Pharmacokinetics of Nitrate and Nitrite following Beetroot Juice Consumption

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

Master of Science

in

Nutrition and Dietetics

at Massey University, Albany, New Zealand.

Emily Margaret Jakubcik

2019

Abstract

Background: Nitrate (NO_3^-) rich beetroot juice (BR) supplementation has been shown to improve cardiovascular function via reduction to nitrite (NO_2^-) and thus the signalling molecule nitric oxide (NO). However, limited research exists for the role of inorganic NO_2^- contained within BR.

Objective: To evaluate the individual effects of NO_3^- and NO_2^- consumed from BR on plasma [NO_3^-]/[NO_2^-] and various cardiovascular measures.

Design: Eleven adults completed four trials; whereby they consumed 250 mL of BR containing one of the following; i) High-NO_3^- (572 mg NO_3^-, 32 mg NO_2^-); ii) Med-NO_3^-/NO_2^- (280 mg NO_3^-, 237 mg NO_2^-); iii) Med-NO_2^- (43 mg NO_3^-, 262 mg NO_2^-); iv) Placebo (PL; 8 mg NO_3^-, 5.8 mg NO_2^-). Plasma [NO_3^-]/[NO_2^-], blood pressure (BP), heart rate (HR), mean arterial pressure (MAP), cardiac output (CO) and stroke volume (SV) were measured at baseline and every hour or second hour for 6 h post BR consumption.

Outcomes: Ingestion of the high-NO_3^- and med-NO_3^-/NO_2^- BR increased plasma [NO_2^-] and [NO_3^-] from 2 h, with both remaining elevated after 6h ($p<0.05$). Med-NO_2^- increased plasma [NO_3^-] ($p<0.05$), but did not increase plasma [NO_2^-] compared to PL ($p=0.177$). MAP was lower following the consumption of high-NO_3^- at 4 h and med-NO_2^- at 6 h ($p<0.05$). However, there were no differences in SBP, DBP, HR, CO and SV between trials.

Conclusion: Inorganic NO_3^- consumption is the critical factor in elevating plasma [NO_3^-] or [NO_2^-], however, both NO_2^- and NO_3^- show potential to reduce MAP. The known reduction of SBP/DBP following NO_3^- supplementation was not observed, making it unclear if NO_2^- contributes to a reduction in SBP/DBP alongside NO_3^-.

Key words: Nitric Oxide; Cardiovascular Disease; Cardio-protective; Blood Pressure.

Acknowledgements

My sincere appreciation goes out to the numerous people below for their help in this research.

Assoc Prof Ajmol Ali and Assoc Prof Kay Rutherfurd-Markwick, both of my academic supervisors, thank you for the organization and encouragement over the last two years. With your support, passion and knowledge you allowed my interest to become a reality, and I will always be thankful.
Marsanne Chabert, thank you for facilitating the trials and for playing a huge role in the planning of the research. I am truly thankful for your efforts.

To the eleven participations involved in this study, thank you so much for your time and for being such a vital part of my research.

My friends and family, the support you have shown me doesn't go unrecognized. I am so very thankful for each and every one of you.
And to Mum, Dad and my partner Patrick, thank you for helping me believe in myself and thank you for the times you encouraged me to never give up.

Dedication

This thesis is dedicated to my Grandfather Raymond, you taught me to dream big and are my greatest inspiration. I hope I made you proud.

I love you Pop.

29.08.1929 – 25.09.2016.

Table of Contents

List of Figures

List of Tables

Abbreviations

ANOVA	Analysis of Variance
BR	Beetroot Juice
BP	Blood Pressure
CO	Cardiac Output
CVD	Cardiovascular Disease
DASH	Dietary Approach to Stop Hypertension
DBP	Diastolic blood pressure
FAS	Felt Arousal Scale
FS	Feeling Scale
HPLC	High Performance Liquid Chromatography
HR	Heart rate
MAP	Mean Arterial Pressure
NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric Oxide Synthases
NZ	New Zealand
POMS	Profile of Mood States
PT	Perceptual Tests
RPM	Revolutions per minute
SBP	Systolic blood pressure
SD	Standard Deviation
SV	Stroke Volume
USCOM	Ultrasonic Cardiac Output Measure

1. INTRODUCTION, AIM AND OBJECTIVES

1.1 Background

Cardiovascular disease (CVD) accounts for approximately one-third of all deaths in New Zealand, placing a significant burden on the New Zealand health system (Heart Foundation NZ, 2017). A major risk factor for the development of CVD is increased blood pressure (BP) or hypertension (Deb and Dasgupta, 2008, Wang et al., 2006) and is often the target for CVD based interventions.

Epidemiological evidence suggests a diet rich in fruit and vegetables reduces BP and therefore the subsequent risk of CVD (Borgi et al., 2016, Challa and Uppaluri, 2018, Dauchet et al., 2007, Liu., 2003). These benefits have been previously attributed to the large amount of antioxidants and vitamins present in vegetables (Borgi et al., 2016, Liu., 2003). However, recent studies fail to replicate the same cardio-protective benefits of antioxidants and vitamins (Lee et al., 2005, Sesso et al., 2008, Waters et al., 2002). This has prompted researchers to investigate the potential of non-antioxidant factors present in fruit and vegetables.

While it is likely that the cardio-protective effects observed with fruit and vegetable intake are multi-factorial, the consumption of high nitrate (NO_3^-) vegetables (green leafy vegetables and beetroot) (Remington and Winters, 2017, Santamaria, 2006) reduced BP more in comparison to other

vegetables (Hord et al., 2009, Joshipura et al., 1999). As a result, there has been increased interest in the potential of NO_3^- derived from fruit and vegetables and its ability to reduce BP (Hord et al., 2009).

Beetroot juice (BR) contains varying amounts of both inorganic NO_3^- and inorganic nitrite (NO_2^-), which in the human body can reduce to the signalling molecule nitric oxide (NO). NO has a role in the relaxation and dilation of the smooth muscle surrounding the blood vessel, and through this, a reduction in BP (d'El-Rei et al., 2016). As NO has a half-life of only milliseconds, it cannot be supplemented and needs to be formed within the body to elicit these benefits (Thomas, 2015). The production of NO in the human body can occur through either an endogenous or exogenous pathway. The endogenous pathway utilises nitric oxide synthase (NOS) to catalyse the production of NO from endogenous L-arginine (Clifford et al., 2015, Kelly et al., 2012, Wylie et al., 2013). Whereas the exogenous pathway is dependent on the consumption of NO_3^-. After NO_3^- is ingested, it is absorbed in the gastrointestinal tract and distributed to various locations, including the mouth (Habermeyer et al., 2015). The anaerobic bacteria found in saliva in the mouth reduce NO_3^- to NO_2^- (Bailey et al., 2009). Following the reduction of NO_3^-, the NO_2^- produced and remaining NO_3^- are swallowed and both re-enter the gastrointestinal tract. When NO_2^- reaches the acidic environment of the stomach it reduces to NO (Bailey et al., 2012). NO then relaxes and dilates the smooth muscle surrounding the blood vessels which in turn reduces BP (Carlström et al., 2015, Contreras et al., 2000, d'El-Rei et al., 2016).

After the discovery of the exogenous NO pathway, the potential of NO_3^- was widely studied as a BP reducing agent. Due to the high content of NO_3^- in BR (~476 µg/g), BR was often used in research. As expected multiple studies have shown that following supplementation with BR, both SBP (5-22 mmHg) and DBP reduced (2.4-18.3 mmHg) (Bailey et al., 2009, Hobbs et al., 2012, Jajja et al., 2014, Kapil et al., 2015, Vanhatalo et al., 2010, Wylie et al., 2013). Unexpectedly however, some studies have failed to show any change in either SBP or DBP (Bondonno et al., 2015, Floyd et al., 2019). Floyd et al. (2019) attributed the inability for NO_3^- to reduce BP within their study to their participants' raised plasma glucose and insulin, while Bondonno et al. (2015) attributed the lack of effect to their use of a hypertensive population. However, this conclusion made by Bondonno et al. (2015) is inconsistent with other studies which show that BR does have an effect on a hypertensive population (Kapil et al., 2015). Furthermore, it appears that the higher initial or baseline SBP, the larger the decrease in SBP (Bahadoran et al., 2017), suggesting that including a hypertensive population would enhance as opposed to prevent a reduction in BP. There is limited research undertaken on the effects of plasma glucose and insulin and their interaction with NO_3^-. However, Floyd et al. (2019) included participants with a low baseline SBP (111 mmHg), this, as eluded to, may prevent a decrease in BP via a self-limiting effect of NO. Furthermore, NO_3^- has been shown to reduce BP in a dose dependent manner, with a larger dose of NO_3^- reducing BP more significantly than a lower dose (Hobbs et al., 2012, Kapil et al., 2010, Wylie et al., 2013). Bondonno et al. (2015) included a low NO_3^- dose (1.4

mmol/day) which was likely not sufficient to result in a reduction in BP. It can be concluded that a reduction in BP is likely dependent on both the individuals' baseline BP and the NO_3^- dose. Importantly, the dose-dependent effect suggests that when supplementing with BR, it is very important to consider and control the NO_3^- dose.

Investigation into acute supplementation with 800 mg NO_3^- from different sources concluded SBP decreased significantly following supplementation with BR (5 mmHg), spinach juice (7 mmHg) and rocket salad (6 mmHg), but there was no reduction in SBP following sodium nitrate ($NaNO_3^-$) supplementation (Jonvik et al., 2016). BR, spinach juice and rocket salad all contain antioxidants, vitamins and traces of NO_2^- which $NaNO_3^-$ does not (Habermeyer et al., 2015). The cardio-protective effect of antioxidants and vitamins alone has been questioned (Lee et al., 2005, Sesso et al., 2008, Waters et al., 2002), however, several studies support NO_2^- having a cardio-protective effect (Brunton., 1867, Reichert and Mitchell., 1880, Gladwin et al., 2000). Specifically, NO_2^- in the form of amyl NO_2^- has been previously used as a therapeutic treatment of angina (Brunton., 1867) and NO_2^- has been shown to reduce BP in cats and dogs (Reichert and Mitchell., 1880). Much more recently an arterial-venous plasma NO_2^- gradient was discovered indicating that a change in plasma NO_2^- concentration can have a beneficial effect on the arteries and hence BP (Gladwin et al., 2000). This is consistent with the relationship seen between a rise in plasma [NO_2^-] and a reduction in BP in recent studies (Jonvik et al., 2016, Ormesher et al., 2018, Webb et al., 2008). The supplementation of $NaNO_2^-$ capsules elicits an elevated plasma

NO_2^- (Hunault et al., 2009), inferring that through the arterial-venous plasma NO_2^- gradient, $NaNO_2^-$ capsules will reduce BP. Despite this, a study investigating the cardio-protective effect of $NaNO_2^-$ capsules (80 mg NO_2^- or 160 mg NO_2^-) showed an improvement in brachial artery flow but interestingly, no change in BP (DeVan et al., 2015). Contrasting, mice studies have shown that NO_2^- infusion (48 nmol) led to a reduction in mean arterial pressure (MAP) (Duranski et al., 2005, Rifkind et al., 2007) and reversed endothelial dysfunction (Sindler et al., 2011). BR contains lower amounts of NO_2^- (38 µg/g) than both the $NaNO_2^-$ capsules and NO_2^- infusions, so these effects seen are not directly comparable. A potential does however arise for inorganic NO_2^- present in BR to reduce BP, and hence contributing to the cardio-protective benefits previously seen with BR supplementation.

Acute supplementation with BR has been concluded to reduce BP, secondary to the high levels of NO_3^- present. However, BR contains varying amounts of both NO_3^- and NO_2^- (Tamme et al., 2006), which via the NO_3^--NO_2^--NO pathway may both be contributing to the cardio-protective effects. The potential of NO_2^- is further highlighted with a reduction of BP occurring only when NO_3^- is supplemented through sources which also contain NO_2^- (beetroot, spinach, rocket salad) and not when the source does not contain NO_2^- ($NaNO_3^-$ supplements) (Jonvik et al., 2016). Despite the potential of NO_2^-, there is little scientific and/or medical interest in the NO_2^- present naturally in BR and its contribution to cardio-protective effects. Consequently,

it is unclear if a variation of NO_2^- content within BR will differ the cardio-protective effect.

The literature review presented in Chapter 2 will expand on the points raised throughout this introduction and further lay foundations for the present study.

1.2 Aims, Objectives and Hypothesis

1.2.1 Aim:

The aim of the current study was to evaluate the individual and combined effects of NO_3^- and NO_2^- consumed from BR on plasma $[NO_3^-]/[NO_2^-]$ and various cardiovascular, mood and perceptual measures.

1.2.2 Objectives

Specific objectives include investigating;

- The change in plasma NO_3^- and NO_2^- over 6 h following the ingestion of BR containing, i) high NO_3^-, low NO_2^-, ii) medium NO_3^- and NO_2^- iii) medium NO_2^-, low NO_3^- or iv) low NO_3^- low NO_2^-
- The change in BP, heart rate (HR), mean arterial pressure (MAP) cardiac output (CO) and stroke volume (SV) over 6 h following the ingestion of BR containing i) high NO_3^-, low NO_2^-, ii) medium NO_3^- and NO_2^- iii) medium NO_2^-, low NO_3^- or iv) low NO_3^- low NO_2^-.
- The change in mood over 6 h following the ingestion of BR containing i) high NO_3^-, low NO_2^-, ii) medium NO_3^- and NO_2^- iii) medium NO_2^-, low NO_3^- or iv) low NO_3^- low NO_2^-
- The association between plasma NO_3^- and NO_2^- and cardiovascular and mood measures.

1.2.3 Hypothesis

It was hypothesized that acute supplementation of:

- BR rich in NO_3^- would increase plasma [NO_2^-] and plasma [NO_3^-] more than BR low in NO_3^-.
- BR rich in NO_2^- would increase plasma [NO_2^-] and plasma [NO_3^-] more than BR low in NO_2^-.
- BR rich in NO_3^- would lead to a greater decrease in BP (SBP, DBP, MAP), CO and SV compared to BR low in NO_3^-.
- BR rich in NO_2^- would lead to a greater decrease in BP (SBP, DBP, MAP), CO and SV compared to BR low in NO_2^-.

To examine these hypotheses, we conducted a randomized, double-blind crossover trial to investigate and compare the pharmacokinetics of acute supplementation with BR containing high and/or low NO_3^- and/or NO_2^- and med NO_3^-/NO_2^-, and its the relationship with mood and perceptual responses, and cardiovascular measures (SBP, DBP, HR, CO, MAP and SV).

1.2.4 Thesis Structure

This thesis contains four key chapters with additional appendices and references. Chapter 1 outlines the background, scope and justification of this study, focusing on the current research of both NO_3^- and NO_2^- and their ability to elicit cardio-protective effects. Included in this chapter are also the study aims, objectives and researcher contributions. Chapter 2 contains a review of the current literature on the cardio-protective effects of beetroot juice with a specific focus on the potential of NO_3^- and NO_2^- to elicit these effects. Chapter 3 is the manuscript of the empirical data collected as part of this thesis, comprising of an abstract, introduction, methods, results, discussion and conclusions. Chapter 4 provides the conclusion to the thesis, outlining

the strengths and limitations of the study and recommendations for future research. Following this are a series of appendices containing ethics consent, the various questionnaires used, as well as supplementary methods.

1.2.5 Researcher Contribution

Table 1.1 Researchers Contributions to thesis study

Contributors	**Contribution to Research**
Emily Jakubcik	Wrote the research proposal, recruited participants, data collection, literature review, blood assays, analyzed and completed statistical analysis, interpretation of results and wrote thesis manuscript.
Associate Professor Ajmol Ali	Academic Supervisor, completed ethics application, planned research design, assisted in revision and approval of the thesis manuscript.
Associate Professor Kay Rutherfurd-Markwick	Co-Academic Supervisor, blood assays, planned research design, assisted in revision and approval of the thesis manuscript.
Marsanne Chabert	Pilot trial, data collection, research design, analysis of data.
Cameron Haswell	Performed phlebotomy and processing of blood samples.

Luke Stanaway	Performed phlebotomy and processing of blood samples

2. REVIEW OF THE LITERATURE

2.1 Introduction

Research indicates that diet plays a large role in the development of cardiovascular disease (CVD). In particular, nutritional interventions which focus on high intakes of fruit and vegetables, such as the DASH (Dietary Approach to Stop Hypertension) diet, reduce the subsequent risk of developing CVD (Challa and Uppaluri, 2018). While the benefits of fruit and vegetables in the diet are likely to be multi-factorial, there has recently been an increased interest in the effect of nitrate (NO_3^-) and its cardio-protective effect. Most research investigating the effects of NO_3^- have been undertaken with beetroot juice (BR) supplementation, secondary to its high NO_3^- content (Lidder and Webb., 2013). The benefits of NO_3^- occur due to its ability to form the vasodilator NO via an exogenous nitric oxide (NO) pathway (Hunault et al., 2009). Specifically, NO_3^- is reduced to nitrite (NO_2^-) which is then reduced to the vasodilator NO (Hunault et al., 2009). BR however, also contains NO_2^- (Shah et al., 2013), which through the same exogenous NO pathway, may be eliciting cardio-protective effects. Despite this potential, there is little scientific and/or medical interest in the NO_2^- in BR.

2.2 Cardiovascular Disease (CVD)

CVD generally refers to conditions involving narrowed or blocked blood vessels, which are commonly associated with heart attacks, chest pain (angina) and/or stroke (Clinic, 2019). CVD is a huge burden on the NZ health system, accounting for approximately 33% of deaths (Heart Foundation NZ, 2017) and comprising of approximately 11% of the total health budget (Coppell et al., 2013).

A poor dietary pattern, in particular the over-consumption of foods high in saturated fats, refined sugars, cholesterol and low in fibre (Siri-Tarino et al., 2010) contribute to CVD through the development of hypertension, or high blood pressure (BP) (Deb and Dasgupta, 2008). High blood pressure is defined as a systolic blood pressure (SBP) greater than 140 mmHg and a diastolic blood pressure (DBP) greater than 90 mmHg) (Deb and Dasgupta, 2008). Secondary to this, nutritional interventions focusing on a high intake of fruit and vegetables, such as the DASH diet have become a very popular and important strategy to achieve normal blood pressure and control CVD risk (Challa and Uppaluri, 2018). Normal blood pressure is defined as SBP 90 - 120 mmHg and DBP 60 - 80 mmHg (Deb and Dasgupta, 2008). The cardio-protective effects associated with the DASH diet have been previously attributed to the abundance of antioxidants and vitamins present in fruit and vegetables (Borgi et al., 2016, Liu., 2003). Interestingly however, when investigated alone, antioxidants and vitamins show no cardio-protective benefit (Lee et al., 2005, Sesso et al., 2008, Waters et al., 2002). This infers

that another factor present may be contributing to the cardio-protective effects associated with fruit and vegetables.

While it is likely that the cardio-protective effect observed with fruit and vegetable intake is multi-factorial, the consumption of high NO_3^- vegetables (green leafy vegetables and beetroot) (Remington and Winters, 2017, Santamaria, 2006) lead to the greatest reduction of BP compared to other vegetables (Hord et al., 2009, Joshipura et al., 1999). In the human body NO_3^- reduces to the vasodilator NO, which via the dilation of the blood veseel, reduces BP (d'El-Rei et al., 2016). Collectively, this research has resulted in an increased interest into the potential of NO_3^- derived from fruit and vegetables leading to a reduction in BP (Hord et al., 2009).

2.3 Nitric Oxide (NO)

NO is an essential molecule in maintaining cardiovascular health due to its ability to relax and dilate the smooth muscle surrounding the blood vessels (Carlström et al., 2015, Contreras et al., 2000, d'El-Rei et al., 2016) and from this, lower BP (d'El-Rei et al., 2016). Furthermore, NO deficiency promotes the development of CVD through platelet activation and plaque formation (Bian et al., 2008). Due to a half-life of only milliseconds (Thomas, 2015) NO cannot be given as a supplement, and instead needs to be formed within the body.

2.3.1 Formation of NO

Both an endogenous and exogenous pathway exist for NO production in the human body. The endogenous pathway was originally thought to be the sole

pathway, whereby nitric oxide synthase (NOS) catalyses the production of NO from L-arginine (Clifford et al., 2015, Kelly et al., 2012, Wylie et al., 2013). An exogenous pathway was later discovered, where inorganic anions NO_3^- and NO_2^- reduce to NO via simple electron reductions (Carlström et al., 2015).

The exogenous pathway begins with the ingestion of NO_3^-. Following the consumption of NO_3^- it becomes absorbed in the upper gastrointestinal tract and then distributed via the blood circulation to multiple locations including the salivary glands (Habermeyer et al., 2015). When NO_3^- reaches the salivary glands, it concentrates at least 10 fold (McIlvenna et al., 2017). Once concentrated 6-7% of the NO_3^- is reduced to NO_2^- by anaerobic bacteria in the mouth (Bailey et al., 2012). The remaining NO_3^- and NO_2^- are then swallowed and both re-enter the gastrointestinal tract (Habermeyer et al., 2015). NO_2^- reaches a peak plasma concentration 2.5-3 h post NO_3^- ingestion while NO_3^- reaches a peak plasma concentration 1-1.5h post ingestion (James et al., 2015). When NO_2^- reaches the acidic environment of the stomach it reduces to NO via a simple one-electron reduction (Bailey et al., 2012, Hunault et al., 2009). If NO_3^- is unable to be reduced to NO_2^- in the mouth (e.g. spitting of saliva or antibacterial mouthwash) NO cannot be formed and therefore the exogenous pathway is reliant on the reduction of NO_3^- to NO_2^- (Bailey et al., 2012, Butler and Feelisch, 2008, McIlvenna et al., 2017, Webb et al., 2008). This exogenous NO pathway is able to confirm the cardio-protective potential of NO_3^-, as well as highlighting a potential for NO_2^- to form NO, and hence for NO_2^- to also elicit cardio-protective effects.

2.4 Dietary sources of NO_3^- and NO_2^-

Generally, cress, lettuce, radish, rocket spinach and red beetroot contain the highest amounts of NO_3^- (Santamaria, 2006). Beetroot is often used in research when identifying the effects of NO_3^-, due to its high NO_3^- content, independent of form (raw, juiced or supplement) (Lidder and Webb, 2013, Remington and Winters, 2017). However, due to the ease of supplementation, beetroot juice (BR) is often used in research.

The quantity of NO_3^- within BR is estimated to be approximately 476 µg/g (Shah et al., 2013). However, the amount is influenced by many factors including the use of nitrogen fertilisers (Ahluwalia et al., 2016, Miyazaki, 1975, Pussemier et al., 2006, Santamaria, 2006, Van Cleemput and Samater, 1995), type of soil, biological properties of the plant, plant maturity, harvesting time, lighting conditions, humidity (Bose and Srivastava, 2001, Tamme et al., 2006) and temperature (dos Santos Baião et al., 2016). Therefore, the quantity of NO_3^- may vary considerably between BR even when given the same quantity.

The quantity of NO_2^- within BR is significantly lower than NO_3^-, and is estimated to be approximately 38 µg/g (Habermeyer et al., 2015, Lee et al., 1971, Shah et al., 2013, Tamme et al., 2006). However, like NO_3^- the amount of NO_2^- present in BR varies considerably dependent on many of the same external factors. Specifically, NO_3^- breaks down to NO_2^- over time and hence the quantity of NO_2^- increases with the storage of beetroot and its juice (Ahluwalia et al., 2016, Hunault et al., 2009, Lee et al., 1971, Lundberg and

Govoni, 2004, Vasconcellos et al., 2016). Furthermore, the NO_2^- formed via NO_3^- consumption will vary between individuals, depending on the properties of the consumer's mouth (pH, quantity of oral and gut bacteria and saliva flow rate), the fitness status of the consumer and environmental factors such as ambient oxygen (James et al., 2015). Additionally, females have a higher baseline NO_2^- as well as a greater oral NO_3^- reduction ability compared to males (Kapil et al., 2018). Therefore, not only does the quantity of NO_2^- in BR varies between BR supplements, but the amount of NO_2^- that is formed following the consumption of NO_3^- can also vary considerably.

As BR contains a variable amount of NO_3^- the amount of NO that is formed from various supplements and hence the subsequent cardio-protective effect will differ. The NO_2^- content within BR is also variable, however the consequence of this is unclear as the cardio-protective effect of NO_2^- is not confirmed.

2.5 NO_3^- ingestion and CVD

Through the exogenous formation of NO, consumption of NO_3^- has the potential to reduce BP in the human body (Lundberg and Govoni, 2004). A selection of randomised crossover studies which explore the cardio-protective effect following acute supplementation with NO_3^- are included in Table 2.2 as well as a small selection of parallel study designs investigating the potential of non-acute supplementation period (~4 weeks). Studies were chosen to include both normotensive and hypertensive populations. Key highlighted outcomes were the effect of BR supplementation on various health based outcomes such as SBP, DBP and ambulatory blood pressure

(ABPM) (BP measures at regular intervals over a period of time), and the correlating plasma [NO_3^-] and [NO_2^-].

Table 2.2 Clinical trials investigating the effects of NO_3^- on cardiovascular health

Reference	Participants	Study Design	Supplementation Protocol	Testing Protocols	Blood Measures (NO Indices)	Health-Based Outcomes
Vanhatalo et al. (2010)	8 healthy adults 3 F, 5 M. (age 29 +/- 6)	Randomised, double-blind, crossover.	15-day supplementation. 0.5 L BEET-IT BR/day (5.2 mmol /day NO_3^-)	BP measured 2.5 h post supplement, day 2, 5, 12 & 15.	↑ plasma [NO_3^-] ↑ plasma [NO_2^-]	↓ SBP. ↓ DBP.
Bailey et al. (2009)	8 healthy M. (age 26 +/- 6)	Randomised, double-blind crossover.	6-day supplementation 0.5 L BEET-IT BR/day (5.5 mmol/day NO_3^-)	BP measured day 4, 5 & 6.	↑ plasma [NO_2^-]	↓ SBP.
Larsen et al. (2006)	17 healthy adults. 2 F, 15 M. (age 24)	Randomised, double-blind crossover.	3-day supplement period. 0.1 mmol/kg $NaNO_3^-$. * similar levels found in 150-250 g of NO_3^- rich vegetables.	BP measured end day 3.	↑ plasma [NO_3^-] ↑ plasma [NO_2^-]	↓ DBP. ↔ SBP.
Wylie et al. (2013)	10 healthy M. (age 23 +/- 5)	Randomised, double-blind crossover.	Acute supplementation with; 70 mL BR (4.2 mmol NO_3^-) 140 mL BR (8.4 mmol NO_3^-) 280 mL BR (16.8 mmol NO_3^-) *PL = 0 mL BR.	BP measured 1, 2, 4, 8, 12 and 24 h post ingestion.	↑ plasma [NO_3^-] ↑ plasma [NO_2^-] Note: this was dose-dependent.	↓ SBP ↓ DBP. (both are dose-dependent but no difference between 8.4 & 16.4).
Bondonno et al. (2015)	27 hypertensive adults 10 M, 17 F. (age 63.2 +/- 4.4)	Randomised, double-blind, crossover.	7-day supplementation. 2 x 70 mL BEET-IT BR/day.	BP measured daily. ABPM day 7.	↑ plasma [NO_3^-] ↑ plasma [NO_2^-]	↔ BP or AMBP.
Coles et al. (2012)	30 healthy adults. 15 F, 15 M. (age 42.5 +/- 3.4)	Randomised, double-blind, crossover.	Acute supplementation 500g BR (15 mmol NO_3^-).	ABPM 24 h BP.	Not measured.	↓ SBP in men.
Jajja et al. (2014)	21 subjects, 12 M, 9 F.	2-arm parallel, open-label, randomised.	21-day supplementation. 70 mL BEET-IT BR (300-400 mg NO_3^- /day).	ABPM 24 h baseline and at 21 days. Daily BP measuring.	Not measured.	↓ SBP. ↔ DBP or AMBP.

	(age 62.7 +/- 1.5 BR group; 61.4 +/- 1.3 PL).					
Kapil et al. (2010)	Study 1: 21 healthy adults. (age 22.5 +/- 0.8) Study 2: 6 healthy adults (age 28.8 +/- 1.7)	Randomised, double-blind crossover	Study 1: Acute supplementation 24 mmol KNO_3^- capsule (1488 mg NO_3^-). Study 2: Acute supplementation 4 mmol KNO_3^- capsule (248 mg NO_3^-) or 12 mmol KNO_3^- capsule (744 mg NO_3^-).	BP measured 24 h post-supplement.	↑ plasma [NO_3^-] ↑ plasma [NO_2^-] Effect was dose dependent.	↓ SBP. ↓ DBP. Effect was dose dependent.
Kapil et al. (2015)	64 hypertensive participants. PL group N=32; 22 F, 10 M (age 56.3 +/- 16.4). BR group N=32; 16 F, 16 M (age 57.6 +/- 13.9)	Double-blind, parallel, randomised.	4-week daily supplementation. 250 mL BR (6.4 mmol NO_3^-)	Pre-supplement; 2-week at home BP measuring & 1 x 24 h AMBP. Daily BP 4 weeks and 1 x AMBP. Post-supplement 2 week at home BP measuring & 1 x 24 h AMBP.	↑ plasma [NO_3^-] ↑ plasma [NO_2^-]	↓ SBP. ↓ DBP. ↓ AMBP
Floyd et al. (2019)	33 participants. 15 M; 18 F. (age 27 +/- 6.5)	Randomised, double-blind crossover	Acute supplementation high (24 mmol NO_3^-) or low (8 mmol NO_3^-) dose KNO_3^-. OGTT 1 h post supplement.	BP measured every 15 m 4 h post supplement.	↑ plasma [NO_2^-]	↔ DBP or SBP.
Hobbs et al. (2012)	18 normotensive M (age 31.4 +/- 3.0)	Randomised, controlled, single blind, cross-over.	Acute supplementation O % BR (<0.05 mmol NO_3^-) 20% BR (2.3 mmol NO_3^-) 50% BR (5.7 mmol NO_3^-) 100% BR (11.4 mmol NO_3^-)	24 h AMBP post supplement.	Not measured.	↓ SBP. ↓ DBP.
Velmurugan et al. (2013)	24 healthy adults. 12 M, 12 F. (age 26.1 +/- 0.8)	Single blind, randomised, crossover.	Acute supplementation BR (3.1 +/- 0.35 mmol NO_3^-).	Measured baseline platelet reactivity and 3 h post supplement.	↑ plasma [NO_3^-] ↑ plasma [NO_2^-]	↓ platelet reactivity.

Kukadia et al. (2019)	15 participants. 11 F, 4 M. (age 29 +/- 8.3)	Randomised, double-blind crossover	Acute supplementation 70mL BEET-IT BR (6.5 – 7.3 mmol NO_3^-)	BP 20, 10 and 5 min pre-supplement. 5, 10, 15, 30, 60 min post-supplement. 24 h AMBP after 60 min.	Not measured.	↓ aortic SBP (at 30 m and 60 m (smaller)) ↔ brachial BP or 24 h AMBP.

NO_3^-, nitrate; NO_2^-, nitrite; BR, beetroot juice; PL, placebo; BP, blood pressure; SBP, systolic BP; DBP, diastolic BP; AMBP, ambulatory blood pressure; M, male, F, female, ↔ no significant difference, ↓ significant decrease, ↑ significant increase.

Inorganic NO_3^- supplementation has been shown to elicit various vascular benefits in a number of studies (Kapil et al., 2015, Velmurugan et al., 2013). In particular, a reduction of SBP (5-22 mmHg) and DBP (2.4-18.3 mmHg) is commonly observed following BR supplementation (Bailey et al., 2009, Hobbs et al., 2012, Jajja et al., 2014, Kapil et al., 2015, Vanhatalo et al., 2010, Wylie et al., 2013). Consistent with the exogenous NO pathway, the reduction in BP was seen in conjunction with a rise in plasma [NO_3^-] and [NO_2^-]. Unexpectedly, however, some studies show no change in SBP (Bondonno et al., 2015, Floyd et al., 2019, Larsen et al., 2006) or DBP (Bondonno et al., 2015, Floyd et al., 2019, Jajja et al., 2014) following acute (Floyd et al., 2019) and non-acute (3 – 21 days) (Bondonno et al., 2015, Jajja et al., 2014, Larsen et al., 2006) supplementation with NO_3^--rich BR.

The absence of cardio-protective effects observed in a selection of studies was proposed to be secondary to the health status of participants included (Bondonno et al., 2015, Floyd et al., 2019). Bondonno et al. (2015) attributed the lack of effect to their use of a hypertensive population. However, a larger study showed a pronounced decrease in BP when including only hypertensive individuals (Kapil et al., 2015). Furthermore, it appears that the higher initial or baseline SBP, the larger the observed decrease in SBP (Bahadoran et al., 2017), inferring that a hypertensive population would enhance rather than prevent a reduction in BP. Floyd et al. (2019) utilised potassium NO_3^- capsules as their supplement source, and attributed the lack of effect on BP to participants elevated plasma [glucose] and [insulin] levels (mimicking a diabetic state). A lack of effect has also previously been

associated with the use of capsules as opposed to BR, independent of NO_3^- dose (Jonvik et al., 2016). This is because unlike capsules, BR contains various other components which may help promote a cardio-protective effect (Jonvik et al., 2016). While there is no study to disprove that a diabetic state will prevent the cardio-protective effect of NO_3^-, the use of capsules may be preventing a cardio-protective effect. Additionally, Floyd et al. (2019) included participants with a low baseline SBP (111 mmHg), which may preclude a decrease in BP via a self-limiting effect of NO. Therefore, there are several external factors which may be preventing a reduction of BP following NO_3^- supplementation, including, but not limited to, a low baseline BP, an elevated plasma [glucose] and [insulin] and the supplement source.

NO_3^- reduces BP in a dose-dependent manner in human subjects, with a larger dose of NO_3^- reducing BP more significantly than a lower dose of NO_3^- (Hobbs et al., 2012, Kapil et al., 2010, Wylie et al., 2013). Bondonno et al. (2015) used hypertensive participants who consumed BR containing ~4.3 mmol NO_3^- (140 mL of 3.1 g NO_3^-/ L), while Kapil et al. (2015) used hypertensive participants who consumed BR containing ~6.4 mmol NO_3^-. Based on these results, it is likely that the dose supplemented by Bondonno et al. (2015) was not sufficient to result in a significant reduction in BP. The minimum NO_3^- needed to provide an cardio-protective effect is unclear, and appears to differ between studies and participants. It can be concluded however, that the NO_3^- dose will alter the subsequent cardio-protective effect associated with BR.

Beet-It Shot is the most common BR products commercially available, it is formulated as a 70 mL shot which contains approximately 300-400 mg NO_3^- (White, 2018). This 70 mL Beet-It Shot has been utilized as the supplement source in multiple studies (Bondonno et al., 2015, Jajja et al., 2014, Kukadia et al., 2019). In studies that solely utilize this 70mL Beet-It Shot as their BR supplementation, no cardio-protective effect is seen (Bondonno et al., 2015, Jajja et al., 2014, Kukadia et al., 2019). Researchers are often reliant on the accuracy of the Beet-It Shot containing the estimated 300-400 mg NO_3^- for the quantification of the NO_3^- in their test supplement. Jajja et al. (2014) analysed a small sample (N = 3) of the Beet-It Shots that were used in their study, and found these shots only contained 165 +/- 2 mg NO_3^-, which is significantly lower than the estimated 300 – 400 mg. While Beet-It should be meeting the minimum NO_3^- content listed on the packaging, this finding suggests that researchers cannot be reliant on the Beet-It composition being accurate. To ensure accurate conclusions are made researchers will need to measure the actual level of NO_3^- in their own BR supplement. Those studies who failed to complete their own analysis of the Beet-It product used, may have been supplementing with an insufficient dose for cardio-protective effects (Bondonno et al., 2015, Jajja et al., 2014, Kukadia et al., 2019). These results provide further evidence that the dose of NO_3^- present in BR is crucial when eliciting cardio-protective effects, and it cannot be assumed that all BR supplementation will have the same cardio-protective effect.

The cardio-protective effects associated with the consumption of NO_3^- occur through its ability to produce NO. However, through the same exogenous NO

pathway, NO_2^- consumption may also form NO and hence be responsible for some of the cardio-protective effects seen. As previously discussed BR contains both NO_3^- and NO_2^-, with the content differing significantly depending on a number of factors. The importance of quantifying NO_3^- in BR has been discussed above. If NO_2^- will also elicit a cardio-protective effect, researchers must also quantify the amount of NO_2^- present in BR. However, a large gap in the research exists, as to whether NO_2^- naturally present in BR is able to also elicit a cardio-protective effect.

2.6 NO_2^- ingestion and CVD

When reviewing the exogenous NO pathway, NO_3^- reduces to NO_2^- which then further reduces to NO (Lidder and Webb, 2013). This shows that like NO_3^-, NO_2^- can exogenously produce the vasodilator NO (Hunault et al., 2009). Despite this, little consideration has been given as to whether the inorganic NO_2^- within BR contributes significantly to the lowering of BP; instead the focus has been solely on the inorganic NO_3^- content.

Cardio-protective effects have previously been associated with ingestion of NO_2^-, in forms other than BR. Organic NO_2^- in the form of amyl NO_2^- was previously used as a therapeutic treatment of angina (Brunton, 1867), however the value of this was compromised secondary to its short duration of effect and therefore its use was discontinued. Following this, the oral supplementation with inorganic KNO_2^- was tested, showing that NO_2^-, even at a small dose (~30 mg) caused a moderate decrease in arterial BP in healthy human volunteers (Reichert and Mitchell, 1880). However, multiple other researchers found the same decrease in BP following inorganic NO_3^-

supplementation as well as concluding NO_3^- had a longer therapeutic effect than organic NO_2^- (Atkinson, 1888, Densham, 1927). Secondary to the abundance of side-effects (nausea, stomach ache and diarrhoea) associated with potassium and $NaNO_2^-$ ingestion (Blumgarten, 1934), there was little further research on the effects of NO_2^- ingestion.

More recently however, an arterial-venous plasma NO_2^- gradient was discovered highlighting that any change in plasma [NO_2^-] has a dilating effect on arteries, and hence can alter BP (Gladwin et al., 2000). A dilation of blood vessels, and hence a reduction in BP was seen post NO_2^- infusion, confirming the arterial-venous plasma NO_2^- gradient (Cosby et al., 2003). A dilation of blood vessels was still observed when the endogenous NO pathway was blocked (via NOS synthesis inhibition), thus confirming the formation of exogenous NO via a NO_2^- infusion (Cosby et al., 2003). However, when comparing inorganic NO_3^- to inorganic NO_2^-, NO_3^- has a longer half-life (5-8 h vs. 1-5 min) and greater stability in the plasma (Wang et al., 2013). Therefore, inorganic NO_3^- through BR supplementation became the main focus to elevate plasma [NO_2^-] and to produce the cardio-protective effects seen with BR consumption. However, inorganic NO_2^- still has the potential to be contributing to the cardio-protective effects seen with BR consumption.

2.6.1 Cardio-protective effect of NO_2^- in mice

Recent studies on the cardio-protective effect of NO_2^- are much more scarce than studies investigating the cardio-protective effect of NO_3^-. Research in mice showed the administration of a $NaNO_2^-$ solution (48 nmol NO_2^-) had a

greater cardio-protective effect than the administration of a $NaNO_3^-$ solution (48 nmol NO_3^-) (Duranski et al., 2005), suggesting NO_2^- may have a larger cardio-protective effect than NO_3^-. Further evidence supporting an effect of NO_2^- was shown in mice, with a biphasic fall in mean arterial pressure (MAP; 1 min post and 4 min post) occurring following a 10 min $NaNO_2^-$ infusion at a rate of 1 μmol/kg body wt/min (Rifkind et al., 2007). Additionally, a 3-week supplementation with $NaNO_2^-$ (50 mg/L in drinking water) was shown to reverse age-associated endothelial dysfunction in mice (Sindler et al., 2011). Similarly, the endothelial dysfunction which occurred when mice were fed a cholesterol-enriched diet for 3 weeks was reversed with both 33 and 99 mg/L ad libitum NO_2^- in their drinking water (Stokes et al., 2009). This research shows that not only does NO_2^- appear to reverse age-associated effects in mice, but there is emerging evidence that it can also reverse the effects of a cholesterol-rich diet. It remains unclear if these benefits would continue with long-term supplementation, or if NO_2^- could reverse the damage following a long-term cholesterol-rich diet. It is also unclear if inorganic NO_2^- present in BR will have the same effect as the NO_2^- infusion utilised in these studies, as well as if these benefits would be replicable in humans. Therefore, while these studies further suggest a cardio-protective potential of NO_2^-, there is no evidence surrounding the effect of inorganic NO_2^- present in BR.

2.6.2 Potential of inorganic NO_2^- within BR

Evidence regarding ingestion of NO_2^- through BR supplementation is scarce. The effects of NO_2^- ingestion in humans has primarily been tested through the ingestion of $NaNO_2^-$ capsules. A recent study supplemented with $NaNO_2^-$ capsules (80 mg NO_2^- or 160 mg NO_2^- per day for 10 weeks), concluded that

NO_2^- was both safe and improved brachial artery flow in humans (DeVan et al., 2015). Furthermore, 80 mg NO_2^-/day but not 160 mg NO_2^-/day increased arterial diameter and decreased arterial stiffness (DeVan et al., 2015). This highlights that similarly to NO_3^- there appears to be an optimal dose of NO_2^- to elicit the largest cardio-protective effect. However, it also infers that NO_2^-, unlike NO_3^- will provide a larger cardio-protective effect with a smaller dose (Hobbs et al., 2012, Kapil et al., 2010, Wylie et al., 2013). The results obtained from the ingestion of NO_2^- via NO_2^- capsules cannot be extrapolated to the ingestion of inorganic NO_2^- within BR. This is because the NO_2^- content differs between a cup of BR (~5 mg) and the NO_2^- capsules studied (80 mg/ 160 mg), as well as BR containing various other antioxidants and vitamins which may along with NO_3^- contribute to a cardio-protective effect.

When investigating results from recent clinical trials on BR, studies have shown a 3 to 4 h delay in the reduction of BP post-BR consumption. This delay relates to an increase in plasma NO_2^- which occurs once NO_3^- is reduced to NO_2^- (James et al., 2015, Wylie et al., 2013), suggesting that the formation of NO_2^- is a key contributor to cardio-protective effects. A summary of these findings is provided in Table 2.3.

Table 2.3 Clinical trials investigating the effects of NO_2^- on cardiovascular health.

Reference	Participants	Study Design	Supplementation Protocol	Testing Protocols	Blood Measures (NO Indices)	Health-Based Outcomes
Webb et al. (2008)	14 healthy participants 9 M, 5 F. (age 25.5 +/- 4.5)	Open-label crossover design.	Acute supplementation with 500mL BR.	BP measured 1 h pre and 3 h post supplement.	↑ plasma [NO_3^-] ↑ plasma [NO_2^-]	SBP peak reduction 2.5 h post. DBP peak reduction 3 h post. Related to peak plasma [NO_2^-]
Jonvik et al. (2016)	18 healthy adults. 11 M, 7 F. (age 28 +/- 1)	Semi randomised crossover design.	Acute supplementation with different beverages each containing 800 mg (~12.9 mmol) NO_3^-. Spinach juice. BR. Rocket salad. $NaNO_3^-$. supplement.	BP measured before and up to 300 minutes after supplement ingestion.	↑ plasma [NO_3^-] ↑ plasma [NO_2^-] Note: plasma NO_2^- levels differed dependent on beverage. (Spinach: 980 +/- 160 nmol/L; rocket salad 643 +/- 63 nmol/L; BR 557 +/- 57 nmol/L; NaNO3 580 +/- 58 nmol/L).	↓ SBP at 150 minutes BR and rocket salad. ↓ SBP at 300 minutes spinach. ↔ SBP $NaNO_3^-$. ↓ DBP at 150 minutes with all beverages. ↓ DBP at 300 minutes rocket salad and spinach.
Ormesher et al. (2018)	40 pregnant women between 22 and 35 + 6 weeks gestation.	Double blind randomised control trial.	8-day supplementation. 70 mL BR (~400 mg NO_3^-)	BP measured 0 h, 3 h and 24 h and on day 8.	↑ plasma [NO_3^-] ↑ plasma [NO_2^-]	↔ BP. Highly significant correlation between changes in DBP and plasma [NO_2^-] in NO_3^- treated arm.

NO_3^-, nitrate; NO_2^-, nitrite; BR, beetroot juice; PL, placebo; BP, blood pressure; SBP, systolic BP; DBP, diastolic BP; M, male, F, female, ↔ no significant difference, ↓ significant decrease, ↑ significant increase.

Table 2.3 highlights that despite varying supplementation protocols (e.g. 500 mL acute BR supplementation; 8-day 70 mL BR supplementation) the same inverse correlation between plasma NO_2^- and BP is observed, which is in line with the arterial-venous plasma NO_2^- gradient. While this correlation is seen with NO_3^- consumption, consumption of $NaNO_2^-$ also leads to a rise in plasma NO_2^- (Hunault et al., 2009, James et al., 2015, Jansson et al., 2008), which is not dependent on the consumption of NO_3^-. These findings suggest that $NaNO_2^-$ supplementation is an efficient way to increase plasma [NO_2^-] and hence elicit cardio-protective effects. Furthermore, previous literature has shown that following BR supplementation, if NO_3^- is unable to be reduced to NO_2^- (through the removal of saliva or use of mouthwash) cardio-protective effects are not seen (Butler and Feelisch, 2008, Webb et al., 2008). This finding suggests that the consumption of NO_2^- may be beneficial as it removes the reliance of NO_3^- reducing to NO_2^- in the mouth.

When investigating the potential of the inorganic NO_2^- present in BR, it should be noted that BR contains a significantly lower amount of NO_2^- compared to NO_3^- (Shah et al., 2013). However, following the supplementation with $NaNO_2^-$, the bioavailability of NO_2^- was concluded to be 98% after oral administration of 0.12 mmol $NaNO_2^-$ (0.1176 mmol), and 95% after oral administration of 0.06 mmol $NaNO_2^-$ (0.057 mmol) (Hunault et al., 2009). This is significantly higher than the amount of NO_2^- which would be formed from NO_3^- following consumption of BR (4-8 %) (Hunault et al., 2009, James et al., 2015, Jansson et al., 2008). If the bioavailability of NO_2^- is also accurate for the inorganic NO_2^- present in BR, it would appear that despite

an approximate 12 times lower NO_2^- content (~38 µg/g) than NO_3^- (~476 µg/g) in BR, NO_2^- would be contributing to a similar rise in plasma [NO_2^-] as the NO_3^- content. This is because for the same dose, NO_2^- is absorbed 23 times greater than the amount of NO_2^- formed from the reduction of NO_3^- (95-98 % vs. 4 – 8 %) (Hunault et al., 2009). Furthermore, secondary to the arterial-venous plasma NO_2^- gradient, this would also infer that in BR the NO_2^- would elicit similar cardio-protective effects to the NO_3^- present.

Investigation into acute supplementation with 800 mg NO_3^- from different sources suggested a beneficial effect of inorganic NO_2^- present in vegetables (Jonvik et al., 2016). Specifically, SBP decreased significantly following supplementation with BR (5 mmHg), spinach juice (7 mmHg) and rocket salad (6 mmHg), but not following $NaNO_3^-$ supplementation (Jonvik et al., 2016). BR, spinach juice and rocket salad all contain similar antioxidants, vitamins and importantly traces of NO_2^- which $NaNO_3^-$ does not (Habermeyer et al., 2015, Shah et al., 2013). While the cardio-protective effect of antioxidants and vitamins alone is unclear (Lee et al., 2005, Sesso et al., 2008, Waters et al., 2002), as discussed previously, NO_2^- may be having a cardio-protective effect (DeVan et al., 2015). Although the potential of NO_2^- through sodium and potassium NO_2^- capsules has suggested, Jonvik et al. (2016) provides insight into the potential of naturally occurring inorganic NO_2^- within vegetables, including BR.

Through the supplementation with NO_2^- capsules and NO_2^- infusions, NO_2^- has been shown to elicit cardio-protective effects. Similarly, this suggests

that the inorganic NO_2^- present in BR may also produce cardio-protective effects, an idea that has not yet been researched. Despite this potential, a negative perception towards the ingestion of NO_2^- exists secondary to its role in meat preservation and the subsequent cancer risk (Clifford et al., 2015) and to a lesser extent a change in mood (Khambadkone et al., 2018). The potential negative effects of inorganic NO_2^- present in BR must be further investigated.

2.7 Adverse effects of NO_2^- and NO_3^-

Both NO_3^- and NO_2^- have been used for decades in the preservation of meat products (Binkerd and Kolari, 1975). NO_2^- through meat consumption has been linked to an increased cancer risk secondary to the conversion of NO_2^- to N-nitrosamines in the human body (Clifford et al., 2015). Due to this, the Joint Food and Agriculture Organisation and World Health Organisation set the acceptable daily intake of NO_3^- to 3.7 mg/kg and NO_2^- to 0.001 mg/kg (Hord et al., 2009). In context, this would be equivalent to a 70kg adult consuming approximately 2 L of commercial BR/day. However, meat contains other compounds which in conjunction with NO_2^- speed up and promote the formation of N-nitrosamines (Mills et al., 2017, Song et al., 2015). As vegetables do not have these same compounds, there is no evidence to suggest an increased cancer risk with prolonged NO_3^- and NO_2^- consumption from vegetable sources (Clements et al., 2014, Clifford et al., 2015). This infers that while NO_2^- derived from meats may be detrimental in high doses, the consumption of inorganic NO_2^- within vegetables, including BR, is not concerning.

An individual's diet can influence their subsequent mood state (Firth et al., 2019, Jørgensen et al., 2014, O'neil et al., 2014). Specifically, the long-term consumption of NO_3^- cured meat products was associated with a 3.31-fold increase in hospitalized acute mania, after adjusting for age, sex, race, socioeconomic status, BMI, smoking and multiple comparisons (Khambadkone et al., 2018). Furthermore, mania-like changes were seen in rats, following the consumption of a commercially available cured meat product (BJ-Comm) containing approximately 33.9 mg/kg (70kg human = 2373 mg) NO_3^- (Khambadkone et al., 2018). While there is strong evidence linking an unhealthy dietary pattern to a poorer mood in both humans (Richard et al., 2015) and in rats (Gancheva et al., 2017), when controlling for all variables, excluding NO_3^- additives, NO_3^- was still linked to an increased hyperactivity (Khambadkone et al., 2018). This allowed the researchers to attribute the effects to NO_3^- and not the meat-rich diet pattern (Khambadkone et al., 2018). Inconsistent with these findings however, an acute supplementation with spinach (182 mg NO_3^-) showed no effect on mood 8 h post consumption in healthy men and women (via the Bond-Lader mood scale) (Bondonno et al., 2014). Collectively this suggests that NO_3^- rich meat products may contribute to a change in mood following chronic high exposure. However, it does not appear that an acute supplementation, via a vegetable source will alter mood.

2.8 Future Directions

Previous literature has concluded that BR consumption is effective in reducing BP within humans. Furthermore, studies have begun investigating the cardio-protective effects associated with various inorganic NO_3^- doses

within BR. However, there has been no consideration of a possible cardio-protective effect of inorganic NO_2^- found naturally in BR. NO_2^- like NO_3^- goes on to produce the vasodilator NO via the exogenous pathway, and hence has potential to also lead to cardio-protective benefits through BR supplementation. As BR contains different quantities of both NO_3^- and NO_2^- it is vital to understand the effect of NO_2^- as well as NO_3^- present in BR.

2.9 Summary of the literature

CVD accounts for approximately 33% of deaths in NZ, making it a huge burden on the NZ health system. Knowledge surrounding a cost-efficient intervention is crucial to reduce the prevalence of CVD in NZ and its subsequent burden.

BR supplementation is a potential CVD prevention intervention secondary to the NO_3^- content. In the human body NO_3^-, via an exogenous pathway, forms NO, a natural vasodilator which can reduce BP (Carlström et al., 2018). Consistent with this, BR supplementation has been linked to a reduction in BP in a number of studies (Bailey et al., 2009, Hobbs et al., 2012, Kapil et al., 2015, Larsen et al., 2006, Vanhatalo et al., 2010, Wylie et al., 2013), which has led to the conclusion that BR consumption exhibits a cardio-protective effect in the human body.

One study investigating the effects of supplementation with 800 mg NO_3^- from various sources (BR, spinach juice, rocket salad and $NaNO_3^-$ capsules) showed that consumption of the three vegetable sources resulted in similar reductions in BP (Jonvik et al., 2016). However, consumption of the $NaNO_3^-$

capsules led to no reduction in BP (Jonvik et al., 2016, Larsen et al., 2006). BR, spinach juice and rocket salad contain many compounds that $NaNO_3^-$ does not, including NO_2^-. The presence of NO_2^- within these three vegetable sources is of high importance as NO_2^- through the same exogenous pathway produces NO. Therefore, NO_2^- may also be contributing to the reduction in BP. Supporting this, in mice NO_2^- infusions have shown various cardio-protective benefits including a decrease in MAP and reversed endothelial dysfunction (Duranski et al., 2005, Rifkind et al., 2007, Sindler et al., 2011). Furthermore, in human subjects, $NaNO_2^-$ supplements improved various vascular outcomes.

BR supplementation has shown promising cardio-protective effects, which have been attributed to the NO_3^- content and its ability to form the vasodilator NO. However, BR also contains NO_2^-, which also has the ability to form the vasodilator NO. The cardio-protective effects of NO_2^- have historically and again more recently been shown via NO_2^- infusions and $NaNO_2^-$ capsules. However, the cardio-protective effect of inorganic NO_2^- present in BR has not been investigated.

3. RESEARCH STUDY MANUSCRIPT

The following chapter is presented as a manuscript prepared for the international journal *Nutrients*. *Nutrients* has no restrictions on the length of manuscripts, full experimental details are required so results may be reproduced. Additional methods are found in Appendix I.

Pharmacokinetics of Nitrate and Nitrite following Beetroot Juice Consumption

Jakubcik, Emily Margaret[1]; Ali, Ajmol[1,3], Rutherfurd-Markwick, Kay[2,3].

[1]School of Sport, Exercise and Nutrition, Massey University, New Zealand.

[2]School of Health Sciences, Massey University, New Zealand

[3]Centre for Metabolic Health Research, Massey University, New Zealand

3.1 ABSTRACT

Background: Nitrate (NO_3^-) rich beetroot juice (BR) supplementation has been shown to improve cardiovascular function via reduction to nitrite (NO_2^-) and thus the signalling molecule nitric oxide (NO). However, limited research exists for the role of inorganic NO_2^- contained within BR.

Objective: To evaluate the individual and combined effects of NO_3^- and NO_2^- consumed from BR on plasma $[NO_3^-]/[NO_2^-]$ and various cardiovascular measures.

Design: Eleven adults completed four trials one week apart. In each trial they consumed 250 mL of BR containing one of the following i) High-NO_3^- (572 mg NO_3^-, 32 mg NO_2^-); ii) Med-NO_3^-/NO_2^- (280 mg NO_3^-, 237 mg NO_2^-); iii) Med-NO_2^- (43 mg NO_3^-, 262 mg NO_2^-); iv) Placebo (PL; 8 mg NO_3^-, 5.8 mg

NO_2^-). Plasma [NO_3^-]/[NO_2^-], blood pressure (BP), heart rate (HR), mean arterial pressure (MAP), cardiac output (CO) and stroke volume (SV) were measured at baseline and every hour or second hour for 6 h post BR consumption.

Outcomes: Ingestion of the high-NO_3^- and med-NO_3^-/NO_2^- BR increased plasma [NO_2^-] and [NO_3^-] from 2 h, with both remaining elevated after 6h ($p<0.05$). Med-NO_2^- increased plasma [NO_3^-] ($p<0.05$) but did not increase plasma [NO_2^-] compared to PL ($p=0.177$). MAP was lower following the consumption of high-NO_3^- at 4 h and med-NO_2^- at 6 h ($p<0.05$). However, there were no differences in SBP, DBP, HR, CO and SV between trials.

Conclusion: Inorganic NO_3^- consumption is the critical factor in elevating plasma [NO_3^-] or [NO_2^-], however, both NO_2^- and NO_3^- show potential to reduce MAP. The known reduction of SBP/DBP following NO_3^- supplementation was not observed, making it unclear if NO_2^- will contribute to a reduction in SBP/DBP when NO_3^- does.

Key words: Nitric Oxide; Cardiovascular Disease; Cardio-protective; Blood Pressure.

3.2 BACKGROUND

Cardiovascular disease (CVD) places a significant burden on the New Zealand health system, accounting for approximately one-third of all deaths (Heart Foundation NZ, 2017). A major risk factor for the development of CVD is increased blood pressure (BP) or hypertension (Deb and Dasgupta, 2008, Wang et al., 2006) which is often the target for CVD-based interventions.

Epidemiological evidence suggests a diet rich in fruit and vegetables reduces BP and therefore the subsequent risk of CVD (Borgi et al., 2016, Challa and Uppaluri, 2018, Dauchet et al., 2007, Liu, 2003). This effect has been previously attributed to the abundance of antioxidants and vitamins which are present in fruit and vegetables (Borgi et al., 2016, Liu, 2003). Recently however, the cardio-protective effect of antioxidants and vitamins alone has been questioned (Lee et al., 2005, Sesso et al., 2008, Waters et al., 2002). This has prompted investigation of potential cardio-protective effects of other factors present in fruits and vegetables.

While it is likely that the cardio-protective effect observed with fruit and vegetable intake is multi-factorial, the consumption of high NO_3^- vegetables (green leafy vegetables and beetroot) (Remington and Winters, 2017, Santamaria, 2006) has been shown to lead to the greatest reduction in BP compared to other vegetables (Hord et al., 2009, Joshipura et al., 1999). As a result, there has been increased interest in the potential of NO_3^- derived from fruit and vegetables leading to a reduction in BP (Hord et al., 2009).

Beetroot juice (BR) contains varying amounts of both inorganic NO_3^- and inorganic NO_2^- (Habermeyer et al., 2015, Lee et al., 1971, Lidder and Webb, 2013) which in the human body can reduce to the signaling molecule NO through an exogenous pathway (Carlström et al., 2018). This exogenous pathway begins with the absorption of NO_3^- in the gastrointestinal tract, which is then distributed to various locations, including the mouth (Habermeyer et al., 2015). In the mouth NO_3^- is reduced to NO_2^- by anaerobic bacteria (Bailey et al., 2009). NO_2^- is then swallowed and re-enters the gastrointestinal tract, when it reaches the stomach, the acidic environment reduces NO_2^- to NO (Bailey et al., 2012). NO enters the circulation and is then able to reduce BP by relaxing and dilating the smooth muscle surrounding the blood vessel (Bian et al., 2008, Contreras et al., 2000, d'El-Rei et al., 2016).

Consistent with the exogenous pathway, multiple studies have reported a reduction of systolic blood pressure (SBP) (5-22 mmHg) and diastolic blood pressure (DBP) (2.4-18.3 mmHg) following supplementation with BR (Bailey et al., 2009, Hobbs et al., 2012, Jajja et al., 2014, Kapil et al., 2015, Vanhatalo et al., 2010, Wylie et al., 2013). Other studies however, show no change in either SBP or DBP (Bondonno et al., 2015, Floyd et al., 2019). NO_3^- has been shown to reduce BP in a dose-dependent manner, with a larger dose of NO_3^- reducing BP more significantly than a lower dose (Hobbs et al., 2012, Kapil et al., 2010, Wylie et al., 2013). Floyd et al. (2019) supplemented a sufficient NO_3^- dose (8 or 24 mmol/day / 680 or 2040 mg/day), therefore the increased plasma glucose/insulin was concluded to

prevent the reduction in BP. Bondonno et al. (2015) however provided a low NO_3^- dose (1.4 mmol/day / 119 mg/day) which was likely insufficient to result in a reduction in BP. It can be concluded that the reduction in BP that is seen following consumption of BR is likely dependent on the NO_3^- dose, or the plasma glucose/insulin concentration.

Investigation into the acute supplementation with 800 mg (9.4 mmol) NO_3^- from different sources concluded SBP decreased significantly following supplementation with BR (↓5 mmHg), spinach juice (↓7 mmHg) and rocket salad (↓6 mmHg), but not following $NaNO_3^-$ supplementation (Jonvik et al., 2016). BR, spinach juice and rocket salad all contain antioxidants, vitamins and traces of NO_2^- which $NaNO_3^-$ does not (Habermeyer et al., 2015). The cardio-protective effect of antioxidants and vitamins alone is questioned (Lee et al., 2005, Sesso et al., 2008, Waters et al., 2002). Interestingly, several studies have emerged which support NO_2^- having a cardio-protective effect. In human subjects, the oral consumption of NO_2^- via $NaNO_2^-$ elicits an elevated plasma NO_2^- (Hunault et al., 2009), which correlates with a reduction in BP (Jonvik et al., 2016, Ormesher et al., 2018, Webb et al., 2008). When specifically investigating the cardio-protective effect of $NaNO_2^-$ capsules (80 mg or 160 mg NO_2^- / 1.15 mmol or 2.3 mmol NO_2^-) an improvement in brachial artery flow was seen but interestingly no change in BP (DeVan et al., 2015). Studies in mice have shown that a NO_2^- infusion led to a reduction in mean arterial pressure (MAP) (Duranski et al., 2005, Rifkind et al., 2007) and reversed endothelial dysfunction (Sindler et al., 2011). The cardio-protective effects of NO_2^- can be explained via the exogenous NO

pathway, and the ability for NO_2^- to also produce the vasodilator NO (Bailey et al., 2012, DeVan et al., 2015). While there is potential for NO_2^- to reduce the BP via the exogenous NO pathway, to date there has been no consideration whether the NO_2^- in BR contributes to the observed reductions in BP.

Here, we determine the impact of different doses of NO_3^- and NO_2^- from BR on plasma levels of NO_3^- and NO_2^-, and explore the effect of these difference doses on cardiovascular as well as various perceptual measures. In addition, correlations between the plasma NO_3^- and NO_2^- levels and physiological outcomes were investigated.

3.3 METHODS AND MATERIALS

3.3.1 Sample Size

Previous literature in healthy adults has shown a mean change in BP of 10 mmHg (9 SD) between BR supplementation and a control (Ashor et al., 2015). Using this information a power analysis (G.Power 3.1) was conducted to determine an adequate sample size with the outcome measure of BP. This analysis concluded a sample size of 10 would be sufficient, using a SD of 4.5, with a statistical power of 0.87 and an alpha level of $p < 0.05$.

3.3.2 Participants

Eleven healthy, normotensive adults (18 – 50 years old) were recruited for the study (5 M; 6 F). All participants completed a health-screening

questionnaire (Appendix A) to determine eligibility. Those who regularly consumed NO_3^--based dietary supplements, were unable to participate in blood collection, had a beetroot allergy, were pregnant or had known health issues e.g. hypertension or cardiovascular disease were excluded. Prior to their involvement, the study was explained to all participants and an information sheet was provided (Appendix B). Following this, written consent was obtained (Appendix C). The study was approved by the Massey University Human Ethics Committee (SOA 18/35) (Appendix D)

3.3.3 Intervention

Freshly prepared BR was blended with other solutions (fruit juices/water), to produce two standardized drinks of a constant soluble solid concentration (11ºBrix). Drink C (Med-NO_2^-) was made to a standardized low NO_3^- (43 mg/250 mL) and medium NO_2^- (262 mg/250 mL) concentration. Drink D (Placebo) was made to a standardized low NO_3^- (8 mg/250 mL) and low NO_2^- (5.8 mg/250 mL) concentration. These drinks were pasteurized and filled into 250 mL plastic bottles and stored frozen and then thawed immediately prior to use. Drinks C and D became the base drinks for drink B and drink A respectively.

To prepare drink B (Med-NO_3^-/NO_2^-) 30 mL of drink C was removed immediately prior to consumption (leaving 220 mL) and replaced with 30 mL of a commercially available solution (‘Beet-it shot’) with analysed NO_3^- and NO_2^- content. This produced a 250 mL beverage of 11ºBrix containing standardized medium NO_2^- (237 mg/250 mL) and medium NO_3^- (280 mg/250 mL). Similarly, to prepare drink A (High-NO_3^-) 70mL of drink D was removed immediately prior to consumption (leaving 180 mL) and replaced with 70 mL

of a ‘Beet-it shot’. This produced a 250 mL beverage of 11^{o}Brix containing standardized low NO_2^- (32 mg/250 mL) and high NO_3^- (572 mg/250 mL). The final concentrations of both NO_3^- and NO_2^- within the drinks were determined using high-performance liquid chromatography (HPLC) as described in Section 3.3.6

3.3.4 Study Design and Procedures

Participants were required to visit the laboratory on five occasions. During the first visit participants were familiarized with the testing procedures and equipment, including perceptual and mood questionnaires, ultrasonic cardiac output monitor (USCOM1A; Uscom Ltd; Sydney, Australia) and automated sphygmomanometer (deluxe HEM-7310; OMRON Healthcare CO. Ltd; Kyoto, Japan). Height (m) and weight (kg) were measured via a stadiometer and electronic calibrated scales, respectively.

For 24 h prior to visit two, participants recorded their food and fluid intake and were asked to replicate these diets 24 h prior to the remaining visits (three to five). Participants were instructed to arrive at the laboratory in a fasted state, and to have refrained from caffeine ingestion, strenuous exercise and alcohol for 24 h pre-trial. Each trial commenced in the morning at approximately the same time of day (+/- 1 h).

Visits two, three, four and five were experimental trials. Each participant was randomly allocated the order which the supplemented beverages would be consumed in a double-blinded, randomized crossover design. Visits two, three, four and five were each separated by a one-week washout period to allow for complete normalization of NO_3^- levels in the body (Coles and Clifton, 2012, Cosby et al., 2003, Lansley et al., 2011). In each of the

experimental trials, 250 mL of juice containing either i) High NO_3^- (572 mg NO_3^-, 32 mg NO_2^-; 6.72 mmol NO_3^-, 0.46 mmol NO_2^-), ii) MedNO_3^-/NO_2^- (280 mg NO_3^-, 237 mg NO_2^-; 3.29 mmol NO_3^-, 3.43 mmol NO_2^-) iii) med NO_2^- (43 mg NO_3^-, 262 mg NO_2^-; 0.51 mmol NO_3^-, 3.79 mmol NO_2^-) iv) placebo (8 mg NO_3^-, 5.8 mg NO_2^-; 0.09 mmol NO_3^-, 0.08 mmol NO_2^-) was consumed. Each drink was similar in both appearance and taste, and was not perceived as significantly different ($p>0.05$) when presented to a consumer sensory panel. The dose chosen for the high-NO_3^- (572 mg) and med-NO_3^-/NO_2^- (280 mg) drinks have been shown to be sufficient to elicit cardio-protective effects (Wylie et al., 2013). Therefore, the med-NO_3^-/NO_2^- drink and med-NO_2^- drinks were utilized to analyze the interaction of NO_3^- and NO_2^-.

The drink was consumed within a 10-min period alongside a standardized, isocaloric breakfast with a macronutrient distribution of 15 g protein, 30 g carbohydrate and 10 g fat. Three hours' post-supplement intake the participants received a standardized, isocaloric lunch with a macronutrient distribution of 25 g protein, 45 g carbohydrate, 16 g fat.

On experimental trial days, baseline measures of blood pressure, perceptual and mood tests and a resting blood sample was taken on arrival prior to supplementation. The allocated drink and breakfast were consumed, and the timer started immediately after the drink was finished. Blood samples, mood/perceptual tests, BP and HR were measured each hour and other hemodynamic measurements (SV, MAP, CO) were carried out via the USCOM every second hour (0 h, +2 h, +4 h, +6 h). Participants were asked to remain in the laboratory for the duration of the trial and were able to complete office work between sampling periods.

The study protocol is illustrated in **Figure 3.1;** for further information, see Appendix E which outlines the full procedure.

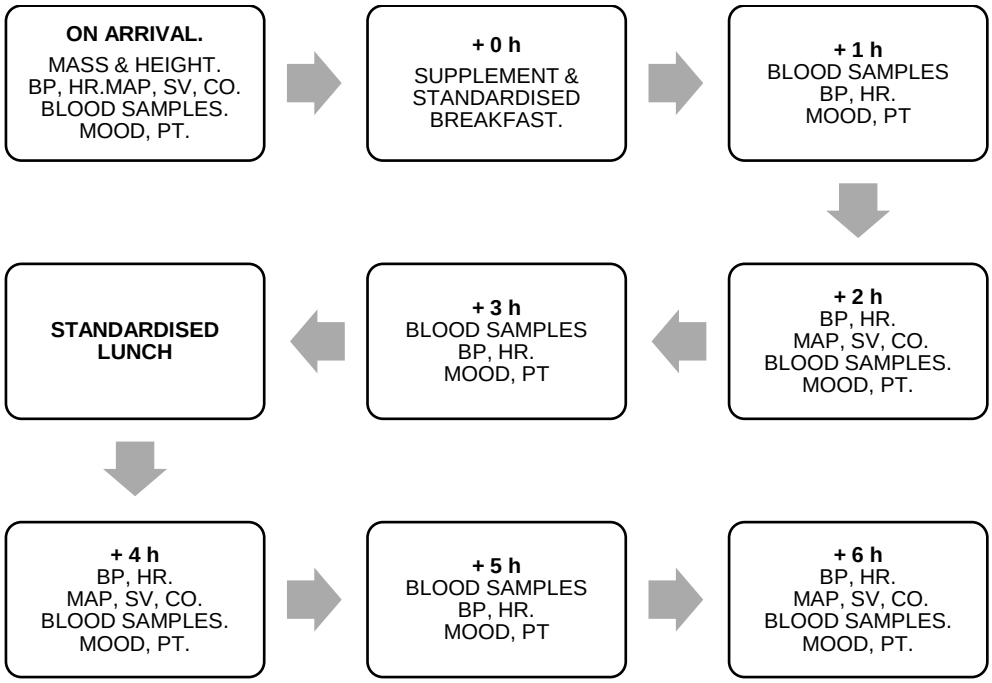

Figure 3.1 Protocol of the experimental trials**.** BP, blood pressure; HR, heart rate; SV, stroke volume; MAP, mean arterial pressure; CO, cardiac output; PT, perceptual tests

3.3.5 Blood Measurements

Six-milliliter venous blood samples were taken via a cannula (where possible) or venepuncture within the antecubital area, and collected into heparinized tubes. Following cannula blood sampling, 5 mL of a non-coagulant saline solution was then injected through the cannula to prevent clotting and blockage of the line. Samples were mixed and immediately centrifuged (MF-50 Hanil Science Industrial, Korea) at 3500 rpm (1300 g) for 10 min, and the collected plasma was aliquoted and stored at -80°C for later HPLC analysis of NO_3^- and NO_2^-.

3.3.6 HPLC analysis

The quantification of NO_3^- and NO_2^- in beetroot juice was based on the method described by Chein-Chung et al. (2003) and Cheng & Tsang. (1998). Briefly samples were centrifuged, filtered (0.45 µm) diluted, and separated on a Gracesmart C_{18} column using a mobile phase of 0.01 M Octylammonium orthophosphate (pH 3-3.5) at a flow rate of 0.8 mL/min. Detection occurred at a wavelength of 193 nm for NO_2^- and 213 nm for NO_3^-. The quantification of NO_2^- in plasma was based on the method described by Li et al. (2000). The plasma samples were filtered using 4 x washed 10 kD cut off filters (Amicon Ultra 2 10 KDa 2 mL Thermo LPN 00288739 UFC201024). The determination of NO_2^- occurs through its derivitisation with 2,3-diaminoaphthalene (DAN) to yield the highly fluorescent 2,3-naphthotriazole (NAT). NAT was then separated on a 5 µm reversed-phase C_8 column (Nova-Pak) with a 40 µm reversed-phase C_{18} guard column. The mobile phase was programmed at 1.3 ml/min and followed the sequence of 15 mM sodium phosphate buffer (pH 7.5) with 50% methanol (0.0 – 3.0 min), Milli-Q water (3.1 – 5.0 min), 100% methanol (5.1 – 8.0 min), Milli-Q water (8.1 – 10.0 min), and finally the 15 mM sodium phosphate buffer (pH 7.5) with 50% methanol again (10.1 – 15.0 min). Fluorescence was monitored with excitation at 375 nm and emission at 415 nm.

This same filter process was utilized for plasma NO_3^- analysis. Following the filtration, the method previously described to quantify the NO_3^- within juice was used to quantify the NO_3^- within plasma with suitable dilution.

3.3.7 Mood and Perceptual Measurements

The degree of arousal was determined via the felt arousal scale (FAS; Appendix F), ranging from 1 (low arousal) to 6 (high arousal) (Svebak and

Murgatroyd, 1985). The degree of pleasure and/or displeasure was determined via the 11-point feeling scale (FS) (Appendix G) ranging from -5 (very bad) to +5 (very good) (Hardy and Rejeski, 1989). The profile of mood states (POMS) (Appendix H) evaluated 7 mood states: fatigue (fPOMS), anger, vigour (vPOMS), tension, esteem, confusion and depression. The tool ranks the emotions felt towards each mood state, on a 5-point Likert scale ranging from 0 (not at all) to 4 (extremely) (McNair, 1971, Morfeld et al., 2007).

3.3.8 Cardiovascular Measurements

Participants BP and HR were measured via an automated sphygmomanometer on the left arm, taking three measurements and determining a mean value (Ogedegbe and Pickering, 2010): deluxe HEM-7310; OMRON Healthcare CO. Ltd; Kyoto, Japan) (Appendix I).
The USCOM was used to measure velocity time integral (VIT), heart rate (HR), systolic blood pressure (SBP) and diastolic blood pressure (DBP). Utilizing these measurements and various algorithms the mean arterial pressure (MAP), stroke volume (SV) and cardiac output (CO) were obtained (Appendix I).

3.3.9 Statistical Analysis

All statistical analyses were completed using IBM SPSS package version 22 (IBM Corporation, Chicago, IL, USA). Data was expressed as mean +/- SD and all analyses were completed on raw data and delta-changed data to eliminate the effect of baseline differences. $P < 0.05$ was considered to be statistically significant. For groups showing statistical differences, an effect

size was calculated, with 0.2 being considered a ‘small’ effect size, 0.5 being considered a ‘medium’ effect size and 0.8 a ‘large’ effect size (Cohen, 1992).

Any differences between individual baseline values for all variables for both different drinks and different trial numbers were determined using a one-way ANOVA.

The main effect of drink, main effect of time and an interaction effect of drink * time were determined for each variable (SBP, DBP, HR, MAP, SV, CO, FPOMS, VPOMS, POMS, FS, FAS) using a repeated measures two-way ANOVA. To ensure accuracy, both the measured values (absolute value) and the change from baseline (delta-change) were analyzed. Additionally, to exclude the effect of lunch, analysis of each variable was completed taking into account pre-lunch time points (0 h to 3 h) along with the analysis on all time points (0 h to 6 h) for both forms of data. To limit cofounders this was repeated for a main effect of trial, time and an interaction effect of trial * time. When a significant interaction was found, Holm-Bonferroni post-hoc tests were used to find out where differences lay.

Pearson correlation was used to investigate relationships between plasma $[NO_2^-]$ and $[NO_3^-]$ and other cardiovascular and perceptual variables.

3.4 RESULTS

3.4.1 Participants

All 11 participants completed the BP, HR, USCOM, mood, and perceptual tests. Of the 11, only eight participants completed all blood samples due to difficulties obtaining blood. Participant characteristics are reported in **Table 3.4.**

Table 3.4 Mean baseline characteristics of participants +/- SD

Participant characteristics	**Total (n=11)**
Age (y)	24 +/- 5.7
Height (cm)	173 +/- 8.9
Body mass (kg)	67.6 +/- 13.3
Plasma NO_2^- (μM)	0.77 +/- 0.086 *
Plasma NO_3^- (μM)	36.74 +/- 1.89 *
SBP (mmHg)	111.36 +/- 1.73*
DBP (mmHg)	70.83 +/- 1.22 *

Systolic Blood Pressure (SBP); Diastolic Blood Pressure (DBP).
* Values are taken as a mean of eligible participants 0 h measure at each trial.

3.4.2 Baseline and trial effect

There was no difference in the baseline (0h) values of any variables between different drink trials or between different trial numbers ($p>0.05$). Additionally, there was no interaction effect of trial * time for most variables ($p<0.05$), except for CO which showed an interaction effect ($p=0.015$).

3.4.5 Plasma NO_2^-

There was an interaction of drink * time for plasma [NO_2^-] ($p<0.001$). Specifically, plasma [NO_2^-] was elevated in the high-NO_3^- trial relative to med-NO_2^- (3 h and 6 h post-consumption) and PL (2 h and 6 h post consumption; $p<0.05$; Figure 3.2). PL and med-NO_2^- consumption showed

no increase in plasma [NO_2^-] at any time point ($p>0.05$). The delta change values also showed an elevation in plasma [NO_2^-] following consumption of high-NO_3^- relative to PL and med-NO_2^- (3 h and 6 h; $p<0.05$). Plasma [NO_2^-] had a higher increase in the med-NO_3^-/NO_2^- trial relative to PL (3 h and 6 h) and med-NO_2^- (3 h; $p<0.05$), however still lower than high-NO_3^- at 6 h ($p<0.05$) but not at 3 h ($p=0.16$).

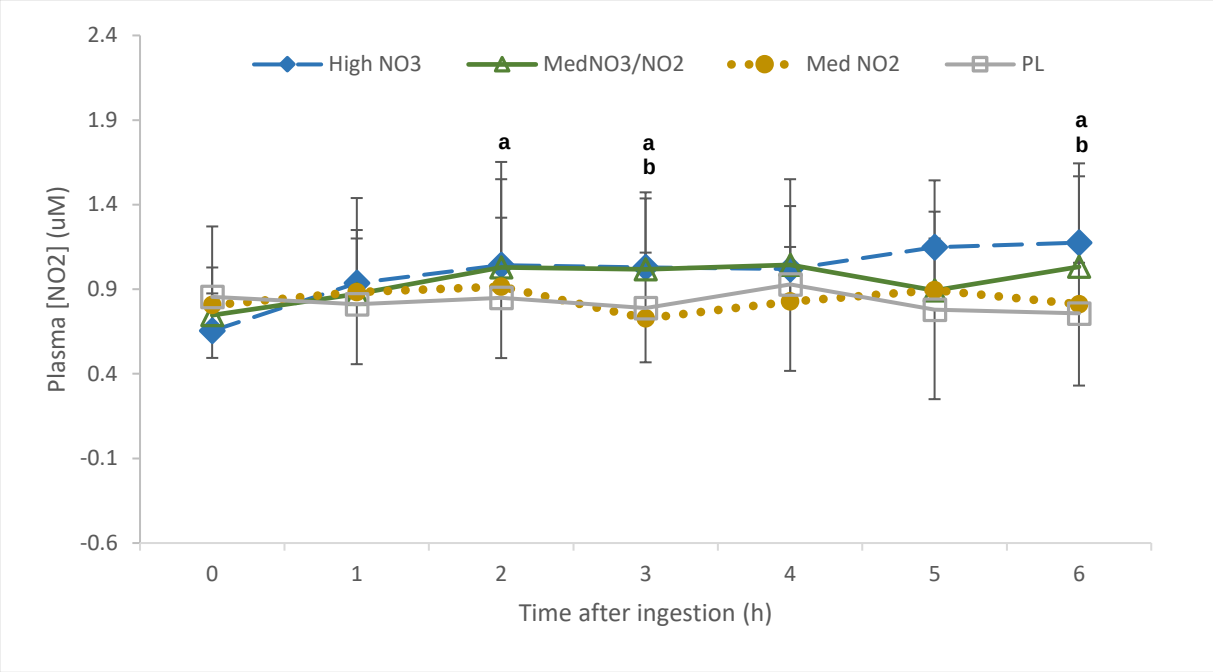

Figure 3.2 Plasma nitrite concentration ([NO_2^-]) (uM) over a 6h period following the ingestion of high-NO_3^-, med-NO_3^-/NO_2^-, med-NO_2^- and PL (placebo) drinks.

[a] Significant difference between high-NO_3^- and PL ($p<0.05$).
[b] Significant difference between high-NO_3^- and med-NO_2^- ($p<0.05$).

3.4.6 Plasma NO_3^-

There was an interaction of drink * time for plasma [NO_3^-] ($p<0.001$) Specifically, plasma [NO_3^-] was elevated in the high-NO_3^-, med-NO_2^- and med-NO_3^-/NO_2^- trials relative to PL (2 h and 6 h post-consumption; $p<0.01$; Figure 3.3). In all drink trials, time was shown to increase plasma [NO_3^-] rising from 1 h ($p<0.001$) and plateauing at 5 h (5 h vs 6 h $p=0.80$). The delta change values showed the highest increase in plasma [NO_3^-] occurred

following the consumption of high-NO_3^- ($p<0.001$), with the second highest increase occurring following the consumption of med-NO_3^-/NO_2^- ($p<0.001$).

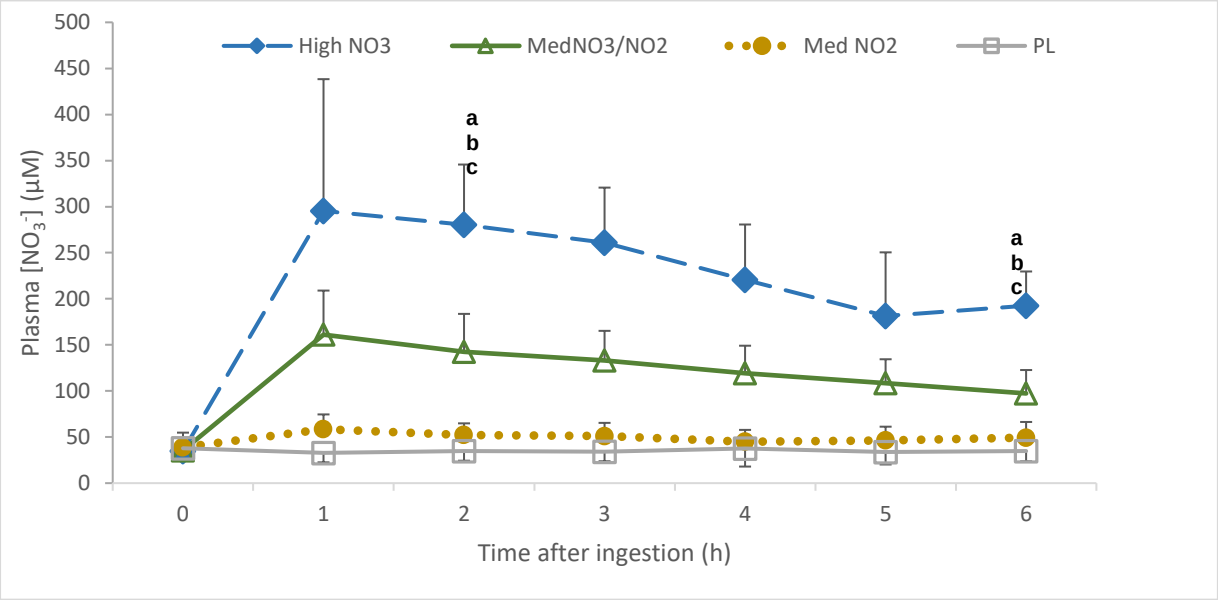

Figure 3.3 Plasma nitrate concentration ([NO_3^-]) (uM) over a 6h period following the ingestion of high-NO_3^-, med-NO_3^-/NO_2^-, med-NO_2^- and PL (placebo) drinks.

[a] Significant difference between high-NO_3^- and PL ($p<0.05$).
[b] Significant difference between high-NO_3^- and med-NO_2^- ($p<0.05$).
[c] Significant difference between high-NO_3^- and med-NO_3^-/NO_2^- ($p<0.05$).

3.4.6 Blood Pressure (BP)

There was no interaction of drink * time for systolic BP (SBP) ($p=0.325$). SBP was reduced in all trials (2 h, 3 h, 5 h, 6 h post-consumption; $p<0.001$; Figure 3.4), with no difference occurring between trials ($p=0.783$). These findings were consistent when analysing delta change and pre-lunch data.

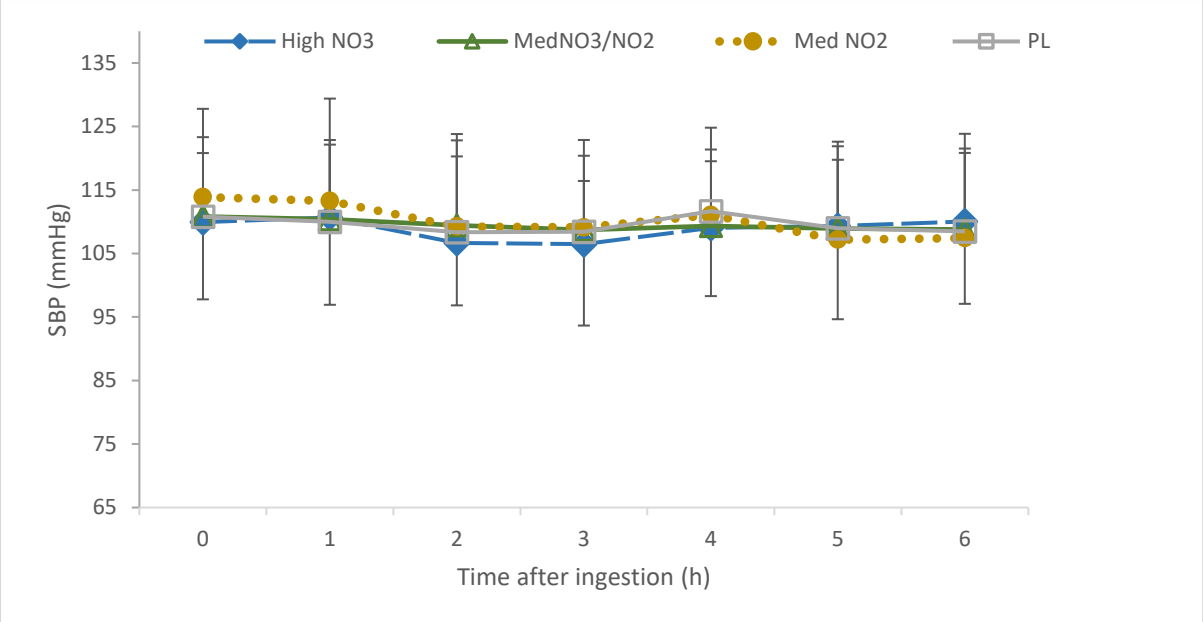

Figure 3.4 Systolic Blood Pressure (SBP) (mmHg) over a 6 h period following the ingestion of high-NO_3^-, med-NO_3^- /NO_2^-, med-NO_2^- and PL (placebo) drinks.

There was a trend for interaction of drink * time for absolute and change in diastolic blood pressure (DBP; p=0.059; p=0.058). This trend was not present pre-lunch (p>0.05). There was no difference in DBP between trials (p=0.692; Figure 3.5), and no effect of time on DBP (p=0.124; Figure 3.5).

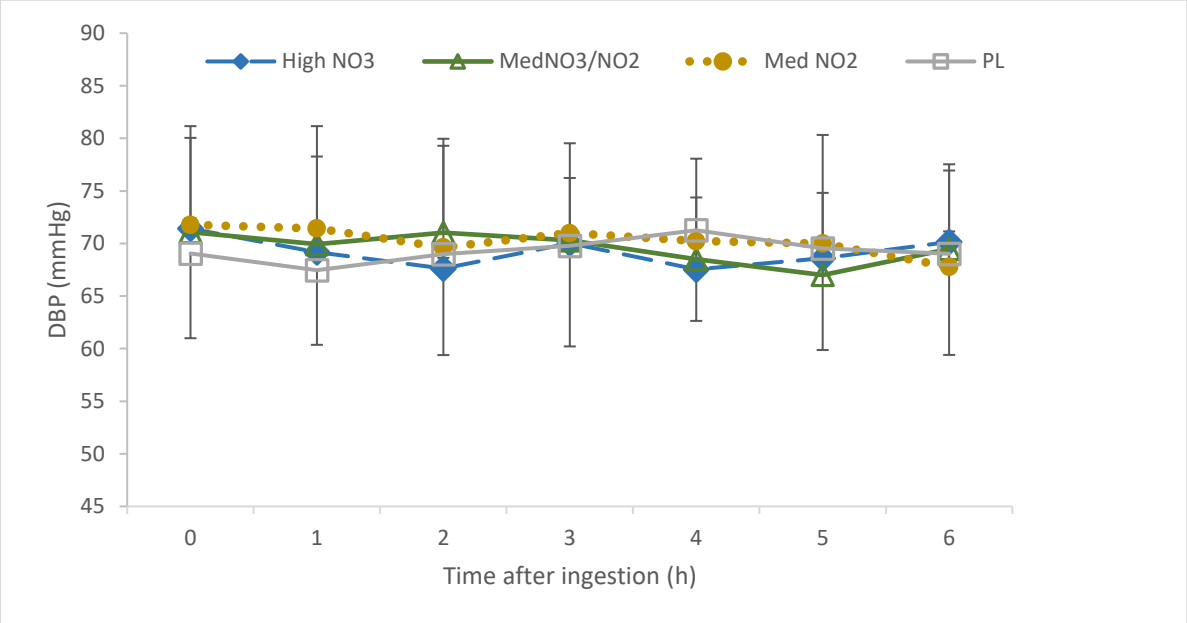

Figure 3.5 Diastolic Blood Pressure (DBP) (mmHg) over a 6 h period following the ingestion of high-NO_3^-, med-NO_3^- /NO_2^-, med-NO_2^- and PL (placebo) drinks

3.4.7 Heart Rate

There was no interaction of drink * time for heart rate (HR) in absolute or delta change data ($p>0.05$). However, an interaction of drink * time for HR exists pre-lunch. Specifically, HR was reduced in the PL trials relative to med-NO_3^-/NO_2^- (3 h post-consumption, $p<0.05$; Figure 3.6).

There was a main effect of time for HR ($p=0.008$); HR was reduced 1 h, 4 h, 5 h, 6 h post-consumption ($p<0.05$), with the greatest decrease in HR occurring at 3 h ($p<0.001$; Figure 3.6).

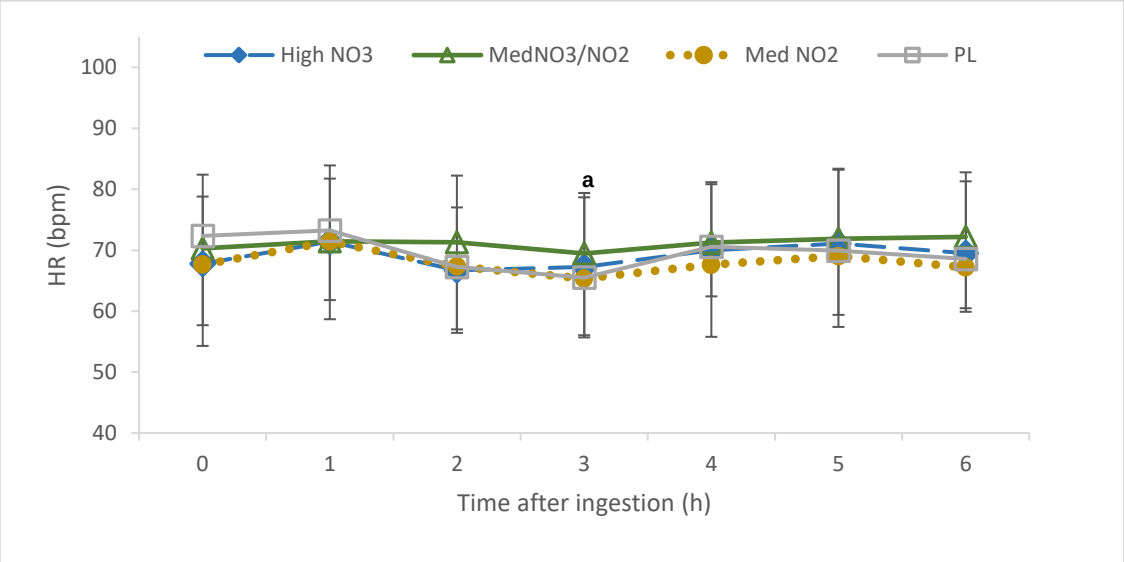

Figure 3.6 Heart Rate (HR) (bpm) over a 6 h period following the ingestion of high-NO_3^-, med-NO_3^-/NO_2^-, med-NO_2^- and PL (placebo) drinks.

[a] Significant difference between med-NO_3^-/NO_2^- and PL ($p<0.05$).

3.4.8 USCOM Measures

There was no interaction of drink * time for cardiac output (CO), or stroke volume (SV) ($p>0.05$). Time had no effect on CO or SV ($p>0.05$), and no difference occurred between trials ($p>0.05$; Figure 3.7; 3.8). These findings were consistent when analysing delta change and pre-lunch data.

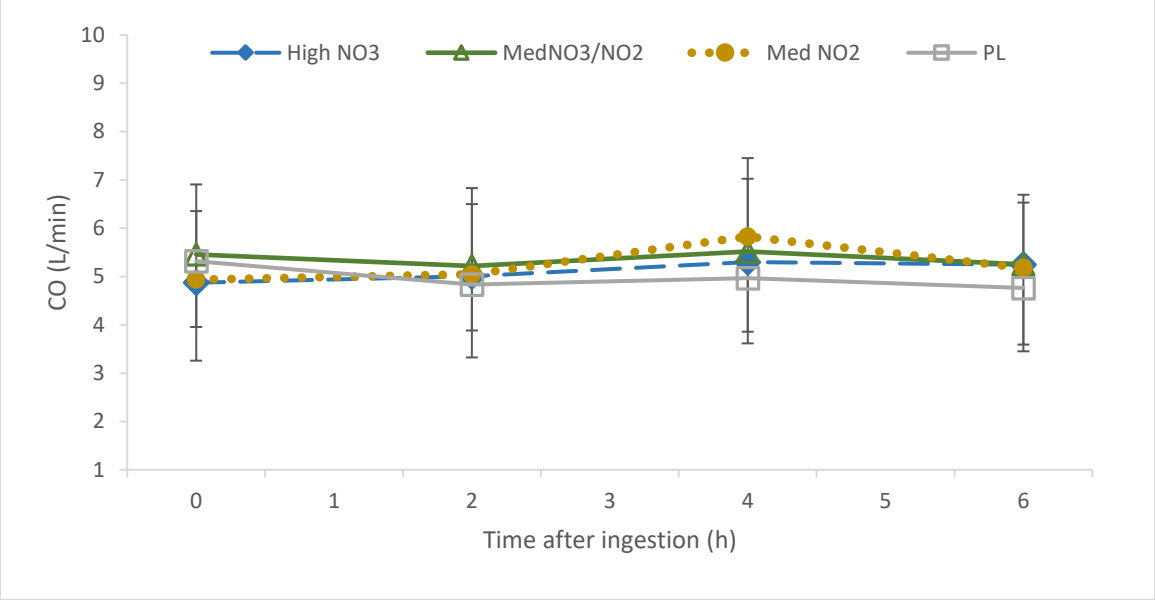

Figure 3.7 Cardiac Output (CO) (L/min) over a 6h period following the ingestion of high-NO_3^-, med-NO_3^-/NO_2^-, med-NO_2^- and PL (placebo) drinks.

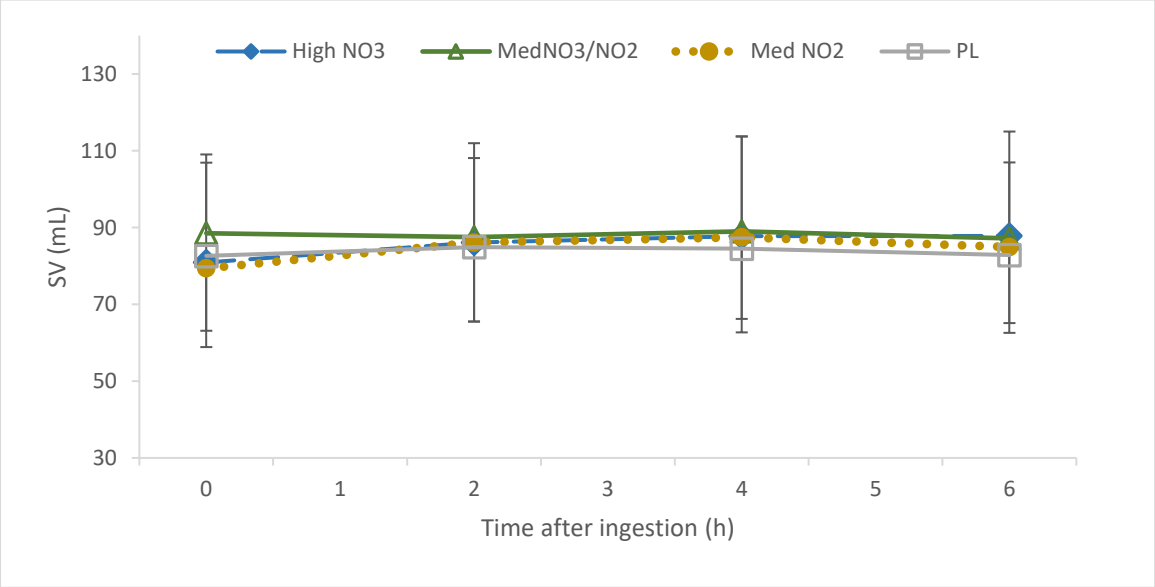

Figure 3.8 Stroke Volume (SV) (mL) over a 6h period following the ingestion of high-NO_3^-, med-NO_3^-/NO_2^-, med-NO_2^- and PL (placebo) drinks.

There was an interaction of drink * time for mean arterial pressure (MAP; p=0.013), specifically, MAP was reduced in the high-NO_3^- trial relative to med-NO_2^- (4 h post-consumption; p<0.05; Figure 3.9). The delta change values also showed an interaction of drink * time for MAP, which was reduced in the high-NO_3^- and med-NO_3^-/NO_2^- trial relative to PL (4 h post consumption; p<0.05; Figure 3.10) and in the med-NO_2^- group relative to high-NO_3^- and PL (6 h post consumption; p<0.05; Figure 3.10)

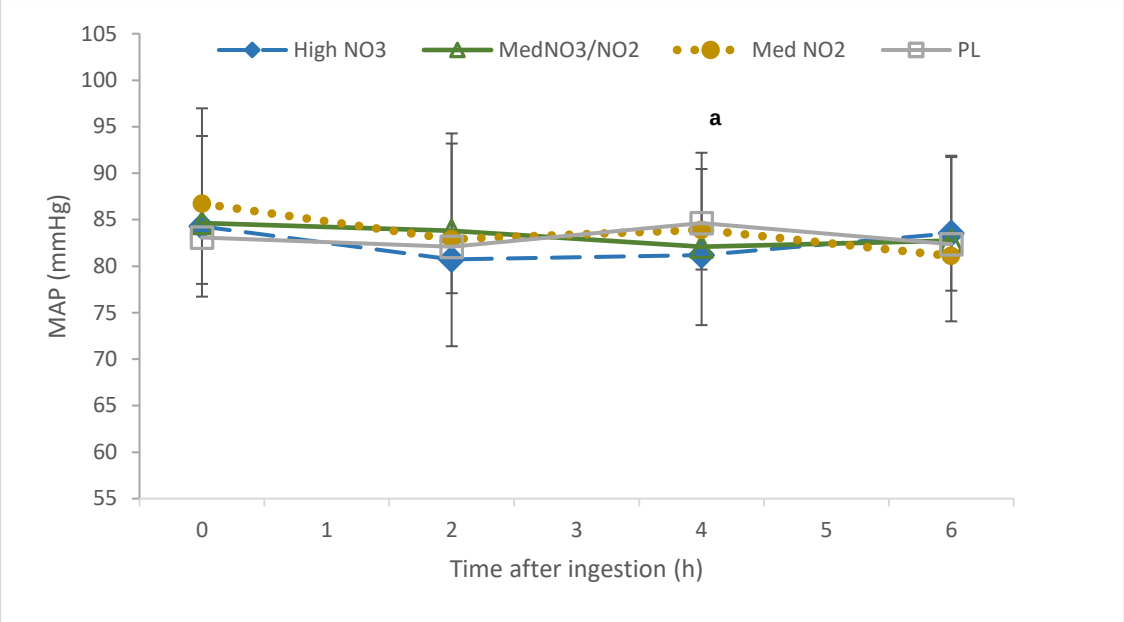

Figure 3.9 Mean Arterial Pressure (MAP (mmHg)) over a 6h period following the ingestion of high-NO_3^-, med-NO_3^-/NO_2^-, med-NO_2^- and PL (placebo) drinks.

[a] Significant difference between high-NO_3^- and med-NO_2^- ($p<0.05$).

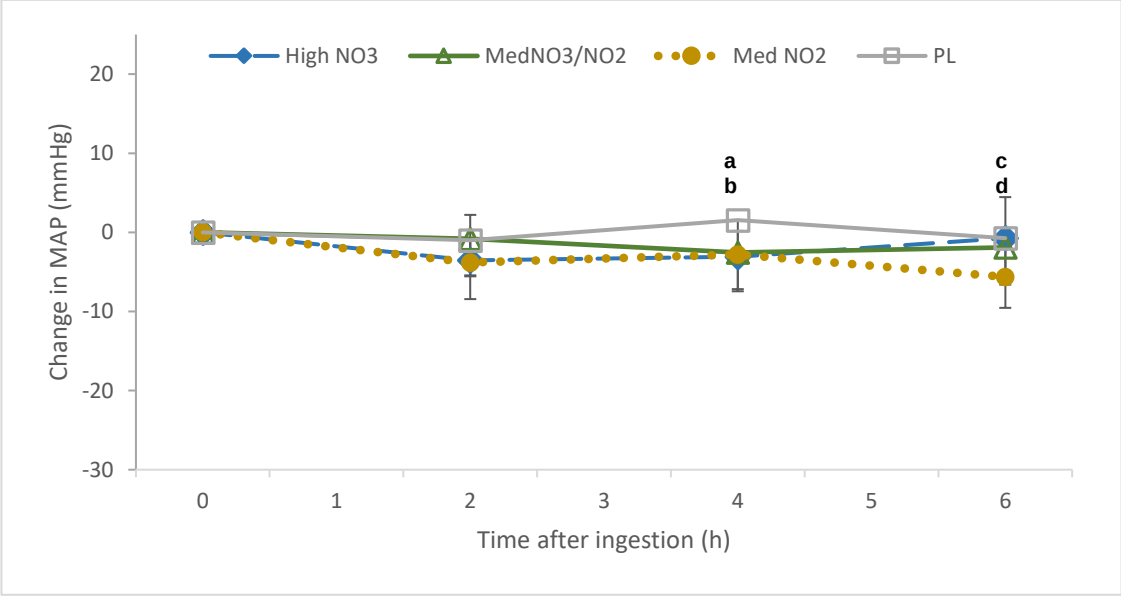

Figure 3.10 Change in Mean Arterial Pressure (MAP (mmHg)) over a 6h period following the ingestion of high-NO_3^-, med-NO_3^- /NO_2^-, med-NO_2^- and PL (placebo) drinks.

[a] Significant difference between high-NO_3^- and PL ($p<0.05$).
[b] Significant difference between med-NO_3^-/NO_2^- and PL ($p<0.05$).
[c] Significant difference between high-NO_3^- and med-NO_2^- ($p<0.05$).
[d] Significant difference between med-NO_2^- and PL ($p<0.05$).

3.4.9 Perceptual Responses and Mood

There was a trend for an interaction of drink * time for the fatigue subcomponent of POMS (fPOMS; p=0.063; Figure 3.11), but no interaction of drink * time for vigour POMS (vPOMS) overall POMS (oPOMS), feeling scale (FS) or felt arousal scale (FAS; p>0.05; Figure 3.12, Figure 3.13, Figure 3.14, Figure 3.15). Additionally, neither time, nor drink had an effect on any of the above perceptual and mood measures (p>0.05). Delta change data highlighted an increased fatigue score following high-NO_3^- and med-NO_3^-/NO_2^- consumption (p<0.05) and a decreased fatigue score following med-NO_2^- and PL consumption (p<0.05). PL led to the largest decrease in fatigue score (p<0.01).

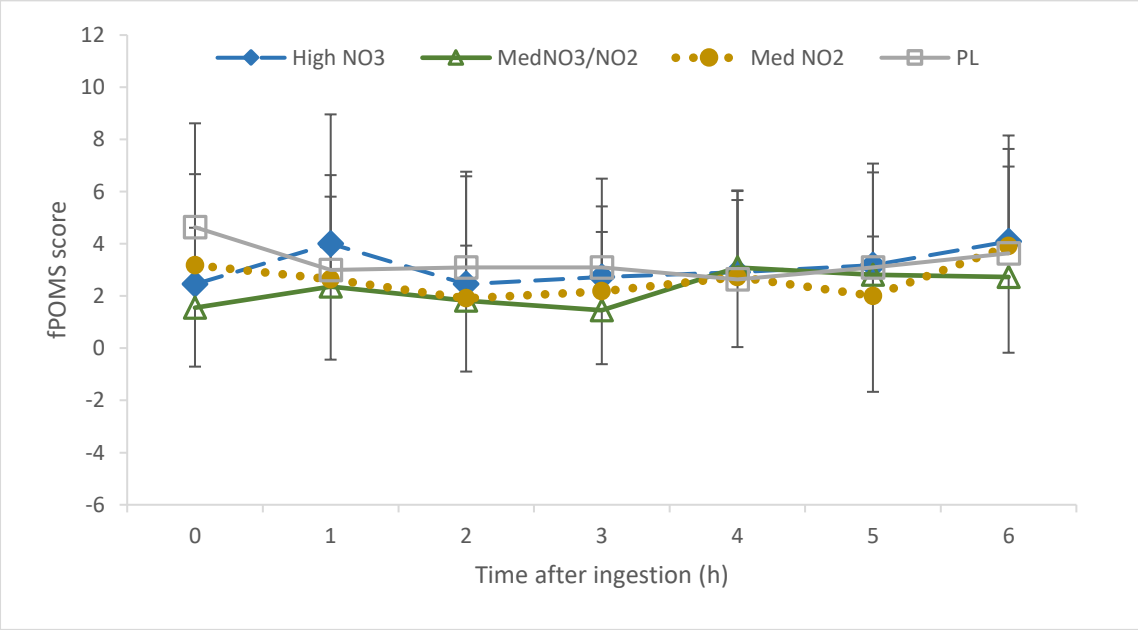

Figure 3.11 Fatigue POMS (fPOMS) score over a 6h period following the ingestion of high-NO_3^-, med-NO_3^-/NO_2^-, med-NO_2^- and PL (placebo) drinks.

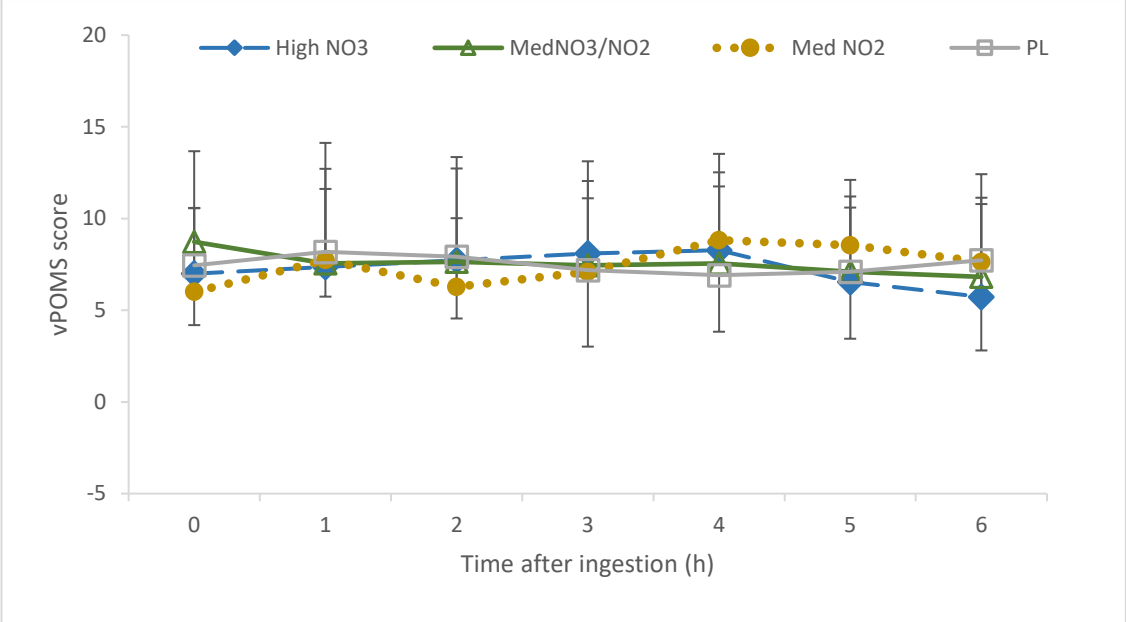

Figure 3.12 Vigour POMS (vPOMS) score over a 6h period following the ingestion of high-NO_3^-, med-NO_3^-/NO_2^-, med-NO_2^- and PL (placebo) drinks.

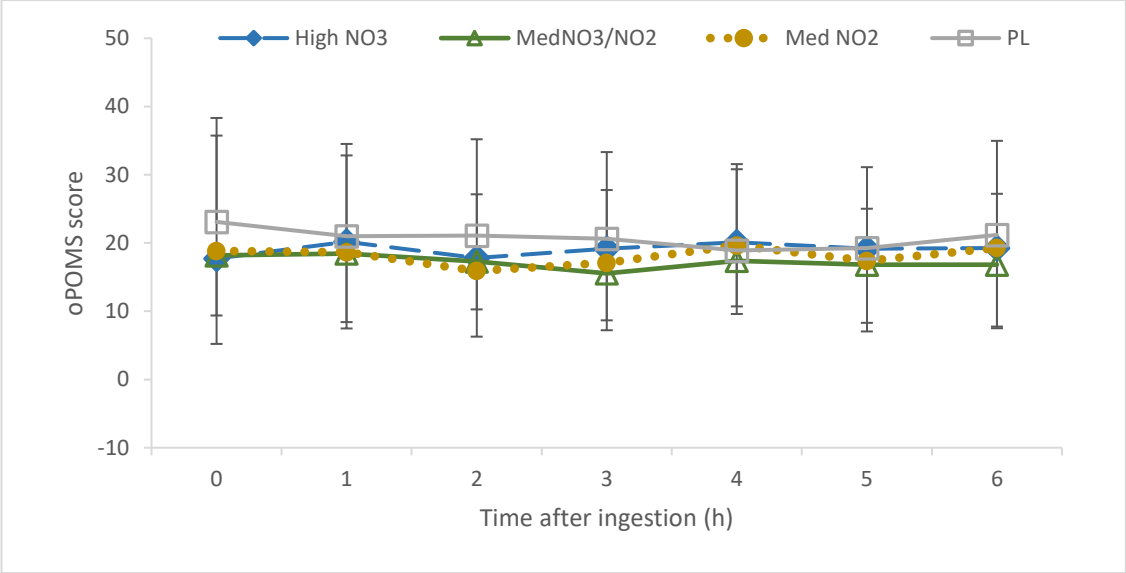

Figure 3.13 Overall POMS (oPOMS) score over a 6h period following the ingestion of high-NO_3^-, med-NO_3^-/NO_2^-, med-NO_2^- and PL (placebo) drinks.

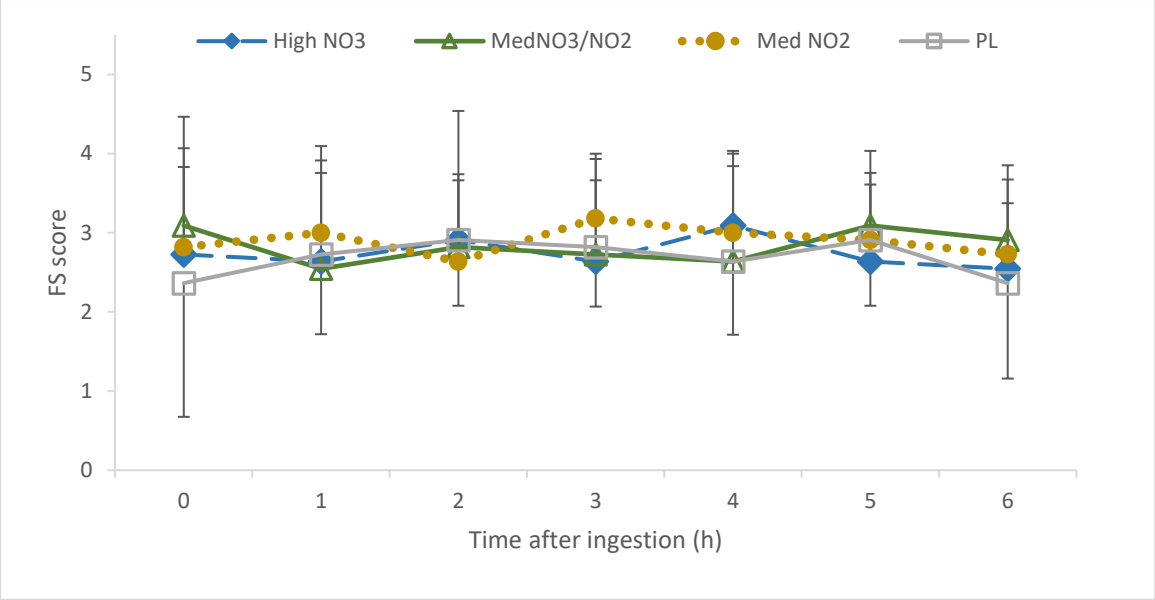

Figure 3.14 Feeling Scale (FS) score over a 6h period following the ingestion of high-NO_3^-, med-NO_3^-/NO_2^-, med-NO_2^- and PL (placebo) drinks.

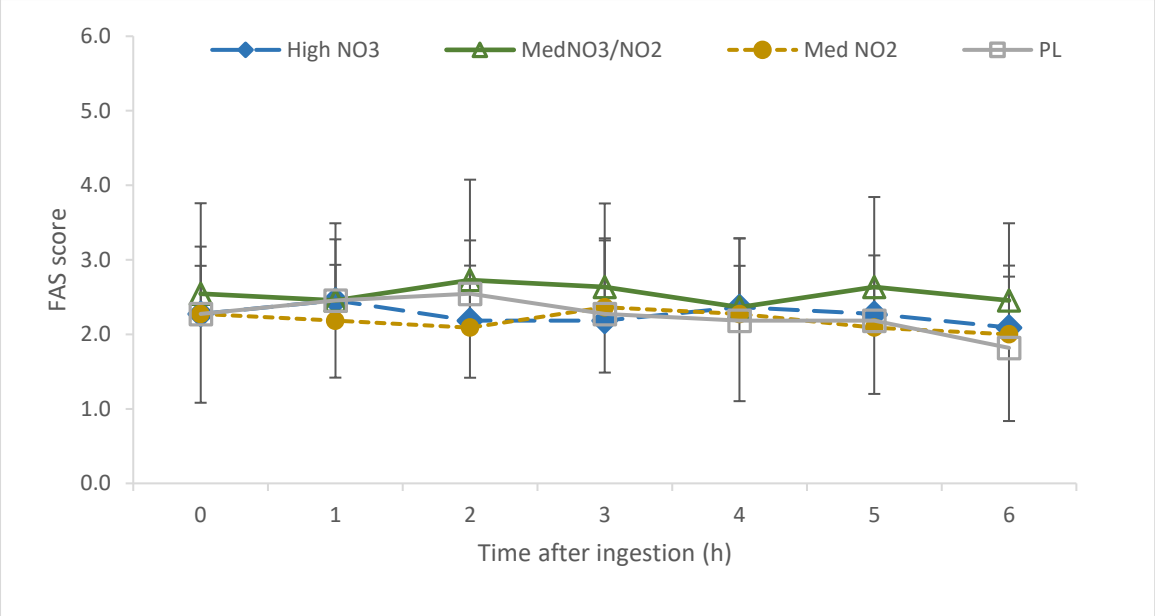

Figure 3.15 Felt Arousal Scale (FAS) score over a 6h period following the ingestion of high-NO_3^-, med-NO_3^-/NO_2^-, med-NO_2^- and PL (placebo) drinks.

3.4.10 Correlations

No correlations were found between plasma [NO_2^-] and cardiovascular, mood and perceptual measures at any time point, which was consistent with the delta change values.

No significant correlations were found between plasma [NO_3^-] and cardiovascular, mood and perceptual measures at any time point. Delta change values showed two positive correlations where plasma [NO_3^-] correlated with SBP at 6 h ($r=0.428$; $p<0.006$) and fPOMS at 3 h post consumption ($r=0.322$; $p=0.042$). Additionally, a negative correlation was also seen with the delta change data, where plasma [NO_3^-] correlated with DBP at 4 h ($r=-0.364$; $p=0.021$).

3.5 DISCUSSION

This study examined the effects of acute supplementation with beetroot juice (BR) rich in either NO_3^- or NO_2^-, or a combination of both, on plasma [NO_3^-], plasma [NO_2^-], cardiovascular responses, mood and perception in normotensive adults. The main findings of this study demonstrate that; 1) NO_3^- consumption is the critical factor in elevating both plasma [NO_3^-] and [NO_2^-]; 2) NO_3^- ingestion reduces MAP 4 h post-consumption while NO_2^- reduces MAP 6 h post-consumption; 3) an increase in plasma [NO_3^-] leads to a decreased DBP after 4 h regardless of BR consumption.

3.5.1 Plasma [NO_2^-] and [NO_3^-]

This is the first study to directly evaluate the effects of NO_2^- present in BR on plasma [NO_2^-] and [NO_3^-]. Both high-NO_3^- and med-NO_3^-/NO_2^- increased plasma [NO_3^-] and [NO_2^-] to a greater extent than med-NO_2^- and PL, inferring that NO_3^- consumption is the critical factor for the rise in plasma [NO_3^-] and [NO_2^-]. Furthermore, high-NO_3^- caused a higher rise in both plasma [NO_3^-] and [NO_2^-] than med-NO_3^-/NO_2^-. Both of these drinks contained similar combined quantities of NO_3^- and NO_2^- (604 mg; 517 mg), but different quantities of NO_3^- (572 mg; 280 mg) and NO_2^- (32 mg; 237 mg), leading to the conclusion that there is little effect of NO_2^- on plasma [NO_3^-] or [NO_2^-].

The results show that, following the consumption of high-NO_3^- peak plasma [NO_3^-] occurred after 1 h, and a peak plasma [NO_2^-] occurred after 2 h remaining plateaued till 6 h. These findings are consistent with literature which showed NO_3^- rich BR led to a peak plasma [NO_3^-] 1-1.5 h post ingestion (Clements et al., 2014) and a peak plasma [NO_2^-] 2.5-3 h post

ingestion (McIlvenna et al., 2017). Furthermore, the current study showed that following the consumption of med-NO_2^- there was a peak plasma [NO_3^-] 1 h post ingestion but no increase in plasma [NO_2^-]. This result is inconsistent with previous studies, where the ingestion of $NaNO_2^-$ capsules increased both plasma [NO_3^-] and [NO_2^-] leading the authors to conclude that NO_2^- was 95-98% bioavailable (in relation to IV dose) (Hunault et al., 2009). Although the source of supplement was different between the studies, there were comparable doses of NO_2^- (193 – 253 mg vs. 262 mg in current study). Specific research comparing the effect of NO_2^- supplementation between BR and capsules does not exist. However, studies have shown that synthetic nutrients delivered in capsules are unlikely to be used by the body in the same way as their natural counterparts (Liu, 2003). This is directly in contrast to work by Kapil et al. (2010) who observed the same cardio-protective effect with BR and KNO_3^- capsules when the dose of NO_3^- was the same. This may infer that NO_3^- works alone to elicit benefits, opposed to other nutrients who work synergistic to one another.

A reduction in BP following BR consumption (400 mg-800 mg NO_3^-) is reliant on a rise in plasma [NO_2^-] (0.5-0.9 µmol/L) (Jonvik et al., 2016, Ormesher et al., 2018, Webb et al., 2008). As high-NO_3^- led to the greatest rise in plasma [NO_2^-], NO_3^- consumption may be the critical factor in producing a reduction in BP following BR supplementation. Furthermore, when BR is stored at room temperature, NO_3^- begins to convert to NO_2^- (Lee et al., 1971). This implies that when BR is left at room temperature for a period of time, it may not lead to the same increase in plasma [NO_2^-] and therefore may have a

reduced ability to lower BP. However, the current study does not show the same correlation between BP and plasma [NO_2^-] seen in previous literature despite similar NO_3^- ingestion in the current study. However, a relationship between a rise in plasma [NO_3^-] and a decrease in DBP was seen. Investigating this relationship, high-NO_3^- led to the greatest rise in plasma [NO_3^-] and therefore should lead to the greatest reduction in DBP.

Due to the novelty of the findings, further research is required to confirm the pharmacokinetics of NO_2^- naturally present in BR and any correlation with cardiovascular measures.

3.5.2 Blood Pressure

3.5.2.1 Systolic and Diastolic Blood Pressure

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) decreased following the consumption of all drinks, including PL suggesting that participants were becoming more relaxed with time. Interestingly between drinks, the reduction of SBP was not different, leading to the conclusion that neither NO_3^- or NO_2^- had an influence on SBP. Similarly, DBP did not differ between drinks, however there was a trend for high-NO_3^- to reduce DBP more than the other drinks.

In previous literature only a small number of studies fail to show a reduction of BP following NO_3^- supplementation (Bondonno et al., 2015, Floyd et al., 2019). More commonly, it is concluded that NO_3^- supplementation (comparable doses to the present study) will elicit a reduction in SBP (Bailey

et al., 2009, Coles and Clifton, 2012, Hobbs et al., 2012, Jajja et al., 2014, Kapil et al., 2015, Kapil et al., 2010, Stanaway et al., 2019, Vanhatalo et al., 2010, Wylie et al., 2013) and DBP (Hobbs et al., 2012, Kapil et al., 2015, Kapil et al., 2010, Larsen et al., 2006, Vanhatalo et al., 2010, Wylie et al., 2013).

Most research suggests that NO_3^- supplementation does elicit a reduction in BP. In the studies where an effect is not observed, it is suggested that a cofounding factor is present, which prevents NO_3^- from reducing BP. Floyd et al. (2019) suggested that an elevation in plasma [glucose] and [insulin] can prevent a reduction in BP in a younger population (27 +/- 6.5 years) following potassium NO_3^- supplementation (24 mmol NO_3^-). In the current study, 15 g of carbohydrate was consumed alongside the supplement, with a further 30 g consumed at lunch (+4 h) which may have prevented a reduction in BP due to the rise in [glucose] and [insulin]. However, the low baseline SBP of 113 mmHg within Floyd et al. (2019) and in the current study (111 mmHg) may better explain the lack of effect. A meta-regression including 19 eligible randomised clinical trials investigating the effects of BR supplementation (316 – 860 mg NO_3^-/day) over a duration ranging from 2 – 56 d, showed a larger decrease in SBP occurred in participants with a higher baseline SBP measure (Bahadoran et al., 2017). Most previous studies which have shown BR consumption to have an effect on SBP had participants with baseline SBP measures ranging between 127-149 mmHg.

Investigating the effect of participants' baseline SBP measure further, Wylie et al. (2013) included participants with a baseline SBP of 118mmHg, which is lower than most other studies researched (127-149 mmHg). Consistent with the finding by Bahadoran et al. (2017), Wylie et al. (2013) also showed a lower reduction in SBP (5 mmHg) compared to the other studies investigated (7.3-22.2 mmHg). It is likely that the low baseline BP has limited the cardio-protective effect seen in the current study secondary to a potential self-limiting effect of NO_3^-.

NO_3^- rich supplementation has been concluded to reduce BP more significantly in those with a higher baseline BP. As the current study failed to show any BP lowering effect associated with NO_3^-, it is unlikely that any effect of NO_2^- on BP would be shown. Further research into the potential effect of NO_2^- on SBP and DBP is required in a population with a higher baseline SBP.

3.5.2.2 Mean Arterial Pressure

NO_3^-, independent of dose (high-NO_3^- and med-NO_3^-/NO_2^-) reduced MAP after 4 h, and NO_2^- (med-NO_2^-) reduced MAP after 6 h. Due to the direct conversion of NO_2^- to NO in the body, med-NO_2^- was proposed to provide an early onset of cardio-protective effects compared to high-NO_3^- (Bailey et al., 2012, Zand et al., 2011).

Previous literature has shown a decrease in MAP (-8.2 +/- 7.6 mmHg; -7 +/- 1 mmHg) following NO_3^- rich BR consumption (300 mg; 450 mg) (Keen et al., 2015, Kemmner et al., 2017), specifically occurring 4 h post supplementation

(Kemmner et al., 2017), which is consistent with the current study (-3 +/- 3 mmHg). However, the delayed effect of NO_2^- on MAP in comparison to NO_3^- cannot be explained by the current literature, and is not consistent with the exogenous NO pathway. Further studies are required to ensure this is an accurate and replicable outcome with a larger sample size to appropriately rule out any effect of time on MAP.

3.5.3 Cardiovascular measures

The current study showed no change in HR, SV or CO following NO_3^- rich BR consumption, which is consistent with a selection of the literature (Kapil et al., 2015, Kelly et al., 2012, Oggioni et al., 2018). The present study has also shown that consumption of NO_2^- rich BR also had no effect on these variables.

The cardio-protective effects observed in the literature are likely mediated by the relaxation and dilation of the smooth muscle surrounding the blood vessel associated with NO production (Bailey et al., 2012, Carlström et al., 2015). Additionally, the endogenous formation of NO, via L-arginine supplementation, has shown to limit noradrenaline production, depressing sympathetic activity, and increasing the parasympathetic tone (Lee et al., 2009). A main characteristic of the sympathetic drive is an increased HR, CO and SV (White and Raven, 2014), inferring endogenous NO has the ability to reduce HR, CO and SV (Thorin and Thorin-Trescases, 2009). Exogenous NO_3^- has not been investigated, but the current study, along with previous literature suggest that this does not have the same effect on noradrenaline

and hence not the same effect on HR, CO and SV that endogenous NO_3^- has been shown to.

3.5.4 Mood and Perception

The POMS questionnaire provides an overall assessment of mood but can also be used to assess different subcomponents. A higher fatigue or higher vigour score would infer a higher degree of each emotion. All drinks influenced the fatigue score, but had no other changes on mood or perceptual measures. Interestingly, high-NO_3^- and med-NO_3^-/NO_2^- increased fatigue score, while med-NO_2^- and PL decreased the fatigue score which could not be attributed to time or trial number. This is a novel finding which suggests NO_3^- and NO_2^- in BR do not have the same effect on mood following acute supplementation and therefore may have varying psychological effects within the body.

Previous literature concluded no change in mood following acute NO_3^- rich (182 mg) spinach consumption (Bondonno et al., 2014). Therefore, the increase in fatigue following NO_3^- rich BR was not expected. Fatigue is affected by changes in blood flow to the brain and increases in neuro-excitation (Ishizaki et al., 2008). NO_3^- through the production of NO causes cerebral vasodilation which should increase blood flow to the brain and reduce levels of fatigue (Thompson et al., 2014). In support of this finding, previous literature has shown an association between a high consumption of NO_3^- rich foods and admission into hospital with mania (elevated energy) over a 10-year period (Khambadkone et al., 2018). Further research is

required to confirm the effect of BR on fatigue, specifically the alternating effect of BR high in NO_3^- compared to BR high in NO_2^-.

The current findings indicate a potential for NO_2^- which is naturally present in BR to reduce MAP in normotensive adults and improve fatigue levels. Further research investigating the pharmacokinetics of the NO_2^- present in BR, and the subsequent correlations of both plasma [NO_3^-] and [NO_2^-], is warranted. The inconsistent findings of SBP and DBP associated with NO_3^- rich BR in the current study indicate a need for further research to confirm the effect of NO_2^- present in BR. Future studies should include participants with a higher baseline SBP as this has been associated with a larger reduction in SBP.

3.5.6 Conclusion

Plasma [NO_3^-] increased in a dose-dependent manner following the ingestion of NO_3^- and plasma [NO_3^-] increased following the ingestion NO_2^- independent of dose. While, plasma [NO_2^-] increased following the ingestion of NO_3^- only, with a significantly higher increase following high-NO_3^- supplementation compared to med-NO_3^-/NO_2^- supplementation. There was no effect of NO_3^- or NO_2^- ingestion on SBP, DBP, SV or CO compared to PL. MAP decreased following the consumption of high-NO_3^-, med-NO_3^-/NO_2^- and med-NO_2^-, however in comparison med-NO_2^- had its effect 2 h later. Collectively, these results indicate that consumption of NO_3^- is the critical factor in elevating plasma [NO_3^-] and [NO_2^-], although the consumption of either NO_3^- or NO_2^- is able to reduce MAP. Future research is required in a

population with a higher baseline BP so effects of NO_2^- consumption on SBP/DBP can be observed.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1 Summary

Acute supplementation with beetroot juice (BR) has emerged as an effective approach to reducing blood pressure (BP). These effects have been attributed to the large content of NO_3^- present, which via an exogenous pathway, forms the natural vasodilator NO. BR however contains NO_2^- which via the same exogenous pathway can form NO. Limited research currently exists on the ability of inorganic NO_2^- contained within BR to reduce BP.

The primary objective of this study was to determine the pharmacokinetics of NO_3^- and NO_2^- present in BR, by examining the subsequent changes in plasma [NO_3^-] and plasma [NO_2^-] following the consumption of different BR drinks. The results show a pronounced increase in both plasma [NO_3^-] and plasma [NO_2^-] following the consumption of high-NO_3^- and med-NO_3^-/NO_2^- compared to PL and med-NO_2^- BR. Additionally, plasma [NO_3^-] but not plasma [NO_2^-] increased following consumption of med-NO_2^- compared to PL.

The secondary objective was to determine the individual effects of NO_3^- and NO_2^- present in BR on various cardiovascular measures, including, SBP, DBP, MAP, HR, CO and SV. The results show that neither NO_3^- nor NO_2^- influenced these variables compared to PL, except for MAP. Specifically, NO_3^- reduced MAP after 4 h, and NO_2^- reduced MAP after 6 h ($p<0.05$).

The final objective was to determine the individual effects of NO_3^- and NO_2^- present in BR on various mood and perceptual measures, including, FS, FAS, POMS (fatigue, vigour and overall). The results show that neither NO_3^-

nor NO_2^- influenced any of these measures compared to the PL, except for fatigue. Fatigue was increased following the consumption of high-NO_3^- and med-NO_3^-/NO_2^-, and decreased following the consumption of med-NO_2^- and PL.

4.2 Conclusion

This study highlights an increase in both plasma [NO_3^-] and plasma [NO_2^-] occurs following the consumption of NO_3^--rich BR. In comparison the consumption of NO_2^--rich BR led to a significantly lower rise in plasma [NO_3^-] and no rise in plasma [NO_2^-]. These findings suggest that NO_3^- consumption is the critical factor in BR to elicit an increased plasma [NO_2^-]. Interestingly in the current study an increase in plasma [NO_2^-] did not correlate with a decrease in SBP/DBP as suggested in previous literature. However, an increase in plasma [NO_3^-] reduced DBP 4 h post-consumption.

In line with the second objective, this study highlights a potential for both NO_3^- and NO_2^- present in BR to reduce MAP, specifically NO_3^- has its greatest potential 4 h post-consumption and NO_2^- 6 h post-consumption. Despite the ability to reduce MAP, both NO_3^- and NO_2^- failed to elicit a change in the various other CVD measures compared to PL. Previous literature has reported a profound decrease in SBP/DBP following the supplementation with NO_3^--rich BR. As the current study was unable to replicate these results it is unclear if NO_2^- has an effect on SBP/DBP, or if, like NO_3^- the effect is prevented in the current study. Further research is required to confirm the effect of consumption of NO_2^--rich BR on SBP/DBP.

In line with the final objective, acute supplementation with BR, independent of NO_3^- or NO_2^- content did not influence any mood measures except fatigue

within the POMS questionnaire. Consumption of NO_3^--rich BR increased fatigue levels, while the consumption of NO_2^--rich BR decreased fatigue levels. This effect is not consistent with previous literature and warrants further research.

4.3 Strengths

To date, no research has investigated the individual effect of consumption of inorganic NO_2^- present in BR on plasma $[NO_3^-]/[NO_2^-]$ and on various cardiovascular measures. Furthermore, there is limited research which controls for and limits the amount of inorganic NO_2^- present in BR to investigate the individual effect of inorganic NO_3^-. The present study builds on the research concluding a cardio-protective effect of BR and attempts to attribute these effects more accurately to NO_3^- or NO_2^-.

A further strength of the study was the use of multiple drinks which together encapsulated a range of NO_3^- and NO_2^- doses. The individual effect of NO_3^- and then NO_2^- could be made by ensuring that when NO_3^- was high, NO_2^- was low and vice versa. The med-NO_3^-/NO_2^- drink was made to have a similar combined content of both NO_3^- and NO_2^- to the high-NO_3^- drink. Due to the dose-dependent effect of NO_3^- seen in previous literature (Hobbs et al., 2012, Kapil et al., 2010, Wylie et al., 2013) the difference between these two drinks is able to show the researcher the relative effect of NO_2^- compared to NO_3^-.

Furthermore, some studies investigating BR have not been double-blinded (Hobbs et al., 2012, Jajja et al., 2014, Velmurugan et al., 2013) or did not use an appropriate placebo. Water has been previously used as a placebo, which makes participants aware they are not receiving the treatment, which

potentially biases the results. Water has been shown to increase brachial BP (Jordan et al., 2000), likely through the sympathetic nervous system and hypo-osmolality. This makes water an inappropriate control for BR. The PL along with the treatment BR was presented to a consumer sensory panel and were not perceived as significantly different which ensured our PL was appropriate. Additionally, the current study was double-blinded, preventing any chance of bias.

Lastly, the double-blinded, randomised crossover design made the study robust and eliminated bias in treatment assignment. Furthermore, due to this study design all variables were tested to ensure there was no significant effect of trial order, this ensured any result obtained was secondary to the consumption of the drink.

4.4 Limitations

Despite the strengths of the study, it is important to acknowledge the limitations. Firstly, the small sample size of this study limited the ability to extrapolate the results to broader populations and hence make population-based recommendations. However, the sample size was based on previous studies and was designed to be large enough to detect a clinically important reduction in BP.

Another limitation was the food intake throughout the study period. Participants were fed breakfast along with the BR supplementation, and then lunch 4 h post BR supplementation. This provided a realistic eating pattern and produced applicable findings. Consequently, a rise in HR was seen after lunch was given. Furthermore, a small rise was seen in plasma [NO_3^-] 2 h post-lunch, inferring that there may have been traces of NO_3^- present in

lunch. Subsequently, an inverse correlation between plasma [NO_3^-] and SBP was seen at 6 h post BR consumption.

Another limitation was that only eight participants were able to provide all blood samples, and hence be included in the later pharmacokinetic analysis. This did not appear to compromise the results as significant differences were found in plasma [NO_3^-] and [NO_2^-] between drink trials as expected. Furthermore, the method of blood collection was not consistent (venepuncture or cannula) due to the difficulties obtaining blood. It is unclear the effect this has on plasma [NO_3^-] and [NO_2^-] specifically.

The final limitation was the lower NO_2^- content in the med-NO_2^- drink compared to the NO_3^- content in the high-NO_3^- drink. Due to the variation in these quantities, when comparing these two drinks the effect of NO_2^- was unable to be directly compared to the effect of NO_3^- However, in BR NO_2^- is found in significantly lower quantities than NO_3^-, and therefore the drinks investigated are realistic and ecologically valid. Furthermore, the med-NO_3^-/NO_2^- had a comparable overall NO_3^- and NO_2^- content to high-NO_3^- with the difference being a higher content of NO_3^- and lower content of NO_2^- in the high-NO_3^- drink. Therefore, the difference between these two drinks allowed for a more direct comparison of NO_3^- to NO_2^- as well as providing a representation of stored BR where NO_3^- breaks down to NO_2^-

4.5 Use of the findings of this research study

With the high prevalence of CVD in New Zealand, a simple intervention to actively reduce CVD risk is essential. Supplementation with BR has been identified to reduce BP, which may lead to a large reduction in the incidence

of CVD on a population level (Strachan and Rose, 1991). However, BR is not all the same and contains varying quantities of NO_3^- and NO_2^- depending on many environmental and genetic factors. It is important that any given intervention aimed at reducing CVD risk is able to effectively reduce BP. Therefore, with the use of BR, it is crucial to investigate the individual ability of NO_3^- and NO_2^- to reduce BP.

The current study shows that as NO_2^- does not lead to the same increase in plasma [NO_3^-] and [NO_2^-] as NO_3^-. If NO_2^- is higher and NO_3^- is lower (e.g. through the storage of BR) the BR will be less efficient in increasing the plasma concentration of NO_3^- and NO_2^-. Likewise, if the NO_2^- content increases and NO_3^- decreases in BR, the reduction in MAP associated with the consumption will occur to a similar extent, but it will occur approximately 6 h post-consumption as opposed to 4 h post-consumption. The current study cannot accurately conclude the effect of NO_3^- nor NO_2^- on SBP/DBP. This is secondary to the inconsistency seen between the current findings and the findings seen in previous literature on the reduction of SBP/DBP following NO_3^- consumption. However, if the increase in plasma [NO_2^-] caused a decrease in SBP/DBP as previous literature suggests then NO_3^- would be the crucial factor to include within BR. This infers that stored BR or BR high in NO_2^- and lower in NO_3^- would not be an appropriate intervention to reduce CVD risk.

The current study highlights that the consumption of different BR (with varying NO_3^- and NO_2^- contents) will cause different cardio-protective effects and pharmacokinetics. This may provide guidance for food manufacturers to

more consciously provide BR which has the highest cardio-protective effects, and ensure both NO_3^- and NO_2^- are controlled for and consistent in their products. Additionally, it will ensure that consumers are aware of the benefits of various different BR, and how to best select and store their BR.

4.6 Recommendations

1. Further studies are required to accurately conclude the effect of NO_2^- on SBP and DBP. Specifically, the effect of NO_2^- should be investigated in a population with a higher baseline BP so reductions in BP are more likely to be observed.
2. This research was conducted in primarily University students with most participants between the age of 21-24 years old. An investigation with a more diverse population is required to produce applicable findings to the general population.
3. Further research is required in a larger sample size to ensure the pharmacokinetics and pharmacodynamics of both NO_3^- and NO_2^- was accurately concluded.
4. Future research investigating not only acute supplementation, but also chronic supplementation may be advantageous, as CVD is a chronic condition.

References:

Ahluwalia, A., Gladwin, M., Coleman, G. D., Hord, N., Howard, G., Kim-Shapiro, D. B., Lajous, M., Larsen, F. J., Lefer, D. J. & McClure, L. A. 2016. Dietary Nitrate and the Epidemiology of Cardiovascular Disease: Report from a National Heart, Lung, and Blood Institute Workshop. *Journal of the American Heart Association,* 5**,** 3402.

Ashor, A., Jajja, A., Sutyarjoko, A., Brandt, K., Qadir, O., Lara, J. & Siervo, M. 2015. Effects of Beetroot Juice Supplementation on Microvascular Blood Flow in Older Overweight and Obese Subjects: A Pilot Randomised Controlled Study. *Journal of Human Hypertension,* 29**,** 511.

Atkinson, G. A. 1888. The Pharmacology of the Nitrites and Nitro-Glycerine. *Journal of Anatomy and Physiology,* 22**,** 351.

Bahadoran, Z., Mirmiran, P., Kabir, A., Azizi, F. & Ghasemi, A. 2017. The Nitrate-Independent Blood Pressure–Lowering Effect of Beetroot Juice: A Systematic Review and Meta-Analysis. *Advances in Nutrition,* 8**,** 830-838.

Bailey, S. J., Vanhatalo, A., Winyard, P. G. & Jones, A. M. 2012. The Nitrate-Nitrite-Nitric Oxide Pathway: Its Role in Human Exercise Physiology. *European Journal of Sport Science,* 12**,** 309-320.

Bailey, S. J., Winyard, P., Vanhatalo, A., Blackwell, J. R., DiMenna, F. J., Wilkerson, D. P., Tarr, J., Benjamin, N. & Jones, A. M. 2009. Dietary Nitrate Supplementation Reduces the O2 Cost of Low-Intensity

Exercise and Enhances Tolerance to High-Intensity Exercise in Humans. *Journal of Applied Physiology,* 107**,** 1144-1155.

Bian, K., Doursout, M. F. & Murad, F. 2008. Vascular System: Role of Nitric Oxide in Cardiovascular Diseases. *Journal of Clinical Hypertension,* 10**,** 304-310.

Binkerd, E. & Kolari, O. 1975. The History and Use of Nitrate and Nitrite in the Curing of Meat. *Food and Cosmetics Toxicology,* 13**,** 655-661.

Blumgarten, A. S. 1934. *Textbook of Materia Medica and Therapeutics,* New York, USA, Macmillan.

Bondonno, C. P., Downey, L. A., Croft, K. D., Scholey, A., Stough, C., Yang, X., Considine, M. J., Ward, N. C., Puddey, I. B. & Swinny, E. 2014. The Acute Effect of Flavonoid-Rich Apples and Nitrate-Rich Spinach on Cognitive Performance and Mood in Healthy Men and Women. *Food and Function,* 5**,** 849-858.

Bondonno, C. P., Liu, A. H., Croft, K. D., Ward, N. C., Shinde, S., Moodley, Y., Lundberg, J. O., Puddey, I. B., Woodman, R. J. & Hodgson, J. M. 2015. Absence of an Effect of High Nitrate Intake from Beetroot Juice on Blood Pressure in Treated Hypertensive Individuals: A Randomized Controlled Trial. *American Journal of Clinical Nutrition,* 102**,** 368-375.

Borgi, L., Muraki, I., Satija, A., Willett, W. C., Rimm, E. B. & Forman, J. P. 2016. Fruit and Vegetable Consumption and the Incidence of Hypertension in Three Prospective Cohort Studies. *Hypertension,* 67**,** 288-293.

Bose, B. & Srivastava, H. 2001. Absorption and Accumulation of Nitrate in Plants: Influence of Environmental Factors. *Indian Journal of Experimental Biology*.

Brunton, T. L. 1867. On the Use of Nitrite of Amyl in Angina Pectoris. *Lancet,* 90**,** 97-98.

Butler, A. R. & Feelisch, M. 2008. Therapeutic Uses of Inorganic Nitrite and Nitrate: From the Past to the Future. *Circulation,* 117**,** 2151-2159.

Carlström, M., Liu, M., Yang, T., Zollbrecht, C., Huang, L., Peleli, M., Borniquel, S., Kishikawa, H., Hezel, M. & Persson, A. E. G. 2015. Cross-Talk between Nitrate-Nitrite-No and No Synthase Pathways in Control of Vascular No Homeostasis. *Antioxidants & Redox Signaling,* 23**,** 295-306.

Carlström, M., Lundberg, J. O. & Weitzberg, E. 2018. Mechanisms Underlying Blood Pressure Reduction by Dietary Inorganic Nitrate. *Actual Physiological,* 224**,** e13080.

Challa, H. J. & Uppaluri, K. R. 2018. Dash Diet (Dietary Approaches to Stop Hypertension). *Statpearls* USA: StatPearls Publishing.

Clements, W. T., Lee, S.-R. & Bloomer, R. J. 2014. Nitrate Ingestion: A Review of the Health and Physical Performance Effects. *Nutrients,* 6**,** 5224-5264.

Clifford, T., Howatson, G., West, D. J. & Stevenson, E. J. 2015. The Potential Benefits of Red Beetroot Supplementation in Health and Disease. *Nutrients,* 7**,** 2801-2822.

Clinic, M. 2019. Heart Disease. *Mayo Clinic - Patient Care & Health Information*, https://www.mayoclinic.org/diseases-conditions/heart-disease/symptoms-causes/syc-20353118

Coles, L. T. & Clifton, P. M. 2012. Effect of Beetroot Juice on Lowering Blood Pressure in Free-Living, Disease-Free Adults: A Randomized, Placebo-Controlled Trial. *Nutrition Journal,* 11**,** 106.

Contreras, F., Rivera, M., Vasquez, J., De la Parte, M. & Velasco, M. 2000. Endothelial Dysfunction in Arterial Hypertension. *Journal of Human Hypertension,* 14**,** S20.

Coppell, K. J., Mann, J. I., Williams, S. M., Jo, E., Drury, P. L., Miller, J. C. & Parnell, W. R. 2013. Prevalence of Diagnosed and Undiagnosed Diabetes and Prediabetes in New Zealand: Findings from the 2008/09 Adult Nutrition Survey. *NZ Med Journal* 126**,** 23-42.

Cosby, K., Partovi, K. S., Crawford, J. H., Patel, R. P., Reiter, C. D., Martyr, S., Yang, B. K., Waclawiw, M. A., Zalos, G. & Xu, X. 2003. Nitrite Reduction to Nitric Oxide by Deoxyhemoglobin Vasodilates the Human Circulation. *Nature Medicine,* 9**,** 1498.

d'El-Rei, J., Cunha, A. R., Trindade, M. & Neves, M. F. 2016. Beneficial Effects of Dietary Nitrate on Endothelial Function and Blood Pressure Levels. *International Journal of Hypertension,* 2016**,** 6 Pages.

Dauchet, L., Kesse-Guyot, E., Czernichow, S., Bertrais, S., Estaquio, C., Péneau, S., Vergnaud, A.-C., Chat-Yung, S., Castetbon, K. & Deschamps, V. 2007. Dietary Patterns and Blood Pressure Change

over 5-Y Follow-up in the Su. Vi. Max Cohort. *American Journal of Clinical Nutrition,* 85**,** 1650-1656.

Deb, S. & Dasgupta, A. 2008. A Study on Risk Factors of Cardiovascular Diseases in an Urban Health Center of Kolkata. *Indian Journal of Community Medicine: Official Publication of Indian Association of Preventive & Social Medicine,* 33**,** 271.

Densham, B. 1927. The Adjuvant Action of the Lactate Ion on the Vaso-Dilator Effect of Sodium Nitrite. *The Journal of Physiology,* 63**,** 175.

DeVan, A. E., Johnson, L. C., Brooks, F. A., Evans, T. D., Justice, J. N., Cruickshank-Quinn, C., Reisdorph, N., Bryan, N. S., McQueen, M. B. & Santos-Parker, J. R. 2015. Effects of Sodium Nitrite Supplementation on Vascular Function and Related Small Metabolite Signatures in Middle-Aged and Older Adults. *Journal of Applied Physiology,* 120**,** 416-425.

dos Santos Baião, D., Conte-Junior, C. A., Paschoalin, V. M. F. & Alvares, T. S. 2016. Quantitative and Comparative Contents of Nitrate and Nitrite in Beta Vulgaris L. By Reversed-Phase High-Performance Liquid Chromatography-Fluorescence. *Food Analytical Methods,* 9**,** 1002-1008.

Duranski, M. R., Greer, J. J., Dejam, A., Jaganmohan, S., Hogg, N., Langston, W., Patel, R. P., Yet, S.-F., Wang, X. & Kevil, C. G. 2005. Cytoprotective Effects of Nitrite During in Vivo Ischemia-Reperfusion of the Heart and Liver. *Journal of Clinical Investigation,* 115**,** 1232-1240.

Firth, J., Marx, W., Dash, S., Carney, R., Teasdale, S. B., Solmi, M., Stubbs, B., Schuch, F. B., Carvalho, A. F. & Jacka, F. 2019. The Effects of Dietary Improvement on Symptoms of Depression and Anxiety: A Meta-Analysis of Randomized Controlled Trials. *Psychosomatic Medicine,* 81**,** 265-280.

Floyd, C. N., Lidder, S., Hunt, J., Omar, S. A., McNeill, K. & Webb, A. J. 2019. Acute Interaction between Oral Glucose (75 G as Lucozade) and Inorganic Nitrate: Decreased Insulin Clearance, but Lack of Blood Pressure-Lowering. *British Journal of Clinical Pharmacology***,** 1443-1453.

Gancheva, S., Galunska, B. & Zhelyazkova-Savova, M. 2017. Diets Rich in Saturated Fat and Fructose Induce Anxiety and Depression-Like Behaviours in the Rat: Is There a Role for Lipid Peroxidation? *International Journal of Experimental Pathology,* 98**,** 296-306.

Gladwin, M. T., Shelhamer, J. H., Schechter, A. N., Pease-Fye, M. E., Waclawiw, M. A., Panza, J. A., Ognibene, F. P. & Cannon, R. O. 2000. Role of Circulating Nitrite and S-Nitrosohemoglobin in the Regulation of Regional Blood Flow in Humans. *Proceedings of the National Academy of Sciences,* 97**,** 11482-11487.

Habermeyer, M., Roth, A., Guth, S., Diel, P., Engel, K. H., Epe, B., Fürst, P., Heinz, V., Humpf, H. U. & Joost, H. G. 2015. Nitrate and Nitrite in the Diet: How to Assess Their Benefit and Risk for Human Health. *Molecular Nutrition & Food Research,* 59**,** 106-128.

Hardy, C. J. & Rejeski, W. J. 1989. Not What, but How One Feels: The Measurement of Affect During Exercise. *Journal of Sport and Exercise Psychology,* 11**,** 304-317.

Heart Foundation NZ. 2017. *General Health Statistics in New Zealand* [Online]. Available: https://www.heartfoundation.org.nz/statistics - 2 [Accessed].

Hobbs, D. A., Kaffa, N., George, T. W., Methven, L. & Lovegrove, J. A. 2012. Blood Pressure-Lowering Effects of Beetroot Juice and Novel Beetroot-Enriched Bread Products in Normotensive Male Subjects. *British Journal of Nutrition,* 108**,** 2066-2074.

Hord, N. G., Tang, Y. & Bryan, N. S. 2009. Food Sources of Nitrates and Nitrites: The Physiologic Context for Potential Health Benefits. *American Journal of Clinical Nutrition,* 90**,** 1-10.

Hunault, C. C., van Velzen, A. G., Sips, A. J., Schothorst, R. C. & Meulenbelt, J. 2009. Bioavailability of Sodium Nitrite from an Aqueous Solution in Healthy Adults. *Toxicology Letters,* 190**,** 48-53.

Ishizaki, J., Yamamoto, H., Takahashi, T., Takeda, M., Yano, M. & Mimura, M. 2008. Changes in Regional Cerebral Blood Flow Following Antidepressant Treatment in Late-Life Depression. *International Journal of Geriatric Psychiatry,* 23**,** 805-811.

Jajja, A., Sutyarjoko, A., Lara, J., Rennie, K., Brandt, K., Qadir, O. & Siervo, M. 2014. Beetroot Supplementation Lowers Daily Systolic Blood Pressure in Older, Overweight Subjects. *Nutrition Research,* 34**,** 868-875.

James, P. E., Willis, G. R., Allen, J. D., Winyard, P. G. & Jones, A. M. 2015. Nitrate Pharmacokinetics: Taking Note of the Difference. *Nitric Oxide,* 48**,** 44-50.

Jansson, E. Å., Huang, L., Malkey, R., Govoni, M., Nihlén, C., Olsson, A., Stensdotter, M., Petersson, J., Holm, L. & Weitzberg, E. 2008. A Mammalian Functional Nitrate Reductase That Regulates Nitrite and Nitric Oxide Homeostasis. *Nature Chemical Biology,* 4**,** 411.

Jonvik, K. L., Nyakayiru, J., Pinckaers, P. J., Senden, J. M., van Loon, L. J. & Verdijk, L. B. 2016. Nitrate-Rich Vegetables Increase Plasma Nitrate and Nitrite Concentrations and Lower Blood Pressure in Healthy Adults–3. *Journal of Nutrition,* 146**,** 986-993.

Jordan, J., Shannon, J. R., Black, B. K., Ali, Y., Farley, M., Costa, F., Diedrich, A., Robertson, R. M., Biaggioni, I. & Robertson, D. 2000. The Pressor Response to Water Drinking in Humans: A Sympathetic Reflex? *Circulation,* 101**,** 504-509.

Jørgensen, B. P., Hansen, J. T., Krych, L., Larsen, C., Klein, A. B., Nielsen, D. S., Josefsen, K., Hansen, A. K. & Sørensen, D. B. 2014. A Possible Link between Food and Mood: Dietary Impact on Gut Microbiota and Behavior in Balb/C Mice. *PloS One,* 9**,** e103398.

Joshipura, K. J., Ascherio, A., Manson, J. E., Stampfer, M. J., Rimm, E. B., Speizer, F. E., Hennekens, C. H., Spiegelman, D. & Willett, W. C. 1999. Fruit and Vegetable Intake in Relation to Risk of Ischemic Stroke. *Jama,* 282**,** 1233-1239.

Kapil, V., Khambata, R. S., Robertson, A., Caulfield, M. J. & Ahluwalia, A. 2015. Dietary Nitrate Provides Sustained Blood Pressure Lowering in

Hypertensive Patients: A Randomized, Phase 2, Double-Blind, Placebo-Controlled Study. *Hypertension,* 65**,** 320-327.

Kapil, V., Milsom, A. B., Okorie, M., Maleki-Toyserkani, S., Akram, F., Rehman, F., Arghandawi, S., Pearl, V., Benjamin, N. & Loukogeorgakis, S. 2010. Inorganic Nitrate Supplementation Lowers Blood Pressure in Humans: Role for Nitrite-Derived NO. *Hypertension,* 56**,** 274-281.

Kapil, V., Rathod, K. S., Khambata, R. S., Bahra, M., Velmurugan, S., Purba, A., Watson, D. S., Barnes, M. R., Wade, W. G. & Ahluwalia, A. 2018. Sex Differences in the Nitrate-Nitrite-NO Pathway: Role of Oral Nitrate-Reducing Bacteria. *Free Radical Biology and Medicine,* 126**,** 113-121.

Keen, J. T., Levitt, E. L., Hodges, G. J. & Wong, B. J. 2015. Short-Term Dietary Nitrate Supplementation Augments Cutaneous Vasodilatation and Reduces Mean Arterial Pressure in Healthy Humans. *Microvascular Research,* 98**,** 48-53.

Kelly, J., Fulford, J., Vanhatalo, A., Blackwell, J. R., French, O., Bailey, S. J., Gilchrist, M., Winyard, P. G. & Jones, A. M. 2012. Effects of Short-Term Dietary Nitrate Supplementation on Blood Pressure, O2 Uptake Kinetics, and Muscle and Cognitive Function in Older Adults. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 304**,** R73-R83.

Kemmner, S., Lorenz, G., Wobst, J., Kessler, T., Wen, M., Günthner, R., Stock, K., Heemann, U., Burkhardt, K. & Baumann, M. 2017. Dietary Nitrate Load Lowers Blood Pressure and Renal Resistive Index in

Patients with Chronic Kidney Disease: A Pilot Study. *Nitric Oxide,* 64**,** 7-15.

Khambadkone, S. G., Cordner, Z. A., Dickerson, F., Severance, E. G., Prandovszky, E., Pletnikov, M., Xiao, J., Li, Y., Boersma, G. J. & Talbot, C. C. 2018. Nitrated Meat Products Are Associated with Mania in Humans and Altered Behavior and Brain Gene Expression in Rats. *Molecular Psychiatry***,** 1.

Kukadia, S., Dehbi, H., Tillin, T., Coady, E., Chaturvedi, N. & Hughes, A. 2019. A Double-Blind Placebo-Controlled Crossover Study of the Effect of Beetroot Juice Containing Dietary Nitrate on Aortic and Brachial Blood Pressure over 24 Hours. *Frontiers in Physiology,* 10**,** 47.

Lansley, K. E., Winyard, P. G., Bailey, S. J., Vanhatalo, A., Wilkerson, D. P., Blackwell, J. R., Gilchrist, M., Benjamin, N. & Jones, A. M. 2011. Acute Dietary Nitrate Supplementation Improves Cycling Time Trial Performance. *Medicine and Science in Sports and Exercise,* 43**,** 1125-1131.

Larsen, F. J., Ekblom, B., Sahlin, K., Lundberg, J. O. & Weitzberg, E. 2006. Effects of Dietary Nitrate on Blood Pressure in Healthy Volunteers. *New England Journal of Medicine,* 355**,** 2792-2793.

Lee, C., Shallenberger, R., Downing, D., Stoewsand, G. & Peck, N. 1971. Nitrate and Nitrite Nitrogen in Fresh, Stored and Processed Table Beets and Spinach from Different Levels of Field Nitrogen Fertilisation. *Journal of the Science of Food and Agriculture,* 22**,** 90-92.

Lee, C.-W., Li, D., Channon, K. M. & Paterson, D. J. 2009. L-Arginine Supplementation Reduces Cardiac Noradrenergic Neurotransmission in Spontaneously Hypertensive Rats. *Journal of Molecular and Cellular Cardiology,* 47**,** 149-155.

Lee, I.-M., Cook, N. R., Gaziano, J. M., Gordon, D., Ridker, P. M., Manson, J. E., Hennekens, C. H. & Buring, J. E. 2005. Vitamin E in the Primary Prevention of Cardiovascular Disease and Cancer: The Women's Health Study: A Randomized Controlled Trial. *Jama,* 294**,** 56-65.

Lidder, S. & Webb, A. J. 2013. Vascular Effects of Dietary Nitrate (as Found in Green Leafy Vegetables and Beetroot) Via the Nitrate-Nitrite-Nitric Oxide Pathway. *British Journal of Clinical Pharmacology,* 75**,** 677-696.

Liu, R. H. 2003. Health Benefits of Fruit and Vegetables Are from Additive and Synergistic Combinations of Phytochemicals. *American Journal of Clinical Nutrition,* 78**,** 517S-520S.

Lundberg, J. O. & Govoni, M. 2004. Inorganic Nitrate Is a Possible Source for Systemic Generation of Nitric Oxide. *Free Radical Biology and Medicine,* 37**,** 395-400.

McIlvenna, L. C., Monaghan, C., Liddle, L., Fernandez, B. O., Feelisch, M., Muggeridge, D. J. & Easton, C. 2017. Beetroot Juice Versus Chard Gel: A Pharmacokinetic and Pharmacodynamic Comparison of Nitrate Bioavailability. *Nitric Oxide,* 64**,** 61-67.

McNair, D. M. 1971. *Manual Profile of Mood States,* San Diego, CA, USA, Educational & Industrial Testing Service.

Mills, C. E., Khatri, J., Maskell, P., Odongerel, C. & Webb, A. J. 2017. It Is Rocket Science–Why Dietary Nitrate Is Hard to 'Beet'! Part Ii: Further Mechanisms and Therapeutic Potential of the Nitrate-Nitrite-No Pathway. *British Journal of Clinical Pharmacology,* 83**,** 140-151.

Miyazaki, M. 1975. Studies on the Accumulation of Ntrate in Tomato Fruit for Canning. *Scientia Horticulturae,* 3**,** 109-128.

Morfeld, M., Petersen, C., Krüger-Bödeker, A., Von Mackensen, S. & Bullinger, M. 2007. The Assessment of Mood at Workplace-Psychometric Analyses of the Revised Profile of Mood States (Poms) Questionnaire. *GMS Psycho-Social Medicine,* 4**,** 06.

Myers, M. G., McInnis, N. H., Fodor, G. J. & Leenen, F. H. 2008. Comparison between an Automated and Manual Sphygmomanometer in a Population Survey. *American Journal of Hypertension,* 21**,** 280-283.

O'neil, A., Quirk, S. E., Housden, S., Brennan, S. L., Williams, L. J., Pasco, J. A., Berk, M. & Jacka, F. N. 2014. Relationship between Diet and Mental Health in Children and Adolescents: A Systematic Review. *American Journal of Public Health,* 104**,** e31-e42.

Ogedegbe, G. & Pickering, T. 2010. Principles and Techniques of Blood Pressure Measurement. *Cardiology Clinics,* 28**,** 571-586.

Oggioni, C., Jakovljevic, D., Klonizakis, M., Ashor, A., Ruddock, A., Ranchordas, M., Williams, E. & Siervo, M. 2018. Dietary Nitrate Does Not Modify Blood Pressure and Cardiac Output at Rest and During Exercise in Older Adults: A Randomised Cross-over Study. *International Journal of Food Sciences and Nutrition,* 69**,** 74-83.

Ormesher, L., Myers, J. E., Chmiel, C., Wareing, M., Greenwood, S. L., Tropea, T., Lundberg, J. O., Weitzberg, E., Nihlen, C. & Sibley, C. P. 2018. Effects of Dietary Nitrate Supplementation, from Beetroot Juice, on Blood Pressure in Hypertensive Pregnant Women: A Randomised, Double-Blind, Placebo-Controlled Feasibility Trial. *Nitric Oxide,* 80**,** 37-44.

Pussemier, L., Larondelle, Y., Van Peteghem, C. & Huyghebaert, A. 2006. Chemical Safety of Conventionally and Organically Produced Foodstuffs: A Tentative Comparison under Belgian Conditions. *Food Control,* 17**,** 14-21.

Reichert, E. T. & Mitchell, S. W. 1880. The Physiological Action of Potassium Nitrite *American Journal Medical Science,* 156**,** 158 - 180.

Remington, J. & Winters, K. 2017. Effectiveness of Dietary Inorganic Nitrate in Lowering Blood Pressure in Hypertensive Adults: A Systematic Review Protocol. *JBI Database of Systematic Reviews and Implementation Reports,* 15**,** 2445-2452.

Richard, A., Rohrmann, S., Vandeleur, C. L., Mohler-Kuo, M. & Eichholzer, M. 2015. Associations between Fruit and Vegetable Consumption and Psychological Distress: Results from a Population-Based Study. *BMC Psychiatry,* 15**,** 213.

Rifkind, J. M., Nagababu, E., Barbiro-Michaely, E., Ramasamy, S., Pluta, R. M. & Mayevsky, A. 2007. Nitrite Infusion Increases Cerebral Blood Flow and Decreases Mean Arterial Blood Pressure in Rats: A Role for Red Cell No. *Nitric Oxide,* 16**,** 448-456.

Santamaria, P. 2006. Nitrate in Vegetables: Toxicity, Content, Intake and Ec Regulation. *Journal of the Science of Food and Agriculture,* 86**,** 10-17.

Sesso, H. D., Buring, J. E., Christen, W. G., Kurth, T., Belanger, C., MacFadyen, J., Bubes, V., Manson, J. E., Glynn, R. J. & Gaziano, J. M. 2008. Vitamins E and C in the Prevention of Cardiovascular Disease in Men: The Physicians' Health Study Ii Randomized Controlled Trial. *Jama,* 300**,** 2123-2133.

Shah, I., Petroczi, A., James, R. A. & Naughton, D. P. 2013. Determination of Nitrate and Nitrite Content of Dietary Supplements Using Ion Chromatography. *Journal of Analytical & Bioanalytical Techniques,* 12.

Sindler, A. L., Fleenor, B. S., Calvert, J. W., Marshall, K. D., Zigler, M. L., Lefer, D. J. & Seals, D. R. 2011. Nitrite Supplementation Reverses Vascular Endothelial Dysfunction and Large Elastic Artery Stiffness with Aging. *Aging Cell,* 10**,** 429-437.

Siri-Tarino, P. W., Sun, Q., Hu, F. B. & Krauss, R. M. 2010. Saturated Fat, Carbohydrate, and Cardiovascular Disease. *American Journal of Clinical Nutrition,* 91**,** 502-509.

Song, P., Wu, L. & Guan, W. 2015. Dietary Nitrates, Nitrites, and Nitrosamines Intake and the Risk of Gastric Cancer: A Meta-Analysis. *Nutrients,* 7**,** 9872-9895.

Stanaway, L., Rutherfurd-Markwick, K., Page, R., Wong, M., Jirangrat, W., Teh, K. H. & Ali, A. 2019. Does Acute Supplementation with Nitrate-

Rich Beetroot Juice Benefit Older Adults More Than Younger Adults. *Multidisciplinary Digital Publishing Institute Proceedings,* 8**,** 26.

Stokes, K. Y., Dugas, T. R., Tang, Y., Garg, H., Guidry, E. & Bryan, N. S. 2009. Dietary Nitrite Prevents Hypercholesterolemic Microvascular Inflammation and Reverses Endothelial Dysfunction. *American Journal of Physiology-Heart and Circulatory Physiology,* 296**,** H1281-H1288.

Strachan, D. & Rose, G. 1991. Strategies of Prevention Revisited: Effects of Imprecise Measurement of Risk Factors on the Evaluation of “High-Risk” and “Population-Based” Approaches to Prevention of Cardiovascular Disease. *Journal of Clinical Epidemiology,* 44**,** 1187-1196.

Svebak, S. & Murgatroyd, S. 1985. Metamotivational Dominance: A Multimethod Validation of Reversal Theory Constructs. *Journal of Personality and Social Psychology,* 48**,** 107.

Tamme, T., Reinik, M., Roasto, M., Juhkam, K., Tenno, T. & Kiis, A. 2006. Nitrates and Nitrites in Vegetables and Vegetable-Based Products and Their Intakes by the Estonian Population. *Food Additives and Contaminants,* 23**,** 355-361.

Thomas, D. D. 2015. Breathing New Life into Nitric Oxide Signaling: A Brief Overview of the Interplay between Oxygen and Nitric Oxide. *Redox Biology,* 5**,** 225-233.

Thompson, K. G., Turner, L., Prichard, J., Dodd, F., Kennedy, D. O., Haskell, C., Blackwell, J. R. & Jones, A. M. 2014. Influence of Dietary Nitrate Supplementation on Physiological and Cognitive Responses to

Incremental Cycle Exercise. *Respiratory Physiology & Neurobiology,* 193**,** 11-20.

Thorin, E. & Thorin-Trescases, N. 2009. Vascular Endothelial Ageing, Heartbeat after Heartbeat. *Cardiovascular Research,* 84**,** 24-32.

Van Cleemput, O. & Samater, A. H. 1995. Nitrite in Soils: Accumulation and Role in the Formation of Gaseous N Compounds. *Fertilizer Research,* 45**,** 81-89.

Vanhatalo, A., Bailey, S. J., Blackwell, J. R., DiMenna, F. J., Pavey, T. G., Wilkerson, D. P., Benjamin, N., Winyard, P. G. & Jones, A. M. 2010. Acute and Chronic Effects of Dietary Nitrate Supplementation on Blood Pressure and the Physiological Responses to Moderate-Intensity and Incremental Exercise. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 299**,** R1121-R1131.

Vasconcellos, J., Conte-Junior, C., Silva, D., Pierucci, A. P., Paschoalin, V. & Alvares, T. S. 2016. Comparison of Total Antioxidant Potential, and Total Phenolic, Nitrate, Sugar, and Organic Acid Contents in Beetroot Juice, Chips, Powder, and Cooked Beetroot. *Food Science and Biotechnology,* 25**,** 79-84.

Velmurugan, S., Kapil, V., Ghosh, S. M., Davies, S., McKnight, A., Aboud, Z., Khambata, R. S., Webb, A. J., Poole, A. & Ahluwalia, A. 2013. Antiplatelet Effects of Dietary Nitrate in Healthy Volunteers: Involvement of Cgmp and Influence of Sex. *Free Radical Biology and Medicine,* 65**,** 1521-1532.

Wang, W., Lee, E. T., Fabsitz, R. R., Devereux, R., Best, L., Welty, T. K. & Howard, B. V. 2006. A Longitudinal Study of Hypertension Risk Factors and Their Relation to Cardiovascular Disease: The Strong Heart Study. *Hypertension,* 47**,** 403-409.

Wang, Y., Townsend, M. K., Eliassen, A. H. & Wu, T. 2013. Stability and Reproducibility of the Measurement of Plasma Nitrate in Large Epidemiologic Studies. *North American Journal of Medicine & Science,* 6**,** 82.

Waters, D. D., Alderman, E. L., Hsia, J., Howard, B. V., Cobb, F. R., Rogers, W. J., Ouyang, P., Thompson, P., Tardif, J. C. & Higginson, L. 2002. Effects of Hormone Replacement Therapy and Antioxidant Vitamin Supplements on Coronary Atherosclerosis in Postmenopausal Women: A Randomized Controlled Trial. *Jama,* 288**,** 2432-2440.

Webb, A. J., Patel, N., Loukogeorgakis, S., Okorie, M., Aboud, Z., Misra, S., Rashid, R., Miall, P., Deanfield, J. & Benjamin, N. 2008. Acute Blood Pressure Lowering, Vasoprotective, and Antiplatelet Properties of Dietary Nitrate Via Bioconversion to Nitrite. *Hypertension,* 51**,** 784-790.

White, D. W. & Raven, P. B. 2014. Autonomic Neural Control of Heart Rate During Dynamic Exercise: Revisited. *The Journal of Physiology,* 592**,** 2491-2500.

White, J. 2018. *Beet-It Insight* [Online]. England Available: https://www.beet-it.com/ [Accessed].

Wylie, L. J., Kelly, J., Bailey, S. J., Blackwell, J. R., Skiba, P. F., Winyard, P. G., Jeukendrup, A. E., Vanhatalo, A. & Jones, A. M. 2013. Beetroot

Juice and Exercise: Pharmacodynamic and Dose-Response Relationships. *Journal of Applied Physiology,* 115**,** 325-336.

Zand, J., Lanza, F., Garg, H. K. & Bryan, N. S. 2011. All-Natural Nitrite and Nitrate Containing Dietary Supplement Promotes Nitric Oxide Production and Reduces Triglycerides in Humans. *Nutrition Research,* 31**,** 262-269.

Appendices

APPENDIX A – HEALTH SCREENING QUESTIONNAIRE

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Pharmacokinetics of beetroot juice supplementation

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 infectious disease that may be transmitted in blood?

Yes ☐ No ☐

Qu 11. Do you have any issues wearing a face mask while sitting at rest?

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 ☐

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

APPENDIX B – INFORMATION SHEET

Pharmacokinetics of beetroot juice supplementation

PARTICIPANT INFORMATION SHEET

Researcher Introduction

We, Associate Professors Ajmol Ali and Kay Rutherfurd-Markwick, are researchers at Massey University (Albany) interested in further examining the role of nitrate and nitrite supplementation (contained within beetroot juice) on human health.

Invitation to Participate in Research Study

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 and nitrite) 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 interaction of nitrate and nitrite within the beverage on subsequent health outcomes is unclear. Therefore, the aim of this study is to examine nitrate and nitrite concentration in blood following ingestion of various drinks containing high and/or low nitrate and/or nitrite.

Participant Recruitment

If you are aged 18-50 years, we'd like to invite you to participate in this study. However, if you have any of the conditions listed on the "health checklist" you 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. You 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.

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). 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 complete 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 one of the four beetroot juice beverages; i) high nitrate (572mg), low nitrite (96mg); ii) low nitrate (43mg), medium nitrite (262mg); iii) medium nitrate (280mg), medium nitrite (243mg); iv) low nitrite (93mg), low nitrate (8mg). You will be asked to consume a different beverage 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 (e.g. sleep, activity) 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 researchers.

For the test days, you will be asked to arrive at the laboratory in the morning after observing an overnight fast; we will then insert a cannula, take a resting blood sample, and measure your weight, resting blood pressure, resting metabolic rate, and heart rate. You will then be asked to 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. We will then take blood samples and cardiovascular measures after every hour for 6 hours. 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. We will take 4 ml blood sample every 1 hour, thus 28 ml (6 teaspoons) of blood will be taken in total per testing day.

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. 24.5 hours (30 min for familiarisation and 6 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:

Principle Researchers :

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

Data collection team:

Luke Stanaway (PhD candidate)

luke.stanaway.1@uni.massey.ac.nz

Marsanne chabert (MSc student intern)

marsannechabert@orange.fr

Kyle Southward (PhD candidate)

K.A.Southward@massey.ac.nz

Emily Jakubcik (MSc student)

emilyjakubcik@outlook.com

Dr Wendy O'Brien (Lab Manager)

W.J.OBrien@massey.ac.nz

Committee Approval Statement

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application xx/xx. 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.

APPENDIX C – PARTICIPANT CONSENT FORM

Pharmacokinetics of beetroot juice supplementation

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 D: ETHICS APPROVAL

Date: 31 July 2018

Dear A/Pro Aj Ali

Re: Ethics Notification - **SOA 18/35** - **Pharmacokinetics of nitrate and nitrite ingestion**

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 **Tuesday, 31 July, 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

Professor Craig Johnson
Chair, Human Ethics Chairs' Committee and Director (Research Ethics)

APPENDIX E – RUNNING SHEET

On arrival.	❑ Mass + Height ❑ BP, HR ❑ SV, MAP, SVR, CO ❑ Blood samples ❑ Mood & PT
near 9:30 am	Breakfast. **Supplementation** *(timer begins when supplement finished).*
+1 (10:30am)	❑ Blood samples ❑ BP, HR ❑ Mood & PT
+2 (11:30pm)	❑ Blood samples ❑ BP ❑ SV, MAP, SVR, CO ❑ Mood & PT
+3 (12:30pm)	❑ Blood samples ❑ BP, HR ❑ Mood & PT → LUNCH
+4 (13:30pm)	❑ Blood samples

	❑ BP, HR ❑ SV, MAP, SVR, CO ❑ Mood & PT
+5 (14:30pm)	❑ Blood samples ❑ BP, HR ❑ Mood & PT
+6 (15:30pm)	❑ Blood samples ❑ BP, HR ❑ SV, MAP, SVR, CO ❑ Mood & PT

Study protocol**.** BP, blood pressure; HR, heart rate; SV, stroke volume; MAP, mean arterial pressure; SVR systemic vascular resistance; CO, cardiac output; PT, perceptual tests

APPENDIX F – FELT AROUSAL SCALE

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 G – FEELING SCALE

FEELING SCALE

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

APPENDIX H – POMS

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

APPENDIX I – SUPPLEMENTARY METHODS

Sphygmomanometer

The use of an automated sphygmomanometer has been validated against a manual sphygmomanometer and was shown to be a significant predictor of both manual systolic and diastolic blood pressure (Myers et al., 2008). A manual sphygmomanometer gives rise to an increased time and ambiguity of results.

USCOM

USCOM is a derivative of echocardiography utilizing CW Doppler ultrasound to provide various hemodynamic measurements. The machine measures the velocity time integral (VTI) of the aortic valve (via the suprasternal insonation window) and of the pulmonary valve (via the left parasternal window) blood flow as it leaves the heart. An algorithm based on height determines a cross-sectional area (CSA) of the two valves allowing stroke volume (SV) to be calculated as SV = CSA x VTI.

The interval between systolic ejections determines the heart rate (HR). SV and HR together allows cardiac output to be calculated as CO = SV x HR.

The systolic and diastolic blood pressure (SBP, DBP) allow the mean arterial pressure (MAP) to be calculated MAP = DBP + ({SBP – DBP]/3).