cognition shown throughout this PhD study add to the foundation and justification for future studies to investigate the potential clinical significance of supplementation with NO_3^--rich beetroot juice in older adults with cardiovascular and cognitive risk factors or disease.

5. Future research in the field of dietary NO_3^- supplementation should also use significantly larger sample sizes to provide more acute mean changes and to investigate key outcome variables, in particular plasma [NO_3^-] and [NO_2^-], and BP. Due to the nature of clinical research into supplementation with NO_3^--rich beetroot juice, participant time commitment is generally high and thus larger sample sizes can be difficult to obtain. Therefore, by minimising the number of variables being investigated it can reduce the participant demand, thus increasing the chance of higher subject recruitment. Additionally, with larger sample sizes and key measures of plasma [NO_3^-] and [NO_2^-], and BP it will be possible to complete detailed correlation analysis to examine whether larger increases in plasma [NO_3^-] and [NO_2^-] result in greater reductions in BP, and whether this is affected by age, gender, or ethnicity.

7.5 Concluding Remarks

Consumption of NO_3^--rich beetroot juice has the potential to improve cardiovascular and cognitive health and performance, particularly in older adults. The findings from this PhD project highlight that supplementation with dietary NO_3^- can improve BP, MAP, and cognitive function in both younger and older adults. To the authors' knowledge, this research is the first to show an age difference for the effects of dietary NO_3^- supplementation on cardiovascular function and cognition, resulting in greater reductions in BP and SVR, and improvements in aspects of cognitive

performance in older adults. In addition, chronic supplementation with dietary NO_3^- was also shown to reduce BP in older adults in the absence of acute increases in plasma NO_2^-. Collectively, the work presented in this PhD warrants future investigation into the use of NO_3^--rich beetroot juice as a potential preventative and intervention therapy to combat CVD and neurological disorders and maintain cardiovascular and cognitive health during ageing.

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Appendices

Appendix 1 – Publication, Presentation, and Awards List

Peer-reviewed Journal Publications
Stanaway, L.; Rutherfurd-Markwick, K.; Page, R.; Ali, A. Performance and Health Benefits of Dietary Nitrate Supplementation in Older Adults: A Systematic Review. *Nutrients* 2017, *9*, 1171.
Stanaway, L.; Rutherfurd-Markwick, K.; Page, R.; Wong, M.; Jirangrat, W.; Teh, K. H.; Ali, A. Acute Supplementation with Nitrate-Rich Beetroot Juice Causes a Greater Increase in Plasma Nitrite and Reduction in Blood Pressure of Older Compared to Younger Adults. *Nutrients* 2019, *11*, 1683.
Conference and Symposium presentations
Stanaway, L.; Rutherfurd-Markwick, K.; Page, R.; Ali, A. Oral presentation: The Effects of a Nitrate-rich, Vegetable Beverage on Cognition, Mood and Cardiovascular Responses in Younger and Older Adults. *Research Week at Counties Manukau.* June 2017. Middlemore Hospital, Auckland, New Zealand.
Stanaway, L.; Rutherfurd-Markwick, K.; Page, R.; Ali, A. Oral presentation: The Effects of a Nitrate-rich, Vegetable Beverage on Cognition, Mood and Cardiovascular Responses in Younger and Older Adults. *Auckland Nutrition Research Network Student Presentations*. December 2017. Auckland, New Zealand.
Stanaway, L.; Rutherfurd-Markwick, K.; Page, R.; Ali, A. Oral presentation: An Insight into Evolving Technology: The USCOM, NIRS and iSTAT. *Centre of Metabolic Health Research Symposium*. June 2018. Massey University, Auckland, New Zealand.
Stanaway, L.; Rutherfurd-Markwick, K.; Page, R.; Wong, M.; Jirangrat, W.; Teh, K. H.; Ali, A. Oral presentation: Does Acute Supplementation with Nitrate-Rich Beetroot Juice Benefit Older Adults More than Younger Adults. *Nutrition Society of New Zealand Annual Conference*. December 2018. Massey University, Auckland, New Zealand.
Stanaway, L.; Rutherfurd-Markwick, K.; Page, R.; Wong, M.; Jirangrat, W.; Teh, K. H.; Ali, A. Oral presentation: Acute Supplementation with Nitrate-rich Beetroot Juice Causes a Greater Increase in Plasma Nitrite and Reduction in Blood Pressure of Older Compared to Younger Adults. *20th International Congress on Nutrition & Health*. March 2019. Stockholm, Sweden.

Stanaway, L.; Rutherfurd-Markwick, K.; Page, R.; Wong, M.; Jones, B.; Ali, A. Poster presentation: Effects of Chronic Supplementation with Nitrate-rich Beetroot Juice on Cardiovascular Responses in Healthy Adults. *25th European Nutrition and Dietetics Conference*. April 2019. Rome, Italy.
Stanaway, L.; Rutherfurd-Markwick, K.; Page, R.; Wong, M.; Jones, B.; Ali, A. Oral presentation: Effects of Chronic Supplementation with Nitrate- Rich Beetroot Juice on Cardiovascular Responses in Healthy Adults. *Nutrition Society of New Zealand Annual Conference*. December 2019. Napier, New Zealand.
Awards
Three Minute Thesis winner Albany, 2018, Massey University, New Zealand.
First Time Speaker Award - Second Place, Nutrition Society of New Zealand Annual Conference, December 2018.
Recipient of the Massey University Foundation Grant, 2018/2019.
Recipient of the Claude McCarthy Fellowship, January to June 2019.
Recipient of the Nutrition Society of New Zealand Student Travel Scholarship, November to December 2019.

Appendix 2 – Systemic review published version

Appendix 2.1 – Chapter 3 published article

nutrients

MDPI

Review

Performance and Health Benefits of Dietary Nitrate Supplementation in Older Adults: A Systematic Review

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Abstract: Supplementation with nitrate (NO_3^-)-rich beetroot juice has been shown to improve exercise performance and cardiovascular (CV) responses, due to an increased nitric oxide (NO) availability. However, it is unclear whether these benefits are greater in older adults who have an age-related decrease in NO and higher risk of disease. This systematic review examines 12 randomised, crossover, control trials, investigating food-based NO_3^- supplementation in older adults and its potential benefits on physiological and cognitive performances, and CV, cerebrovascular and metabolic health. Four studies found improvements in physiological performance (time to exhaustion) following dietary NO_3^- supplementation in older adults. Benefits on cognitive performance were unclear. Six studies reported improvements in CV health (blood pressure and blood flow), while six found no improvement. One study showed improvements in cerebrovascular health and two found no improvement in metabolic health. The current literature indicates positive effects of dietary NO_3^- supplementation in older adults on physiological performance, with some evidence indicating benefits on cardiovascular and cerebrovascular health. Effects on cognitive performance were mixed and studies on metabolic health indicated no benefit. However, there has been limited research conducted on the effects of dietary NO_3^- supplementation in older adults, thus, further study, utilising a randomised, double-blind, control trial design, is warranted.

Keywords: nitric oxide; beetroot juice; older adults; cognition; cardiovascular; blood pressure

1. Introduction

Over the past decade, there has been a decrease in levels of functional activity (physiological performance) and an increase in frailty, in the aging population, alongside increased rates of disease and age-related dysfunction [1,2]. An individual's performance and health in their later years of life can be influenced by many lifestyle factors; some are more controllable and have greater impact on performance and health-related outcomes than others [2]. For example, dietary habits have been indicated as a dominant influencer of healthy aging and age-related dysfunction, which can be easily improved to promote beneficial outcomes [3]. Due to this significant influence of diet, recent research has begun to focus on food-based supplements, which contain key bio-active compounds, proposed to promote performance and health benefits [4].

One key nutritional ingredient that has gained recent attention is inorganic nitrate (NO_3^-). Nitrate is found in high concentrations in green leafy vegetables, such as spinach and rocket, and

root vegetables such as beetroot [2]. Additionally, nitrate is a precursor for nitric oxide (NO)—the bio-active form of nitrate—which has many functions in the human body, ranging from regulation of neurotransmission, immunity and blood flow, to alterations in oxygen consumption, and has been shown to have many performance (both physiological and cognitive) [5,6] and health benefits [7]. It is important to note that an acceptable daily intake of nitrate has been set at 3.7 $mg \cdot kg^{-1}$, as a small percentage of nitrate is also converted to N-nitrosamines in the human body, which have been implicated in certain cancers, such as gastric, pancreatic and thyroid cancers [4]. Negative side effects are commonly associated with consumption of nitrate salts, such as those found in cured meats [2,4]. Despite this, there is currently no evidence to indicate negative effects of prolonged nitrate consumption from vegetable sources, such as beetroot [2,4].

There are two known pathways for NO production in the human body. The first is the endogenous pathway, where L-arginine is converted to NO by nitric oxide synthases (NOS), which until recently was believed to be the sole pathway [4,8]. The second pathway is the exogenous pathway. Following consumption of dietary NO_3^-, it is rapidly absorbed from the stomach and duodenum of the small intestine and enters the blood stream [6]. About 20–25% of the NO_3^- is absorbed from the circulation and concentrated (10 to 20-fold) in the salivary glands [9]. Facultative anaerobes, located in the crypts on the dorsal surface of the tongue, reduce NO_3^- to the bioactive nitrite (NO_2^-), which is swallowed again [4]. Small amounts of this NO_2^- will be reduced to NO in the acidic environment of the stomach, while most re-enters the systemic circulation and is transported to specific locations in the body, where it can be reduced to NO, via one or more potential non-enzymatic or enzymatic pathways [10]. The exact mechanism behind how NO^{2-} enters the circulation and is reduced is still unclear; however, this reduction tends to occur in hypoxic tissue and conditions of low pH [4,10]. This reduction occurs predominately in the vasculature [11]. It is also important to note that through this NO^3–NO^2–NO pathway, endogenous NO^{3-} can be recycled by oral bacteria to act as a reservoir of precursors for NO production and that interruption of this recycling (such as from use of antiseptic mouthwash) has been shown to decrease plasma nitrite levels and raise blood pressure (BP) in individuals in the absence of nitrate intake [4,10].

The ability for consumption of dietary NO_3^- to result in an increased production of NO, via the aforementioned exogenous pathway, has led to greater interest in dietary NO_3^- (particularly in the form of beetroot juice), as a food-based supplement with the potential to improve performance and health [2]. Furthermore, it has been shown that NO production in the body decreases with age and is believed to be a contributor to age-related dysfunction and increased risk of disease [4]. Therefore, more recently, specific interest has been directed at the benefits of NO_3^- supplementation in the older population.

Previous studies have shown that both acute and prolonged supplementation of dietary NO_3^- in older adults can significantly improve oxygen uptake ($\dot{V}O_2$) responses during exercise [8], and increase time to fatigue [5,12,13], thus promoting improved exercise performance. Furthermore, it has also been shown that NO_3^- supplementation can significantly improve cognitive performance, as shown by enhanced reaction times [6] in older adults. However, the investigation into cognitive performance benefits with dietary NO_3^- supplementation is scarce, with equivocal findings [8]. In addition to the potential performance benefits, studies have shown positive health-related outcomes in older adults, with acute and prolonged dietary NO_3^- supplementation, including reduced blood pressure [7,12] and improved blood flow to the muscle and brain [13,14], indicative of potential cardiovascular and cerebrovascular benefits, which may result in a lower risk of certain diseases. As older adults have age-related diminishments in functional capacity of epithelial vasodilation (reducing blood flow), cardiovascular function, cognitive function, and mood, there is greater potential for improvements in these areas with NO_3^- supplementation, relative to younger adults who generally possess optimal functional capabilities [13].

Therefore, the purpose of this systematic review was to critically evaluate the effects of dietary NO_3^- supplementation on performance (physiological and cognitive) and health factors (specifically,

cardiovascular, cerebrovascular and metabolic health) in older adults. This is the first systematic review of its kind, evaluating potential benefits of dietary NO_3^- supplementation, specifically in older adults.

2. Materials and Methods

A systematic literature search was conducted by one researcher (L.S.) to identify primary research in the area of inorganic dietary NO_3^- supplementation in older adults, with the aim of examining performance and health benefits associated with dietary NO_3^- consumption.

2.1. Types of Outcome Measures

The primary focuses of this systematic review were any performance and health parameters affected by dietary NO_3^- supplementation in older adults. This included changes in physiological and cognitive performance, as well as cardiovascular, cerebrovascular, metabolic, and bone health.

2.2. Search Method

The following online databases were systematically searched: PubMed, Ovid, Science Direct and Web of Science, from inception to May 2017. The keywords chosen for the search included: nitrate OR nitrates OR beetroot AND older adults OR elderly. This search was enhanced via manual examination of the reference lists of all retrieved papers in attempts to detect any other potentially eligible studies.

2.3. Inclusion and Exclusion Criteria

The inclusion and exclusion criteria for all retrieved papers were established by the researchers (L.S., A.A., K.R. and R.P.). This systematic review only included primary research published in peer-reviewed journals, in English, using a randomised, crossover, placebo-controlled design. In addition, studies had to meet a set criteria including: (1) participants had to be older adults (aged 50+) or involve older adults in the study as a separate age group, i.e., those comparing younger with older adults; (2) participants could be healthy or possess one or more morbidities (e.g., hypertension, diabetes, peripheral arterial diseases); (3) studies had to investigate the effects of inorganic dietary NO_3^- supplementation (such as beetroot juice or high nitrate diet interventions) on performance and/or health parameters; (4) studies using multiple supplementation protocols, involving other supplements in addition to nitrate, had to show a clear separation in the effects of NO_3^- and the other supplements.

2.4. Study Selection

Following the removal of duplicate studies from the different search engines, independent screening of the title and abstract of the remaining articles was undertaken to determine eligibility. Selected papers were then read in full to finalise eligibility. A summary of this process is outlined in Figure 1.

2.5. Quality Assessment

Risk of bias was assessed informally, using a bias hierarchy checklist, as described by Wright et al. [15], including the following assessment of key areas of bias: selection, performance, detection and attrition bias. Each article was individually assessed by the reviewer, L.S.

2.6. Data Extraction

Data were extracted independently by one of the researchers (L.S.) and reviewed by the other researchers (A.A., K.R. and R.P.), following a systematic selection list. This included: (1) participant data (number of participants, sex distribution, health stasis (i.e., healthy or diagnosed patients), and age); (2) study design; (3) supplementation protocol; (4) testing protocol used; and (5) primary outcome

measures (physiological performance, cognitive function, and health-based parameter). Mean values were extracted for all data, where appropriate.

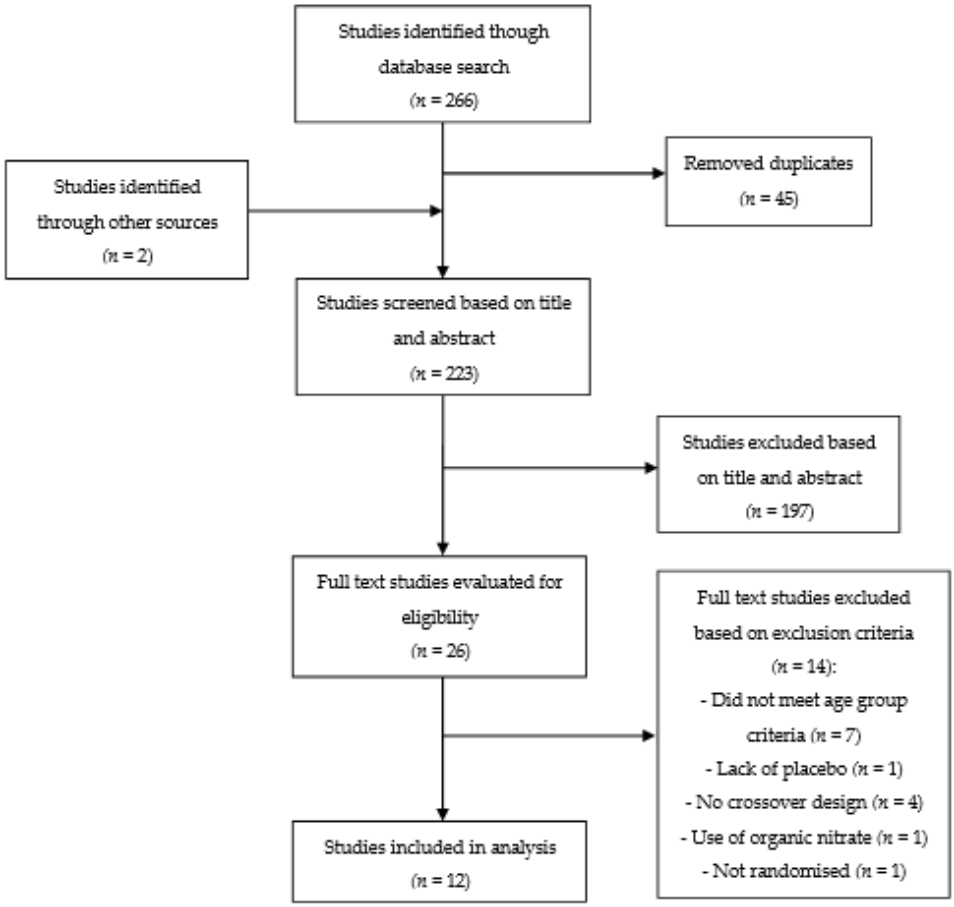

Figure 1. Flowchart showing study selection procedure and results.

3. Results

Twelve studies [5–8,12–14,16–20] out of a total of 266 studies identified from preliminary research of online databases and other sources after reference list screening, were included in this systematic review (Figure 1).

3.1. Participant Characteristics

A summary of the characteristics of the studies is outlined in Table 1. From the 12 included studies, there were a total of 202 participants (95 males, 93 females, and 14 not stated). The number of participants in each trial ranged from 8 [13,18] to 27 [6,17], with mean ages ranging from 59.2 [19] to 74.7 years [14]. These 12 studies targeted older adult participants of varying health status. Five studies recruited healthy older adults [8,14,18–20], two studies recruited older adults with type 2 diabetes [6,17], one study recruited participants with chronic obstructive pulmonary disease (COPD) [5], one recruited participants with heart failure with preserved ejection function (HFpEF) [12], one with peripheral arterial disease (PAD) [13], one with chronic kidney disease (CKD) [7] and one with risk factors for cardiovascular disease [16].

Table 1. Summary of studies investigating the effects of dietary nitrate supplementation on physical performance, cognitive performance, and health-based outcomes in older adults.

Reference	Participants	Study Design	Supplementation Protocol	Testing Protocols	Blood Measures (NO Indices)	Physiological Performance Outcomes	Cognitive Performance Outcomes	Health-Based Outcomes
Berry et al., 2015 [5]	15 COPD patients (m/f 12/3) (age 69.6 ± 8.5)	Randomised, single-blind crossover	Acute supplementation, 2.5 h prior to exercise, 140 mL BR (7.58 mmol NO_3]) (Note PL used was prune juice)	Constant work rate test at 75% max on cycle ergometer	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	↑ Time to exhaustion during submaximal cycle exercise	NT	↓SBP ↓DBP
Eggebeen et al., 2016 [12]	20 HFpEF patients (m/f 3/17) (age 69 ± 7)	Randomised, double-blind crossover	Phase 1: Acute supplementation, 1.5–2 h prior to exercise, 70 mL BR (6.1 mmol NO_3^-) Phase 2: 7 days supplementation, 70 mL·day^{-1} BR (6.1 mmol·day^{-1} NO_3^-)	Constant work rate test at 75% max on cycle ergometer	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	↑ Time to exhaustion during submaximal cycle exercise	NT	↓SBP
Kenjale et al., 2011 [13]	4 male and 4 female PAD patients (age 67 ± 13)	Randomised, open-label, crossover	Acute supplementation, 3 h prior to exercise, 500 mL BR (9 mmol NO_3^-) (Note: orange juice was used as PL)	Walking maximal cardiopulmonary exercise (CPX) test	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	↑ Walking duration longer before onset of pain (18%) ↑ Maximum walking time (17%)	NT	↑ Tissue blood flow (NIRS) ↓DBP ↓SBP and HR during recovery
Siervo et al., 2016 [20]	19 healthy older adults (m/f 9/10) (age 64.7 ± 3)	Randomised, double-blind crossover	7 days supplementation, 2 × 70 mL·day^{-1} BR (12 mmol·day^{-1} NO_3^-)	Incremental cycle test to voluntary exhaustionHand-grip strength testTime up-and-go testRepeated chair raising test10-m walking test	↑ Plasma [NO_3^-] ↑ Urinary [NO_3^-]	↔ O_2 consumption ↔ Hand-grip strength ↔ Up-and-go test ↔ Repeated chair raising test ↔ 10-m walking test	NT	↔ BP
Kelly et al., 2013 [8]	6 male and 6 female older adults (age 64 ± 4; 63 ± 2)	Randomised, double-blind crossover	3 days supplementation, 140 mL·day^{-1} BR (9.6 mmol·day^{-1} NO_3^-)	Walking treadmill test and leg ergometer. Cognitive function tests	↑ Plasma [NO_2^-]	↓$\dot{V}O_2$ response time from rest to walking ↔ 6 m walking test	↔ Cognitive tests	↓SBP ↓DBP
Gilchrist et al., 2014 [6,12]	27 older adults with type 2 diabetes (m/f 18/9) (age 67.2 ± 4.9)	Randomised, double-blind crossover	14 days supplementation, 250 mL·day^{-1} BR (7.5 mmol NO_3^-)	5 cognitive tests on E-Studio software	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	NT	↓ Simple reaction time (improvement)	NT
Presley et al., 2011 [14]	14 healthy older adults (sex not stated) (age 74.7 ± 6.9)	Placebo-controlled, crossover	2 days high NO_3^- diet with BR (~12.4 mmol NO_3^-) Low NO_3^- diet (~0.089 mmol NO_3^-)	NT	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	NT	NT	↑ Cerebral perfusion (MRI)
de Oliveira et al., 2016 [16,17]	20 older adults with risk factors for cardiovascular disease (m/f 7/13) (age 70.5 ± 5.6)	Randomised, double-blind crossover	Acute supplementation, 2 h prior to measurements, 100 g BG (12.2 mmol NO_3^-) (PL used was NO_3^- depleted gel)	NT	↑ Urinary [NO_2^-] ↑ Urinary [NO_3^-]	NT	NT	↑ Endothelial function ↔ BP ↔ Arterial stiffness
Gilchrist et al., 2013 [17]	27 older adults with type 2 diabetes (m/f 18/9) (age 67.2 ± 4.9)	Randomised, double-blind crossover	14 days supplementation, 250 mL·day^{-1} BR (7.5 mmol NO_3^-)	NT	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	NT	NT	↔ BP ↔ MAP ↔ Endothelial function ↔ Insulin sensitivity
Kemmner et al., 2017 [7]	17 older adults with CKD (m/f 7/10) (age 72 ± 6)	Randomised, open-label crossover	Acute supplementation, 4 h prior to repeat measures, 200 mL BR (300 mg NO_3^-) (Note PL used was water)	Hemodynamic parameters and renal resistance index measure	↑ Plasma [NO_3^-]	NT	NT	↓SBP ↓DBP ↓MAP ↓RRI

Table 1. *Cont.*

Reference	Participants	Study Design	Supplementation Protocol	Testing Protocols	Blood Measures (NO Indices)	Physiological Performance Outcomes	Cognitive Performance Outcomes	Health-Based Outcomes
Miller et al., 2012 [18]	8 normotensive older adults (m/f 3/5) (age 72.5 ± 4.7)	4-arm, randomised, crossover	3 days low NO_3^- diet (0.70 mmol NO_3^-) Low NO_3^- diet + supplementation, 500 mL BR (8.5 mmol NO_3^-) High NO_3^- diet (2.50 mmol NO_3^-) High NO_3^- diet + supplementation 500 mL BR	NT	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	NT	NT	↔ BP
Shepherd et al., 2016 [19]	15 healthy older adults (m/f 8/7) (age 59.2 ± 6)	Randomised, double-blind crossover	Acute supplementation, 140 mL BR (11.91 mmol [NO_3^-])	NT	↑ Plasma [NO_2^-] ↑ Plasma [NO_3^-]	NT	NT	↔ BP ↔ Hepatic blood flow (MRI) ↔ Plasma glucose ↔ C-peptide ↔ [Incretin]

All values are mean ± SD. ↑ significant increase;↓ significant decrease; ↔ no significant difference; m/f, males/females; NT, not tested; [NO_2^-], nitrite concentration; [NO_3^-], nitrate concentration; BR, beetroot juice; PL, placebo; BG, beetroot gel; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; RRI, renal resistive index; $\dot{V}O_2$, oxygen uptake; HR, heart rate; NIRS, near infrared spectroscopy; MRI, magnetic resonance imaging; COPD, chronic obstructive pulmonary disease; HFpEF, heart failure with preserved ejection function; CKD, chronic kidney disease; PAD, peripheral arterial disease.

3.2. Study Characteristics

All studies implemented a randomised, crossover design, involving a placebo treatment and at least one NO_3^- treatment. Of the 12 studies, six used a NO_3^- depleted beetroot juice as the placebo [6,8,12,17,19,20], two used a low NO_3^- diet [14,18], and single studies used NO_3^- depleted gel [16], water [7], prune juice [5], or orange juice [13]. Seven studies were double-blinded [6,8,12,16,17,19,20], one was single-blinded [5], and four were open-label trials [7,13,14,18].

3.3. Study Quality

Based on an informal assessment of bias, the studies included in this systemic review were considered to be good to excellent, based on the above-mentioned study characteristics. All studies were placebo controlled, randomised crossover experiments (low selection/detection bias), with the majority being double- or single-blinded (low performance/detection bias).

3.4. Nitrate Supplementation

The majority of the studies (n = 10) utilised supplemented inorganic NO_3^- in the form of beetroot juice, while the remaining two studies used beetroot gel and a high NO_3^- diet, where beetroot juice was included in the diet. The difference in the NO_3^- dose used between studies ranged from 6.1 to 12.4 mmol·day^{-1} (approx. 350–700 mg·day^{-1}). In addition, the supplementation period varied between studies: five studies investigated acute supplementation (consumption 2–4 h prior to testing [5,7,13,16,19]), six investigated chronic supplementation (2.5–14 days of NO_3^- loading [6,8,14,17,18,20]), and one study investigated both acute (1.5–2 h prior) and chronic (7 days) supplementation [12].

3.5. Adverse Effects of Supplementation

Five of the 12 studies [14–18,20] reported minor adverse effects from dietary NO_3^- supplementation; two studies reported beeturia (red urine) [16,20], two studies reported both beeturia and red stools [14,17], and one study reported beeturia, gastrointestinal discomfort and headaches [18]. No chronic adverse effects from NO_3^- supplementation were stated in any of the 12 studies.

3.6. Characteristics of Nitric Oxide Measures

The 12 studies incorporated various measures to investigate the potential changes in NO concentrations in the body following supplementation with dietary NO_3^-, including plasma [NO_3^-] and [NO_2^-], and urinary [NO_3^-] and [NO_2^-] measures. Seven studies reported both plasma [NO_3^-] and [NO_2^-] [5,6,12–14,17–19], one measured solely plasma [NO_2^-] [8], one measured solely plasma [NO_3^-] [7], one measured both plasma and urinary [NO_3^-] [20], and one measured both urinary [NO_3^-] and [NO_2^-] [16]. Similarly, various methods were used to determine the aforementioned values. Five studies used a Sievers NO analyser (chemiluminescence-based) [6,13,14,17,19]; three used an ENO-20 NO analyser (based on colorimetric Griess assay) [5,12,18]; one used high-performance liquid chromatography (HPLC) [16]; one used gas chromatography-mass spectroscopy (GC-MS) [20]; one used an enzyme-linked immunosorbent assay [7]; and one used a modified chemiluminescence method [8].

3.7. Outcomes

3.7.1. Nitric Oxide Indices

Despite the different NO indices used between studies, dietary NO_3^- supplementation resulted in a significant increase in all NO indices (compared to placebo) in all 12 studies. Specifically, increases in plasma [NO_3^-] ranged from 150% [20] to 938% [5], while increases in plasma [NO_2^-] ranged from

168% [17] to 503% [8] and increases in urinary [NO_3^-] were 283% [16] and 979% [20], while the increase in urinary [NO_2^-] was 214% [16], at pre- versus post-supplementation, with dietary NO_3^-.

3.7.2. Performance Outcomes

Physiological Performance

Five of the 12 studies investigated the effects of dietary NO_3^- supplementation on physiological performance in older adults [5,8,12,13,20]. Acute supplementation with dietary NO_3^- (7.58 mmol/400 mg NO_3^-), 2.5 h prior to exercise, significantly improved time to exhaustion during submaximal cycling ($p = 0.031$) [5]. Likewise, 7 days of supplementation with NO_3^- rich beetroot juice (6.1 mmol·day^{-1}/350 mg NO_3^-) also improved time to exhaustion during submaximal cycling exercise ($p = 0.02$) [12]; however, this improvement was not seen during the acute supplementation protocol in the study. Additionally, acute supplementation with NO_3^- rich beetroot juice (9 mmol/500 mg NO_3^-) improved walking duration prior to onset of pain by 18%, and maximal walking time by 17% in older adults with chronic obstructive pulmonary disease (COPD) [13]. Similarly, an increase in the $\dot{V}O_2$ response time from rest to mod rate intensity walking ($p < 0.05$) was shown, following 3 days of supplementation with beetroot juice (9.6 mmol·day^{-1}/550 mg NO_3^-) [8]. Despite this, the same study found no change in a 6-min walking test [8]. Additionally, it was reported that 7 days of supplementation with NO_3^- rich beetroot juice (12 mmol·day^{-1}/700 mg NO_3^-) had no effect on O_2 consumption, a 10-m walking test, a hand-grip strength test, an up-and-go test, or a repeated chair raising test, in healthy older adults [20].

Cognitive Performance

Of the 12 studies, only three investigated the effects of dietary NO_3^- supplementation on cognitive performance in older adults [6,8,14], with only two directly measuring cognitive function [6,8]. The potential for dietary NO_3^- as a cognitive performance aid in older adults consuming a high NO_3^- diet, including beetroot juice (total NO_3^- ~12.4 mmol/700 mg), for 2 days, was shown by an increase in cerebral blood flow to the white matter of the frontal lobe ($p < 0.005$) [14]. Additionally, 14 days of supplementation with NO_3^- rich beetroot juice (250 mL·day^{-1}, ~7.5 mmol/400 mg NO_3^-) in older adults (67.2 ± 4.9 years) with type 2 diabetes, resulted in a significant improvement in simple reaction time, compared to a placebo [6]. Despite this, there was no difference between treatments for other cognitive tests, including decision time, rapid processing, and spatial and shape memory [6]. Investigation of the effects of NO_3^- rich beetroot juice supplementation over 3 days (2 × 70 mL·day^{-1}, ~9.6 mmol/550 mg of NO_3^-) on cognitive performance, in healthy, older adults (aged 60–70), showed no significant differences in memory, attention and information processing capabilities between the beetroot juice and placebo treatments [8].

3.7.3. Health Outcomes

Cardiovascular Health

Ten studies investigated the potential benefits of dietary NO_3^- supplementation on BP in older adults. Five of these studies showed that dietary NO_3^- supplementation resulted in a significant decrease in systolic blood pressure (SBP) [5,7,8,12,13], with four of the five studies also showing a significant decrease in diastolic blood pressure (DBP) following NO_3^- supplementation, compared to a placebo [5,7,8,13]. In contrast, five studies found no change in the BP of older adults after supplementation with dietary NO_3^- [16–20]. There were two studies that did not measure changes in BP [6,14]. Furthermore, two studies also investigated mean arterial pressure (MAP) (the average pressure in arteries from one cardiac cycle) following beetroot juice supplementation. Gilchrist et al. [17] found no difference between treatments (BR, 7.5 mmol/400 mg NO_3^- vs. nitrate-depleted PL) in older adults with type 2 diabetes, whereas Kemmner et al. [7] showed a

decrease in MAP in older adults with CKD, following acute supplementation with NO_3^- rich beetroot juice (200 ml, 6 mmol/350 mg NO_3^-) ($p = 0.012$) [7]. Three studies investigated endothelial function and blood flow in older adults in response to dietary NO_3^- supplementation [13,16,17]. Acute beetroot juice supplementation in older adults (67 ± 13 years) with PAD was found to increase blood flow to the working muscles, using near infrared spectroscopy (NIRS) [13]. Acute supplementation with 100 g of NO_3^- rich beetroot gel (12.2 mmol/700 mg NO_3^-) in older adults with risk factors of cardiovascular disease has also been reported to significantly improve brachial flow-mediated dilation (FMD) (77%), blood flow velocity (BFV) (31%), and reactive hyperaemia (RH) (18%), thus improving endothelial function [16]. However, 14 days of supplementation with NO_3^- rich beetroot juice in older adults with type 2 diabetes had no significant effect on FMD or Doppler perfusion, and thus, did not improve endothelial function [17].

The study by Kemmner et al. [7] also reported a decrease in the renal resistance index (RRI) ($p = 0.017$) following acute supplementation with NO_3^- rich beetroot juice in older adults with CKD.

Cerebrovascular Health

Only one study has investigated the potential cerebrovascular health benefits of dietary NO_3^- supplementation in older adults [14]. This study used magnetic resonance imagining (MRI) to show increased perfusion in the frontal cortex of the brain ($p < 0.005$) of older adults following 2 days of a high NO_3^- diet (including beetroot juice), compared to a diet low in NO_3^- [14].

Metabolic Health

Of the 12 studies, two investigated various metabolic health parameters following dietary NO_3^- supplementation in older adults [17,19]. Chronic supplementation with NO_3^- rich beetroot juice in older adults with type 2 diabetes had no significant effect on insulin sensitivity, measured via the hyperinsulinemic isoglycemic clamp technique [17]. Furthermore, acute supplementation with NO_3^- rich beetroot juice was also shown to have no significant effect on acute plasma glucose, hepatic blood flow, C-peptide concentration, or incretin concentration (glucagon-like peptide-1 (GLP-1)), in older adults with type 2 diabetes [19].

4. Discussion

This systematic review investigated the effects of NO_3^- supplementation on performance and health-related outcomes in older adults. The current literature indicates positive effects of dietary NO_3^- supplementation in older adults on physiological performance, with some evidence indicating benefits on cardiovascular and cerebrovascular health. Effects on cognitive performance were mixed and studies on metabolic health indicated no benefit.

4.1. Nitrate Supplementation and Physiological Performance

Dietary NO_3^- supplementation has the potential to improve physiological performance, by prolonging time to exhaustion and increasing the $\dot{V}O_2$ response time in older adults. Four of the five studies that investigated physiological performance in older adults found positive outcomes in time to exhaustion during submaximal exercise [5,12,13], and in $\dot{V}O_2$ response time [8], while only one study showed no significant effects on physiological performance parameters [20]. This is a similar finding to the systematic review and meta-analysis conducted by Hoon et al. [21] on the effects of dietary NO_3^- supplementation in exercise performance of healthy, young individuals, who reported a pooled effect size of 0.79 for time to exhaustion trials, with favourable benefits from beetroot juice supplementation.

Two studies reported an increase in total exercise capacity following acute supplementation of NO_3^- rich beetroot juice, during submaximal cycling and walking exercise in older adults with COPD and PAD, respectively [5,13]. In contrast, another study found no significant effect of acute beetroot juice supplementation on time to exhaustion during submaximal cycling exercise in older adults with

HFpEF [12]. The disparity in findings between these studies may have been due to variances in study design. The two studies which reported improvements in time to exhaustion used an open-label design, where the drinks for the control trials were prune juice [5] and orange juice [13], which could have produced a 'placebo' effect; however, Eggebeen et al. [12] used a double-blind design with NO_3^- depleted beetroot juice as the control. Moreover, Berry et al. [5] and Kenjale et al. [13] also utilised higher dosages of NO_3^- and longer absorption periods (7.58 mmol NO_3^-, 2.5 h prior, and 9 mmol NO_3^-, 3 h prior, respectively), while Eggebeen et al. [12] had participants consume 6.1 mmol NO_3^-, 1.5–2 h prior to exercise. These reductions in dosage and absorption times could have prevented adequate concentration and/or time for the supplementation to elicit an effect, especially when, in the same study by Eggebeen et al. [12], while no acute effect was seen, they did show an increased time to exhaustion, after 7 days of dietary NO_3^- supplementation. Nevertheless, another study showed no effect of 7 days supplementation (12 mmol·day^{-1} NO_3^-) on time to exhaustion during an incremental cycle test [20]; thus, the discrepancies between the studies could be due to other factors.

Siervo et al. [20] also found no significant improvements in O_2 consumption, grip strength, up-and-go test, repeated chair raises, or 10-m walking time. However, upon closer examination of the results there was a trend ($p = 0.10$) for improved time to exhaustion and an improvement in all tests, following beetroot versus placebo treatment [20]. This may be a result of insufficient sample sizes to portray the full effects ($n = 19$). Additionally, the absence of significant differences in this study may have been due to the use of healthy older adults, where the aforementioned studies who showed improvements utilised COPD [5], PAD [13], and HFpEF [12] patients who already had diminished physiological functions, and thus had a greater potential to benefit from dietary NO_3^- supplementation [4].

The aforementioned improvements in time to fatigue following NO_3^- supplementation may be due to enhanced blood flow to the working muscle, improvements in mitochondrial efficiency, and/or improved muscle contractile function [22]. There is strong evidence to suggest that supplementation of dietary NO_3^- results in a significant reduction in both systolic and diastolic blood pressure in older adults, which is indicative of increased vasodilation and therefore enhanced blood flow [7,8]. Kenjale et al. [13] showed that, alongside the physiological performance benefits of beetroot juice supplementation, participants also experienced significant drops in DBP, SBP and heart rate (HR), indicating NO-mediated vasodilation and increased blood flow to the working muscles. Near infrared spectroscopy (NIRS) also showed increased muscle tissue oxygenation following supplementation with NO_3^- rich beetroot juice [13]. Increased blood flow to the working muscle allows for greater transport of oxygen, which can result in more efficient metabolic control and prolonged time to fatigue at a given workload, thus improving physiological performance [22].

Furthermore, NO_3^- rich beetroot juice has been shown to improve mitochondrial efficiency via up-regulation of the protein, adenosine nucleotide translocase (ANT), which is involved in the control of flow of mitochondrial protons, resulting in a reduction in proton leakage and increased efficiency of oxidative phosphorylation [23]. In addition, dietary NO_3^- supplementation increased the mitochondrial phosphate to oxygen (P/O) ratio (the amount of ATP produced from a given amount of oxygen reduced), thus reducing the amount of O_2 required to maintain a given workload and prolonging time to exhaustion [23].

Bailey et al. [24] used ^{31}P-magnetic resonance spectroscopy to show a decrease in utilisation of skeletal muscle phosphocreatine (PCr), along with a decrease in production of adenosine diphosphate (ADP) and inorganic phosphate (P_i), indicating a reduction in the rate of ATP consumption following consumption of NO_3^- rich beetroot juice. This effect indicated that supplementation with dietary NO_3^- (via NO mediated effects) may alter the ATP consumption rates of actomyosin-ATPase and Ca^{2+}-ATPase during muscle contraction [24]. Therefore, the reductions in ATP utilisation from these two ATPases during contraction would result in the ability to perform a given work rate for longer, thus prolonging time to exhaustion [25].

Collectively, the results indicate that dietary NO_3^- supplementation can result in improved blood flow and mitochondrial efficiency, and a reduced rate of ATP utilisation, leading to a prolonged time to exhaustion during submaximal exercise [22]. It is important to note that some of the results were based on studies using younger adults [23–25]; however, it has been suggested that the same underlying mechanisms would be present in the older population [2]. Additionally, this emphasises the need for further research into the effects on dietary NO_3^- supplementation in older adults.

4.2. Nitrate Supplementation and Cognitive Performance

Older adults show reductions in cerebral blood flow (CBF), which may be due to an age-related decrease in the production of NO [4]. This reduction in blood flow to the brain has been indicated as a major risk factor for the impairment of cognitive function and development of neurodegenerative diseases, such as dementia [8]. Therefore, supplementation with NO_3^- rich beetroot juice could have greater benefits on cognitive function in older adults, via its NO mediated increase in CBF [14]. However, in comparison to the effects of dietary NO_3^- supplementation on physiological performance, the effects on cognitive performance in older adults have only just begun to be investigated, thus the effects are less clear [4].

Only two of the 12 studies directly measured the effects of dietary NO_3^- supplementation on cognitive performance in older adults [6,8]. One study showed an improvement in cognitive performance, via a reduction in simple reaction time, with beetroot juice consumption compared to placebo, following a chronic dosing protocol (14 days of NO_3^- rich beetroot juice, 250 mL·day^{-1}, ~7.5 mmol NO_3^-), in type 2 diabetic, older adults (67.2 ± 4.9 years) [6]. However, they found that performances in decision time, rapid processing, spatial and shape memory tests were not significantly different. The earlier study by Kelly et al. [8] found no effect of dietary NO_3^- supplementation on cognitive performance following 3 days of supplementation with beetroot juice (2 × 70 mL·day^{-1}, ~9.6 mmol of NO_3^-) in healthy, older adults (males 64 ± 4 and females 63 ± 2 years). Specifically, they reported no significant differences in memory, attention and information processing capabilities between beetroot juice and placebo treatments.

The differences in cognitive performance results seen between these two studies could be attributed to methodological variations, including the duration of supplementation and the cognitive tests used. Gilchrist et al. [6] used a longer supplementation period (14 days) and included a simple reaction test; however, Kelly et al. [8] used a much shorter supplementation period (3 days) and did not include a simple reaction test. Additionally, participant selection may have impacted results as one study used older adults with type 2 diabetes [6], while the other used healthy older adults [8]. Therefore, the findings of this systematic review suggest that there may be some potential cognitive performance benefits for older adults following supplementation of dietary NO_3^-; however, these benefits may be specific to certain brain functions, and may be affected by the duration of supplementation as well as participant health status. Nevertheless, with only two studies to draw conclusions from, which provide conflicting results, more research is required to investigate these potential effects.

4.3. Nitrate Supplementation and Health

4.3.1. Cardiovascular

Investigations into the possible cardiovascular health benefits of NO_3^- supplementation in older adults has grown over the past 5 years, with a significant amount of research examining the effects on BP [2]; more recently, the effects on MAP [7], changes in blood flow [13], and endothelial function [16] have become additional areas of examination.

Due to age-related losses in NO production from decreased activity of NOS, older adults are at a higher risk for endothelial dysfunction and cardiovascular disease, of which, high BP and MAP are major risk factors [2]. Supplementation with dietary NO_3^- increases NO production, which, in

turn, has been shown to activate the enzyme, guanylate cyclase, which catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) [24]. Cyclic guanosine monophosphate acts on smooth muscle, causing relaxation, which results in vasodilation of arteries and veins, thus decreasing BP and reducing the risk of cardiovascular disease [8]. Despite this, the findings of this review indicate mixed results for the effects of acute and chronic dietary NO_3^- supplementation on BP and MAP in older adults. Whilst five of the reviewed studies reported a reduction in SBP [5,7,8,12,13], with four of these also showing a reduction in DBP [5,7,8,13], a further five studies found no benefits [16–20]. Additionally, a more recent study found a reduction in MAP [7]; however, an earlier study showed no significant change [17].

The reasons for these conflicting results for BP may be due to differences in the health statuses of the participants. Of the five studies that reported a reduction in BP, only one used healthy older adults [8], while the others used older adults with COPD [5], CKD [7], HFpEF [12], or PAD [13]. Comparatively, three of the five studies which showed no significant change in BP, used healthy older adults [18–20], while one [16] used participants with risk factors for cardiovascular disease (not yet morbid), and one [17] used type 2 diabetic older adults. These differences could suggest that supplementation with dietary NO_3^- may be more beneficial for reducing BP in older adults with morbidities closely related to an already reduced endothelial function, beyond that of age-related changes. Furthermore, the study by de Oliveira et al. [16] used a shorter absorption time of 2 h compared to the more common 2.5–4 h, used by studies which have shown significant reductions in BP [5,7]. This may not have provided sufficient time to allow for significant reductions in BP. In addition, Gilchrist et al. [17] investigated effects on BP while participants were taking antihypertensive and hypoglycaemic medication, which is likely to have impacted the results, as the participants' baseline BPs would have already been lowered, due to the antihypertensive medication. However, one study has shown a decrease in BP in hypertensive patients, aged 18–85, following supplementation with NO_3^--rich beetroot juice, while still adhering to their antihypertensive medication protocol, suggesting improvements may still be possible with supplementation in conjunction with drug protocols [26].

Alternatively, the conflicting results seen between these studies may have been due to differences in study design. Three of the studies which reported beneficial outcomes on BP in older adults following dietary NO_3^- supplementation used prune juice [5], water [7] and orange juice [13] as the placebo controls, while all other studies [8,12,16–20], utilised a NO_3^- depleted beetroot control, so there was potential for a 'placebo' effect. Also, as these three studies [5,7,13] utilised beetroot juice as the NO_3^- source, but did not utilise a NO_3^- depleted beetroot control, it is possible that other bioactive compounds found in beetroot juice (e.g., antioxidants and polyphenols) may have contributed to the reduction in BP [27]. However, other studies using a NO_3^- rich beverage and NO_3^- depleted beetroot juice (placebo) crossover design have still shown reductions in BP of older adults [8]. Furthermore, an investigation into the interruption of the NO_3^-–NO_2^-–NO pathway, via the use of mouthwash or spitting of saliva following beetroot juice consumption, has been shown to negate the reductive effects on BP, indicating high concentrations of NO_3^- as the major contributor to a decreased BP [4]. The reasons for mixed results in MAP are likely similar to those mentioned above; including the continued use of participants' antihypertension medications [17], or the use of an inappropriate study design (open-labelled with the placebo being water [7]).

In addition to reductions in BP and MAP, supplementation with dietary NO_3^- in older adults has also been proposed to benefit cardiovascular health of older adults through its effects on blood flow and endothelial function [12]. Two studies found that acute supplementation with NO_3^- rich beetroot gel (12.2 mmol NO_3^-, 2 h prior) [16] or juice, (9 mmol NO_3^-, 3 h prior) [13] improved endothelial function and blood flow in older adults. This was expected, as foods high in NO_3^- have been shown to inhibit blood platelet aggregation and reverse or prevent further degradation in endothelial function [28], which is particularly important in older adults, as aging is associated with reduced blood flow and endothelial dysfunction [14]. Therefore, NO_3^- supplementation plays an important role in maintaining NO homeostasis when endogenous production may be diminished, thus

maintaining adequate blood flow and distribution [28]. Despite these results, Gilchrist et al. [17] found no significant differences in endothelial function following 14 days of NO_3^- supplementation in older adults with type 2 diabetes, contradicting the aforementioned findings. As mentioned above, a major limitation in the study by Gilchrist et al. [17] was the continued use of participants' antihypertensive medications, which are designed to promote vasodilation and subsequently increase blood flow, [2,4]. Therefore, it is unlikely that supplementation with dietary NO_3^- would promote further benefits to result in improved endothelial function. Based on this, the lack of improvements in blood flow and endothelial function seen by Gilchrist et al. [17] are as expected.

Kemmner et al. [7] reported a reduced RRI in older adults with CKD following supplementation with 200 mL of NO_3^- rich beetroot juice (~6 mmol/300 mg NO_3^-). Chronic kidney disease is another major risk factor for CVD, where hypertension can both result from, or be a large contributor to, the development of the disease [7]. Furthermore, a high RRI is associated with an increased risk of cardiovascular dysfunction [7]. Therefore, as older adults are at a greater risk of kidney dysfunction, due to age-related changes, a reduced RRI following supplementation with dietary NO_3^- is likely to result in reduced risk of a cardiovascular event, thus improving cardiovascular health [7]. It is also important to note that CKD and high RRI can impact type 2 diabetes, potentially affecting metabolic heath as well.

Despite the mixed results reported for changes in the BP and MAP of older adults following dietary NO_3^- supplementation, the review of the published data indicates that there is potential for beneficial effects which can result in improved cardiovascular health. Furthermore, some evidence from the reviewed studies also indicates potential benefits to cardiovascular health of older adults, via improved blood flow, endothelial function and/or RRI post-supplementation with dietary NO_3^-. Therefore, the conflicting results warrant further research, to fully evaluate the extent to which dietary NO_3^- supplementation impacts cardiovascular health in older adults. Additionally, specific attention should be drawn to participant health status and the use of a double-blind study design and NO_3^- depleted control.

4.3.2. Cerebrovascular

Only one study [14] met the inclusion criteria for examining the relationship between dietary NO_3^- consumption and cerebrovascular health. Presley et al. [14] showed increased perfusion in the frontal cortex of the brain (using MRI), of older adults following a diet high in NO_3^- with additional beetroot juice supplementation. This suggests that dietary NO_3^- supplementation may attenuate the age-related drop in NO activity and loss of cerebral blood flow (a major contributor to neurogenerative diseases) [5], thus potentially slowing or even preventing the development of cerebrovascular dysfunction. However, with only one study to draw conclusions from, cause and effect cannot be confirmed. Additionally, no study to date has directly investigated the long-term effects of dietary NO_3^- supplementation on the development of neurodegenerative diseases. Therefore, further research is required to investigate the effects of dietary NO_3^- supplementation on specific diseases of the brain and determine its possible benefits to cerebrovascular health in older adults.

4.3.3. Metabolic

The current systematic review found two studies which examined the relationship between metabolic health and dietary NO_3^- supplementation in older adults, both focusing predominately on insulin sensitivity and changes in plasma glucose levels [17,19]. Nitric oxide helps shuttle glucose absorbed in the small intestine into skeletal muscle [29]. A reduction in NO bioavailability has been shown to result in a decrease in insulin secretion and reduced activation of GLUT4 transporter proteins, and thus a subsequent drop in glucose uptake [19,27]. Therefore, older adults may have an age-related drop in insulin sensitivity, due to a reduction in NO production [17]. Therefore, supplementation with dietary NO_3^- in older adults may improve insulin sensitivity, improving metabolic health. However, the results from the studies included in this review do not support this suggestion.

Neither acute supplementation of NO_3^- rich beetroot juice (140 mL, ~11.91 mmol/600 mg NO_3^-) in healthy older adults [19] nor chronic supplementation (250 mL·day^{-1}, ~7.5 mmol/400 mg NO_3^-) in type 2 diabetic older adults [17] resulted in significant differences in plasma glucose levels or insulin sensitivity. These results suggest that dietary NO_3^- supplementation in older adults does not affect metabolic health via improvements in insulin sensitivity. However, it is important to note that there were some limitations to these studies. Gilchrist et al. [17] had participants continue their prescribed hypoglycaemic medications, which are designed to increase insulin secretion or improve insulin action [30], thereby potentially masking any potential metabolic effects of dietary NO_3^- supplementation. Additionally, Shepherd et al. [19] used healthy older adults with a younger mean age (59.2 ± 6 years) compared to other studies, which may have impacted results, as age-related NO dysfunction may not become an issue until individuals are in their mid-60 s [4], thus, the potential benefits would not be as great in the early stages of older adulthood. With only two studies examining the metabolic health benefits of dietary NO_3^- supplementation in older adults, further investigation is required to provide a stronger evidence base, to allow more in-depth analyses and conclusions to be made.

Certain limitations for this systematic review should be noted. Firstly, there was incomplete retrieval of identified research, as only English articles were included in this systematic review. Secondly, studies were generally characterised by small sample sizes and there were few studies investigating some outcome variables, which may have affected the reliability of the conclusions drawn in this systematic review. A third limitation to our analysis was the lack of a meta-analysis. Due to the small number of studies for each outcome variable, completing a meta-analysis would have resulted in weak representation of the data, thus, it is suggested that a meta-analysis be conducted in the future, once more studies have been published. This would be beneficial because it would provide greater statistical evidence on the effects of dietary NO_3^- supplementation in older adults.

5. Conclusions

In summary, the findings of this systematic review provide convincing evidence for improved physiological performance (increased time to fatigue), in older adults following dietary NO_3^- supplementation. Some evidence suggests positive outcomes of dietary NO_3^- supplementation on cardiovascular health (reduced BP and MAP, increased blood flow and improved endothelial function, and reduced RRI) and cerebrovascular health (increased CBF), while results are mixed as to whether dietary NO_3^- supplementation has benefits on cognitive performance. Metabolic health in older adults does not seem to be improved via changes in insulin sensitivity or plasma glucose levels following dietary NO_3^- supplementation; however, more research is required before firm conclusions can be made. Furthermore, it is evident that these effects may be influenced by the dosage of NO_3^-, duration of supplementation, blinding/placebo-control, and health status of participants. Therefore, further research is required in this area, with a focus on randomised, double-blind, NO_3^- depleted control-based studies, with particular attention to participant health status.

Author Contributions: L.S., A.A., K.R. and R.P. all contributed to the concept of the review; L.S. designed method of the review, which was reviewed by A.A., K.R. and R.P.; L.S. performed the database search and paper selection for the review; L.S. wrote the review; A.A., K.R. and R.P. edited and revised manuscript; L.S., A.A., K.R. and R.P. approved final version of manuscript.

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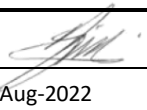
Primary Supervisor's Signature:	
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Appendix 3 - Acute study published version

Appendix 3.1 – Chapter 5 published article

nutrients

MDPI

Article

Acute Supplementation with Nitrate-Rich Beetroot Juice Causes a Greater Increase in Plasma Nitrite and Reduction in Blood Pressure of Older Compared to Younger Adults

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Abstract: Nitrate-rich beetroot juice supplementation has been shown to improve cardiovascular and cognitive function in younger and older adults via increased nitric oxide production. However, it is unclear whether the level of effects differs between the two groups. We hypothesized that acute supplementation with nitrate-rich beetroot juice would improve cardiovascular and cognitive function in older and younger adults, with the potential for greater improvements in older adults. Thirteen younger (18–30 years) and 11 older (50–70 years) adults consumed either 150 mL of nitrate-rich beetroot juice (BR; 10.5 mmol nitrate) or placebo (PL; 1 mmol nitrate) in a double-blind, crossover design, 2.25 h prior to a 30-min treadmill walk. Plasma nitrate and nitrite concentrations, blood pressure (BP), heart rate (HR), cognitive function, mood and perceptual tests were performed throughout the trial. BR consumption significantly increased plasma nitrate ($p < 0.001$) and nitrite ($p = 0.003$) concentrations and reduced systolic BP ($p < 0.001$) in both age groups and reduced diastolic BP ($p = 0.013$) in older adults. Older adults showed a greater elevation in plasma nitrite ($p = 0.038$) and a greater reduction in diastolic BP ($p = 0.005$) following BR consumption than younger adults. Reaction time was improved in the Stroop test following BR supplementation for both groups ($p = 0.045$). Acute BR supplementation increased plasma nitrite concentrations and reduced diastolic BP to a greater degree in older adults; whilst systolic BP was reduced in both older and younger adults, suggesting nitrate-rich BR may improve cardiovascular health, particularly in older adults due to the greater benefits from reductions in diastolic BP.

Keywords: nitric oxide; beetroot juice; blood pressure; cognition; aging

1. Introduction

The world's population is aging, with the number of people aged over 60 years expected to increase from 901 million to 1.4 billion by 2030 [1]. Rates of disease and age-related dysfunction are increasing rapidly [2] and are expected to cause a dramatic increase in costs in the health and disability services areas [1]. This has led to an increased interest in the use of food-based supplements and bioactive compounds to improve or maintain health and body functions [3].

Beetroot juice contains high levels of nitrate (NO_3^-) which can be converted into the bioactive form, nitric oxide (NO) [4]. Nitric oxide plays a major role in many signaling pathways and biological processes, including improving neurotransmission and blood flow, alterations in mitochondrial oxygen consumption, promotion of cognitive benefits, mood, and cardiovascular function [2,4]. Currently, two major pathways are known to result in the generation of NO in humans. First, through the conversion of l-arginine to NO by the enzyme nitric oxide synthase (NOS), which is considered the dominant pathway. Secondly, via the reduction of dietary NO_3^- to NO_2^- (nitrite), which occurs in the mouth by lingual anaerobic bacteria [4]. Nitrite is then swallowed and ultimately enters the bloodstream where it can be reduced further to NO [5]. It is through this second, exogenous pathway of NO production that nitrate-based food supplements such as BR are likely to exert their potential health benefits [4,6].

Acute supplementation with BR can improve physiological responses such as cardiovascular function in younger adults [7–9]. Specifically, consumption of inorganic NO_3^-, either in the form of sodium nitrate or BR, has been shown to result in a significant increase in plasma NO_2^- levels and reduction of blood pressure in both younger (8 and 6 mmHg; systolic and diastolic blood pressure; respectively) [10] and older [4] (5 and 3 mmHg; systolic and diastolic blood pressure; respectively) adults. This positive effect on blood pressure has led to the suggestion that beetroot could potentially be used in medical settings as an alternative to traditional blood pressure-lowering drugs [11].

Acute supplementation with BR (7.5 mmol nitrate) has been shown to improve simple reaction times in older adults [12]. This effect may be due to an increase in blood flow to the brain which has been observed in older adults following consumption of dietary NO_3^- [13]. Cognitive diseases have been linked to age-related reductions in cerebral blood flow (CBF) therefore nitrate supplementation may slow the process of age-related cognitive decline due to the aforementioned increased perfusion to the brain [13,14]. However, other studies have shown no improvement in cognitive function or mood with acute or 3 days of NO_3^- supplementation in older adults [4,6]. The equivocal findings are likely due to methodological differences in the cognitive tests used, the duration of supplementation (from 2 h to 14 days), and amount of NO_3^- consumed (6–12 mmol NO_3^-).

Aging leads to a decline in body processes and functions resulting in increased blood pressure, reduced blood flow, and reduced oxygen delivery to the muscle. All of these measures have been shown to be improved following BR supplementation in both younger and older adults, however, data presented for older adults is limited [4,5]. There may be greater potential for improvement in cardiovascular and cognitive function, and mood following NO_3^- supplementation in older adults relative to younger adults due to age-related changes [15]. Few studies have examined the health benefits of BR supplementation in older adults, and, to our knowledge, only one study directly compared the effects in younger versus older adults [16], therefore further research in this area is required.

We hypothesized that acute supplementation with nitrate-rich beetroot juice would provide a greater improvement in cardiovascular responses, cognition, mood and perception in older adults (50–70 years) compared to younger adults (18–30 years). To examine this hypothesis we conducted a randomized, double-blind crossover trial to investigate and compare the effects of acute supplementation with nitrate-rich beetroot juice on plasma nitrate and nitrite concentrations, blood pressure and cognitive performance measures in older and younger adults.

2. Materials and Methods

2.1. Sample Size

A power analysis was conducted (G-Power 3.1) to calculate the sample size based on the primary outcome measure of blood pressure. Previous literature in healthy adults has shown a mean change in BP of 10 mmHg (9 SD) between BR (9.6 mmol nitrate) and control [4]. Based on this, the required sample size was 11 per group, using an SD of 9, with a statistical power of 0.82 and α-level set at 0.05.

2.2. Participants

Twenty-four healthy, normotensive, recreationally active adults (13 younger, 18–30 years, and 11 older, 50–70 years) volunteered for this study. Participants completed a health-screening questionnaire to ensure suitability. We did not recruit tobacco smokers, users of nitrate-based dietary supplements or well-trained/elite athletes (defined as those completing >4 training sessions per week, or >6 h of intense exercise per week, or having played/playing for a national or age-equivalent sports team). Those with known diseases (e.g., dementia and cardiovascular disease) were also excluded. Prior to testing, all participants were informed of the procedures, associated risks, and the possible benefits of participation, after which written informed consent was obtained. The study was approved by the Massey University Human Ethics Committee (SOA 16/27) and registered with ACTRN (ACTRN12618001466235).

2.3. Intervention

Beetroot juice, extracted from fresh washed beetroots (beta vulgaris 'Pablo'), was blended with other fruit juices to provide a standardized beetroot juice drink with a constant soluble solid concentration (11° Brix) and nitrate concentration (10.5 mmol/150 mL). The placebo beetroot drink was produced using beetroot juice concentrate and standardized to the same constant soluble solids concentration (11° Brix) but contained a low nitrate concentration (1 mmol/150 mL). The BR and placebo juice drinks had previously been presented to a consumer sensory panel using a triangle test, with 25 participants, and were not perceived as significantly different ($p > 0.05$). The nitrate concentration was analyzed using high-performance liquid chromatography (HPLC) based on the method of Cheng and Tsang [17]. Briefly, isocratic separation was achieved using a Gracesmart C-18 column; mobile phase 0.01 M Octylammonium orthophosphate (pH 3–3.5) at a flow rate of 0.8 mL/min. Detection was carried out at 193 nm for nitrite and 213 nm for nitrate.

2.4. Study Design and Procedures

Participants were required to visit the laboratory on three separate occasions. During the first visit, they were familiarized with the testing procedure and equipment, including cognitive, perceptual and mood tests, and body weight (kg) and height (m) were measured using digital scales and a stadiometer, respectively. Participants were instructed to complete a food diary for two days prior to the second study visit and to replicate their diet and lifestyle factors for the third study visit (dietary nitrate was not restricted, in an attempt to investigate additional benefits of BR in conjunction with normal daily intake; however, the food intake diary was used to evaluate intake of dietary nitrate and correlated to baseline plasma nitrate and nitrite concentrations). Participants were asked to arrive at the laboratory in a well-rested, fasted state and to refrain from excessive exercise and alcohol consumption 24 h prior and caffeine 6 h prior to each study visit. All trials were performed in the morning at approximately the same time of day (±2 h).

The second and third visits were the supplementation trials, with each participant randomly allocated (using a random number generator) the order of consumption of the two beverages in a double-blind, crossover design. For these two study visits, participants consumed 150 mL of beetroot juice (BR; containing 10.5 mmol NO_3^-) during one visit and 150 mL of a placebo (PL; containing 1 mmol NO_3^-), on the other visit. A 150-mL beverage was used to obtain approximately 10.5 mmol of nitrate as this amount has been shown to be a sufficient dose to elicit beneficial effects [4]. Drinks were consumed with a standardized, isocaloric breakfast (cereal or toast) with the same macronutrient breakdown (15 g protein, 30 g carbohydrates and 10 g fat). Participants consumed the same breakfast type for both supplementation trials. Visits two and three were separated by a one-week washout period providing sufficient time for normalization of NO_3^- and NO_2^- levels in the body [8].

The protocol for visits two and three is illustrated in Figure 1. Prior to consumption of the beverage, baseline measures of blood pressure (mean value of three measurements taken from the

left arm: deluxe HEM-7130; OMRON Healthcare CO. Ltd.; Kyoto, Japan), heart rate (A1 polar chest transducer), cognitive function, perceptual and mood tests were completed followed by collection of a resting blood sample. Participants then consumed the allocated drink with breakfast, within a 10-min period. Participants were asked to remain in the laboratory for a 2.25 h absorption period while a home development show was aired for entertainment purposes (has minimal effect on cognitive stimulation or arousal levels [4]), or participants could complete computer work or study. The 2.25 h absorption period was chosen as previous research has shown that peak plasma nitrite concentrations occur 2–3 h after beetroot juice consumption [18]. After 2.25 h, the aforementioned measurements were repeated. Blood samples were also taken again 3.25 h post-supplementation.

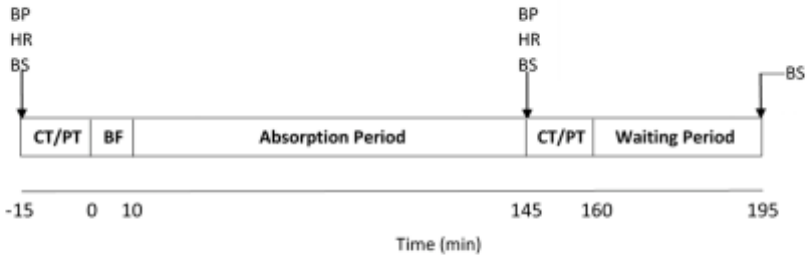

Figure 1. Schematic of the study protocol. CT, cognitive tests; PT, perceptual tests; BF, breakfast; BP, blood pressure; BS, blood sample; HR, heart rate.

2.5. Blood Measurements

Plasma nitrite and nitrate were used as biomarkers for nitric oxide availability [19]. Six milliliter venous blood samples were taken by venepuncture, from a vein within the antecubital area, and collected into heparinized tubes. Samples were mixed, centrifuged (MF–50 Hanil Science Industrial, Incheon, South Korea) at 3500 rpm (1330 g) for 10 min, and the collected plasma aliquoted into Eppendorf tubes (0.5 mL per tube) and stored at −80 °C for later analysis of nitrite and nitrate concentrations by HPLC [20].

2.6. Cognitive Measurements

Cognitive tests were completed in a quiet, isolated room using a computer placed at eye level (PsychoPy software, version 1.83.04 [21]). The cognitive tasks included the choice reaction test (CRT), rapid visual information processing (RVIP), and Stroop tests. Each task was performed a total of four times per participant (two per trial; pre- and 2.5 h post-supplementation). These tests have been used to investigate the effects of nutritional supplementation on executive function, attention, and information processing speed [4,22].

2.6.1. Choice Reaction Test (CRT)

The CRT task involves participants pressing the left-hand key when the word 'LEFT' appears and the right-hand key when the word 'RIGHT' appears.

2.6.2. Rapid Visual Information Processing (RVIP)

The RVIP test involves participants following a chain of single numbers from 1 to 9, which appear in a pseudo-random order at the rate of 100 per minute. The aim of the test is to correctly identify target sequences (2–4–6, 4–6–8, and 3–5–7) as quickly as possible.

2.6.3. Stroop Test

The Stroop test involves the display of different color names ('RED', 'GREEN', and 'BLUE') which appear on the screen, one at a time in different colored fonts (red, green, or blue). The aim of this test is to press the key corresponding to the color of the font that the present word was displayed in.

2.7. Mood and Perceptual Measurements

The feeling scale (FS) was used to measure the degree of displeasure or pleasure based on an 11-point scale ranging from −5 (very bad) to +5 (very good) [23]. The felt arousal scale (FAS) was used to measure the degree of arousal-activation based on a 6-point scale ranging from 1 (low arousal) to 6 (high arousal) [24]. The profile of mood states (POMS) was used to evaluate mood based on ranking how one feels, on a 5-point Likert scale ranging from 0 (not at all) to 4 (extremely) for each word presented under the 7 mood states (fatigue, anger, vigor, tension, esteem, confusion, and depression) [25]. The FS, FAS and POMS measures were all completed before and 2.5 h post-supplementation.

2.8. Statistical Analysis

Statistical analyses were completed using IBM SPSS (Version 22.0). All data except for physical characteristics and baseline measurements were analyzed using mixed-method repeated-measures analysis of variance (ANOVA) with treatment and time as within-subject factors, and age as the between-subject factor. Sphericity was tested using the Mauchly's test to ensure the assumption of sphericity was not violated, and multivariate models were applied if these assumptions were not met. Where significant differences were found, post-hoc tests with Holm-Bonferroni correction were undertaken to assess multiple comparisons. Outliers were excluded based on the Tukey test. Relative effect sizes were calculated using partial eta squared and defined as small (η^2_p = 0.01), medium (η^2_p = 0.06), or large (η^2_p = 0.14). Independent Student's *t*-tests were used to examine differences in physical characteristics (height, weight, age) between age groups. Data are presented as mean ± standard deviation. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Participants

The physical characteristics and baseline nitrate, nitrite and blood pressure measurements for the younger and older group participants are reported in Table 1. All 24 participants completed the blood pressure, mood, perceptual and cognitive tests, and treadmill walking exercise. However, blood samples were only successfully obtained from 19 of the 24 (11 younger and 8 older) participants. One older participant was excluded from the Stroop test as their result was determined to be an outlier (using Tukey test) falling outside the interquartile range.

Table 1. Physical characteristics and baseline nitrate, nitrite and blood pressure measurements of younger and older adult group participants. [1]

	Younger Adults (n = 13)		Older Adults (n = 11)		p-Value	
Male	9		3			
Female	4		8			
Age (years)	25 ± 3		56 ± 6		<0.001 *	
Height (cm)	178.4 ± 10.1		169.8 ± 7.0		0.026 *	
Body Mass (kg)	75.3 ± 14.9		74.7 ± 7.9		0.90	
	Beetroot	**Placebo**	**Beetroot**	**Placebo**	**Beetroot (Between Groups)**	**Placebo (Between Groups)**
Plasma NO_2^- (nM)	193.9 ± 77.5	153.8 ± 57.7	145.4 ± 40.2	143.1 ± 60.3	0.19	0.86
Plasma NO_3^- (μM)	111.8 ± 112.5	116.0 ± 80.9	90.3 ± 62.1	127.0 ± 71.1	0.90	0.27
SBP (mmHg)	116.8 ± 8.1	114.9 ± 9.2	125.2 ± 9.6	120.9 ± 10.8	0.061	0.003 *
DBP (mmHg)	68.5 ± 6.0	68.8 ± 7.3	80.9 ± 6.9	77.9 ± 6.3	0.81	0.042 *

[1] Values are means ± SD. * indicates significant difference ($p < 0.05$).

3.2. *Plasma Nitrite and Nitrate*

There was no significant difference ($p < 0.05$) between baseline plasma [NO_2^-] and [NO_3^-] between the groups (Table 1). Plasma [NO_2^-] was elevated 2.25 and 3.25 h post-supplementation following BR consumption compared to PL) in both groups ($p = 0.003$, $\eta^2_p = 0.41$, Figure 2A). Older adults had a greater increase in plasma [NO_2^-] 2.5 and 3.5 h post-supplementation compared to younger adults following BR supplementation versus PL ($p = 0.038$, $\eta^2_p = 0.23$). Plasma [NO_3^-] was also elevated 2.25 and 3.5 h post-supplementation with BR consumption compared to PL in both groups ($p < 0.001$, $\eta^2_p = 0.67$, Figure 2B). There was a trend for a greater increase in plasma [NO_3^-] in older adults, 3.5 h post BR supplementation versus PL ($p = 0.064$, $\eta^2_p = 0.19$).

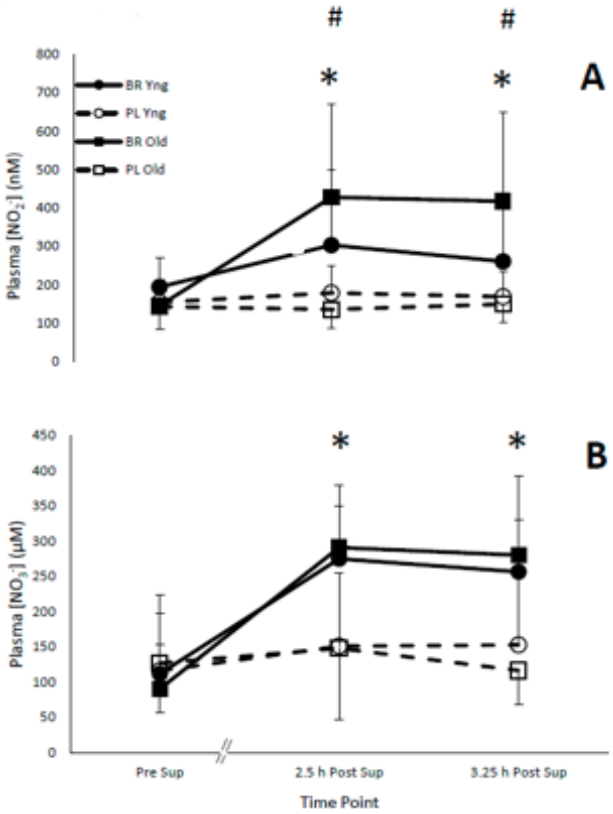

Figure 2. (**A**) Plasma nitrite concentration ([NO_2^-]) (nM) pre- (Pre Sup), post-supplementation (Post Sup) and post-exercise (Post Ex) with beetroot juice (BR) and placebo (PL) in younger (Yng) ($n = 13$) and older (Old) adults ($n = 11$). (**B**) Plasma nitrate concentration ([NO_3^-]) (μM) pre- (Pre Sup), post-supplementation (Post Sup) and post-exercise (Post Ex) with beetroot juice (BR) and placebo (PL) in younger (Yng) and older (Old) adults. Values are expressed as means ± SD. * Significant mean difference between treatment (placebo vs. beetroot), and time (pre- vs. post-supplementation) ($p < 0.05$). # Significant mean difference between treatment, time, and age ($p < 0.05$).

3.3. *Blood Pressure*

Systolic blood pressure (SBP) was reduced in both younger and older adults following consumption of BR ($p < 0.001$, $\eta^2_p = 0.45$, Figure 3A). However, there was no interaction effect for SBP of treatment*time*age ($p = 0.11$, $\eta^2_p = 0.11$). There was a reduction in DBP post-supplementation compared to pre-supplementation following consumption of BR versus PL for older but not younger adults ($p = 0.013$, $\eta^2_p = 0.25$, Figure 3B). Older adults had a greater reduction in DBP post- versus pre-supplementation with BR versus PL, compared to younger adults ($p = 0.005$, $\eta^2_p = 0.31$, Figure 3B).

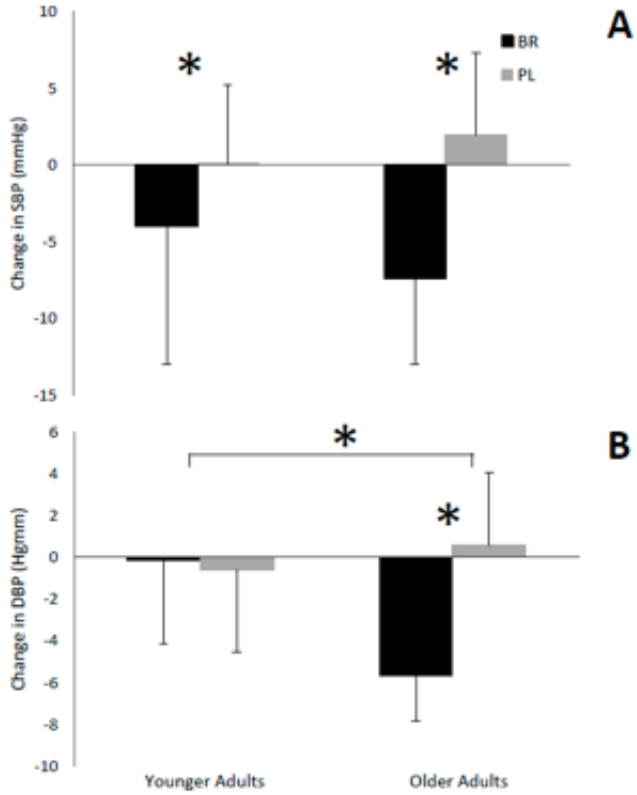

Figure 3. (**A**) Change in systolic blood pressure (SBP) (mmHg) pre- and post-supplementation with beetroot juice (BR) and placebo (PL) in younger (n = 13) and older adults (n = 11). (**B**) Change in diastolic blood pressure (DBP) (mmHg) pre- and post-supplementation with beetroot juice (BR) and placebo (PL) in younger and older adults. Values are expressed as means ± SD. * Significant mean difference between change in blood pressure (**A**, **B**) and change in blood pressure between older and younger adults (**B**) ($p < 0.05$).

3.4. *Heart Rate*

There was no interaction of treatment*time (pre- and post-supplementation) ($p = 0.99$, $\eta^2_p < 0.001$) or interaction of treatment*time*age ($p = 0.62$, $\eta^2_p = 0.011$) on average HR in either group.

3.5. *Cognitive Performance*

Supplementation with BR showed a trend for improved correct response percentage in the Stroop test from pre- to post-supplementation compared to PL ($p = 0.075$, $\eta^2_p = 0.14$). Beetroot juice improved reaction time during the Stroop test from pre- to post-supplementation compared to PL ($p = 0.045$, $\eta^2_p = 0.18$, Table 2). There was no interaction of treatment*time*age for correct responses ($p = 0.99$, $\eta^2_p < 0.001$) or reaction time ($p = 0.38$, $\eta^2_p = 0.037$) in the Stroop test. Placebo improved correct response percentage for CRT from pre- to post-supplementation compared to BR ($p = 0.008$, $\eta^2_p = 0.28$). There was no effect of treatment*time for reaction time in the CRT ($p = 0.14$, $\eta^2_p = 0.094$) or interaction effect of treatment*time and age for percentage correct responses ($p = 0.96$, $\eta^2_p < 0.001$) or reaction time ($p = 0.076$, $\eta^2_p = 0.14$) in the CRT ($p = 0.96$, $\eta^2_p < 0.001$). There were no interaction effects of treatment*time or treatment*time*age for RVIP percentage correct responses ($p = 0.33$ and 0.89, $\eta^2_p = 0.043$ and 0.001, respectively), percentage of errors ($p = 0.92$ and 0.65, $\eta^2_p < 0.001$ and 0.010, respectively), and reaction time ($p = 0.31$ and 0.99, $\eta^2_p = 0.047$ and <0.001, respectively).

Table 2. Cognitive performance tests and mood analysis pre- and post-supplementation with beetroot juice and placebo in younger and older adults. [1]

	Younger Adults				Older Adults			
	Placebo		Beetroot		Placebo		Beetroot	
	Pre	*Post*	*Pre*	*Post*	*Pre*	*Post*	*Pre*	*Post*
Choice Reaction Test								
Correct Response (%)	96.2 ± 2.3	97.5 ± 1.7 *	96.2 ± 2.1	95.6 ± 2.6	98.6 ± 2.0	99.2 ± 0.4 *	98.7 ± 1.7	97.4 ± 2.2
Reaction Time (ms)	433 ± 48	426 ± 34	435 ± 46	421 ± 45	637 ± 125	588 ± 59	596 ± 96	615 ± 134
Rapid Visual Information Processing								
Correct Response (%)	87.2 ± 9.5	90.1 ± 8.8	81.1 ± 16.7	89.5 ± 7.7	57.8 ± 20.3	63.1 ± 21.4	55.9 ± 20.6	65.4 ± 16.9
Errors	1.2 ± 1.2	1.2 ± 1.1	1.4 ± 1.3	1.1 ± 1.1	2.4 ± 3.4	1.3 ± 1.7	2.6 ± 2.0	1.8 ± 1.6
Reaction Time (ms)	422 ± 34	402 ± 32	445 ± 67	409 ± 49	481 ± 65	495 ± 61	498 ± 68	496 ± 76
Stroop Test								
Correct Response (%)	96.5 ± 2.6	95.8 ± 3.6	95.6 ± 3.0	96.3 ± 2.8	98.0 ± 2.2	98.6 ± 1.1	96.7 ± 3.0	98.7 ± 1.6
Reaction Time (ms)	644 ± 112	607 ± 102	690 ± 204	616 ± 117 *	965 ± 194	970 ± 167	978 ± 189	891 ± 138 *
Mood Measures								
FS	1.9 ± 1.1	2.2 ± 1.1	2.6 ± 1.3	2.9 ± 1.3	2.7 ± 1.2	2.8 ± 1.5	2.2 ± 1.8	2.4 ± 1.3
FAS	1.9 ± 0.8	2.4 ± 1.0	2.2 ± 0.6	2.3 ± 1.0	1.9 ± 0.9	2.0 ± 1.1	2.1 ± 1.2	1.9 ± 1.3
POMS	22 ± 11	17 ± 7	22 ± 9	19 ± 8	18 ± 8	16 ± 7	25 ± 10	20 ± 10

[1] Values are expressed as means ± SD. Younger ($n = 13$), Older [choice reaction, rapid visual information processing and mood tests ($n = 11$) Stroop test ($n = 10$). * Significant mean difference between treatment (placebo vs. beetroot), and time (pre- vs. post-supplementation) ($p < 0.05$). FS, feeling scale; FAS, felt arousal scale; POMS, profile of mood states.

3.6. Perceptual Responses and Mood

There was no treatment or treatment*age effects between pre- and post-supplementation for ratings of pleasure-displeasure (FS) ($p = 0.93$ and 0.73, $\eta^2_p < 0.001$ and 0.006, respectively; Table 2) and perceived activation (FAS) ($p = 0.12$ and 0.78, $\eta^2_p = 0.11$ and 0.003 respectively; Table 2). There was no treatment or treatment*age effects for individual categories ($p > 0.05$) or total POMS score ($p = 0.62$ and 0.077, $\eta^2_p = 0.012$ and 0.14, respectively) between pre- and post-supplementation.

4. Discussion

We examined the effects of acute supplementation of NO_3^- rich beetroot juice (BR) on cardiovascular responses, cognition, mood, and perceptual responses in younger and older adults. The main findings of this study demonstrate that consumption of NO_3^- rich BR; (1) significantly elevated plasma NO_2^- and NO_3^- concentrations and reduced BP for both younger and older adults, (2) improved reaction time during the Stroop test pre- versus post-supplementation, and (3) elevated plasma NO_2^- to a greater degree and resulted in a greater reduction in DBP in older adults.

4.1. Plasma $[NO_2^-]$ and $[NO_3^-]$

This is the first study to directly compare plasma $[NO_2^-]$ and $[NO_3^-]$ in younger and older adults following BR supplementation, with consumption of BR significantly elevating both plasma $[NO_2^-]$ and $[NO_3^-]$ in both age groups. Previous studies have found similar increases in plasma $[NO_2^-]$ and $[NO_3^-]$ following dietary NO_3^- supplementation in older [15] and younger adults [26]. Supplementation with BR resulted in a greater increase in plasma $[NO_2^-]$ and showed a trend for a greater increase in plasma $[NO_3^-]$ in older compared to younger adults (Figure 2). This finding was expected as older adults (60–70 years) have been suggested to have an age-related decrease in NO activity, suggesting that NO_3^- supplementation may have a greater impact on the older population, resulting in larger increases in plasma $[NO_2^-]$ and $[NO_3^-]$ [4]. Due to the novelty of this finding, further research is required directly comparing plasma $[NO_2^-]$ and $[NO_3^-]$ in younger and older adults following BR supplementation.

4.2. Blood Pressure

Systolic blood pressure was reduced in both age groups following BR supplementation, while DBP was reduced only in the older adults. Studies have shown that reductions in SBP and DBP of as little as 2 mmHg can greatly reduce the incidence of CVD in both hypertensive and normotensive individuals [27]. Consequently, reductions such as those observed in this study are considered clinically meaningful [28].

The reductions in BP observed in this study were likely due to an increase in NO production [8], which has been shown to activate the enzyme guanylate cyclase which catalyzes the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) [8,29]. cGMP acts on smooth muscle causing relaxation, resulting in vasodilation of arteries and veins, thus decreasing BP [4]. It would be expected that NO_3^- supplementation would also reduce DBP via this same mechanism; however, this only occurred in older adults. Previous studies have indicated that a reduction in DBP in younger adults following BR consumption is less common compared to a reduction in SBP [8,30], while studies in older adults more commonly show reductions in DBP following BR supplementation [4,15]. Although it has been suggested that the BP-lowering effects of BR may be related to other bioactive compounds or micronutrients found in beetroot juice; studies using a nitrate depleted placebo (by ion-exchange chromatography), and thus containing all other compounds, have still shown similar reductions in BP, indicating the nitrate content is the determining factor [8,9].

Interestingly, older adults were found to have a larger reduction in DBP following supplementation with BR compared to younger adults; however, this was not seen for SBP. The interaction effect on DBP is a relevant finding as older adults are at a higher risk of endothelial dysfunction and cardiovascular

disease, of which high blood pressure is a major risk factor, due to age-related reductions in NO production [2]. This finding also supports the theory that older adults have a lower initial level of NO and reduced endothelial functioning capacity, thus NO_3^- supplementation may have a greater potential for reducing BP in older versus younger adults [4,16]. In contrast to our findings, one study comparing the effects of dietary NO_3^- supplementation in younger versus older adults found no significant difference in SBP or DBP between the two age groups [16], however, they did not use a placebo-controlled, crossover design which could have impacted the results.

4.3. Cognitive Performance

Current findings suggest only certain aspects of cognitive performance (Stroop but not CRT, RVIP) were improved following BR supplementation. This is consistent with the findings of others [22], which showed significant improvements in Stroop test mean reaction time post BR (12.8 mmol of NO_3^-) consumption compared to PL (0.08 mmol of NO_3^-), but no changes in other cognitive performance tests. Supplementation with NO_3^- rich BR has been shown to significantly increase cerebral perfusion, specifically to the prefrontal cortex [13], therefore, improvements in reaction time in the Stoop test (a task that specifically targets the prefrontal cortex) could be expected. This may be due to increases in cerebral oxygenation allowing for a decrease in the O_2 cost of mental processing [31]. The variation in results from the different cognitive tests may be due to differences in the cognitive demands of the tests, with the Stroop test placing greater strain on an individual's mental processing capacity compared to simple reaction and number recall tests. Thus, BR supplementation may only improve cognitive performance when a large degree of cognitive difficulty is imposed [22]. It is also important to note that placebo supplementation improved the correct response percentage for the CRT, however, due to the extremely high percentages of correct responses and small standard deviations, it is possible this result lacks biological significance and indicates the need for further investigation.

While it has been proposed that supplementation with NO_3^- rich BR could have greater benefits on cognitive function in older adults due to its ability to compensate for age-related decreases in NO production and CBF [13], our findings did not support this. The lack of difference between age groups may have been due to the small sample size used and slightly younger mean age (56 years) of the older adults, as results from the CRT showed a trend for greater reduction in reaction time for older adults following BR supplementation ($p = 0.076$). Another major limitation in this area was the lack of a direct measure of CBF, thus it was not possible to determine whether or not BR supplementation increased blood flow to the brain and whether any increase was greater in the older adults.

4.4. Mood and Perception

Supplementation with NO_3^- rich BR did not improve any mood or perceptual measures (Table 2), supporting the findings of others [31]. Mood is affected by changes in blood flow to the brain and increases in neuro-excitation [32], thus, supplementation with dietary NO_3^- has been suggested as a mood enhancer due to its effects on cerebral vasodilation and stimulation of NO production [6,14]. Studies have also shown that age-related depression and alterations in mood are related to a reduction in CBF, and changes in neurotransmitter concentrations [32]. As BR supplementation increases CBF and stimulates increased NO production, it is possible that older adults may experience greater improvements in mood following consumption [4,13]. The lack of change in mood in this study could be due to insufficient sample size. In addition, it has been shown that age-related depression tends to occur in the mid-to-late 60s, [32] hence the lack of significance in mood and perception values between age groups seen here may have been due to the mean age of the older group being only 56 y.

4.5. Future Directions

The current findings indicate the potential for NO_3^- rich BR as an intervention for reducing BP in normotensive younger and older adults, with further research needed to assess its viability in a medical setting; for example, being used in conjunction with antihypertensive medication or to help

delay the need for pharmaceutical treatment in populations with hypertension. The findings also highlight the need for further research, with specific attention to direct comparisons of measures in younger and older adults, larger sample size for cognitive and mood measures, and more sensitive and specific measures for potential cognitive and mood benefits from supplementation with NO_3^- rich BR (e.g., near-infrared spectroscopy (NIRS) of the brain to monitor oxygenation and CBF [15]). Additionally, as this study showed a benefit on BP following NO_3^- rich BR supplementation in the healthy population this may suggest greater benefits in hypertensive groups, which should be an area for future research. Moreover, further research is needed to examine the effects of long-term consumption (chronic) of NO_3^- rich BR supplementation on health benefits. To date, most longer-term studies, involve daily consumption for periods of between 6 to 14 days [9,12] so future studies should focus on investigating longer supplementation periods of at least 28 days duration.

5. Conclusions

Acute supplementation with NO_3^- rich BR increased plasma [NO_2^-] and [NO_3^-] and reduced SBP in both age groups. The increase in plasma NO_2^- was greater in older adults, and DBP was reduced in older compared to younger adults. There was no difference in HR. Reaction time was improved in the Stroop test following BR supplementation; however, no difference was seen between age groups or for any other cognitive tests. Supplementation with BR did not improve perceptual or mood measures pre- versus post-supplementation. Collectively, these results indicate that acute supplementation with BR can reduce BP and improve aspects of cognitive performance; thus having potential health benefits for both younger and older adults.

Author Contributions: L.S., A.A., K.R.-M. and R.P. designed the research study; L.S. conducted the research trials; L.S. performed the database search and paper selection for the review; A.A., K.R.-M., K.H.T., M.W. and W.J. produced, analyzed and stored the beetroot juice; L.S., K.S. and K.R.-M. performed blood analysis; L.S. performed data and statistical analysis; L.S. wrote the paper. All authors contributed to the discussion, edited and revised the manuscript, and accepted the final manuscript.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Luke Allen Stanaway
Name/title of Primary Supervisor:	Prof Ajmol Ali
In which chapter is the manuscript /published work:	Chapter 5

Please select one of the following three options:

(●) The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

Stanaway, L.; Rutherfurd-Markwick, K.; Page, R.; Wong, M.; Jirangrat, W.; Teh, K. H.; Ali, A. Acute Supplementation with Nitrate-Rich Beetroot Juice Causes a Greater Increase in Plasma Nitrite and Reduction in Blood Pressure of Older Compared to Younger Adults. Nutrients 2019, 11, 1683.

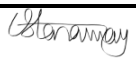
( ) The manuscript is currently under review for publication – please indicate:

- The name of the journal:

- The percentage of the manuscript/published work that was contributed by the candidate:

- Describe the contribution that the candidate has made to the manuscript/published work:

( ) It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	
Date:	01-Aug-2022
Primary Supervisor's Signature:	
Date:	01-Aug-2022

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

Appendix 4 – Dose-response study documents

Appendix 4.1 – Dose-response study ethics approval letter

Human Ethics Application SOA 18/30 Approved

HoU Review Group
A/Pro Andrew Foskett
ReviewerGroup
A/Pro Aj Ali
A/Pro Rachel Page
Dr Kay Rutherfurd

Researcher:
Title:Dose-response relationship of dietary nitrate-rich beetroot juice on cardiovascular and cognition function in older adults.

Dear

Thank you for the above application that was considered by the Massey University Human Ethics Committee: Human Ethics Southern A Committee at their meeting held on 31/07/2018.
On behalf of the Committee I am pleased to advise you that the ethics of your application are approved.

Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.
If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely
Associate Professor Tracy Riley, Dean Research
Acting Director (Research Ethics)

Appendix 4.2 – Dose-response study recruitment poster

What dose of nitrate-rich vegetable juice best improves your performance and cardiovascular health ?

Take part in an exciting study to find out!

Who we are after:

- Males and females aged 50 -80 years.
- Available between January and May 2019

Benefits of participation include:

- Find out what dose of nitrate works best for you.
- General health measures.
- $25 MTA voucher per visit (4 visits)

Contact:

Luke Stanaway

PhD Student

Massey University

- **02102965220**
- **vegetablestudy@hotmail.com**

Dose-response relationship of nitrate-rich beetroot juice on cardiovascular and cognitive function in older adults

PARTICIPANT INFORMATION SHEET

Researcher Introduction
My name is Luke and I am a PhD student at Massey University. My supervisors are A/Prof Ajmol Ali, A/Prof Kay Rutherfurd-Markwick and A/Prof Rachel Page.

Invitation to Participate in Research Study
With increasing rates of disease and age-related decline, there is a growing interest in the use of food-based supplements to help improve or maintain one's health and body functions. This has led to interest in beetroot juice (which contains nitrate) as a health food, as it has the potential to improve cognition, mood and cardiovascular function. We have recently found that consumption of beetroot juice reduces blood pressure and improves cognitive performance in younger and older adults; however, the optimal dose of dietary nitrate needed to for optimal health benefits is still unclear. Therefore, the aim of this study is to examine the dose-response effects of four different concentrations of dietary nitrate-rich beetroot juice on cardiovascular function, cognition, and mood in older adults (50 – 80 years).

Participant Recruitment
Men and women (aged 50–80 years) are invited to participate in this study. Individuals with any of the conditions listed on the "health checklist" should not volunteer to participate. If you are unsure about any of the listed conditions, then you should consult with the researchers. Please note that consumption of beetroot juice may cause beeturia (red or pink urine) or red stool but this effect does not last long. Each participant will receive a $25 MTA voucher (per visit) travel expenses for participation in this study on a pro-rata basis upon completion of the study.

Project Procedures and Participant Involvement
If you agree to participate, you will be asked to come to the Sport and Exercise Laboratory five times (Building 60 Massey University Oteha Rohe Campus, Albany Highway, Albany). You will be asked to wear appropriate comfortable clothing. The first visit (30 min) will be for a familiarisation of the procedures and equipment to be used for the main trials. You will also be asked to fill out a health screening checklist and consent form. The four test days will be completed on days 1, 7, 14, and 21. On the first test day you will be randomly allocated to an order of consumption of the four beetroot juices; placebo (a drink that tastes the same but contains minimal (60mg) nitrate), low (150mg), moderate (300mg) or high (600mg) nitrate concentration. You will be asked to consume a different concentration on each of the four test days. You will also be asked to refrain from consuming caffeine and alcohol, and not to exercise for 24 hours prior to the main trials. You will also need to record your diet for two days prior to the first test day and replicate that diet and lifestyle factors for the following three test days. If you become sick or injured during the 21 days and cannot complete the experiment, please contact the researcher.

For the test days, you will be asked to arrive at the laboratory in the morning after observing an overnight fast; we will then take a resting blood sample and measure your weight, resting blood

pressure, resting metabolic rate, heart rate, and blood flow via USCOM ultrasound. You will then be asked to complete three cognitive tests (on a computer) with near-infrared spectroscopy to measure brain blood flow and complete three survey and questionnaire type mood and perceptual scales (profile of mood states, feeling scale and felt arousal scale). Afterwards, we will ask you to consume a standardised breakfast (cereal with milk or toast with peanut butter) with 250 ml of one of the four drinks. A 2.25 hour waiting period is required for sufficient absorption of the beverage. Within this time, you may do some work, check emails or watch videos in the lab; however, it is important that no food is consumed (you can drink water only) during this time. After the waiting period we will take another blood sample and repeat measures of resting metabolic rate, heart rate, blood pressure, cognitive tests and mood scales. In total, we will take two 10-ml (two teaspoons) blood samples per trial.

The above procedures will be repeated for the second, third and fourth test days. The total time commitment for the five days will be approx. 12.5 hours (30 min for familiarisation and 3 hours per test day trial).

Participant's Rights

You are under no obligation to accept this invitation. Should you choose to participate, you have the right to:

• decline to answer any particular question

• withdraw from the study at any time, even after signing a consent form (if you choose to withdraw you cannot withdraw your data from the analysis after the data collection has been completed)

• ask any questions about the study at any time during participation

• provide information on the understanding that your name will not be used unless you give permission to the researcher

• be given access to a summary of the project findings when it is concluded

Good Practice and Cultural Safety for Massey University Research

The study was discussed with Messina Shaw (Student Recruitment Adviser - Māori Academic Support). We have considered the inclusion of Māori and indigenous values and concepts, allowing for the use of whānau support and appropriate Māori protocols. We acknowledge the concept of manaakitanga, respecting the participant`s inherent dignity and acting in a caring manner towards them by way of:

- Taking full responsibility to perform research in a safe and ethical manner (aroha)
- Providing the participant with all of the critical information regarding the study in a clear way, so they can make informed decisions (tūmanako and whakapono)
- An awareness of the cultural significance and sensitivity for a culturally safe implementation of the study (māhaki)
- Respect for the privacy and confidentiality of Māori participants
- Acknowledging the tapu (sacred) nature of blood/human tissue by offering remaining blood samples (if appropriate) back to the donor and keeping human samples secured and separated from other biological material, to ensure that the tapu māheuheu is not mixed with or contaminated by other tapu or noa (profane) substances.

Confidentiality

All data collected will be used solely for research purposes and has the possibility of being presented in a professional journal. All personal information will be kept confidential by assigning numbers to each participant. No names will be visible on any papers on which you provide information. All data/information will be dealt with confidentiality and will be stored in a secure location for five years

on the Massey University Albany Campus. After this time, it will be disposed of by an appropriate staff member from the School of Sport and Exercise.

Project Contacts
If you have any questions regarding this study, please do not hesitate to contact either of the following people for assistance:

Student researcher:
Luke Stanaway.
luke.stanaway.1@uni.massey.ac.nz
02102965220

Supervisors:
A/Prof Ajmol Ali (School of Sport, Exercise and Nutrition, Massey University)
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(09) 213 6414

A/Prof Kay Rutherfurd-Markwick (School of Health Sciences, Massey University)
k.j.rutherfurd@massey.ac.nz
(09) 213 6646

A/Prof Rachel Page (School of Health Sciences, Massey University)
r.a.page@massey.ac.nz
(04) 801 5799 ext. 63462

Committee Approval Statement

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application SOA 18/30. If you have any concerns about the conduct of this research, please contact Dr Lesley Batten, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 356 9099 x 85094, email humanethicsoutha@massey.ac.nz .

Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted, you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.

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Dose-response relationship of nitrate-rich beetroot juice on cardiovascular and cognitive function in older adults

Health Screening Questionnaire

Name:_____

Address: _____

Phone: _____

Age: _____

Gender:_____

Please read the following questions carefully. If you have any difficulty, please advise the medical practitioner, nurse or exercise specialist who is conducting the exercise test.

Please answer all of the following questions by ticking only one box for each question:

The questions are based upon the Physical Activity Readiness Questionnaire (PAR-Q), originally devised by the British Columbia Dept of Health (Canada), as revised by [1]Thomas *et al.* (1992) and [2]Cardinal *et al.* (1996), and with added requirements of the Massey University Human Ethics Committee. The information provided by you on this form will be treated with the strictest confidentiality.

Qu 1. Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?

Yes ☐ No ☐

Qu 2. Do you feel a pain in your chest when you do physical activity?

Yes ☐ No ☐

Qu 3. In the past month have you had chest pain when you were not doing physical activity?

Yes ☐ No ☐

Qu 4. Do you lose your balance because of dizziness, or do you ever lose consciousness?

Yes ☐ No ☐

Qu 5. Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?

Yes ☐ No ☐

Qu 6. Have you been hospitalised in the past few months?

Yes ☐ No ☐

Qu 7. Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?

Yes ☐ No ☐

Qu 8. Have any immediate family had heart problems prior to the age of 60?

Yes ☐ No ☐

Qu 9. Do you have any issues with having your blood taken?

Yes ☐ No ☐

Qu 10. Do you have any issues wearing a face mask while sitting at rest?

Yes ☐ No ☐

Qu 11. Do you have any allergic reactions, or issues consuming beetroot juice?

Yes ☐ No ☐

Qu 12. Do you have any of the following conditions, which may be affected by consumption of beetroot juice? (Kidney stones or a history of kidney stones, liver or kidney dysfunction, or diabetes).

Yes ☐ No ☐

I have read, understood and completed this questionnaire.

Signature ***(Participant):*** _____ Date: _____

References

1. Thomas S, Reading J and Shephard RJ. Revision of the Physical Activity Readiness Questionnaire (PAR-Q). *Can J Sport Sci* 17(4): 338-345.
2. Cardinal BJ, Esters J and Cardinal MK. Evaluation of the revised physical activity readiness questionnaire in older adults. *Med Sci Sports Exerc* 28(4): 468-472

Dose-response relationship of nitrate-rich beetroot juice on cardiovascular and cognitive function in older adults

CONSENT FORM FOR STUDY VOLUNTEERS

This consent form will be held for a minimum period of five (5) years

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I understand that I have the right to withdraw from the study at any time and to decline to answer any particular questions (if I choose to withdraw I cannot withdraw my data from the analysis after the data collection has been completed).

I agree to provide information to the researcher on the understanding that my name will not be used without my permission. (The information will be used only for this research and publications arising from this research project.)

I agree to participate in this study under the conditions set out in the Information Sheet.

Signature: _____ **Date**_____

Full Name (printed) _____

Phone Number_____ **Age** _____ **Date of Birth** _____

Are you willing to be contacted regarding future research projects within the School of Sport, Exercise and Nutrition? Your name and email address will be saved in a secure location. You will be sent periodic newsletters regarding research studies within the School. You can opt out of this newsletter at any time.

☐ Tick here if you accept.

Appendix 4.6 – Dose-response study data collect sheet

Familiarisation Trial

SUBJECT NAME	**AGE (y)**	**SUBJECT NUMBER**	
MASS (kg)	**DATE**		
HEIGHT (cm)	**BP(mmHg)**		
Breakfast Selection			
Health Screening and Consent Forms Completed			
Feeling Scale Familiarization			
Felt Arousal Scale Familiarization			
PMOS Familiarization			
Cognitive Tests Familiarization	**CB** ☐	**RVIP** ☐	**Stroop** ☐

Blood Pressure	**Systolic**	**Diastolic**
One		
Two		
Three		
Average		
MAP		
SVR		
Heart Rate		
Average		
RMR		

SUBJECT NAME				**DATE**		
MASS (kg)				**AGE (y)**		
HEIGHT (cm)				**BP(mmHg)**		
POMS (Before / After Sup / After Exercise)						
Feeling Scale (Before / After Sup / After Exercise)						
Felt Arousal Scale (Before / After Sup / After Exercise)						
Cognitive Tests (Before / After Sup / After Exercise)						
Blood Samples (Before / After Sup / After Exercise)						
Blood Pressure	**Systolic**			**Diastolic**		
Before Supplementation						
Average						
2.5 h After Supplementation						
Average						
Heart Rate (Before / After Sup)						
Heart Rate Average						
USCOM	**MAP**			**SVR**		
Before Supplementation						
2.5 h After Supplementation						
RMR Before Supplementation						
RMR After Supplementation						
RMR (Meta-analyser)	**Data recorded automatically via computer**					

SUBJECT NAME				**DATE**			
MASS (kg)				**AGE (y)**			
HEIGHT (cm)				**BP(mmHg)**			
POMS (Before / After Sup / After Exercise)							
Feeling Scale (Before / After Sup / After Exercise)							
Felt Arousal Scale (Before / After Sup / After Exercise)							
Cognitive Tests (Before / After Sup / After Exercise)							
Blood Samples (Before / After Sup / After Exercise)							
Blood Pressure	**Systolic**			**Diastolic**			
Before Supplementation							
Average							
2.5 h After Supplementation							
Average							
Heart Rate (Before / After Sup)							
Heart Rate Average							
USCOM	**MAP**			**SVR**			
Before Supplementation							
2.5 h After Supplementation							
RMR Before Supplementation							
RMR After Supplementation							
RMR (Meta-analyser)	**Data recorded automatically via computer**						

SUBJECT NAME			**DATE**			
MASS (kg)			**AGE (y)**			
HEIGHT (cm)			**BP(mmHg)**			
POMS (Before / After Sup / After Exercise)						
Feeling Scale (Before / After Sup / After Exercise)						
Felt Arousal Scale (Before / After Sup / After Exercise)						
Cognitive Tests (Before / After Sup / After Exercise)						
Blood Samples (Before / After Sup / After Exercise)						
Blood Pressure	**Systolic**			**Diastolic**		
Before Supplementation						
Average						
2.5 h After Supplementation						
Average						
Heart Rate (Before / After Sup)						
Heart Rate Average						
USCOM	**MAP**			**SVR**		
Before Supplementation						
2.5 h After Supplementation						
RMR Before Supplementation						
RMR After Supplementation						
RMR (Meta-analyser)	**Data recorded automatically via computer**					

SUBJECT NAME				**DATE**			
MASS (kg)				**AGE (y)**			
HEIGHT (cm)				**BP(mmHg)**			
POMS (Before / After Sup / After Exercise)							
Feeling Scale (Before / After Sup / After Exercise)							
Felt Arousal Scale (Before / After Sup / After Exercise)							
Cognitive Tests (Before / After Sup / After Exercise)							
Blood Samples (Before / After Sup / After Exercise)							
Blood Pressure	**Systolic**			**Diastolic**			
Before Supplementation							
Average							
2.5 h After Supplementation							
Average							
Heart Rate (Before / After Sup)							
Heart Rate Average							
USCOM	**MAP**			**SVR**			
Before Supplementation							
2.5 h After Supplementation							
RMR Before Supplementation							
RMR After Supplementation							
RMR (Meta-analyser)	**Data recorded automatically via computer**						

Two-Day Food Record

Name: _____

Dates of recorded intake: _____

Instructions for Keeping Your Two-Day Food Record

- Please keep your two-day food record for the two consecutive days prior to the Cycle trial and 3 main trials.
- Try to replicate the same diet and lifestyle factors prior to the second main trial.
- Each time you eat or drink anything (meals, snacks, etc.) during the two days, write down what was eaten.
- To measure how much was eaten; use a set of **measuring cups and spoons** to help estimate amounts. Also see the examples below to estimate portion sizes, as this is much more practical and easier.
- Note if food choices are homemade or purchased. Please include brand names whenever possible.

Amounts and Conversions

1/4 cup = 50 ml or 4 Tablespoons

1/3 cup = 75 ml or 5 1/2 Tablespoons

1/2 cup = 125 ml or 8 Tablespoons

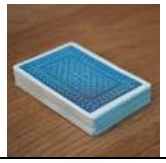
2/3 cup = 150 ml or 10 1/2 Tablespoons

3/4 cup = 175 ml or 12 Tablespoons

1 cup = 250 ml or 16 Tablespoons

1 oz = 1 slice of processed cheese or lunchmeat

How to Estimate Your Portion Size

Meat Three (3) ounces of meat are about the size and thickness of a deck of playing cards or an audiotape cassette.	
Fruit A medium apple or peach is about the size of a tennis ball.	
Grains One cup of rice or pasta is about the size of your fist.	
Cheese One ounce of cheese is about the size of four dice.	

Two-Day Food Record Checklist

Beverages	What kind of milk? homogenised, 2%, 1%, skim, other. Was it fruit juice or fruit beverage or drink?
Breads	Did you spread on butter or margarine?
Cereal	Did you add milk? Did you add sugar or fruit?
Dairy	What brand or kind of yogurt? What brand or kind of cheese?
Vegetables	Was it raw or cooked? Was it fresh, frozen or canned? Did you add any butter, margarine or sauce?
Fruit	Was it a small, medium or large fruit? Was it fresh, frozen or canned?
Grains	Did you add any butter, margarine, peanut butter, jam or honey? Was it a half or whole sandwich? Was it a small or large muffin or bagel?
Fish	Was your canned fish packed in water or oil How did you cook your fish?
Meats	How did you cook your meat? What kind of cut was it e.g. chicken leg or chicken breast?
Soups	Was your soup prepared with milk, water or cream?
Restaurants	What restaurant was it?
Packaged food	What brand was it?

Sample Menu

Day 1: Tuesday, May 14, 2018				
Time of Meal or Snack	**Type of Food or Beverage Offered**	**Amount Eaten**	**Method of Preparation or Brand**	**Comments** (e.g. amount of food served, too tired to eat)
Breakfast	Cereal	½ cup	Honey Nut Cheerios	
	Milk 2%	½ cup		On cereal
	Banana	½ med		
AM Snack	Animal Crackers	10	Christie	
	Apple juice	4 oz	Allen's pure apple juice-canned	
Lunch	Grilled cheese sandwich			
	Whole wheat bread	1 slice	Dempsters	No crusts
	Cheese slice	1 slice	Kraft slices	
	Butter on bread	1 Tbsp		
	Yogurt – strawberry	75 ml	Mini-go	
	Milk	½ cup	2%	
PM Snack	Granola bar	1 bar – 35 g	Quaker Chewy, Trail Mix – tropical fruit	Ate half of it
Dinner	Chicken fingers	1 ½	President's Choice	
	French fries	10	McCain regular	
	Honey	2 Tbsp		For dipping
	Ketchup	2 Tbsp	Heinz	
	Carrots	½ medium	Raw, cut in sticks	
	Milk	½ cup	2%	
Evening Snack	Ice cream	1 cup	Chocolate Nestle	

Was this day's intake considered: [ ] Poor [X**] Average [ ] Very Good**

Trial 1

Day 1	Date:			
Time of Meal or Snack	**Type of Food or Beverage Offered**	**Amount Eaten**	**Method of Preparation or Brand**	**Comments** (e.g. amount of food served, too tired to eat)
Breakfast				
AM Snack				
Lunch				
PM Snack				
Dinner				
Evening Snack				

Was this day's intake considered: [ ] Poor [ ] Average [ ] Very Good

Day 2	**Date:**			
Time of Meal or Snack	**Type of Food or Beverage Offered**	**Amount Eaten**	**Method of Preparation or Brand**	**Comments** (e.g. amount of food served, too tired to eat)
Breakfast				
AM Snack				
Lunch				
PM Snack				
Dinner				
Evening Snack				

Was this day's intake considered: [ ] Poor [ ] Average [ ] Very Good

Trial 2

Day 1	Date:			
Time of Meal or Snack	**Type of Food or Beverage Offered**	**Amount Eaten**	**Method of Preparation or Brand**	**Comments** (e.g. amount of food served, too tired to eat)
Breakfast				
AM Snack				
Lunch				
PM Snack				
Dinner				
Evening Snack				

Was this day's intake considered: [ ] Poor [ ] Average [ ] Very Good

Day 2	**Date:**			
Time of Meal or Snack	**Type of Food or Beverage Offered**	**Amount Eaten**	**Method of Preparation or Brand**	**Comments** (e.g. amount of food served, too tired to eat)
Breakfast				
AM Snack				
Lunch				
PM Snack				
Dinner				
Evening Snack				

Was this day's intake considered: [ ] Poor [ ] Average [ ] Very Good

Trial 3

Day 1	**Date:**			
Time of Meal or Snack	**Type of Food or Beverage Offered**	**Amount Eaten**	**Method of Preparation or Brand**	**Comments** (e.g. amount of food served, too tired to eat)
Breakfast				
AM Snack				
Lunch				
PM Snack				
Dinner				
Evening Snack				

Was this day's intake considered: [ ] Poor [ ] Average [ ] Very Good

Day 2	**Date:**			
Time of Meal or Snack	**Type of Food or Beverage Offered**	**Amount Eaten**	**Method of Preparation or Brand**	**Comments** (e.g. amount of food served, too tired to eat)
Breakfast				
AM Snack				
Lunch				
PM Snack				
Dinner				
Evening Snack				

Was this day's intake considered: [ ] Poor [ ] Average [ ] Very Good

Trial 4

Day 1	Date:			
Time of Meal or Snack	**Type of Food or Beverage Offered**	**Amount Eaten**	**Method of Preparation or Brand**	**Comments** (e.g. amount of food served, too tired to eat)
Breakfast				
AM Snack				
Lunch				
PM Snack				
Dinner				
Evening Snack				

Was this day's intake considered: [ ] Poor [ ] Average [ ] Very Good

Day 2	Date:			
Time of Meal or Snack	**Type of Food or Beverage Offered**	**Amount Eaten**	**Method of Preparation or Brand**	**Comments** (e.g. amount of food served, too tired to eat)
Breakfast				
AM Snack				
Lunch				
PM Snack				
Dinner				
Evening Snack				

Was this day's intake considered: [ ] Poor [ ] Average [ ] Very Good

DRC 16

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Luke Allen Stanaway
Name/title of Primary Supervisor:	Prof Ajmol Ali
In which chapter is the manuscript /published work:	Chapter 4

Please select one of the following three options:

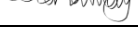
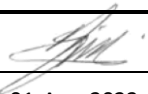
○ The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

○ The manuscript is currently under review for publication – please indicate:

- The name of the journal:
- The percentage of the manuscript/published work that was contributed by the candidate:
- Describe the contribution that the candidate has made to the manuscript/published work:

◉ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	
Date:	01-Aug-2022
Primary Supervisor's Signature:	
Date:	01-Aug-2022

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

Appendix 5 – Acute study documents

Appendix 5.1 – Acute study ethics approval letter

Human Ethics Application SOA 16/27 Approved

HoU Review Group

ReviewerGroup
Dr Aj Ali

Researcher: Luke Stanaway
Title:The effects of a vegetable-based performance beverage as a breakfast food on cognition, mood and cardiovascular responses in younger and older adults.

Dear Luke

Thank you for the above application that was considered by the Massey University Human Ethics Committee: Human Ethics Southern A Committee at their meeting held on 07/07/2016.
On behalf of the Committee I am pleased to advise you that the ethics of your application are approved.

Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.
If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely
Dr Brian Finch, Chair
Massey University Human Ethics Committee

Appendix 5.2 – Acute study recruitment poster

Can beetroot juice improve your cognition, mood, and physical performance?

Take part in an exciting study to find out!

Who we are after:

- Males and females aged 18-30 and 50-70 years.
- Capable of walking on a treadmill at a moderate pace for 30 min.
- Available between November– December 2016.

Benefits of participation include:

- Find out if beetroot juice works for you.
- General health measures.
- $50 MTA voucher.

Contact:

Luke Stanaway
Postgraduate Student
Massey University
- 02102965220
- luke_ruawai@hotmail.co.nz

The effects of beetroot juice intake with breakfast on cognition, mood, and cardiovascular responses in younger and older adults.

PARTICIPANT INFORMATION SHEET

Researcher Introduction

My name is Luke and I am a postgraduate student at Massey University. My supervisors are Dr Ajmol Ali and Dr Kay Rutherfurd-Markwick. I completed my Bachelors of Science majoring in Exercise and Sport Science and Human Nutrition last year and I am undertaking this study for my Honours project.

Invitation to Participate in Research Study

With increasing rates of disease and age-related dysfunction, there is a growing interest in the use of food-based supplements and bioactive compounds to help improve or maintain one's health and body functions. This has led to the interest in beetroot juice (which contains nitrate) as a health food, which has the potential to improve cognition, mood and cardiovascular function. However, further research is required to support this. The aim of this study is to examine the effects of acute nitrate supplementation, from beetroot juice taken with breakfast, on cognition, mood and cardiovascular responses in younger and older adults.

Participant Recruitment

Healthy males and females (aged 18 – 30 or 50 – 70 years) are invited to participate in this study. Individuals who are advanced/elite athletes (someone who trains 4 or more times per week for their sport and competes at a high level), or with any of the conditions listed on the "health checklist" should not volunteer to participate. If you are unsure about any of the listed conditions, then you should consult with the researchers. Please note that consumption of beetroot juice may cause beeturia (red or pink urine) but is short-lived. Each participant will receive a $50 MTA voucher for travel expenses upon completion of the study. Please read this information sheet carefully before deciding whether or not to participate.

Project Procedures and Participant Involvement

If you agree to participate, you will be asked to come in three times to the lab, located in Building 60 Massey University Oteha Rohe, Albany Highway, Albany. You will be asked to wear appropriate exercise clothing, including training shoes. The first visit (30 min) will be for a familiarisation of the procedures and equipment as well as to set your treadmill walking speed for the main trials. You will also be asked to fill out a health screening checklist and consent form. The two main trials will be one week apart. In the first trial you will be randomly allocated to either the beetroot juice supplement or placebo (a drink that tastes the same but does not contain nitrate) trial and in the second trial you will be asked to consume the other beverage. You will also be asked to refrain from caffeine, alcohol and exercise

24 hours prior to main trials and record your diet for two days prior to the first main trial and replicate diet and lifestyle factors for the second trial.

For the main trials you will be asked to arrive at the laboratory in the morning fasted; we will then take blood samples (3 samples per main trial, each of 10 ml) and measure your weight, resting blood pressure, oxygen uptake (using a K4 breath-by-breath analyser) and heart rate. You will then be asked to complete three cognitive tests and complete three mood and perceptual scales (profile of mood states, feeling scale and felt arousal scale). Afterwards we will ask you to consume a standardised breakfast (cereal or toast) with 300 ml of either the beetroot drink or placebo. A 2.5 hour waiting period is required for sufficient absorption of the beverage. Within this time, you may do some work, check emails or watch videos in the lab; however, it is imperative that no food is consumed (you can drink water only). After the waiting period we will take another blood sample and repeat measures of heart rate, blood pressure, cognitive tests and mood scales. You will then be asked to complete a 30 min moderate treadmill walk at your pre-determined speed. We will measure heart rate continuously thought the trial, and oxygen uptake and ratings of perceived exertion (RPE) at regular periods during the 30 min walk. On completion of the exercise all measures will be repeated again.

The above procedures will be repeated for the second trial; however, you will consume the other beverage to that of the first trial. The total time commitment for the three days will be 7.5 – 8.5 hours.

Participant's Rights

You are under no obligation to accept this invitation. Should you choose to participate, you have the right to:

- decline to answer any particular question
- withdraw from the study at any time, even after signing a consent form (if you choose to withdraw you cannot withdraw your data from the analysis after the data collection has been completed)
- ask any questions about the study at any time during participation
- provide information on the understanding that your name will not be used unless you give permission to the researcher
- be given access to a summary of the project findings when it is concluded

Confidentiality

All data collected will be used solely for research purposes and has the possibility of being presented in a professional journal. All personal information will be kept confidential by assigning numbers to each participant. No names will be visible on any papers on which you provide information. All data/information will be dealt with in confidentiality and will be stored in a secure location for five years on the Massey University Albany Campus. After this time, it will be disposed of by an appropriate staff member from the School of Sport and Exercise.

Project Contacts
If you have any questions regarding this study, please do not hesitate to contact either of the following people for assistance:

Student researcher:
Luke Stanaway.
luke_ruawai@hotmail.co.nz
02102965220

Supervisors:
Dr Ajmol Ali (School of Sport and Exercise, Massey University)
a.ali@massey.ac.nz
(09)414-0800 ext.43414

Dr Kay Rutherfurd-Markwick (School of Food and Nutrition, Massey University)
k.j.rutherfurd@massey.ac.nz
(09)414-0800 ext.43646

Committee Approval Statement

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 16/27. If you have any concerns about the conduct of this research, please contact Mr Jeremy Hubbard, Chair, Massey University Human Ethics Committee: Southern A, telephone 04 801 5799 x 63487, email humanethicsoutha@massey.ac.nz.

Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted, you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

The effects of beetroot juice intake with breakfast on cognition, mood and cardiovascular responses in younger and older adults.

Health Screening Questionnaire

Name: _____

Address: _____

Phone: _____

Age: _____ Gender: _____

Emergency contact

Name: _____

Phone: _____

Please read the following questions carefully. If you have any difficulty, please advise the medical practitioner, nurse or exercise specialist who is conducting the exercise test.

Please answer all of the following questions by ticking only one box for each question:

The questions are based upon the Physical Activity Readiness Questionnaire (PAR-Q), originally devised by the British Columbia Dept of Health (Canada), as revised by [1]Thomas *et al.* (1992) and [2]Cardinal *et al.* (1996), and with added requirements of the Massey University Human Ethics Committee. The information provided by you on this form will be treated with the strictest confidentiality.

Qu 1. Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?

Yes ☐ No ☐

Qu 2. Do you feel a pain in your chest when you do physical activity?

Yes ☐ No ☐

Qu 3. In the past month have you had chest pain when you were not doing physical activity?

Yes ☐ No ☐

Qu 4. Do you lose your balance because of dizziness or do you ever lose consciousness?

Yes ☐ No ☐

Qu 5. Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?

Yes ☐ No ☐

Qu 6. Do you take any other medication?

Yes ☐ No ☐

If yes, please state what the medication is for: _____

Qu 7. Have you been hospitalised recently?

Yes ☐ No ☐

Qu 8. Do you have a bone or joint problem (for example back, knee or hip) that could be made worse by a change in your physical activity?

Yes ☐ No ☐

Qu 9. Do you know of any other reason why you should not do physical activity?

Yes ☐ No ☐

Qu 10. Have any immediate family had heart problems prior to the age of 60?

Yes ☐ No ☐

Qu 11. Do you have any issues with having your blood taken?

Yes ☐ No ☐

Qu 12. Do you have any infectious disease that may be transmitted in blood?

Yes ☐ No ☐

Qu 13. Do you have any issues exercising or completing any physical activity with a face mask?

Yes ☐ No ☐

Qu 14. Do you have any allergic reactions, or issues consuming beetroot juice?

Yes ☐ No ☐

Qu 15. Do you have any of the following conditions, which may be affected by consumption of beetroot juice? (Kidney stones or a history of kidney stones, liver or kidney dysfunction).

Yes ☐ No ☐

Qu 16. Are there any issues that may prevent you from completing the 30-min moderate walk on a treadmill? If yes, please explain.

Yes ☐ No ☐

_____

_____

I have read, understood and completed this questionnaire.

Signature ***(Participant):*** _____ Date: _____

References

3. Thomas S, Reading J and Shephard RJ. Revision of the Physical Activity Readiness Questionnaire (PAR-Q). *Can J Sport Sci* 17(4): 338-345.
4. Cardinal BJ, Esters J and Cardinal MK. Evaluation of the revised physical activity readiness questionnaire in older adults. *Med Sci Sports Exerc* 28(4): 468-472

The effects of supplementation with nitrate rich beetroot juice on performance and cardiovascular health of younger and older adults

CONSENT FORM FOR STUDY VOLUNTEERS

This consent form will be held for a minimum period of five (5) years

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I understand that I have the right to withdraw from the study at any time and to decline to answer any particular questions (if I choose to withdraw I cannot withdraw my data from the analysis after the data collection has been completed).

I agree to provide information to the researcher on the understanding that my name will not be used without my permission. (The information will be used only for this research and publications arising from this research project.)

I agree to participate in this study under the conditions set out in the Information Sheet.

If you are interested in hearing about future research studies, please tick here ☐

Signature: _____ **Date**_____

Full Name (printed) _____

Phone Number_____ **Age** _____ **Date of Birth** _____

Participant code ☐

Appendix 5.6 – Acute study data collect sheet

Familiarisation Trial

SUBJECT NAME		**SUBJECT NUMBER**	
MASS (kg)		**DATE**	
HEIGHT (cm)		**BP(mmHg)**	
Breakfast Selection			
Health Screening and Consent Forms Completed			
Feeling Scale Familiarization			
Felt Arousal Scale Familiarization			
PMOS Familiarization			
Cognitive Tests Familiarization		**CRT** ☐	**RVIP** ☐ **Stroop** ☐
Blood Pressure	**Systolic**	**Diastolic**	
One			
Two			
Three			
Average			
Treadmill Speed (km/h)			

Trial 1

SUBJECT NAME									
MASS (kg)				**DATE**					
HEIGHT (cm)	**AGE (y)**			**BP(mmHg)**					
POMS (Before / After Sup / 3 h post-sup)									
Feeling Scale (Before / After Sup / 3 h post-sup)									
Felt Arousal Scale (Before / After Sup / 3 h post-sup)									
Cognitive Tests (Before / After Sup / 3 h post-sup)									
Blood Samples (Before / After Sup / 3 h post-sup)									
Blood Pressure	**Systolic**			**Diastolic**					
Before Supplementation									
Average									
2.25 h After Supplementation									
Average									
3 h Post Supplementation									
Average									
Heart Rate (Before / After Sup / After Exercise)									
Heart Rate Average									

SUBJECT NAME									
MASS (kg)			**DATE**						
HEIGHT (cm)	**AGE (y)**		**BP(mmHg)**						
POMS (Before / After Sup / 3 h post-sup)									
Feeling Scale (Before / After Sup / 3 h post-sup)									
Felt Arousal Scale (Before / After Sup / 3 h post-sup)									
Cognitive Tests (Before / After Sup / 3 h post-sup)									
Blood Samples (Before / After Sup / 3 h post-sup)									
Blood Pressure	**Systolic**			**Diastolic**					
Before Supplementation									
Average									
2.25 h After Supplementation									
Average									
3 h Post Supplementation									
Average									
Heart Rate (Before / After Sup / After Exercise)									
Heart Rate Average									

MOOD AND PERCEPTUAL TESTS

Profile of Mood States-Short Form (POMS-40)

Refer to the definitions below. Consider how you are feeling right now, when circling the appropriate response. Please make sure you have responded to all items.

FATIGUE	Not at all	A little	Moderately	Quite a bit	Extremely
Worn out	0	1	2	3	4
Weary	0	1	2	3	4
Bushed	0	1	2	3	4
Fatigued	0	1	2	3	4
Exhausted	0	1	2	3	4

Anger	Not at all	A little	Moderately	Quite a bit	Extremely
Peeved	0	1	2	3	4
Bitter	0	1	2	3	4
Resentful	0	1	2	3	4
Grouchy	0	1	2	3	4
Angry	0	1	2	3	4
Furious	0	1	2	3	4
Annoyed	0	1	2	3	4

Vigour	Not at all	A little	Moderately	Quite a bit	Extremely
Cheerful	0	1	2	3	4
Powerful	0	1	2	3	4
Full of Pep	0	1	2	3	4
Active	0	1	2	3	4
Energetic	0	1	2	3	4
Lively	0	1	2	3	4

TENSION	Not at all	A little	Moderately	Quite a bit	Extremely
Restless	0	1	2	3	4
Nervous	0	1	2	3	4
On-Edge	0	1	2	3	4
Tense	0	1	2	3	4
Uneasy	0	1	2	3	4
Anxious	0	1	2	3	4

ESTEEM	Not at all	A little	Moderately	Quite a bit	Extremely
Embarrassed	0	1	2	3	4
Ashamed	0	1	2	3	4
Proud	0	1	2	3	4
Competent	0	1	2	3	4
Satisfied	0	1	2	3	4

CONFUSION	Not at all	A little	Moderately	Quite a bit	Extremely
Bewildered	0	1	2	3	4
Forgetful	0	1	2	3	4
Confused	0	1	2	3	4
Unable to Concentrate	0	1	2	3	4
Uncertain About things	0	1	2	3	4

DEPRESSION	Not at all	A little	Moderately	Quite a bit	Extremely
Hopeless	0	1	2	3	4
Helpless	0	1	2	3	4
Sad	0	1	2	3	4
Worthless	0	1	2	3	4
Miserable	0	1	2	3	4
Discouraged	0	1	2	3	4

FEELING SCALE

+5	Very good
+4	
+3	Good
+2	
+1	Fairly good
0	Neutral
-1	Fairly bad
-2	
-3	Bad
-4	
-5	Very bad

"While participating in exercise it is common to experience changes in mood. Some individuals find exercise pleasurable, whereas others find it to be displeasurable. Additionally, feeling may fluctuate across time. That is, one might feel good and bad a number of times during exercise. Scientists have developed this scale to measure such responses."

FELT AROUSAL SCALE (FAS)
(Svebak & Murgatroyd, 1985)

Estimate here how aroused you actually feel. Do this by circling the appropriate number. By "arousal" we meant how "worked-up" you feel. You might experience high arousal in one of a variety of ways, for example as excitement or anxiety or anger. Low arousal might also be experienced by you in one of a number of different ways, for example as relaxation or boredom or calmness.

1 LOW AROUSAL

2

3

4

5

6 HIGH AROUSAL

Appendix 6 – Chronic study documents

Appendix 6.1 – Chronic study ethics approval letter

Human Ethics Application SOA 17/36 Approved

HoU Review Group
Dr Andrew Foskett
ReviewerGroup
A/Pro Rachel Page
Dr Aj Ali
Dr Kay Rutherfurd

Researcher: Luke Stanaway
Title:The effects of supplementation with nitrate rich beetroot juice on performance and cardiovascular health of younger and older adults.

Dear Luke

Thank you for the above application that was considered by the Massey University Human Ethics Committee: Human Ethics Southern A Committee at their meeting held on 24/08/2017.
On behalf of the Committee I am pleased to advise you that the ethics of your application are approved.

Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.
If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely
Dr Brian Finch, Chair
Massey University Human Ethics Committee

Appendix 6.2 – Chronic study recruitment poster

Can nitrate-rich vegetable juice improve your performance and cardiovascular health?

Take part in an exciting study to find out!

Who we are after:

- Males and females aged 18 -30 or 50-80 years.
- Capable of cycling for approximately 20 minutes.
- Available between February and October 2018

Benefits of participation include:

- Find out if nitrate works for you.
- General health measures.
- $20 MTA voucher per visit (5 visits)

Contact:

Luke Stanaway

PhD Student

Massey University

- **02102965220**
- **vegetablestudy@hotmail.com**

The effects of supplementation with nitrate-rich beetroot juice on performance and cardiovascular health of younger and older adults.

PARTICIPANT INFORMATION SHEET

Researcher Introduction
My name is Luke and I am a PhD student at Massey University. My supervisors are A/Profs Ajmol Ali, Kay Rutherfurd-Markwick and Rachel Page.

Invitation to Participate in Research Study
With increasing rates of disease and age-related dysfunction, there is a growing interest in the use of food-based supplements to help improve or maintain one's health and body functions. This has led to the interest in beetroot juice (which contains nitrate) as a health food, as it has the potential to improve cognition, mood and cardiovascular function. For my Honours project I found that beetroot juice reduced blood pressure and improved cognitive performance in younger and older adults; however, further research is required to support this. The aims of this study are to examine the effects of a single dose and prolonged intake of nitrate supplementation, of beetroot juice, on cognition, mood and cardiovascular responses in younger (18-30 years) and older adults (50-80 years).

Participant Recruitment
Men and women (aged 18–30 or 50–80 years) are invited to participate in this study. Individuals who are advanced/elite athletes (someone who trains more than four times per week constantly for their sport and competes at a high level), or with any of the conditions listed on the "health checklist" should not volunteer to participate. If you are unsure about any of the listed conditions, then you should consult with the researchers. Please note that consumption of beetroot juice may cause beeturia (red or pink urine) or red stool but this is short-lived. Each participant will receive a $25 MTA voucher (per visit) for travel expenses for participation in this study on a pro-rata basis. upon completion of the study. Please read this information sheet carefully before deciding whether or not to volunteer to participate.

Project Procedures and Participant Involvement
If you agree to participate, you will be asked to come to the Sport and Exercise laboratory five times. The laboratory is located in Building 60 Massey University Oteha Rohe Campus, Albany Highway, Albany. You will be asked to wear appropriate exercise clothing, including training shoes. The first visit (30 min) will be for a familiarisation of the procedures and equipment to be used as well as to calculate your work rate for the cycle exercise for the main trials and explain the protocol for juice consumption. You will also be asked to fill out a health screening checklist and consent form. The second visit will be a baseline work to completion cycle test (30 min). Visits 3 – 5 will be the three intervention trials and will be completed on days 0, 14 and 28. In the first intervention trial you will be randomly allocated to either the beetroot juice supplement or placebo (a drink that tastes the same but does not contain nitrate) trial.

The second and third intervention trials will be identical to the first but performed after 14 and 28 days of supplementation, respectively. You will be asked to consume the allocated drink in the morning with breakfast everyday apart from the trial days where you will consume the drink in the laboratory with breakfast. You will be provided half the drinks after trial 1 and the other half after trial 2. All empty bottles will need to be returned to the laboratory at each trial. You will also be asked to refrain from caffeine and alcohol consumption and exercise 24 hours prior to main trials. You will also need to record your diet for two days prior to the first main trial and replicate diet and lifestyle factors for the second and third trials. If you become sick or injured during the 28 days and cannot complete the supplementation, please contact the researcher.

For the intervention trials, you will be asked to arrive at the laboratory in the morning after observing an overnight fast; we will then take a resting blood sample and measure your weight, resting blood pressure, oxygen uptake and heart rate. You will then be asked to complete three cognitive tests (on a computer) and complete three mood and perceptual scales (profile of mood states, feeling scale and felt arousal scale). Afterwards we will ask you to consume a standardised breakfast (cereal or toast) with 250 ml of either the beetroot drink or placebo. A 2.25 hour waiting period is required for sufficient absorption of the beverage. Within this time, you may do some work, check emails or watch videos in the lab; however, it is important that no food is consumed (you can drink water only). After the waiting period we will take another blood sample and repeat measures of heart rate, blood pressure, cognitive tests and mood scales. You will then be asked to complete a performance test on a stationary bike based on 20-min at 75% of your pre-determined work max. We will measure heart rate, oxygen uptake, and brain blood flow continuously throughout the cycle, and ratings of perceived exertion (RPE) at regular periods during the time trial. On completion of the exercise all measures will be repeated again. In total, we will take three 10-ml (two teaspoons) blood samples per trial.

The above procedures will be repeated for the second and third trial. The total time commitment for the four days will be approx. 12 hours (30 min for familiarisation, 30 min for baseline cycle trial and 3.5 hours per intervention trial).

Participant's Rights

You are under no obligation to accept this invitation. Should you choose to participate, you have the right to:

- decline to answer any particular question
- withdraw from the study at any time, even after signing a consent form (if you choose to withdraw you cannot withdraw your data from the analysis after the data collection has been completed)
- ask any questions about the study at any time during participation
- provide information on the understanding that your name will not be used unless you give permission to the researcher
- be given access to a summary of the project findings when it is concluded

Confidentiality

All data collected will be used solely for research purposes and has the possibility of being presented in a professional journal. All personal information will be kept confidential by assigning numbers to each participant. No names will be visible on any papers on which you provide information. All data/information will be dealt with confidentiality and will be stored in a secure

location for five years on the Massey University Albany Campus. After this time, data will be disposed by an appropriate staff member from the School of Sport, Exercise and Nutrition.

Project Contacts
If you have any questions regarding this study, please do not hesitate to contact either of the following people for assistance:

Student researcher:
Luke Stanaway.
luke.stanaway.1@uni.massey.ac.nz
02102965220

Supervisors:
A/Prof Ajmol Ali (School of Sport, Exercise and Nutrition, Massey University)
a.ali@massey.ac.nz
(09) 213 6414

A/Prof Kay Rutherfurd-Markwick (School of Health Sciences, Massey University)
k.j.rutherfurd@massey.ac.nz
(09) 213 6646

A/Prof Rachel Page (School of Health Sciences, Massey University)
r.a.page@massey.ac.nz
(04) 801 5799 ext. 63462

Committee Approval Statement

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 17/36. If you have any concerns about the conduct of this research, please contact Dr Lesley Batten, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 356 9099 x 85094, email humanethicsoutha@massey.ac.nz .

Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is

accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted, you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

The effects of supplementation with nitrate rich beetroot juice on performance and cardiovascular health of younger and older adults

Health Screening Questionnaire

Name:_____

Address: _____

Phone: _____

Age: _____

Gender:_____

Please read the following questions carefully. If you have any difficulty, please advise the medical practitioner, nurse or exercise specialist who is conducting the exercise test.

Please answer all of the following questions by ticking only one box for each question:

The questions are based upon the Physical Activity Readiness Questionnaire (PAR-Q), originally devised by the British Columbia Dept of Health (Canada), as revised by [1]Thomas *et al.* (1992) and [2]Cardinal *et al.* (1996), and with added requirements of the Massey University Human Ethics Committee. The information provided by you on this form will be treated with the strictest confidentiality.

Qu 1. Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?

Yes ☐ No ☐

Qu 2. Do you feel a pain in your chest when you do physical activity?

Yes ☐ No ☐

Qu 3. In the past month have you had chest pain when you were not doing physical activity?

Yes ☐ No ☐

Qu 4. Do you lose your balance because of dizziness or do you ever lose consciousness?

Yes ☐ No ☐

Qu 5. Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?

☐ ☐

Yes No

Qu 6. Have you been hospitalised recently?

Yes ☐ No ☐

Qu 7. Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?

Yes ☐ No ☐

Qu 8. Have any immediate family had heart problems prior to the age of 60?

Yes ☐ No ☐

Qu 9. Do you have any issues with having your blood taken?

Yes ☐ No ☐

Qu 10. Do you have any infectious disease that may be transmitted in blood?

Yes ☐ No ☐

Qu 11. Do you have any issues exercising or completing any physical activity while wearing a face mask?

Yes ☐ No ☐

Qu 12. Do you have any allergic reactions, or issues consuming beetroot juice?

Yes ☐ No ☐

Qu 13. Do you have any of the following conditions, which may be affected by consumption of beetroot juice? (Kidney stones or a history of kidney stones, liver or kidney dysfunction).

Yes ☐ No ☐

Qu 14. Are there any issues that may prevent you from completing an approximately 20 min maximal effort cycle (adjusted for your fitness level)? If yes, please explain.

Yes ☐ No ☐

_____

_____

_____

I have read, understood and completed this questionnaire.

Signature ***(Participant):*** _____ Date: _____

References

5. Thomas S, Reading J and Shephard RJ. Revision of the Physical Activity Readiness Questionnaire (PAR-Q). *Can J Sport Sci* 17(4): 338-345.
6. Cardinal BJ, Esters J and Cardinal MK. Evaluation of the revised physical activity readiness questionnaire in older adults. *Med Sci Sports Exerc* 28(4): 468-472

The effects of supplementation with nitrate rich beetroot juice on performance and cardiovascular health of younger and older adults

CONSENT FORM FOR STUDY VOLUNTEERS

This consent form will be held for a minimum period of five (5) years

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I understand that I have the right to withdraw from the study at any time and to decline to answer any particular questions (if I choose to withdraw I cannot withdraw my data from the analysis after the data collection has been completed).

I agree to provide information to the researcher on the understanding that my name will not be used without my permission. (The information will be used only for this research and publications arising from this research project.)

I agree to participate in this study under the conditions set out in the Information Sheet.

Signature: _____ **Date**_____

Full Name (printed) _____

Phone Number_____ **Age** _____ **Date of Birth** _____

Are you willing to be contacted regarding future research projects within the School of Sport, Exercise and Nutrition? Your name and email address will be saved in a secure location. You will be sent periodic newsletters regarding research studies within the School. You can opt out of this newsletter at any time.

☐ Tick here if you accept.

Appendix 6.6 – Chronic study data collect sheet

SUBJECT NAME	**AGE (y)**	**SUBJECT NUMBER**
MASS (kg)	**DATE**	
HEIGHT (cm)	**BP(mmHg)**	
Breakfast Selection		
Health Screening and Consent Forms Completed		
Feeling Scale Familiarization		
Felt Arousal Scale Familiarization		
PMOS Familiarization		
Cognitive Tests Familiarization	**CB** ☐	**RVIP** ☐ **Stroop** ☐

Blood Pressure	**Systolic**	**Diastolic**
One		
Two		
Three		
Average		

Heart Rate			
Average			
***W*max**			
Cycle Work to Complete (KJ)			
Baseline Cycle Trial Time			

Familiarisation (incremental cycle test)

i. Younger adults warm up on 75 W for 5 min. Test starts at 95W increasing by 35 W every 2 min until voluntary exhaustion.
ii. Older adults warm up on 50 W for 5 min. Test starts at 70 W increasing by 25 W every 2 min until voluntary exhaustion.
iii. Calculate Wmax using $Wmax = Wout + [\left(\frac{t}{120}\right) \times 35]$

$$Wmax = Wout + [\left(\frac{t}{120}\right) \times 25]$$

$$Total\ Work\ to\ complete\ (J) = Wmax \times 0.75 \times 1200$$

Time (min)	Watts (W)
Warm up 5min	75
0 – 2	95
2 – 4	130
4 – 6	165
6 – 8	200
8 – 10	235
10 – 12	270
12 – 14	305
14 - 16	340

Time	Watts
Warm up 5min	50
0 – 2	70
2 – 4	95
4 – 6	120
6 – 8	145
8 – 10	170
10 – 12	195
12 – 14	220
14 - 16	245

SUBJECT NAME				**DATE**		
MASS (kg)				**AGE (y)**		
HEIGHT (cm)				**BP(mmHg)**		
POMS (Before / After Exercise)						
Feeling Scale (Before / After Exercise)						
Felt Arousal Scale (Before / After Exercise)						
Cognitive Tests (Before / After Exercise)						
Blood Pressure	**Systolic**			**Diastolic**		
Before Exercise						
Average						
Post Exercise						
Average						
Heart Rate (Before / After Exercise)						
Heart Rate Average						
Cycle Work to Complete (KJ)						
Percentage of Work Done (%)	**20**	**40**	**60**	**80**	**100**	**End**
Work to Complete (KJ)						
Time Completed (min)						
Time to Completion						
O_2 Uptake Kinetics (Meta-analyser)	**Data recorded automatically via computer**					
RPE (6-20)						
Feeling Scale						
Felt Arousal Scale						
Heart Rate 10s						
20s						
30s						
40s						
50s						
60s						
Mean HR						

SUBJECT NAME				**DATE**				
MASS (kg)				**AGE (y)**				
HEIGHT (cm)				**BP(mmHg)**				
POMS (Before / After Sup / After Exercise)								
Feeling Scale (Before / After Sup / After Exercise)								
Felt Arousal Scale (Before / After Sup / After Exercise)								
Cognitive Tests (Before / After Sup / After Exercise)								
Blood Samples (Before / After Sup / After Exercise)								
Blood Pressure	**Systolic**			**Diastolic**				
Before Supplementation								
Average								
2.5 h After Supplementation								
Average								
Post Exercise								
Average								
Heart Rate (Before / After Sup / After Exercise)								
Heart Rate Average								
Cycle Work to Complete (KJ)								
Percentage of Work Done (%)	**20**	**40**	**60**	**80**	**100**	**End**		
Work to Complete (KJ)								
Time Completed (min)								
Time to Completion								
O_2 Uptake Kinetics (Meta-analyser)	**Data recorded automatically via computer**							
RPE (6-20)								
Feeling Scale								
Felt Arousal Scale								
Heart Rate 10s								
20s								
30s								
40s								
50s								
60s								
Mean HR								

SUBJECT NAME				**DATE**				
MASS (kg)				**AGE (y)**				
HEIGHT (cm)				**BP(mmHg)**				
POMS (Before / After Sup / After Exercise)								
Feeling Scale (Before / After Sup / After Exercise)								
Felt Arousal Scale (Before / After Sup / After Exercise)								
Cognitive Tests (Before / After Sup / After Exercise)								
Blood Samples (Before / After Sup / After Exercise)								
Blood Pressure	**Systolic**			**Diastolic**				
Before Supplementation								
Average								
2.5 h After Supplementation								
Average								
Post Exercise								
Average								
Heart Rate (Before / After Sup / After Exercise)								
Heart Rate Average								
Cycle Work to Complete (KJ)								
Percentage of Work Done (%)	**20**	**40**	**60**	**80**	**100**	**End**		
Work to Complete (KJ)								
Time Completed (min)								
Time to Completion								
O_2 Uptake Kinetics (Meta-analyser)	**Data recorded automatically via computer**							
RPE (6-20)								
Feeling Scale								
Felt Arousal Scale								
Heart Rate 10s								
20s								
30s								
40s								
50s								
60s								
Mean HR								

SUBJECT NAME				**DATE**				
MASS (kg)				**AGE (y)**				
HEIGHT (cm)				**BP(mmHg)**				
POMS (Before / After Sup / After Exercise)								
Feeling Scale (Before / After Sup / After Exercise)								
Felt Arousal Scale (Before / After Sup / After Exercise)								
Cognitive Tests (Before / After Sup / After Exercise)								
Blood Samples (Before / After Sup / After Exercise)								
Blood Pressure	**Systolic**			**Diastolic**				
Before Supplementation								
Average								
2.5 h After Supplementation								
Average								
Post Exercise								
Average								
Heart Rate (Before / After Sup / After Exercise)								
Heart Rate Average								
Cycle Work to Complete (KJ)								
Percentage of Work Done (%)	**20**	**40**	**60**	**80**	**100**	**End**		
Work to Complete (KJ)								
Time Completed (min)								
Time to Completion								
O_2 Uptake Kinetics (Meta-analyser)	**Data recorded automatically via computer**							
RPE (6-20)								
Feeling Scale								
Felt Arousal Scale								
Heart Rate 10s								
20s								
30s								
40s								
50s								
60s								
Mean HR								

Appendix 6.7 – Chapter 6 DRC 16 form

DRC 16

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Luke Allen Stanaway
Name/title of Primary Supervisor:	Prof Ajmol Ali
In which chapter is the manuscript /published work:	Chapter 6

Please select one of the following three options:

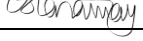
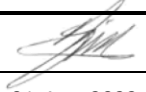
○ The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

○ The manuscript is currently under review for publication – please indicate:

- The name of the journal:
- The percentage of the manuscript/published work that was contributed by the candidate:
- Describe the contribution that the candidate has made to the manuscript/published work:

◉ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	
Date:	01-Aug-2022
Primary Supervisor's Signature:	
Date:	01-Aug-2022

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

GRS Version 5 – 13 December 2019
DRC 19/09/10