

Ageing, dietary nitrate and whole beetroot consumption: acute and long-term effects on metabolic, vascular and cognitive function

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Abstract

Dietary nitrate is a natural component of vegetables that can have profound effects on physiological functions in the body. One vegetable in particular, beetroot (*Beta Vulgaris*), has received attention in the last decade as a source of nitrate and more importantly, nitric oxide (NO). NO is a potent vasodilator that is produced endogenously from L-arginine but can also be produced from the sequential reduction of dietary nitrate to nitrite in the oral cavity and further to NO in the acidic environment of the stomach. The production of NO is essential in maintaining a healthy vascular system and low availability of NO is implicated in vascular dysfunction and thus the onset of cardiovascular and cerebrovascular diseases. Low endogenous NO production is closely associated with the ageing process and could contribute to the increase in high blood pressure and impairment of endothelial and cognitive function seen in older adults. Moreover, it is postulated that age attenuates the production of NO from exogenous nitrate, further contributing to the symptoms of low NO availability. Thus, ways to increase NO availability in an ageing population are imperative.

Although highly concentrated beetroot juice has been used as an effective vehicle of dietary nitrate in several studies, there is a significant gap in the research regarding longer-term intervention, for which whole beetroot may be more applicable. Processed forms of beetroot are more costly, contain more sugar and lack the dietary fibre present in the whole vegetable. Fibre, alongside the range of phenolic compounds, antioxidants and vitamins present in beetroot, may have further health benefits. Therefore, the overarching aim of this thesis was to investigate the effects of whole beetroot consumption in both acute and prolonged studies, particularly in an older population. A number of noteworthy findings emerged from the studies conducted during this research. Firstly, although whole beetroot, providing between 272mg and 816mg nitrate, was found to effectively raise plasma nitrate and nitrite levels in older participants, this did not translate to beneficial physiological outcomes such as blood pressure reduction and improved blood flow, which were evident in a younger population. Larger nitrate doses (1000mg) in the form of potassium nitrate did, however, lower blood pressure in an older population, reducing the disparity between the age groups. Secondly, more prolonged and consistent consumption of

whole beetroot over an eight-week period was found to reduce blood pressure in an older population and led to improvements in gut bacterial diversity, short-chain fatty acid (SCFA) production, cognitive reaction time and memory retrieval speed. This suggests additive effects of dietary nitrate beyond those seen in an acute setting and highlights the benefits of the whole vegetable as a supplement to the diet. In summary, whole beetroot was found to be an acceptable dietary source of nitrate and one that had acute physiological benefits in a young population. Ageing, however, reduced the activity of dietary nitrate in this acute setting. Despite this, the prolonged consumption of whole beetroot has the potential to lower blood pressure, improve cognitive function and potentially impact on gut health in an ageing population.

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Conference abstracts

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List of abbreviations

3MV	3-methyl valeric acid
6MWT	6-minute walk test
AAMI	Age-associated memory impairment
AD	Alzheimer's disease
ADI	Advised daily intake
ADL	Activities of daily living
Alx	Augmentation index
ANOVA	Analysis of variance
AUC	Area under the curve
BMI	Body mass index
CBF	Cerebrovascular blood flow
CDR	Cognitive drug research
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CRF	Clinical research facility
CRP	C-reactive protein
CVD	Cardiovascular disease
DASH	Dietary approaches to stop hypertension
DBP	Diastolic blood pressure
DH ₂ O	Deionized water
DPS	Deproteinising solution
DTPA	Diethylenetriaminepentaacetic acid
eNOS	Endothelial nitric oxide synthase
FeNO	Fractional exhaled nitric oxide
FEV ₁	Forced expiratory volume
FMD	Flow-mediated dilation
FVC	Forced vital capacity
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
GI	Gastrointestinal
HNO ₂	Nitrous acid
HPLC	High-performance liquid chromatography
ICC	Intraclass coefficient
IL-1	Interleukin-1
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IPAQ	International physical activity questionnaire
L-NMMA	NG-monomethyl-L-arginine
LDL	Low density lipoprotein
MCI	Mild cognitive impairment
MET	Metabolic equivalent
MMSE	Mini mental state exam
MPF	Maximal peak blood flow
N ₂ O ₃	Dinitrogen trioxide
NaOH	Sodium hydroxide

<i>NDNS</i>	National Diet and Nutrition Survey
<i>NEM</i>	N-ethylmaleimide
<i>NFκB</i>	Nuclear factor κ B
<i>nNOS</i>	Neuronal nitric oxide synthase
<i>NO</i>	Nitric oxide
<i>NOA</i>	Nitric oxide analyser
<i>NSAID</i>	Non-steroidal anti-inflammatory drug
<i>OTU</i>	Operational taxonomic unit
<i>PAD</i>	Peripheral arterial disease
<i>PN</i>	Potassium nitrate solution
<i>PORH</i>	Post-occlusive reactive hyperaemia
<i>PWV</i>	Pulse wave velocity
<i>RCT</i>	Randomised controlled trial
<i>RH</i>	Reactive hyperaemia
<i>ROS</i>	Reactive oxygen species
<i>RVI</i>	Royal Victoria Infirmary
<i>RVIP</i>	Rapid visual information processing
<i>SBP</i>	Systolic blood pressure
<i>SCFA</i>	Short chain fatty acid
<i>SOD</i>	Superoxide dismutase
<i>TMT</i>	Trail-making task
<i>TNF-α</i>	Tumour necrosis factor- α
<i>TUG</i>	Timed up-and-go
<i>VAS</i>	Visual analogue scale
<i>WHO</i>	World Health Organisation
<i>WMH</i>	White matter hyperintensity

Chapter 1. Introduction

The aim of this introduction and literature review is to explore and critically discuss the research into two important questions that relate beetroot to healthy ageing. Firstly, is whole beetroot an effective means to elicit the beneficial effects of dietary nitrate? Secondly, does ageing stand in the way of these effects? The review will begin with the negative consequences of ageing and highlight the main causes of these detriments, specifically the production, or lack of, nitric oxide (NO). It will describe the processes of NO production, how these are influenced by age and ways in which NO production can be manipulated. This will be followed by a review of the research on beetroot and the physiological responses to its consumption. This is with a view to highlighting the potential benefit of utilising beetroot in its whole and unprocessed form in a population most at risk of the consequences of a low availability of NO.

1.1 Ageing

The UK ageing population is growing, from 10.3 million adults over the age of 65 years in 2013 to 11.8 million in 2016, which equates to around 18% of the total population (Howdon & Rice, 2018; ONS, 2017; Rutherford, 2012). This proportion is expected to rise to 25% in 2050 due to increased longevity and the knock-on effect of the current age structure of the population, as illustrated in Figure 1.1.

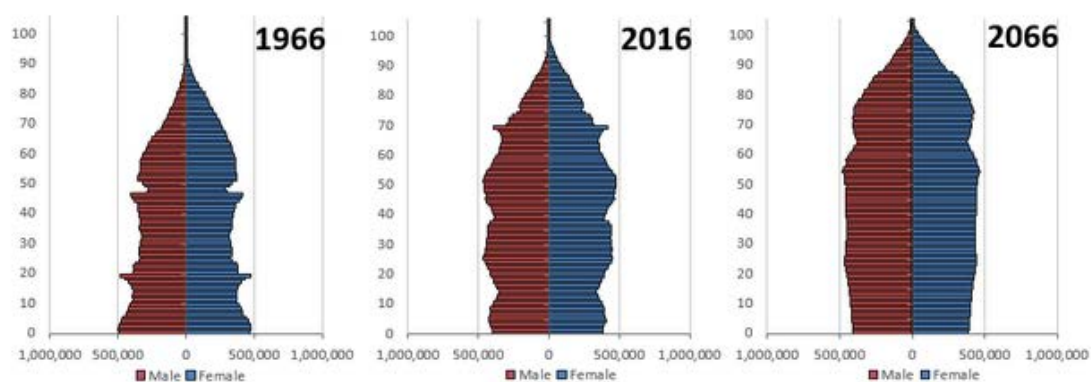


Figure 1.1: Office of National Statistics (ONS) report on principal projections for the population of the UK.

Population age restructuring towards an older population is associated with an increased demand in medical services and thus greater healthcare costs. Ironically, it is these healthcare services, specifically the improvements in recent decades that

have been a driving force in the increased longevity in the present day and allow adults to remain healthier and more independent into old age. The term “successful ageing” is described as remaining free of major chronic disease and maintaining normal physical and cognitive functioning (Newman *et al.*, 2003). Those who age successfully have low numbers of risk factors for prevalent chronic diseases, particularly for cardiovascular disease (CVD) (Newman *et al.*, 2003). Therefore, successful ageing can significantly reduce the medical services cost associated with the current UK population.

1.2 Theories of ageing

Ageing is characterised by the progressive deterioration in physiological function of organ systems and weakening of cell structure and function (Scott-Warren & Maguire, 2017). Two theories of ageing exist: 1) the programmed biological timetable of deterioration and 2) accrued cellular or molecular damage as a result of a build-up of noxious by-products of metabolism or environmental assaults (Scott-Warren & Maguire, 2017). One sub-category of the programmed theory is the immunological theory, which suggests that programmed decline of the immune system leads to an increased susceptibility to infection and thus ageing (Jin, 2010). This dysregulation of the immune system has been linked to CVD, Alzheimer’s disease (AD), inflammation, and cancer (Jin, 2010). Theories of ageing interconnect and have not yet been brought to a consensus. Understanding more about the ageing process and the factors that influence it may help promote healthy ageing, thereby reducing the burden of an ageing population on the healthcare system.

1.3 Features of ageing and age-related disease

Ageing affects the mechanical and contractile efficiency of the cardiovascular system. This presents itself through thickening of the arterial walls and increases in smooth muscle tone, inflammatory markers and total peripheral resistance. Additionally, there are reductions in arterial dilation, endothelial NO release and superoxide dismutase (SOD) activity (Egashira *et al.*, 1993; Ferrari *et al.*, 2003). Consequently, systolic arterial pressure and systemic vascular resistance are elevated (Navaratnarajah & Jackson, 2017). The same detrimental effects of ageing present themselves in vessels in the brain, contributing to neurovascular dysfunction and the onset of cerebrovascular disease. The risk of gastrointestinal (GI) and

oesophageal disorders is heightened with ageing, due to physiological changes in the stomach and oesophagus (Salles, 2007). Alongside this, alterations to the microbiome, both in the oral cavity and the gut, lead to changes in the breakdown and utilisation of nutrients from food and make the body more susceptible to pathogenic bacteria (Woodmansey, 2007). These features of ageing combined or in isolation increase the risk of multiple diseases such as CVD and cancer of the bowel, making older adults an important population in preventative nutritional intervention studies. Some common targets of diet and nutrition research will now be discussed in more detail.

1.3.1 Vascular dysfunction

Vascular endothelial cells line every capillary to artery in the circulatory system where they play a vital role in vascular biology (Rajendran *et al.*, 2013). These cells are paramount in regulating blood flow, blood vessel tone, haemostasis, and neutrophil recruitment. Vascular endothelial dysfunction is a hallmark of disease, with endothelial cells being directly implicated in stroke, heart disease, diabetes, insulin resistance, and tumour growth, amongst others (Rajendran *et al.*, 2013). Dysfunction in the vascular system develops with age and causes changes such as vasoconstriction, inflammation and pro-coagulation (Raubenheimer *et al.*, 2017; Sepúlveda *et al.*, 2015). Vascular dysfunction therefore negatively affects blood flow and has been implicated in the pathogenesis of AD (Dede *et al.*, 2007).

One key feature of vascular endothelial dysfunction is the reduced production of NO by endothelial NO synthase (eNOS) (Kunz, 2000). The release of NO from vascular endothelial cells maintains a background vasodilatory tone and this NO is continually synthesised by eNOS from the precursor L-arginine (Lyons *et al.*, 1997; Palmer *et al.*, 1988). eNOS converts L-arginine to citrulline and then NO, which, when released, disperses to the underlying smooth muscle (Sessa, 2004). Here it stimulates the production of cyclic guanosine monophosphate (cGMP), which results in relaxation of the vessel wall via changes in intracellular calcium concentration (Sessa, 2004). Disruption of the vasodilatory action in the vascular system by NO can lead to hypertension and atherosclerosis. NO production, from L-arginine and a more

recently discovered pathway involving dietary nitrate, will be discussed in more detail in Chapter 1.5, as dietary intervention may enable an increase in NO production.

Animal studies initially linked a reduction in NO-dependent vasodilation to ageing but Lyons *et al.* (1997) also demonstrated that NO-mediated vasodilation in the forearm vascular bed of humans is reduced in old age. The overall availability of L-arginine and the NOS cofactor tetrahydrobiopterin is lower in older adults (Delp *et al.*, 2008), which could explain the reduction in NO production from the process outlined previously. Reactive oxygen species (ROS) can inactivate NO (Schulz *et al.*, 2004), implicating oxidative stress in vascular dysfunction.

1.3.2 Oxidative stress

Harman (1956) first proposed that damage by ROS, or free radicals, might be a key player in the mechanism of ageing. This theory of free radical damage may explain some of the structural changes that occur with ageing, such as DNA damage, decline in mitochondrial function, and lipid peroxidation of membranes (Bar-Shai *et al.*, 2008). ROS are generally unstable molecules that induce the non-enzymatic oxidation of proteins, carbohydrates, lipids and nucleic acids (Canizzo *et al.*, 2011), and in ageing the antioxidant balance shifts towards increased production of these molecules. The excessive production of ROS is evident in many conditions such as hypertension, hypercholesterolaemia and diabetes. Furthermore, vascular oxidative stress has been implicated in alterations to cerebrovascular regulation and an increase in ROS generation has been demonstrated in cerebral ischaemia (Girouard & Iadecola, 2006). Oxidative stress has therefore been associated with the progression of AD.

Oxidative stress is caused by an imbalance in ROS and antioxidant systems, which allows free radicals to pass freely through plasma membranes and cause damage to the cells (Li *et al.*, 2013). The main cellular defence against ROS damage is activity by catalase, SOD, and glutathione peroxidase (Gupta *et al.*, 2001; Wang *et al.*, 2018), and levels of these free radical-scavenging enzymes are all reduced in ageing cells (de la Fuente *et al.*, 2004). SOD, for example, catalyses the dismutation of the radical superoxide into oxygen or hydrogen peroxide, thus reducing the levels of

superoxide, which, when not regulated, can combine with NO to form a potent oxidant peroxynitrite (Pacher *et al.*, 2007). The body also utilises antioxidants such as ascorbic acid, α -tocopherol and flavonoids (Li *et al.*, 2013) to combat ROS. Dietary antioxidants can help to limit the damage of oxidative stress by acting directly on ROS or by stimulating the cells' defence systems (Scalbert *et al.*, 2005). The phenolics in polyphenols, for example, can accept an electron and thereby form stable phenoxyl radicals, disrupting oxidation reactions in cells (Scalbert *et al.*, 2005). Flavonoids donate hydrogen atoms to radicals, terminating free radical chain reactions and stopping the oxidation of lipids and other molecules (Li *et al.*, 2013). Ascorbic acid works either through its direct interaction with superoxide radicals or indirectly by recycling tocopherol (Som *et al.*, 1983).

The central nervous system (CNS) is particularly vulnerable to oxidative stress, especially during ageing (Joseph *et al.*, 1998). This is due to a high content of polyunsaturated fatty acids that are easily oxidised, weaker antioxidative enzymes, and high oxygen consumption in the CNS (Garaschuk *et al.*, 2018; Li *et al.*, 2013 for review). Oxidative stress contributes to motor and cognitive behavioural deficits in the ageing brain (Shukitt-Hale, 1999), which may stem from a deficiency in glucose and oxygen consumption caused by the high levels of ROS (Mosconi, 2013). In a rodent study, supplementation with strawberry or spinach extract for eight months halted decrements in cognitive performance and neuronal function in senescent animals (Shukitt-Hale *et al.*, 2015), demonstrating the potential potency of nutritional compounds when confronting oxidative stress.

Oxidative stress can also contribute significantly to the structural remodelling in blood vessel walls that contribute to arterial stiffness (Patel *et al.*, 2011), which is described in more detail in Chapter 1.4. Polyphenols can attenuate this remodelling and improve endothelial dysfunction before the onset of the formation of plaque (Pandey & Rizvi, 2009), something that is associated with several risk factors for atherosclerosis. Ultimately, the accumulation of oxidative stress is a highly detrimental yet inevitable consequence of ageing but the body has a powerful defence mechanism in the form of antioxidant compounds. Many of these can be supplied or at least enhanced by dietary intake, which may help the body challenge

oxidants and delay or diminish the detrimental effects of oxidative stress on a number of systems. Oxidative stress, when able to infiltrate, has been implicated in the maintenance of the low-grade inflammatory state associated with ageing.

1.3.3 Inflammation

Adaptive immunity declines with age in a phenomenon known as immunosenescence, making individuals more vulnerable to infection and delaying recovery following bouts (Canizzo *et al.*, 2011). Conversely, innate immunity is more active in ageing, inducing a pro-inflammatory state known as “inflamm-ageing” (Franceschi *et al.*, 2007).

The chronic systemic inflammation associated with ageing has been attributed to heightened binding of nuclear factor κ B (NF κ B) to DNA in tissues and organs (Hinojosa *et al.*, 2009). The NF κ B signalling system is the regulator of innate immunity, organising cellular defence in response to pathogens or cellular danger signals (Salminen *et al.*, 2008). The cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α) are key mediators in the innate inflammatory response. In older adults, higher levels of these pro-inflammatory cytokines are often observed in the circulation and in tissue resident macrophages (Salminen *et al.*, 2008) and elevated tissue expression of these pro-inflammatory cytokines indicates chronic low-grade inflammation. Heightened levels of these cytokines are also associated with motor dysfunction and disability in older adults, in addition to reduced cognitive performance (Ferrucci *et al.*, 1999; Szelenyi, 2001).

The innate immune response is associated with an oxidative burst, which up-regulates the formation of ROS and the oxidative stress response. Consequently, cellular antioxidant capacity is reduced. This interplay between reduced antioxidant capacity and chronic inflammation with ageing increases the risk of a variety of diseases including CVD and may accelerate cognitive decline.

1.3.4 Cognitive decline and dementia

Increased longevity in society today brings with it challenges, especially from age-related diseases such as CVD and dementia. Cognitive decline is one of the key

reasons why long-term care is required in old age (Celidoni *et al.*, 2017), with 40% of admissions to care institutions in the UK being due to cognitive failure (Deary *et al.*, 2009).

Age-associated cognitive decline is described as normal or non-pathological cognitive ageing and can affect mental domains such as memory, executive function and processing speed (Deary *et al.*, 2009). There is a slower decline in cognitive function from the age of 50 to 70 years compared to the more pronounced and faster decline from the age of 70 years (Buckner, 2004). Brain ageing is accompanied by low-grade immune activation (Garaschuk *et al.*, 2018), which is characterised by increased levels of the pro-inflammatory cytokines TNF- α and IL-6, for example. The term “inflamm-ageing” thus applies in the brain as well as other tissues. Cognitive decline is a feared aspect of growing old (Corner & Bond, 2004) and a public health goal is to help maintain or enhance cognitive health into older age (Deary *et al.*, 2009). Dementia is not inevitable and there is a clear distinction between normal cognitive ageing and mild cognitive impairment (MCI), the latter of which may convert to Alzheimer’s disease (AD).

By the age of 80, there is estimated to be a 30% loss of brain mass, primarily in grey matter (Scahill *et al.*, 2003). Due to a reduction in neural density, production of central neurotransmitters such as serotonin and catecholamines is reduced and there is a deficiency in dopamine uptake sites and transporters (Peters, 2006). Consequently, ‘fluid’ cognitive abilities such as working memory, speed of processing and executive functioning decline with age, and these may be viable targets for preventative interventions such as nutritional supplementation or dietary changes (Scholey, 2018). For example, brain atrophy has been related to high levels of circulating homocysteine, which can be reduced by compounds such as folic acid and betaine (McRae, 2013).

Reduced blood flow to the brain has been implicated in cognitive impairment and decline, and this diminished cerebral perfusion has been found to precede and likely contribute to the onset of dementia (Ruitenberg *et al.*, 2005). Normal blood flow to the brain is maintained around 50ml per 100g of brain tissue per minute (Cipolla, 2009) and cognitive impairment is evident when cerebral blood flow drops to around

37ml per 100g per minute and below (Marshall *et al.*, 2001). AD shows a stable, progressive decline in cerebral blood flow and cognition. Neurocognitive research points towards white matter hyperintensity (WMH), or leukoaraiosis, occurring with ageing and this appears to lead directly from chronic ischaemia (Holland *et al.*, 2008). The ageing process leads to abnormal arterioles, capillaries and venules, causing or contributing to a chronic ischaemic state in an ageing brain. Age-related white matter degeneration has been linked specifically to reduced executive functioning capacity, which is measured by tests such as task switching and working memory (O'Sullivan *et al.*, 2001; Oosterman *et al.*, 2008). Disrupted neurovascular coupling occurs when cerebrovascular blood flow is not matched to regional metabolic demand (Justice *et al.*, 2015). This destabilises neurons, synapses and neurotransmission and has been linked to a reduction in NO bioavailability (Girouard & Iadecola, 2006).

1.3.5 Hypertension

When blood pressure levels are consistently high (equal to or above 140/90mmHg) an individual is classed as hypertensive (NICE, 2011). Hypertension is currently one of the leading causes of death and disability in the world, and age, obesity, sodium intake and physical inactivity are amongst the main risk factors for high blood pressure (Kannel, 1996). High blood pressure itself is a major risk factor for CVD (Yusuf *et al.*, 2001) and stroke (Robinson *et al.*, 1998). Gee and Ahluwalia (2016) describe blood pressure as a continuum, with increasing pressures leading to increased risk of CVD. Hypertension is more prevalent in the older population and there is a direct relationship between increasing age and higher blood pressure (Chobanian *et al.*, 2003). According to the latest (2015) Health Survey for England statistics, the percentage of those with hypertension aged 55-64, 65-74 and 75+ years are 45.0%, 58.5% and 66.5%, respectively, clearly illustrating the increasing age and blood pressure relationship and the extent of the problem in the population.

Anti-hypertensive medications, such as beta-blockers and diuretics, are prescribed in an attempt to lower blood pressure to normal levels. With every 20mmHg systolic and 10mmHg diastolic increase in blood pressure, mortality from both stroke and ischaemic heart disease doubles (Chobanian *et al.*, 2003). A reduction in diastolic

blood pressure (DBP) by 5mmHg is said to reduce the risk of stroke by one third and coronary heart disease (CHD) by one fifth (Collins *et al.*, 1990) and every 10mmHg reduction in systolic blood pressure (SBP) significantly reduces the risk of major CVD events with a 13% reduction in all-cause mortality (Ettehad *et al.*, 2016).

There are a number of causes of high blood pressure but in relation to ageing it can be caused by structural changes to the cardiovascular system such as stiffening and plaque formation. The role of NO in the structural integrity of the vascular system again puts NO production central to this age-related problem.

1.3.6 Digestion and microbiotal changes

A preponderance in medication use into old age can cause hyposalivation (dry mouth) and changes to the gut microenvironment with antibiotics, each leading to an increased risk of pathogenic bacterial growth (Tiihonen *et al.*, 2010). Modification of the oral microenvironment may influence non-enzymatic, or bacterial, conversion of nitrate into nitrite (Percival *et al.*, 1991); this nitrate conversion process is key in NO production and is described in more detail in Chapter 1.5. Reduced secretion of hydrochloric acid, known as hypochlorhydria (Britton & McLaughlin, 2013) and pepsin in the stomach are also associated with ageing, leading to a rise in gastric pH. Furthermore, the ageing process is associated with alterations in colonic functioning, such as increases in the colonic transit time due to a decline in the peristaltic activity in the colon. This is postulated to be due to a reduction in expression of neural transmitters, such as NO or acetylcholine (Salles, 2007).

In addition to changes that occur as a result of medication use, the ageing process itself can modify the body's microenvironment, affecting the gut microbiome as well as the population of oral bacteria. The gut microbiome plays a pivotal role in the absorption of nutrients, as well as maintaining a natural barrier to pathogens (Britton & McLaughlin, 2013). Age-related alterations in this barrier could lead to a systemic and luminal inflammatory response. Indeed, the elderly show an increased susceptibility to gut infections relative to younger adults (Ogra, 2010). Changes in the bacterial content of the gut have been linked with both inflammatory and metabolic disorders, including inflammatory bowel disease, diabetes, CVD and colorectal

cancer (Frank *et al.*, 2007; Jeffery *et al.*, 2012). Furthermore, it has been proposed that the gut microbiota can influence brain chemistry and function, affecting motor control (Heijtz *et al.*, 2011).

Colonic bacteria are predominantly anaerobes with wide-ranging metabolic activities. Many of these anaerobes can ferment dietary carbohydrates to form short chain fatty acids (SCFAs), for example acetate, propionate and butyrate, which all have a role in gut health (Duncan & Flint, 2013). There are a number of dominant bacterial species in a healthy colon, including *Faecalibacterium prausnitzii*, which possesses anti-inflammatory activities (Duncan & Flint, 2013). The dominant phyla of the gut microenvironment include *Firmicutes*, *Bacteroidetes* and *Actinobacteria*, as well as *Proteobacteria* and *Verrucomicrobia* in lower numbers (Duncan *et al.*, 2008; Karlsson *et al.*, 2013). A decline in bacterial diversity occurs with ageing and in the elderly there seems to be a reduction in *Bifidobacteria* and an increase in *Enterobacteriaceae* and certain *Proteobacteria* (O'Toole & Claesson, 2010). The balance shifts towards higher numbers of *Bacteroidetes* and lower numbers of *Firmicutes* in elderly compared to younger adults (Tiihonen *et al.*, 2010). Additionally, butyrate levels tend to be lower in the elderly compared to younger adults due to a reduction in bacterial species that produce it (Biagi *et al.*, 2010). It is clear that there are changes to the gut microbiota during ageing and disruption to the normal balance of bacteria can lead to both local and systemic inflammation (Saffrey, 2014).

The gut microbiota clearly play a crucial role in the health and functioning of the digestive system specifically and have now also been linked to functioning of other systems in the body such as the gut-brain axis. The digestive and bacterial changes that occur with age are likely to have repercussions on a number of functions in the body, placing dietary aspects such as fibre, prebiotics and antioxidant nutrients in a prominent and influential position. Both short- and long-term dietary changes have been found to have an impact on the gut microenvironment (Thomas *et al.*, 2011), as the microbial community adapts to release the most energy from the available substrates. The diet of an ageing population may therefore be of particular importance not only to the microbiome but also to the overall health status of the population.

1.3.7 Changes in physiological function

The capacity of skeletal muscle to maintain an efficient regenerative pathway is compromised in ageing (Carosio *et al.*, 2011). Muscle tissue diminishes, causing a reduction in mass and strength; this condition is known as sarcopenia (Marcell, 2003). Sarcopenia contributes to a reduction in functional capacity in older adults, contributing to frailty and a loss of independence. Cognitive decline also contributes to this reduction in capacity.

Maintaining independent functioning is key in healthy ageing. A high quality of life is dependent on being able to perform activities of daily living (ADL) into old age and these functions are also related to hospitalisation, surgical outcome and mortality (Gault & Willems, 2013; Penninx *et al.*, 2000). The inability to perform daily tasks is linked to weaker muscles and a reduced capacity to generate force (Close *et al.*, 2005). The cytokines IL-6, TNF- α and C-reactive protein (CRP) have been implicated in this weakness, resulting in poor physical performance in older adults (Legrand *et al.*, 2013).

Changes in vascular function, blood pressure levels and gut microbial diversity, amongst the other features of ageing mentioned, may be subject to intervention studies aimed at slowing the rate of decline into older age. It is therefore important to assess the functioning of these systems to better understand how the ageing process may attenuate their capacity.

1.4 Assessment of vascular function, functional capacity and cognition

In monitoring the integrity and functioning of the vascular system into older age, researchers are afforded the opportunity to carry out an assessment of the potential for beneficial interventions in this area. Both the macrovascular and microvascular system can be examined, allowing comprehension of the overall vascular system's efficiency. At a whole-body level, functional capacity and cognitive functioning measures can reflect an individual's ability to carry out basic daily tasks, thereby helping them to maintain independence into older age.

1.4.1 Macrovascular assessment

The vascular endothelium helps to maintain blood flow and alterations to this can cause the build-up of plaque inside the blood vessels (atherosclerosis) or augment the vasodilatory response. Arterial distensibility, or its opposite arterial stiffness, is an indicator of endothelial function and thus atherosclerosis. Aortic pulse wave velocity (PWV), for example, has an inverse relationship with arterial distensibility and has been found to be a predictor of cardiovascular risk in patients with hypertension (Blacher *et al.*, 1999). PWV is the assessment of the length of time in which pressure pulse waves travel from the carotid to the femoral arteries and measurement of this is considered the gold standard for assessment of arterial stiffness (Ecobici & Stoicescu, 2017). PWV assessment requires accurate measurement of the distance between the two arterial sites and the femoral artery can be an intimate measurement site, which can hinder reliable assessment. Nevertheless, PWV is the most reported marker of arterial stiffness in studies with older adults (Table 1.2). Ageing results in an increase in PWV and this has been found to be associated with CV mortality, stroke and CHD even in healthy older adults (Sutton-Tyrrell *et al.*, 2005). Augmentation index (Alx) is also inversely related to arterial distensibility and it increases with age. Alx is an indirect measure of arterial stiffness derived from central arterial pressure waves (Ecobici & Stoicescu, 2017).

Brachial flow-mediated dilation (FMD) is another non-invasive measure of endothelial function and is considered the gold standard for measurement in this area (Raitakari & Celermajer, 2000). FMD is said to represent arterial functioning following NO release from the endothelium and thus act as a marker for vascular function (Thijssen *et al.*, 2011). It is a non-invasive ultrasound measurement of blood flow following occlusion and is compared to the response following the administration of sublingual nitroglycerin, which is an endothelium-independent dilator. Although FMD benefits from being a non-invasive measurement, it is difficult to perform and requires extensive training and skill (Raitakari & Celermajer, 2000), making it a less appropriate tool in a research setting. There is impairment in brachial FMD with age, which may be due in part to a reduction in NO synthesis by NO synthase (Gates *et al.*, 2007; Yeboah *et al.*, 2007). Similar to PWV and Alx, FMD is a predictor of

cardiovascular events. For every 1% increase in FMD, the risk of experiencing a cardiovascular event is reduced by 13% (Inaba *et al.*, 2010)

1.4.2 Microvascular assessment

The microcirculatory system is made up of the smallest blood vessels such as capillaries and arterioles. It is responsible for adjustments in vascular tone that match tissue perfusion with oxygen demand (Gutterman *et al.*, 2016). The microvasculature has a crucial role in modulating vascular tone by the release of NO, other ROS, and arachidonic acid metabolites from the endothelium (Gutterman *et al.*, 2016). Dysfunction in the microvasculature, similar to the macrovascular system, is a predictor of CV events (Lanza & Crea, 2010).

Reactive hyperaemia (RH) is a phenomenon that occurs following arterial occlusion, in which there is a sudden rise in skin and muscle blood flow above resting levels. Blood flow is initially high but returns to the pre-occlusive level within a few minutes (Imms *et al.*, 1988). Measurement and assessment of this non-invasive yet sensitive post-occlusive reactive hyperaemia (PORH) is used as an indicator of macro- and micro-vascular dysfunction (Yamamoto-Suganuma & Aso, 2009).

Assessment of PORH may be a reflection of microvascular endothelial function, which is dependent on NO production in endothelial cells (Yamamoto-Suganuma & Aso, 2009). These same authors identify two phases of RH: 1) a peak response that occurs within a few seconds post-occlusion and 2) a prolonged total hyperaemia period that reflects the blood flow debt repayment. Results from a study by Tagawa *et al.* (1994) suggest that NO plays a small but significant role in maintaining vasodilation subsequent to the peak or maximal reactive hyperaemia response but a minimal role at the peak or early phase, as characterised by Yamamoto-Suganuma and Aso (2009). The maximal peak blood flow (MPF) and the time to MPF after ischaemia are generally used as indices of microvascular endothelial function (Yamamoto-Suganuma & Aso, 2009).

PORH has been found to be associated with several risk factors for CVD, for example high blood pressure (Carberry *et al.*, 1992). Carberry, Shepherd and

Johnson (1992) outline that the vasodilatory capacity of forearm skin is reduced in individuals with hypertension and that an increased resistance to blood flow is key in the maintenance of this high blood pressure. A more recent study identified a significant correlation between impairment of PORH and cardiovascular risk factors in a healthy female population (Vuilleumier *et al.*, 2002).

Measurement of PORH and other RH markers by laser Doppler is considered a valuable, non-invasive tool to monitor and assess the functioning of the microvascular system. PORH is therefore a sensitive marker of vascular function in both healthy (Vuilleumier *et al.*, 2002) and diseased populations (Yamamoto-Suganuma & Aso, 2009). A major advantage of using laser Doppler is its sensitivity at detecting changes in skin blood flow in response to given stimuli (Cracowski *et al.*, 2006). Cracowski *et al.* (2006) describe a number of ways to minimise the variability of laser Doppler measurement of microvascular function but this variability can be a significant limitation of microvascular assessment.

1.4.3 Assessing functional capacity

A well-used assessment tool into old age is the measurement of ADL performance and capacity. For example, the 6-minute walk test (6MWT) tests exercise tolerance, aerobic capacity and endurance, most often in participants with CVD or pulmonary disease (Steffen *et al.*, 2002). Other tests include hand-grip strength, sit-to-stands and timed-up-and-go (TUG). TUG, for example, is a basic assessment of balance during a simple task that has good reliability within and between investigators (Steffen *et al.*, 2002). Each of these tasks, as well as measures of agility, muscle strength, and lung capacity, may highlight the risk of falls or a lack of independent movement. These are important in tasks that require quick movement, such as getting off the bus in time and answering the phone and are highly relevant in an ageing population. More attention is rightly turning to outcomes relevant to daily living, as opposed to clinical values and measurements. For example, basic ADLs consist of feeding, bathing and using the toilet, whilst instrumental ADLs refer to shopping, cooking and housework (Kingston *et al.*, 2012). These are essential measures of disability in older adults, particularly the very old (Kingston *et al.*, 2012).

Lung function can also be used as a measure of functional capacity and tests such as spirometry are widely used to determine the presence of respiratory disease. Spirometry measures lung volume against time and is quick and easy to use (Ranu *et al.*, 2011). It has been shown that compounds that inhibit the breakdown of cGMP, such as sildenafil, have positive effects on pulmonary vascular resistance (Beghetti *et al.*, 2014). Sildenafil preserves levels of cGMP, which is a downstream target of NO, thus enhancing NO-mediated vasodilation in the pulmonary blood vessels (Chapman *et al.*, 2009).

The presence of NO in the lung specifically is measured by fractional exhaled nitric oxide (FeNO). NO production in the lung has been implicated in the pathophysiology of asthma and other lung diseases due to its key role in lung biology (Dweik *et al.*, 2011). These roles include, but are not limited to, vasodilation, neurotransmission, bronchodilation, and mediation of inflammatory responses (Nathan & Xie, 1994; Ricciardolo, 2004 for review). Guo *et al.* (2000) described the role of NO in asthma, suggesting higher concentrations in asthmatic individuals, as well as an increase in L-arginine, which is the precursor to NO production. Ricciardolo (2004) also describes abnormal concentrations of NO in activated states of inflammatory airway disease. As discussed previously, NOS catalyses the oxidation of L-arginine to generate NO and this occurs in various cell types in the respiratory tract, as with other parts of the body (Ricciardolo, 2004). Exhaled NO is derived primarily from the lower respiratory tract, in particular from the airways of the lung (Kharitonov *et al.*, 1996), and the correlation between it and some measures of airway inflammation implicate FeNO as a measure of NO production (Travers *et al.*, 2007). Both spirometry and FeNO are straightforward and quick tests of lung function and the latter can be influenced by external stimuli such as dietary nitrate.

1.4.4 Assessing cognitive function

Age-associated cognitive decline or memory impairment (AAMI) describes benign and non-clinical cognitive deficits associated with ageing (Werner & Korczyn, 2008). MCI, however, involves cognitive decline to an extent beyond what is expected with age or educational background (Werner & Korczyn, 2008). This is most often diagnosed through the use of a Mini Mental State Exam (MMSE) or equivalent.

Cognitive function, on the other hand, can be assessed by a plethora of cognitive tasks, both computer- and paper-based. Collerton *et al.* (2007) described that computer- and paper-based tests were equally effective for assessing cognitive function in older adults but computerised tests were more acceptable to both participants and investigators. Computer-based batteries are frequently used in cognitive assessment and resources such as the Cambridge Neuropsychological Testing Automated Battery (CANTAB) and CogTrack™ allow the allocation of tests to key areas of interest, such as a healthy cohort or a population at risk of cognitive impairment. A number of computer-based assessment tools have been reviewed by Wild *et al.* (2008). Computerised batteries are more sensitive and precise and can be highly standardised (Luciana, 2003; Wild *et al.*, 2008), and also reduce the time required of the investigator. Paper-based tasks, however, such as the Trail Making Task (TMT) are often utilised in research (LaRoche *et al.*, 2014; Justice *et al.*, 2015), which involves connecting numbered circles with a line or connecting numbered and lettered circles in sequence. Combinations of cognitive tasks can be used to inform functioning in certain domains or areas of the brain, for example in global cognition, executive functioning, attention, or psychomotor speed.

The fact that both computer- and paper-based cognitive assessments are employed, spanning a large variety of tasks, makes it difficult for applicable conclusions to be drawn. Furthermore, confounders such as practice effects have been identified in serial cognitive assessment, which can affect clinical outcomes (Goldberg *et al.*, 2015). Using alternative forms or pre-baseline testing, however, are appropriate solutions to this problem (Goldberg *et al.*, 2015).

1.4.5 The role of NO in age-related diseases

A common theme throughout the preceding sections is the reduction in NO production in the endothelial cells or availability with age, which can manifest itself in vascular dysfunction and dementia. Given the role of endothelial and vascular dysfunction in disease it is beneficial to research NO production and ways to maintain it into older age. NO plays such a large role in maintaining a healthy vasculature and this itself is associated with quality of life into older age (Miller *et al.*, 2012). There is a substantial body of research on the pathways involved in NO

production and a brief summary will be presented here. There is then opportunity to explore ways to manipulate these pathways to ensure the maintenance of NO production with ageing.

1.5 NO production

It wasn't until the discovery of NO as an endothelial-reducing factor that research into the effects of NO and consequently dietary nitrate began. Louis Ignarro, who won the Nobel Prize for demonstrating the properties of NO, elegantly described the signalling roles of NO in a review (Ignarro, 2002); the vasodilatory pathway was also described in brief in Chapter 1.3.1. NO has wide diffusibility in the body and is crucial for endothelial integrity, vascular smooth muscle cell proliferation and the regulation of vascular tone and blood pressure (Ignarro, 2002).

The classical pathway for the generation of NO is via NO synthases, producing NO from the precursor amino acid, L-arginine. Until the 1990s, this was thought the only pathway for the production of NO and the breakdown products, nitrate and nitrite, were thought inert waste products (Moncada & Higgs, 1993). However, two research groups discovered that the pathway could in fact be reversed, with NO being produced from the breakdown of nitrate and nitrite in a stepwise manner (Benjamin *et al.*, 1994; Lundberg *et al.*, 1994). This process does not require NO synthases and has been found to be even more efficient at times of hypoxia in tissues. The non-enzymatic system therefore acts as a back-up system to the enzymatic pathway during periods of low oxygen. It is the non-enzymatic production of NO from nitrate that will be focussed on here, as this system can be manipulated through dietary components.

The conversion of inert nitrate to the bioactive NO spurred research into dietary nitrate and a study by Larsen *et al.* (2006) was the first to discover a positive effect, akin to that exerted by NO, on the cardiovascular system from dietary nitrate intake. The breakthrough by Larsen *et al.* in 2006 led to scientists studying dietary nitrate intake manipulation to induce positive effects on vascular function and blood pressure in the human body.

1.5.1 Nitrate and nitrite

Inorganic nitrate has been long-used as a method of acutely lowering blood pressure, first used by the Chinese to treat coronary artery disease as far back as 700 AD (Butler & Feelisch, 2008). The bioconversion of nitrate to nitrite and further to NO and other secondary reaction products is thought to underlie any protective effects on the cardiovascular system (Lundberg *et al.*, 2006). Nitrate itself serves as the primary plant form of the nutrient and must undergo metabolic activation to an active form i.e. nitrite (Bryan & Ivy, 2015). Nitrite, however, is an active secondary breakdown product of nitrate and although nitrite was long considered to be equally inert, more recent research established that nitrite forms a stable reservoir for the bioactive NO during hypoxia when the oxygen-dependent pathway involving NO synthases is limited (Shiva, 2013).

Nitrate and nitrite have been used for centuries in cheese production and for the curing and preservation of meat and fish. The addition of nitrite in the curing of meats contributes to the flavour by inhibiting rancidity, forms an attractive pink colour through interaction with myoglobin and inhibits the growth of bacteria, specifically *Clostridium botulinum* (Bryan & Ivy, 2015). High nitrate levels accumulate in the leaves of green leafy vegetables and in roots such as beetroot. Vegetables contribute the largest proportion of dietary nitrate to the diet and will be discussed in more detail in Chapter 1.6. Common sources of nitrate and nitrite are shown in Table 1.1. A small amount of dietary nitrate comes from drinking water but the nitrate content does vary across regions. WHO recommendations limited the concentration of nitrate in drinking water to 50mg/L in Europe, due to historical cases of “blue baby syndrome” that were linked to contaminated well water high in nitrate. Blue baby syndrome is caused by methemoglobineamia, which is anaemia resulting from the oxidation of the ferrous iron in haemoglobin, forming methemoglobin (Manassaram *et al.*, 2010). This acute toxicity and its link to nitrate and nitrite is being contested due to the high levels of bacteria present in the well water (Powlson *et al.*, 2008). However, high nitrate and nitrite intake in infants and young children should be cautioned.

Another concern with nitrate and nitrite intake is the potential formation of N-nitrosamines, of which some are carcinogenic. A number of epidemiological studies

have tested the link between nitrate and stomach cancer but have found no conclusive link between the incidence and mortality of the cancer and nitrate intake (Powlson *et al.*, 2008). Processing of nitrite in the acidic stomach produces nitrous acid (HNO_2) and dinitrogen trioxide (N_2O_3), which produce N-nitrosamines when they react with dietary secondary amines (Schoental, 1963). There is equivocal research surrounding the roles, if any, of nitrite and N-nitrosamines in human cancer and a review of these is beyond the scope of this thesis. Studies in this area have been reviewed by a number of authors (Bryan & Ivy, 2015; Omar *et al.*, 2012) and a finding relevant to this thesis has been highlighted: antioxidant compounds, such as those found in fruit and vegetables, inhibit nitrosation pathways that produce N-nitrosamines (Bartsch *et al.*, 1988; Tannenbaum *et al.*, 1991). Furthermore, a diet rich in fruit and vegetables, some of which are known to be high in nitrate, has been found to reduce the risk of some cancers (Benetou *et al.*, 2008; González *et al.*, 2006).

The accurate analysis of nitrate and nitrite in vegetables and biological tissues is undoubtedly important and a number of methods are available, differing in sensitivity. Nitrate and nitrite can be analysed using high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), colorimetry, or chemiluminescence (Jobgen *et al.*, 2007; Moorcroft *et al.*, 2001). Nitrate and nitrite content of vegetables is often analysed using GC-MS, which involves the reduction of nitrate to nitrite followed by derivatization with, for example, sulphanilamide (Campanella *et al.*, 2017). This method is often limited by interference between compounds. HPLC allows for simultaneous assessment of nitrate and nitrite but lacks sensitivity and is, similar to GC-MS, vulnerable to interference from chloride in biological samples. Nitrate in, for example, cured meats and cheeses can be assessed by colorimetry using the Griess Method, which involves again reduction to nitrite and a coupling reaction that provides a red azo chromophore, from which nitrite concentration can be assessed (Moorcroft *et al.*, 2001). This method can be limited by the reduction of nitrate to nitrite and interference with reagents. Chemiluminescence is unaffected by chloride and is deemed the gold standard in analysis of nitrate and nitrite. This method will be discussed in more detail in Chapter 2, as it was the analysis method used in the present study.

Table 1.1: Common sources of dietary nitrate.

<i>Classification</i>	<i>Source</i>	<i>Nitrate (mg/100g)</i>
<i>Very high (>250mg/100g)</i>	Rocket	260
	Spinach	214
<i>High (100-250mg/100g)</i>	Lettuce	189
	Radish	187
	Beetroot	146
	Chinese cabbage	139
<i>Medium (50-100mg/100g)</i>	Turnip	62
<i>Low (20-50mg/100g)</i>	Broccoli	40
	Carrot	22
	Processed sausages	9
	Bacon	6
	Banana	5
	Water	2.6 (mg/100ml)

Data from Hord, Tang and Bryan (2009) and Lidder and Webb (2013).

It is clear that nitrate and nitrite are available both through the diet and through endogenous production from the oxidation of NO via the L-arginine/NOS pathway, as shown in Figure 1.2. Manipulation of these pathways to increase NO production is a focus of current research, specifically regarding the ageing process during which NO has been shown to be less available. There is an extensive body of literature surrounding the production of NO from nitrate but it will be discussed in brief here, paying attention to age-related changes that may affect the efficiency of the pathway.

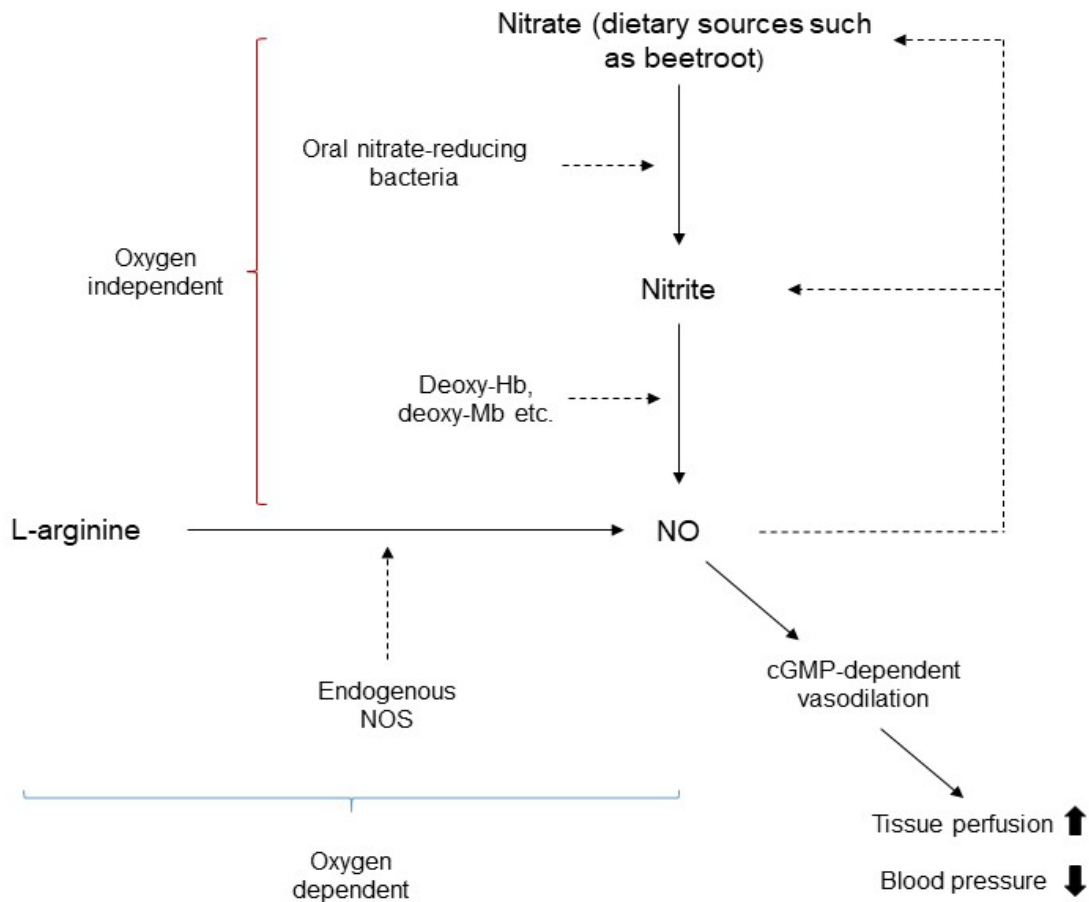


Figure 1.2: Schematic of the nitrate-nitrite-NO pathway. Abbreviations – NO: nitric oxide, NOS: nitric oxide synthase, deoxy-Hb: deoxyhaemoglobin, deoxy-Mb: deoxymyoglobin. Created using information from Weitzberg and Lundberg (2013), Kobayashi *et al.* (2015) and Shannon *et al.* (2017).

1.5.2 Salivary pathway

When consumed and once in the bloodstream, dietary nitrate mixes with endogenously produced nitrate (McKnight *et al.*, 1997), reaching a peak plasma concentration within 60 minutes following ingestion; it is then distributed throughout the body via the circulatory system. 75% of the nitrate is excreted in urine by the kidneys, unprocessed (Spiegelhalder *et al.*, 1976). It is also thought that some nitrate is excreted in sweat and faeces (Saul *et al.*, 1981; Weller *et al.*, 1996). The remaining 25% of the absorbed nitrate is extracted from the blood, transported to the salivary glands and into the oral cavity through the saliva (Duncan *et al.*, 1995; Spiegelhalder *et al.*, 1976). This can be seen in Figure 1.3.

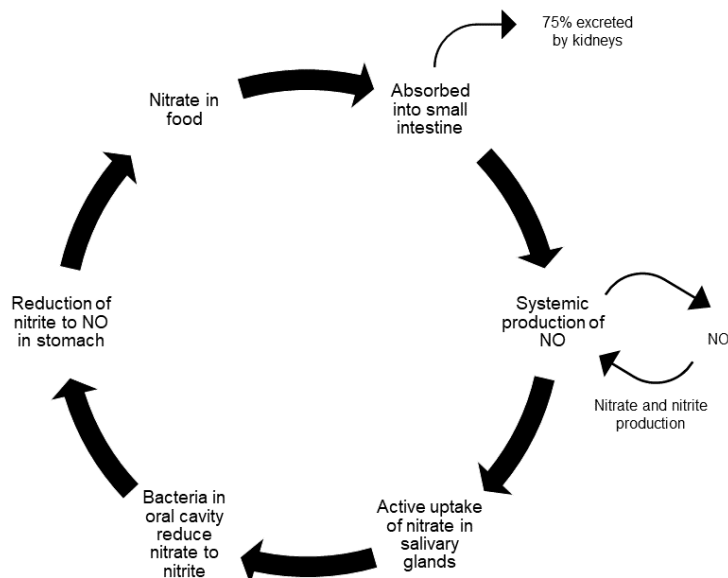


Figure 1.3: Schematic of the enterosalivary nitrate pathway. Adapted from Bondonno *et al.*, 2015.

The process of nitrate breakdown in the mouth has been widely studied and has been found to occur due to a symbiosis between mammals and bacteria designed to convert nitrate to nitrite (Duncan *et al.*, 1995). Microbial reduction of nitrate in the mouth was suggested as early as 1976, with experiments showing that saliva from the salivary glands contained nitrate but not nitrite (Spiegelhalter *et al.*, 1976). Dietary nitrate is broken down in the mouth by commensal bacteria with nitrate reductase activity, for example *Staphylococcus* species. Bacteria are necessary in this process because humans lack the functional enzyme, nitrate reductase, to reduce nitrate to nitrite (Bryan, 2015). The oral bacteria are located in the deep interpapillary clefts of the posterior surface of the tongue, which allow for a greater density of bacteria (Duncan *et al.*, 1997). When these bacteria use nitrate as an alternative electron acceptor in their energy production, nitrite is produced as a by-product (Spiegelhalter *et al.*, 1976).

Without the oral microflora, nitrate would be excreted from the body in an unmodified state, as demonstrated by many studies using antibacterial mouthwash. For example, Govoni *et al.* (2008) demonstrated that rinsing the mouth with antibacterial mouthwash prior to a nitrate load abolished the conversion of nitrate to nitrite in the saliva. This study found that the mouthwash had no effect on the accumulation of

nitrate in the saliva or plasma, indicating that commensal bacteria in the mouth are necessary for the conversion of nitrate to nitrite; antibacterial mouthwash thus stops the nitrate reductase activity of oral bacteria. In addition, avoidance of swallowing following a nitrate load was shown to stop the rise in plasma nitrite (Lundberg & Govoni, 2004). In this study, participants avoided swallowing for one hour after the nitrate load and when swallowing resumed, plasma nitrite levels increased. These studies not only elegantly demonstrate the importance of saliva in the production of plasma nitrite but also indicate that the contribution of other reductase enzymes, such as mammalian xanthine oxidoreductase (Zhang *et al.*, 1998), is very small in the breakdown of nitrate to nitrite.

1.5.3 Nitrite to NO

Upon swallowing, the nitrite produced from nitrate in the saliva enters the circulation (Larsen *et al.*, 2006; Webb *et al.*, 2008). When nitrite comes into contact with the acidic environment in the stomach, some is further converted to NO and the remainder is absorbed back into the bloodstream, raising circulating plasma nitrite levels (Webb *et al.*, 2008). In the stomach, NO has been found to increase the gastric mucosal blood flow and the thickness of mucus in rodents, thereby help to protect against pathogens (Björne *et al.*, 2004). Nitrite has a half-life of around 40-50 minutes (Dejam *et al.*, 2007) and is therefore considered a somewhat stable reserve of NO. Plasma nitrite levels have been found to reflect acute changes in eNOS and act as a reliable marker of NO availability (Lauer *et al.*, 2001). The reduction of nitrite to NO is particularly prevalent in hypoxic tissues, such as contracting skeletal muscle. Nitrite is reduced to NO by members of enzyme families that possess nitrite-reductase activity, for example deoxymyoglobin and NO synthases (Cosby *et al.*, 2003; Vanin *et al.*, 2007).

NO has a short half-life of less than one second (Kelm & Schrader, 1990) but has a number of actions in the body including, but not restricted to, glucose and calcium homeostasis (Stamler & Meissner, 2001), immune function (Bogdan, 2001), vasodilation (Moncada & Higgs, 1993) and neurotransmission (Bult *et al.*, 1990). Remaining nitrate, nitrite and NO from the processes detailed above diffuse to the general circulation and contribute to the NO pool (Lundberg *et al.*, 2006). Therefore,

the ingestion of dietary nitrate, most prominently through vegetable sources, can be viewed as a key modulator of the beneficial activities of NO.

1.5.4 NO formation and ageing

A number of symptoms known to be associated with the ageing process may attenuate the efficiency of the nitrate-nitrite-NO cycle. For example, older adults may suffer from a reduction in or absence of hydrochloric acid secretion (achlorhydia) in the stomach and this may influence the reduction of nitrite to NO in the gastric environment (Lundberg *et al.*, 1994). Furthermore, salivary breakdown of nitrate to nitrite may be influenced by other features of ageing such as a dry mouth, intestinal health and changes to the populations of oral bacteria. Percival, Challacombe and Marsh (1991) found that *Lactobacilli*, opportunist pathogens and *Staphylococci* in saliva increase with age and the prevalence of *Actinomyces* species shifted. *Staphylococci* species are nitrate-reducers, so preponderance in these bacteria in the mouth may work to increase salivary nitrate to nitrite breakdown. However, the work by Percival, Challacombe and Marsh (1991) shows an increase in both pathogenic and pathogenic-protective bacteria in the mouth such as *Lactobacilli*, making it difficult to draw conclusions as to the beneficial or negative effects of bacterial changes with age. Little work has been carried out on salivary bacterial changes with age since but this is an important avenue of research in the context of the nitrate-nitrite-NO pathway. Overall, changes in the environment that support NO production suggest that overall NO production from dietary nitrate will be reduced in older adults compared to a younger population.

Ageing may also be associated with reduced sensitivity to the effects of NO, specifically its dilatory properties. Early rodent studies found an impaired vasodilatory response of vascular smooth muscle in older animals and a reduced responsiveness to endothelium-dependent vasodilators, such as NO (Kung & Luscher, 1995; Winquist *et al.*, 1984). Additionally, as mentioned previously, levels of L-arginine and tetrahydrobiopterin, which is a co-factor for NO production, are reduced in older age, which will have a deleterious effect on overall endogenous NO production. Lyons *et al.* (1997) for example, directly compared 12 young and 12 older participants and postulated that the basal release of NO from the endothelium of resistance vessels in

the older group was lower than the younger group, which explains the blunted response to infusion of NG-monomethyl-L-arginine (L-NMMA), a NO synthase inhibitor.

A meta-analysis by Lara *et al.* (2016) reported that the effect of dietary nitrate on endothelial function was reduced with age. The authors found that improvements in endothelial function following nitrate supplementation were greater in those below the age of 60 years than those above 60 years. A study by Gilchrist *et al.* (2013) for example, demonstrated that, in adults aged 67 ± 5 years, plasma nitrate and nitrite levels increased in response to dietary nitrate supplementation but there was no effect on blood pressure or endothelial function. This suggests that although the nitrate-nitrite-NO salivary pathway may be functioning, basal NO is already low or the resistance vessels are not responding to an increase in NO from dietary nitrate breakdown. However, this study was carried out in patients with Type 2 diabetes and factors associated with the chronic disease may attenuate responses to nitrate loads. Due to a lack of research in more prolonged dietary nitrate supplementation, Lara *et al.* (2016) recommend that longer-term studies are carried out assessing dietary nitrate and endothelial function, especially in older adults and those with higher risk of CVD.

To this point, it is clear that the biology of ageing impacts on resistance vessels, contributing to hypertension and vascular dysfunction. Dietary nitrate has been repeatedly shown to be an effective means of increasing circulating NO levels in the body but it is unclear as to the mechanisms surrounding age-related differences in responses to or production of NO. It is possible that higher doses of dietary nitrate may elicit more favourable responses in an older population but nitrite toxicity demonstrates the need for caution, particularly with nitrate and nitrite use out with the natural context of vegetables i.e. in salt form. Dietary nitrate intake through vegetable consumption remains a safe and effective way to increase NO production in the body, at least in a younger population. What is less clear is the additive effect, if any, of prolonged intake of nitrate-rich vegetables, especially in older adults with a dampened response to an acute dose. Active nutrients in vegetables can have synergistic effects when consumed in the whole form and the increased fibre recommendations in the UK can be achieved more easily through the intake of whole

vegetables. Studies on extended intake of high-nitrate vegetables, consumed in their whole form, will be a valuable addition to the NO research, as they encompass not only the specific role of dietary nitrate but also the benefits of increasing vegetable consumption overall.

1.6 Lifestyle modification and disease

Lifestyle modification is often the first line of prevention of or treatment for many conditions. For example, Public Health England introduced salt reduction targets for convenience foods to reduce the salt content of the diet (PHE, 2017), in an effort to reduce the risk of high blood pressure and consequently stroke and CVD. Another lifestyle factor that can play a role in diseases such as these is a diet rich in fruit and vegetables. This dietary modification, along with a reduction in saturated fat, can substantially lower blood pressure (Appel *et al.*, 2006) and forms the basis of the “Dietary Approaches to Stop Hypertension” or DASH. A high fruit and vegetable diet is also associated with a reduced risk of CHD and ischaemic stroke (Bazzano *et al.*, 2002; Joshipura *et al.*, 1999). More specifically, Joshipura *et al.* (1999) concluded that cruciferous and green leafy vegetables, as well as citrus fruits and juice, were particularly beneficial in this context. Fruit and vegetables can also provide folate, of which low serum levels have been associated with hypertension (Robinson *et al.*, 1998; Forman *et al.*, 2005). Despite this, increasing habitual fruit and vegetable intake in the population has been an ongoing challenge and the five-a-day recommendations, which would supply beneficial nutrients, are not yet being met (Brown *et al.*, 2016).

The WHO recommends that each individual consume 400g of fruit and vegetables per day, which corresponds to five portions of 80g. Those aged 65-74 years eat the most fruit and vegetables but only 24% of men and 27% of women across the age groups consume the recommended five-a-day (Brown *et al.*, 2016). Consequently, the recommended 400g intake of fruit and vegetables is not being achieved and thus micronutrient intake is likely below an adequate level. Rink *et al.* (2013) suggest reduced levels of oxidative stress biomarkers and an increased antioxidant defence in those who meet the five-a-day recommendation, at least in some populations such as pre-menopausal women.

Fruit and vegetables contain an abundance of different vitamins, minerals and other nutrients, which can often work synergistically to have a greater effect together than on their own. Vitamin C, for example, which is found in large quantities in many fruit and vegetables, has been found to greatly enhance the generation of NO from nitrite (Modin *et al.*, 2001) and the absorption of iron from foods (Hallberg *et al.*, 1989). Furthermore, vitamin C is a potent antioxidant that can scavenge radicals by donating electrons (Padayatty *et al.*, 2003). Polyphenols, again through their antioxidant properties, also reduce the scavenging of NO and enhance nitrite reduction to NO (Weitzberg & Lundberg, 2013), thereby increasing the bioavailability of NO. Therefore, the combination of vitamin C, polyphenols and nitrate in food, for example, may contribute to a more potent effect than the nutrients in isolation.

With a reluctance in the general population to consume more fruit and vegetables overall, highlighting the benefits of a smaller number of select products may help consumers initiate the behavioural change towards increasing consumption. There has been a recent turn towards so-called 'functional foods' for their specific effects on health and disease. Functional foods are foods that deliver additional or enhanced benefits beyond the provision of essential nutrients (Hasler, 2002). Functional foods will have an active ingredient or various active components that can positively impact on health. There is good evidence for the application of functional foods in areas such as cardiovascular risk and cognitive function, where researchers have investigated pomegranate, berries and nuts, for example, and shown positive effects (Pribis and Shukitt-Hale, 2014; Asgary, Rastqar and Keshvari, 2018).

The benefits of nitrate have been discussed previously, in addition to the foods that are rich in this nutrient. High-nitrate foods such as beetroot, which is the focus of this thesis, may therefore be classed as functional foods because of their specific effect on vascular function. It has been suggested that a major benefit of a high vegetable intake to CVD comes from the nitrate content of these foods (Lundberg *et al.*, 2006). The Japanese diet, for example, delivers around 18.8mg/kg/day of nitrate, which is significantly higher than a Western diet and this population has one of the lowest incidences of hypertension and CVD in the world (Sobko *et al.*, 2010). A study by Ashworth *et al.* (2015) on healthy female volunteers compared the effects of one week of consumption of high nitrate vegetables to a control group where they were

avoided and found a reduction in blood pressure only in the high nitrate group. This study had a small sample size and is short in duration but highlights that it is not necessarily all vegetables *per se* that provide benefit to the cardiovascular system. It has been proposed that potassium plays a large role in the blood pressure-lowering effects of fruit and vegetables (Adroque and Madias, 2007), however further research has moved away from this theory (Webb *et al.*, 2008).

1.7 Nitrate intake from vegetables

Daily dietary nitrate intake is around 81-106mg (Coles & Clifton, 2012) with up to 80% of this coming from vegetables in a typical Western diet (Lundberg *et al.*, 2004). The rest, 35-44mg/day, comes from cured meats and water, as mentioned previously. The acceptable daily intake (ADI) for nitrate is set at 3.7mg/kg body weight/day, amounting to around 296mg for an average 80kg male. The ADI is an estimate of the maximum amount of a substance that can be ingested daily over a lifetime without presenting an appreciable health risk (EFSA, 2018). However, adherence to the DASH can lead to daily consumption of around 1222mg nitrate (Hord *et al.*, 2009). Furthermore, if consuming a Japanese diet, this would amount to 1504mg/day in an 80kg male, around five times higher than the ADI. A Japanese diet is rich in vegetables that have a high nitrate content and rank highly on the nitrate veg-table created by Lidder and Webb (2013), typically containing both spinach and Chinese cabbage. Vegetables with large green leaves, such as spinach, extract more nitrate from the soil than other vegetables in order to facilitate growth. The six highest-ranking vegetables are as follows in order of content: rocket, spinach, lettuce, radish, beetroot, and Chinese cabbage. The average nitrate content of these vegetables is around 124mg per UK portion of 80g. Of these high-nitrate vegetables, beetroot has become a focus of research because of its availability, low cost, and acceptability as a supplement.

1.8 Beetroot as a functional food

Beetroot is a member of the Chenopodiaceae family, which also includes spinach and chard. It is a widely consumed vegetable in traditional Western cooking (Morgado *et al.*, 2016). Red beetroot (*Beta vulgaris rubra*) has been used as food since 1000 B.C. with the Romans using the leaves for food and the roots for

medicinal purposes (Ninfali & Angelino, 2013). From here on, red beetroot will be referred to as beetroot.

1.8.1 Nitrate levels

Beetroot and other bulbous vegetables store nitrate in their roots in high concentrations. As mentioned previously, beetroot sits within the highest-ranking vegetables for nitrate content; per 100g of fresh weight, beetroot contains more than 250mg of nitrate (Hord *et al.*, 2009) (Table 1.1). However, the nitrate level in beetroot varies greatly during the season, with vegetables harvested in the summer containing substantially lower levels of nitrate; this is due to light reducing the nitrate accumulation (Umar & Iqbal, 2007). The nitrate content of the vegetable is also very dependent on the nitrate concentration of the soil in which it is grown and the presence or lack of nitrogen-based fertilisers (Gee & Ahluwalia, 2016). Although the nitrate content of beetroot can vary, average levels are still high and nitrate remains the most bioactive component of this vegetable. The nutritional profile of beetroot can be seen in Table 4.1.

In addition to nitrate however, beetroot does contain many other active compounds that may prove beneficial to health including phenolic acids, flavonoids, carotenoids, ascorbic acid, and betalains (Georgiev *et al.*, 2010; Wootton-Beard & Ryan, 2011). Phenolic acids and flavonoids are compounds found in many other vegetables but beetroot is quite unique in its betalain content, with only a few other plants containing this compound. Wootton-Beard *et al.* (2014) found that, excluding nitrate, the yellow/orange pigment neobetanin was the predominant secondary metabolite in beetroot juice.

1.8.2 Betalains

Betalains are water-soluble derivatives of betalamic acid (Esatbeyoglu *et al.*, 2015) and there are two subclasses: betacyanins and betaxanthines, which give beetroot its red or yellow colour respectively. Beetroot contains an average of 120mg of betalains per 100g of fresh weight (Lee *et al.*, 2014). Betalains are characteristic of species in the order Caryophyllales, with beetroot and prickly pear being the only edible food products containing these pigments (Kujala *et al.*, 2002). They

accumulate in the cell vacuoles of flowers, fruits and leaves, mainly in epidermal or sub-epidermal tissues and act as pollinator attractants or protective compounds. Betanin is a betacyanin, giving red beetroot its distinctive rich colour and it is therefore widely used as a food colourant (E162) in many dairy products such as milk, ice cream and yoghurt, juices, and sweets.

Betalains are similar to anthocyanins, which give foods such as red cabbage and blackberries their bright red/purple colouration. However, beetroot is one of the few edible and accessible plants to contain the potent betalains. Anthocyanins and betalains play exactly the same role in plants and every species of plant will contain one or the other of these compounds (Wootton-Beard *et al.*, 2014). The antioxidant effects of anthocyanins have been well described in the literature (Basu *et al.*, 2010; Fang, 2014) and it is not unjust to assume a similar effect of betalains if applied in the same conditions. The antioxidant activity of fruits and vegetables is measured on an ORAC scale and beetroot is ranked among the top 10 most potent vegetables with regards to antioxidant activity (Lee *et al.*, 2014). In beetroot, the content of flavonoids and phenolic acids is thought to be too low to account fully for the observed antioxidative effects. Therefore, it is proposed that the betalain, betanin, and its degradation product neobetanin has comparable effects to phenolic compounds (Wootton-Beard *et al.*, 2014). Betanin is a betanidin 5-O-beta-glucoside containing a phenolic and a cyclic amine group, both of which act as antioxidants by donating electrons (Kanner *et al.*, 2001). Both betaxanthins and betacyanins have a radical-scavenging capacity three to four times that of ascorbic acid, catechin and rutin in the DPPH assay (Cai *et al.*, 2003). Sawicki, Bączek and Wiczkowski (2016) demonstrated that the antioxidant capacity of beetroot was positively and significantly associated with betalain content. Oxidative damage to cells, proteins, lipids and DNA accumulates with age and contributes to the pathogenesis of age-related degenerative diseases such as CVD, cancer and osteoporosis (Scalbert *et al.*, 2005). Antioxidants found in foods are therefore widely studied, as they have the potential to limit oxidative damage by acting directly on ROS or by stimulating endogenous defence systems (Scalbert *et al.*, 2005).

Betalains have also been found to have anti-radical and anti-cancer activities (Zielińska-Przyjemska *et al.*, 2012; Esatbeyoglu *et al.*, 2015). They have been found

to protect LDLs from oxidation, even at low concentrations (Kanner *et al.*, 2001). Tesoriere *et al.* (2004) demonstrated that biomarkers of lipid oxidation, for example lipid hydroperoxide, were reduced by betalains. Betalains reach the plasma in relatively high concentrations but have a short half-life in the plasma and erythrocytes of around five hours (Wootton-Beard *et al.*, 2014). These researchers demonstrated a maximum plasma betanin concentration of 0.2 μ mol/L around three hours after a 16mg dose of betanin. Betalains can also be incorporated into red blood cells, protecting them from oxidative haemolysis (Sawicki *et al.*, 2016). Research suggests that phenolic compounds could be beneficial to endothelial function by stimulating the synthesis of NO (Heiss *et al.*, 2003; Schini-Kerth *et al.*, 2010). However, none of the nitrogen-containing glycosylated and phenolic compounds in beetroot, such as betacyanins, betaxanthins, and ferulic acid, have been found to increase NO synthesis (Kujala *et al.*, 2002).

It may be, despite the known specific actions of compounds such as betalains and nitrate that the whole complex of active components in beetroot work synergistically to protect cells and have other beneficial effects on the body. Indeed, Cao *et al.* (1998), for example, suggests that the perceived benefits of a high intake of fruit and vegetables on the risk of disease is not exclusively due to the action of single antioxidants, rather from the combination of various antioxidants present in foods. However, before conclusions can be drawn on the beneficial activities of compounds, their bioavailability within the human body must first be demonstrated.

1.8.3 Bioavailability of the active components in beetroot

The bioavailability of a compound is the proportion that is digested, absorbed and metabolised through normal pathways in the body (Pandey & Rizvi, 2009) and reaches the circulation unchanged. The efficacy of dietary compounds cannot be fully elucidated unless their bioavailability in the body has been determined. Ingested nitrate is quickly and effectively absorbed in the upper GI tract or small intestine with a bioavailability of almost 100% (Bescús *et al.*, 2011). This demonstrates that the nitrate content of beetroot can still be considered functional following ingestion. There is no effect of cooking on the bioavailability of nitrate from vegetables, as evident from comparable values following consumption of raw lettuce and cooked beetroot and spinach (van Velzen *et al.*, 2008).

The bioavailability of nitrate from beetroot is certainly high in young, healthy individuals, on whom bioavailability studies are most often carried out, but Siervo *et al.* (2015) found age differences in plasma nitrite concentrations following consumption of beetroot juice. Variability in plasma nitrite concentrations, as discussed in Chapter 1.5, can allude to either a reduction in dietary nitrate initially reaching the blood or an impaired breakdown of nitrate to nitrite in the saliva, despite high availability of nitrate in the circulation. These authors postulate that age differences could be reflected in, for example, oral microbiota, gastric redox environment and oxygen tension, and that higher doses of nitrate may be necessary in older individuals to make up for the differences in bioavailability. Conversely, Presley *et al.* (2011), in a study on cerebrovascular blood flow (CBF) following a high or low nitrate diet, found rises in plasma nitrite in older adults comparable to those found in younger adults.

It might be the case, however, that normal baseline levels of NO, produced endogenously from L-arginine, are reduced in older adults, regardless of a dietary nitrate load; this was discussed in Chapter 1.5. Indeed, Lyons *et al.* (1997) and Ashor *et al.* (2016) demonstrated a significantly lower baseline NO concentration in older adults compared to younger adults. These are the only two studies that directly compare the availability of and response to dietary nitrate in young and older participants in the same study. The pharmacokinetics of nitrate have not been specifically compared in younger and older adults, taking into account the many processes involved, such as salivary breakdown, urinary output and plasma concentrations. A full picture of this pharmacokinetic profile would aid research, especially surrounding age-related alterations in the pathways involved.

Betalains can be absorbed into the circulation in their unaltered form, allowing them to preserve their molecular structure and biological activity (Ting *et al.*, 2014). In 2001, Kanner, Harel and Granit identified the betacyanins betanin and isobetanin in the urine of healthy volunteers following the consumption of 300ml beetroot juice at levels of 0.5-0.9% of the ingested total, which signifies successful absorption of betalains in humans. A later study by Frank *et al.* (2005) also identified small amounts of betanin and isobetanin, around 0.3% of the total ingested, in the urine of

young, healthy volunteers following consumption of beetroot juice. These authors suggested that a reason for the small traces of betalains detected is that they have low availability or other major pathways of elimination may exist, such as metabolism. Both these studies investigated bioavailability solely through urinary output, which does not take into account the distribution of molecules to other tissues and may underestimate overall bioavailability (Rein *et al.*, 2013).

In a bioavailability study, Clifford *et al.* (2017) were unable to detect betanin in plasma following consumption of whole beetroot or beetroot juice in young, healthy males. Tesoriere *et al.* (2013) investigated absorption rates of betanin from both cactus pear and beetroot and found that epithelial transport in a model of the intestinal wall was slower with beetroot-derived betanin. This could explain the varying bioavailability results from different sources of betanin and suggests that betanin availability from beetroot is lower than from other sources. However, there also lies a difficulty in the lack of available standards for analysis of betanin, thus the true bioavailability of this compound is yet to be quantified.

The bioavailability of the total phenolic compounds within beetroot has been described by Netzel *et al.* (2005) but individual compounds have not been tested to date. Following consumption of 500ml of beetroot juice, Netzel *et al.* (2005) observed ~685mg of phenolic compounds in the urine of healthy females aged 23-24 years, 97% more than following water consumption. This demonstrates that beetroot is a bioavailable source of nitrate and phenolic compounds, at least in a young population. Further work is warranted on the bioavailability in an older population and with other forms of beetroot such as the whole vegetable.

1.9 Forms of beetroot and their use in research

Beetroot is currently widely used in nutrition and health research as a means to provide a source of dietary nitrate. It has been provided in a number of forms including juice, bread, gel and, most recently, powder. These processed products provide a concentrated source of bioactive compounds, some of which can be manipulated to form a nitrate-free “placebo” if nitrate is the primary compound under investigation. Nitrate has been found to be highly bioavailable following consumption in young participants but the pharmacokinetic profiles of nitrate following different

sources may vary, which can be important for the timing or frequency of consumption of certain beetroot products. van Velzen *et al.* (2008), for example, discovered varying plasma nitrate peaks and times to maximum following sodium nitrate, lettuce, spinach, and whole beetroot. Results do, however, appear to point towards a dose response rather than differences between sources, as different nitrate doses were provided with each source.

1.9.1 Beetroot juice

Beetroot juice is the most widely used source of dietary nitrate in research today. It is transportable, practical and has a nitrate-free alternative that can be used as a placebo drink. 70ml concentrated “shots” (Kelly *et al.*, 2013; Jajja *et al.*, 2014; Bondonno *et al.*, 2015) or 250-500ml of regular beetroot juice (Casey *et al.*, 2015; Kapil *et al.*, 2015; Kenjale *et al.*, 2011) are used as doses. Beetroot juice varies in its content from ~310 to 521mg nitrate per 100g (Morgado *et al.*, 2016) due to varying seasons of harvest and processing techniques, as mentioned previously. Furthermore, Jajja *et al.* (2014) found a discrepancy in reported levels on the bottle of beetroot juice shots, with their analysis showing the bottles to contain 165 ± 2 mg of nitrate compared to the 300-400mg written on the bottles. This is a common limitation of research on nutrients within vegetables and it is important that the concentrations of the nutrients of interest are tested regularly.

One major benefit of beetroot juice is that a nitrate-free placebo has recently been developed, allowing a true test of benefits specific to nitrate contained in the juice. These placebo drinks have been developed by manufacturers for research and are not available to the public. However, the effect of betalains, for example, cannot be tested in these placebos and instead requires other varieties of beetroot such as yellow beetroot to determine the role of specific red or yellow pigments. When considering the health benefits of fruit and vegetables, some of these come from their fibre content, which is lacking in juice. Whole food leads to higher satiety ratings due to the fibre content, volume of food, and increased chewing compared to a juice (Haber *et al.*, 1977). For applicability to more prolonged interventions, therefore, whole foods may improve compliance. Furthermore, the sugar content of fruit and vegetable juices is considered high and beetroot is often sweetened or combined with apple juice to improve palatability. This could have consequences on dental

caries and overall sugar consumption in populations if they were to consume sweetened beetroot juice regularly over the long-term.

1.9.2 Whole beetroot

As with the juice, a variety of nitrate contents have been reported in whole beetroot, such as 110-3670mg/kg (Siervo *et al.*, 2013) and 125mg/100g, ranging from 35 to 258mg (van de Avoort *et al.*, 2018). In its whole form, beetroot provides dietary fibre in addition to the bioactive components nitrate and betalains found in the vegetable. One research group examined the components of beetroot grown in organic vs. conventional conditions, demonstrating good levels of sugars, vitamin C, flavonoids, phenolic acids, and betanin in the whole vegetable (Kazimierczak *et al.*, 2014).

A bioavailability study by Clifford *et al.* (2017) demonstrated that both beetroot juice and whole beetroot raised markers of plasma NO concentrations in young, healthy participants, reaching a peak at two hours post-ingestion for both food products. NO levels remained elevated above baseline at eight hours post-ingestion with beetroot juice and five hours post-ingestion with whole beetroot. The reason for the disparity in pharmacokinetics is unclear but as the nitrate content of the whole beetroot was not tested, it could have been due to a smaller nitrate load provided in the whole food compared to the juice. Furthermore, van Velzen *et al.* (2008) demonstrated a 106% bioavailability of nitrate from 300g cooked beetroot that provided 636mg nitrate. Similar to the study by Clifford *et al.* (2017), participants were healthy and aged between 21 and 28 years. Both of these studies provided 300g whole beetroot in a sitting and compared it to a variety of other nitrate sources. Large doses such as this are commonplace in bioavailability studies but limit the applicability of the vegetable to everyday consumption. With an average vegetable portion of 80g, research into smaller and more practical doses are important in the area of public health and nutrition. The population-wide concern surrounding the lack of fruit and vegetable intake demonstrates the importance of research that focuses on whole fruit and vegetables in a real-world situation, even to simply highlight barriers to consumption of these whole foods in a community setting in order for appropriate recommendations to be made.

1.9.3 Other forms of beetroot – gel and enriched bread

Morgado *et al.* (2016) describe the benefits of beetroot gel as being a bio-accessible and convenient source of compounds, with positive acceptance; their beetroot gel contained 620mg of nitrate per 100g. Previously, James *et al.* (2015) found that a high-nitrate gel, made from chard or beetroot, exhibited an earlier plasma nitrate peak compared to beetroot juice suggesting that, although eliciting the same effects, different nitrate sources may affect the overall plasma pharmacokinetics of nitrate. These types of studies need to be replicated, especially with the creation of new beetroot products, and sources of nitrate must be matched for nitrate content to establish a real comparison. The longer-term acceptance of a beetroot gel is unclear and, similar to beetroot juice, concentrated gel contains a higher sugar content than less processed products. This makes the long-term applicability of beetroot gel limited.

A novel beetroot-enriched bread was developed and tested by Hobbs *et al.* in 2012, containing around 68mg nitrate per 100g. Both red and white beetroot have been incorporated into bread for research and red beetroot-enriched bread has been found to significantly increase total urinary nitrate and nitrite following ingestion (Hobbs *et al.*, 2012). This group also found that the beetroot bread reduced DBP and improved endothelium-independent vasodilation over six hours. This acute study was carried out on young males with a mean age of 25 years, so repeat studies are warranted in an older population and also over a more prolonged period if this beetroot bread is to be deemed a cardio-protective food product. Although this is a novel approach to incorporating more vegetables into the diet, it is important to take notice of the additional calories that will be consumed alongside the vegetable; calorie surplus can lead to obesity and increase the risk of a number of non-communicable diseases.

It is clear that beetroot has many beneficial effects on the human body and this is the case when it is used in a variety of forms. There is little research surrounding the benefits of betalains compared to the plethora of studies on the effects of nitrate, or specifically NO, but their poor availability hinders progress in this area. The beneficial effects of beetroot on markers of cardiovascular risk, such as blood pressure and vascular function, stem from the nitrate content of the vegetable. Because of the

effects of ageing on these markers, it is important that research focuses more on the ageing population than young and healthy cohorts, although this has not been the case to date. The following sections explore the application of beetroot in research on blood pressure, vascular function and cognition, and aim to highlight the benefits of targeting populations such as older adults in this area.

1.10 The effects of beetroot on blood pressure

NO concentration in the body is an important factor in the regulation and maintenance of blood pressure and blood pressure has been the focus of research on dietary nitrate to date. Following the discovery of the nitrate-nitrite-NO pathway, Larsen *et al.* (2006) first demonstrated the blood pressure-lowering effect of sodium nitrate, showing an acute reduction of DBP by 3.2mmHg after 6.2mg nitrate/kg body weight. This seminal research directed investigations into other sources of nitrate i.e. beetroot and led researchers to postulate that the blood pressure-lowering effects of the DASH, which can lead to a reduction in SBP by 2.3mmHg, are due to the nitrate content.

1.10.1 Blood pressure in young participants

Webb *et al.* (2008) demonstrated that the blood pressure-lowering effect of beetroot juice comes from the conversion of nitrate to nitrite, evident through the lack of blood pressure reduction associated with spitting after consumption of the nitrate load. This acute study was carried out in healthy volunteers aged between 18 and 45 years and supplemented with 500ml beetroot juice providing ~1395mg nitrate. Jonvik *et al.* (2016) later compared the effects of different sources of 800mg of nitrate, using sodium nitrate, beetroot juice, rocket salad juice and a spinach beverage. All beverages caused an acute rise in plasma nitrate and nitrite but sodium nitrate was the only intervention not to reduce SBP. DBP, however, was reduced following all interventions. The rise in plasma nitrate and nitrite and reduction in DBP following all interventions prompted the researchers to conclude, similar to Webb *et al.* (2008), that nitrate-rich vegetables could be used as dietary supplements and that they have the ability to lower blood pressure. Studies have repeatedly found a correlation between increased plasma nitrite levels and reduced blood pressure (Webb *et al.*, 2008; Siervo *et al.*, 2013 for review; Kapil *et al.*, 2015), which implicates nitrate-rich beetroot in this blood pressure-lowering effect.

Vanhatalo *et al.* (2010) found a reduction in blood pressure following beetroot juice compared to a nitrate-depleted placebo both acutely (2.5 hours) and over a more prolonged period (15 days) when participants consumed 322mg nitrate per day. The amount of nitrate in this study is small compared to the dose provided by Webb *et al.* (2008) and Jonvik *et al.* (2016) but the longer duration of the study necessitates this, as well as demonstrates that blood pressure-lowering effects can be maintained over a longer time period. Hobbs *et al.* (2012) also assessed the effects of smaller doses of beetroot juice on blood pressure and found a trend for blood pressure reduction with increasing beetroot juice doses from 100g to 250g and 500g, providing 143mg, 353mg and 707mg nitrate, respectively. 100g of beetroot juice did result in a significant reduction in blood pressure, indicating that smaller doses of nitrate can have positive effects on young and healthy participants. Hobbs *et al.* (2012) also utilised a novel method of dietary nitrate consumption with the incorporation of beetroot into bread, containing ~112mg nitrate. The maintenance of the blood pressure-lowering effect of beetroot after the processing into bread illustrates that nitrate's biological effects are preserved and again suggests that there is a dose response rather than an effect of source on the activity of nitrate.

Cheng, Cheng and George (2015) investigated whether the varying betalains in yellow and red beetroot had any influence on the blood pressure-lowering effect of beetroot using 24-hour ambulatory blood pressure measurement. There was no significant difference between colour variants on SBP and DBP but both reduced blood pressure overall. This suggests that the different betalains in beetroot make no difference to the blood-pressure lowering effect of the vegetable. Hobbs *et al.* (2012) reiterate this finding by comparing red and white beetroot in their bread products and finding that both lowered blood pressure to a similar extent in young, healthy males.

It is clear that beetroot, due to its nitrate content, has a positive effect on blood pressure in young and healthy participants. The effect of dietary nitrate on plasma nitrite levels is well known in these young participants and the pharmacokinetics of the nitrate has been sufficiently described. In 2013, Siervo *et al.* conducted a systematic review on nitrate and blood pressure and concluded that there was a greater effect of nitrate on SBP but an over-representation of young, healthy men in

the studies. It is important, therefore, to direct research into populations who would benefit most from a reduction in blood pressure i.e. older adults and those with cardiovascular risk factors and chronic disease.

1.10.2 Beetroot on blood pressure in older or diseased populations

From the previous sections in this Chapter it is evident that the ageing process and low-grade inflammation, which is a characteristic of disease, may affect the breakdown of dietary nitrate and the production and response to NO. Despite this, it has been repeatedly demonstrated that dietary nitrate can increase plasma nitrite levels in older adults and those with chronic disease (Table 1.2). Compared to young, healthy adults however, plasma nitrite levels do not always translate to a reduction in blood pressure in older or diseased participants following supplementation with beetroot juice (Miller *et al.*, 2012; Bondonno *et al.*, 2015; Shepherd *et al.*, 2015; de Oliveira *et al.*, 2016). Results on blood pressure in these populations are equivocal, with some research demonstrating a reduction in solely DBP (Kenjale *et al.*, 2011), solely SBP (Eggebeen *et al.*, 2016; Jajja *et al.*, 2014) or both (Kelly *et al.*, 2013). Although there are some positive results following the consumption of dietary nitrate, there is no consensus on the effects, dose or target population.

Kapil *et al.* (2015) found a reduction in both clinic and home blood pressure after four weeks of 250ml of beetroot juice daily in hypertensive adults aged 18-85 years; the dose provided was approximately 397mg daily. Ashworth *et al.* (2015) also found that both raw beetroot juice (250ml) and cooked beetroot (250g) consumed daily for two weeks reduced SBP and DBP in hypertensive participants aged between 25 and 68 years. Nitrate and nitrite levels were not measured in the beetroot or the plasma, so the dose and association with increased NO levels is not clear in this study. A recent study by Blekkenhorst *et al.* (2018) however, found no blood pressure-lowering effects of a four-week high nitrate (~150mg/day) diet in hypertensive adults between the ages of 21 and 75 years. Given that the daily intake of nitrate if people reached the WHO recommended fruit and vegetable intake would be 157mg, 150mg may not be classed as a high nitrate dose in this context, although it is higher than the average intake of 108mg/day (Babateen *et al.*, 2018). Each of these studies had a cohort with a wide range of ages from young adults to elderly. It is therefore unclear

whether there were different effects on those classed as younger or older and if this may explain the presence or lack of a blood pressure-lowering effect.

Table 1.2 also illustrates that beetroot juice is the primary source of dietary nitrate used in research on older adults. The dose varies considerably from 165mg to 1159mg nitrate given acutely or daily up to six weeks and on one occasion the dose provided was lower than described on the product (Jajja *et al.*, 2014). Only four studies to date have supplemented with beetroot juice for longer than seven days, which warrants more focus on a prolonged intervention period, especially on older adults and those at more risk of cardiovascular events. It is unclear whether the acute effects of dietary nitrate, such as blood pressure reduction, are maintained longer term. The effects of oxidative stress and low-grade inflammation on the vascular system into older age open up research into more prolonged intervention with dietary nitrate in an attempt to ameliorate the effects and promote NO production.

Table 1.2: Summary of RCTs assessing the effect of dietary nitrate on a range of biological outcomes in older adults.

Authors	Cohort	Dosage	Duration	Measurements	Study design	Conclusions
<i>Kenjale et al., (2011)</i>	8 adults aged 67±13 with PAD	500ml beetroot juice providing 1116mg nitrate	Single bolus	Plasma nitrate and nitrite, BP, endothelial function	Randomised, open-label, crossover trial	Increase in plasma nitrate and nitrite levels. Reduction in DBP. No effect on endothelial function.
<i>Presley et al., (2011)</i>	5 adults aged 74.7+6.9	High (242mg) and low nitrate diet. High supplemented with 500ml beetroot juice containing 527mg nitrate	4-day diet. 3 hour intervention period	Plasma nitrate and nitrite	2-arm, randomised, crossover trial	Significant increases in plasma nitrate and nitrite levels
<i>Presley et al., (2011)</i>	14 adults aged 74.7+6.9	High (242mg) and low nitrate diet. High supplemented with 500ml beetroot juice containing 527mg nitrate	2-day diet. 3 hour intervention period	Cerebral blood perfusion	2-arm, randomised, crossover trial	Increased CBF within subcortical and deep white matter of the front lobe, but no global increase in CBF
<i>Miller et al., (2012)</i>	8 adults aged 72.5±4.7	500ml (527mg nitrate) beetroot juice . 43mg nitrate in control diet and 155mg in HN diet. HN, HN + juice, control diet and control diet + juice	3-day control diet vs. HN. 3 hour intervention period	Likert scale for acceptance to beetroot, blood pressure, plasma nitrate and nitrite	Randomized four period cross-over controlled design	High nitrate supplement elevated plasma nitrate and nitrite levels throughout day, but high nitrate diet did not. No effect on BP.
<i>Kelly et al., (2013)</i>	12 healthy adults aged 64±2.7	2 x 70ml/day of beetroot juice (595mg of nitrate per day)	3 days	Resting BP and plasma nitrite concentration. 6-minute walk test, brain metabolite concentrations, cognitive tests	Double-blind, randomized, crossover study	Increase in plasma nitrite. SBP and DBP reduced, no improvement in 6MWT, brain metabolite concentrations or

<i>Gilchrist et al., (2014)</i>	27 adults aged 67.2 with Type 2 DM , mean BMI of 30.8	250ml beetroot juice (465mg nitrate) or placebo drink	14 days, 250ml drink per day	Plasma nitrate and nitrite levels. Cognitive function	Double-blind, randomised, placebo-controlled crossover trial	cognitive performance Increase in plasma nitrate and nitrite. Reduction in simple reaction time
<i>Jajja et al., (2014)</i>	21 adults aged 62±1.4 , mean BMI of 30.1	70ml beetroot juice per day (165mg nitrate) or blackcurrant placebo	21 days with 7 day interruption and then continuation	Blood pressure – clinic, daily and 24-hour ABPM. Salivary and urinary nitrate.	2-arm, parallel, randomized	Increase in salivary and urinary nitrate. No changes in resting clinic BP or 24-hr ABPM. Reduced daily SBP after 3 weeks but effect not maintained after interruption
<i>Rammos et al., (2014)</i>	21 adults aged 63±5 with moderate CV risk	Sodium nitrate , 150µmol/kg BW (equivalent to 300g spinach)	4 weeks	Endothelial function – FMD, vascular stiffness – PWV and Alx. Plasma nitrate and nitrite	Randomised, placebo-controlled, double-blind, parallel trial	Improved PWV and Alx compared to control. Lowered SBP but not DBP. Increased plasma nitrate and nitrite levels.
<i>Bondonno et al., (2015)</i>	27 adults aged 63.3±4.4 on anti-hypertensive medication	2 x 70ml beetroot juice per day containing 434mg nitrate	7 days	Blood pressure, plasma, salivary and urinary nitrate	Randomized, placebo-controlled, double-blind, crossover trial	Increase in plasma, salivary and urinary nitrate. No difference in BP
<i>Casey et al., (2015)</i>	12 healthy adults aged 64±2	500ml beetroot juice providing 1159mg nitrate	Single bolus 3 hours prior to exercise	Rhythmic forearm exercise in normoxia and hypoxia. BP and forearm blood flow	Placebo-controlled, crossover trial	Increased compensatory vasodilator response to hypoxic exercise
<i>Justice et al., (2015)</i>	22 old mice	Sodium nitrite supplemented water (50mg/l)	8 weeks	Motor behaviour, forelimb grip strength, open field locomotor activity, accelerating and endurance run	Randomised, parallel, rodent trial	Improved grip strength and open field distance. Reduced low-grade inflammation in

<i>Justice et al., (2015)</i>	30 healthy adults aged 62±7	Sodium nitrite , either 80mg or 160mg capsules/day	10 weeks	ability Motor function. Cognitive function (speed and executive function), plasma nitrate and nitrite.	Randomised, placebo-controlled, double-blind	muscle Small chronic increase in plasma nitrite. Improved maximal exercise capacity with 80mg. Reduced balance errors step test. Improved cognitive performance
<i>Kapil et al., (2015)</i>	64 hypertensive adults aged 57.3±13.9	250ml beetroot juice daily providing 397mg nitrate	4 weeks	BP, vascular function, plasma, salivary and urinary nitrate	Double-blind, randomised, placebo-controlled trial	Increased nitrate. Reduction in SBP, DBP, A1x and PWV. No change in PORH
<i>Shepherd et al., (2015)</i>	14 adults aged 64.7±7.7 with mild-moderate COPD	2 x 70ml beetroot juice (420mg nitrate) or nitrate-depleted beetroot juice	2 days before testing and once on day of testing	Brachial artery BP, 6-minute walk test	Double-blind, placebo-controlled, cross-over design	Rise in plasma nitrate concentration. No improvement in BP or 6MWT
<i>de Oliveira et al., (2016)</i>	20 adults aged 70.5±5.6 with cardiovascular risk factors	100g beetroot gel providing 756mg nitrate	Single bolus	Endothelial function, arterial stiffness and BP. Urinary nitrate.	Randomised, double-blind, crossover and placebo-controlled	Increase in FMD and blood flow velocity. Increased in urinary nitrite and nitrate. RH increased. No effect on BP.
<i>DeVan et al., (2016)</i>	32 healthy adults aged 62±1	Sodium nitrite capsules (80mg or 160mg) daily	10 weeks	PWV, arterial stiffness, plasma concentration, FMD	Double-blind, placebo-controlled, randomized parallel-design	Plasma nitrite increased acutely and chronically. Endothelial function increased, arterial elasticity improved. Particularly with smaller dose
<i>Eggebeen et al., (2016)</i>	20 adults aged 69±7 with heart failure with preserved ejection fraction	70ml beetroot juice , providing 378mg nitrate	Single bolus or daily intake for 7 days	Plasma nitrate and nitrite, BP	Randomised, placebo-controlled,	Increased plasma nitrate and nitrite and reduced SBP both

					crossover trial	acutely and following 7-day supplementation.
<i>Hughes et al., (2016)</i>	12 healthy adults aged 64±5	500ml beetroot juice providing 583mg nitrate	Single bolus	Plasma nitrate and nitrite, aortic BP and Aix	Randomised, parallel design comparing young and old participants	Increased plasma nitrite. Reduction in aortic BP in both young and old but Aix reduced only in young
<i>Velmurugan et al., (2016)</i>	67 adults aged 53.3±10.1 with hypercholesterolemia	250ml beetroot juice daily, providing 372mg	6 weeks	Vascular and platelet function by FMD. Plasma, urine and saliva sampling. Oral microbiome profile.	Randomised, double-blind, placebo-controlled parallel study	Improvement in vascular function. Associated with alterations in oral microbiome and nitrate-reducing genera
<i>Raubenheimer et al., (2017)</i>	12 adults aged 64	140ml beetroot juice providing 800mg nitrate	Single bolus	BP, plasma nitrate and nitrite, vascular inflammation	Randomised, placebo-controlled, crossover trial	Increase in plasma nitrate and nitrite. Reduction in BP, and granulocyte and platelet activation
<i>Oggioni et al., (2018)</i>	19 healthy adults aged 64.7±3.0	2x70ml beetroot juice /day, providing 744mg	7 days	BP, vascular function, inflammatory markers	Double-blind, randomised, crossover placebo-controlled trial	No difference in BP or Aix. No changes in inflammatory markers.

Abbreviations: RCTs = randomised controlled trials, PAD = peripheral arterial disease, BP = blood pressure, DBP = diastolic blood pressure, SBP = systolic blood pressure, CBF = cerebral blood flow, HN = high nitrate, VO₂ = maximum rate of oxygen consumption, 6MWT = 6-minute walking test, DM = diabetes mellitus, BMI = body mass index, ABPM = ambulatory blood pressure monitoring, CV = cardiovascular, BW = body weight, FMD = flow-mediated dilatation, PWV = pulse wave velocity, Aix = augmentation index, PORH = post-occlusive reactive hyperaemia, O₂ = oxygen, RH = reactive hyperaemia.

1.11 The effects of beetroot on vascular function

Enhanced NO production from the consumption of beetroot i.e. dietary nitrate to offset the reduction in endogenous production seen with vascular dysfunction could have a profound effect on vascular function. However, other components of beetroot, namely flavonoids, may also contribute to this benefit. Indeed, flavonoids have been associated with a reduction in vascular oxidative stress due to their interaction with ROS (Torel *et al.*, 1986) and supplementation has been found to have positive effects on vascular tone, NO-mediated endothelium-dependent dilation, and NO formation (review by Schini-Kerth *et al.*, 2010). Overall, enhanced NO bioavailability and reduced oxidative stress in the vascular system may work to reverse macro- and micro-vascular endothelial dysfunction.

Four weeks of supplementation with sodium nitrate has been found to reduce PWV and AIx and improve FMD (Rammos *et al.*, 2014) but importantly FMD is also improved after supplementation with beetroot gel and beetroot juice (Asgary *et al.*, 2016; Kapil *et al.*, 2015; de Oliveira *et al.*, 2016; Velmurugan *et al.*, 2016). These five studies were all carried out in adults with hypertension or other cardiovascular risk factors, which may explain the consistent beneficial results. In contrast to these studies, Gilchrist *et al.* (2013) found no improvements in FMD or microvascular endothelial function after two weeks of 250ml beetroot juice daily (465mg nitrate) in older adults with Type 2 diabetes. Plasma nitrate and nitrite concentrations did increase after the two weeks in the beetroot group compared to the placebo but this did not correlate with endothelial function. This could be attributed to a reduced responsiveness to NO and thus a diminished vascular reactivity with age and Type 2 diabetes.

de Oliveira *et al.* (2016) describe that high nitrate doses i.e. ~744mg are required to obtain positive effects in endothelial function in the elderly; however smaller doses between 350 and 400mg have been found to elicit improvements in older adults (Kapil *et al.*, 2015; Velmurugan *et al.*, 2016). Similar to findings in blood pressure, there is no consensus on the dose of dietary nitrate required to elicit a response, although there appears to be a more positive effect of a range of doses on vascular function overall. FMD, PWV, AIx, and RH can assess functioning of the vascular

function and dietary nitrate has varying effects on these markers, making it difficult to draw conclusions on the effectiveness of beetroot overall.

1.12 The effects of beetroot on cognitive function

Researchers have demonstrated improvements in cognitive performance after a dose of dietary nitrate in young participants (Wightman *et al.*, 2015). In this study, participants received 450ml beetroot juice containing ~341mg nitrate before completing cognitive tasks and CBF analysis. There was an increase in plasma nitrite and an initial rise in CBF following nitrate consumption, which were accompanied by an improvement in performance on the serial 3s subtraction task. In a study on older diabetic participants, 14 days of daily supplementation with 250ml beetroot juice providing 465mg was found to increase plasma nitrate and nitrite concentrations and improve simple reaction time (Gilchrist *et al.*, 2014). Conversely, Kelly *et al.* (2013) found no improvement in cognitive function following three days of supplementation with 140ml concentrated beetroot juice (~595mg nitrate) in older adults, despite rises in plasma nitrite concentrations and the provision of a larger dose compared to Wightman *et al.* (2015) and Gilchrist *et al.* (2014). Diffusion coefficients in the frontal lobes, markers of CBF, were also unchanged. This work contrasts with the study by Presley *et al.* (2011), who found that two days of a high nitrate diet (containing 500ml beetroot juice) increased CBF within the frontal lobe of older adults. The nitrate content of this diet was found to be 527mg, similar to that supplied by Kelly *et al.* (2013), so it is unclear whether ageing or baseline cognitive functioning may play a role here.

Thus far, only one study has investigated more prolonged effects of supplementation on cognitive function. Justice *et al.* (2015) demonstrated that 10 weeks of supplementation with sodium nitrite improved cognitive performance in middle-aged and older adults, as indicated by reductions in time to complete TMT. These authors investigated two doses of sodium nitrite: low (80mg/day) and high (160mg/day). There was a 14% and 18% improvement in task performance following the low and high nitrate dose, respectively. As mentioned previously, more prolonged studies may warrant a more accessible form of nitrate i.e. beetroot in order to ensure compliance. A recent systematic review and meta-analysis on nitrate and cognitive function outlined a lack of overall effect but stipulate that there is insufficient evidence

to make firm conclusions on this (Clifford *et al.*, 2018). These authors postulate that larger sample sizes and longer duration trials are warranted in this area.

Although much of the beetroot research focuses on nitrate, other compounds such as the potent antioxidants betalains and phenolics may elicit benefits. Betalains are very similar to anthocyanins in their structure and function but anthocyanins are much more widely researched due to their preponderance in fruits and vegetables. Anthocyanin-rich cherry juice, for example, has been found to improve verbal fluency and short- and long-term memory in older adults with mild-to-moderate dementia when supplemented for 12 weeks (Kent *et al.*, 2017). Previous short-term supplementation with cherry juice was found to have no effect on cognitive performance in young, old or older adults with dementia (Caldwell *et al.*, 2016). Prolonged supplementation (12 weeks) with anthocyanin-rich blueberry juice was also found to improve memory in older adults with early memory decline, tested by word recall and paired associate learning (Krikorian *et al.*, 2010). The focus on prolonged intervention in these studies suggests that the nutritional compounds may be having their effect through a reduction in oxidative stress, which is not achieved through acute dosing. The combination of nitrate, which can have an acute effect on vascular function, and antioxidant compounds such as betalains that may work over a more prolonged period suggest that beetroot may improve blood flow to the brain and attenuate cognitive decline.

1.13 The effects of beetroot on functional capacity

Executive functioning, such as task switching and working memory, is directly related to motor function and performance-based ADLs. For example, in healthy older adults, lower levels of executive function are associated with a reduction in gait performance (LaRoche *et al.*, 2014). With nitrate supplementation improving blood perfusion to parts of the brain involved in executive functioning i.e. the frontal lobes, it is possible that nitrate supplementation could impact on functional capacity.

Shepherd *et al.* (2015), while assessing the effect of nitrate supplementation on blood pressure and walking performance, measured functional capacity in COPD patients before and after supplementation using the 6MWT. 70ml of concentrated beetroot juice did not improve performance in the 6MWT after 2.5 days of

supplementation and this is postulated to be due to the increased oxidative stress leading to uncoupling of NO synthase and consequently reducing NO availability. Similarly, 140ml concentrated beetroot juice supplementation over three days did not improve 6MWT performance in healthy older adults (Kelly *et al.*, 2013). A study by Siervo *et al.* (2016) found modest but non-significant improvements in measures of physical capability including hand-grip strength, timed up-and-go, repeated chair standing and 10-minute walking test following seven days of beetroot juice supplementation in older adults.

If a lack of effect of beetroot juice on functional capacity in older adults is due to heightened oxidative stress then the short intervention periods in these trials are likely to be a contributing factor. As mentioned previously, Justice *et al.* (2015) investigated the effects of 80mg and 160mg sodium nitrite in middle-aged and older adults and found a reduction in the number of balance errors in the rapid step test. TUG was not affected but there was a trend for improvements in grip strength following the smaller dose of sodium nitrite. This research follows on from a rodent study by the same authors, who found that eight-week supplementation with sodium nitrite in old and young mice improved grip strength and endurance run distance in old mice. This is attributed in part to anti-inflammatory effects on aged skeletal muscle with chronic low-grade inflammation (Justice *et al.*, 2015). It is evident, therefore, that more prolonged intervention studies with beetroot are justified in order to elicit benefits to functional capacity through a reduction in oxidative stress and inflammation. This is particularly relevant to older adults and it is likely that this population will benefit most from improvements in functional capacity.

1.14 The effects of beetroot on gut health

Dietary fibre plays a large role in the digestive system, helping the body to remove fat and creating an environment for healthy gut bacteria to thrive and digest food efficiently, contributing to the overall health of the gut microbiota. Any vegetable with 3g of fibre per 100g is classed as a source of fibre, according to the European Commission, and beetroot falls close to this at 2.8g fibre/100g. The addition of fibrous foods to the diet over a prolonged period may benefit the functioning of the gut and the microbiota that populate it.

Research has illustrated an association between microbiota and cognition in ageing (Leung & Thuret, 2015), being termed the “microbiota-gut-brain” axis. In particular, the production of SCFAs is of interest due to their influence on the blood-brain barrier and the health of the colon (Fava *et al.*, 2018; Natarajan & Pluznick, 2014). In the brain, SCFAs have a neuroprotective effect and butyrate specifically has anti-inflammatory actions in the brain and the gut (Noble *et al.*, 2017). As mentioned previously, SCFAs are produced from fermentation of dietary carbohydrate by anaerobes and the levels of various SCFAs are affected by ageing. It has also been shown that different polyphenols affect the viability of different gut bacterial species, indicating the potential for modulation of the gut microbiota balance through bioactive compounds present in fruit and vegetables (Parkar *et al.*, 2013).

Furthermore, nitrite has been posed as a protective compound against gut pathogens in humans, as it can eradicate *Helicobacter pylori* and other pathogenic bacteria (Duncan *et al.*, 1997), indicating a positive impact on gut microbiota. The nitrite in saliva, which is present due to the bacterial breakdown of nitrate in the oral cavity, has been found to increase gastric mucosal blood flow and mucus thickness in rats, through an increase in NO (Björne *et al.*, 2004). This is then thought to have gastro-protective effects. More recently, Velmurugan *et al.* (2016) showed that persistent dietary nitrate ingestion over six weeks raised numbers of oral bacteria possessing nitrate reduction capacity. Further studies in this area are warranted to establish whether changes in oral bacteria following nitrate ingestion may improve nitrate breakdown long term.

Thus, an increase in nitrite formation from ingested dietary nitrate may have beneficial effects on gut health, helping to control pathogenic bacteria. Furthermore, dietary carbohydrates found in beetroot may help to stimulate SCFA production, leading to anti-inflammatory effects. This may also relate to increased consumption of the potent antioxidants betalains present in beetroot. Changes to the gut microbiota and increases in fibre intake from vegetables may also influence gut motility changes that occur with ageing. No studies, however, have directly investigated the effects of whole beetroot on gut health and the microbiota but this may be an interesting avenue of research.

From the literature, it is clear that there is a large body of research surrounding NO, its effects on the body, and methods in which its production can be manipulated through the diet or with supplementation. There is no clear consensus on the dose of dietary nitrate required to elicit a response or the duration of intervention required, especially regarding vascular function. Age effects are seen in the production of NO and/or the body's sensitivity to it in some but not all studies, leaving age-related differences inconclusive. Where evidence is lacking is in longer-duration trials, particularly on cognitive function, functional capacity and gut health. It is unknown whether dietary nitrate effects are seen only acutely, or whether there is an additional benefit with a more prolonged intervention period. Furthermore, few studies are applicable to real life situations, in which people consume a variety of foods, with little restriction. The diet of an ageing population is becoming increasingly important and there should be more focus on compounds, nutrients and whole food groups that can make a real difference in this area.

1.15 Research questions

Hypotheses, aims and objectives

The hypotheses for this project were:

1. The bioavailability of nitrate from whole beetroot will be less in older adults than in younger adults. There will be a trend towards a dose-response to incremental portions, with higher amounts of beetroot leading to an increased bioavailability of nitrate in both populations.
2. The positive effect on vascular function associated with nitrate will be reduced in the older population. The response to the potassium nitrate will be higher than that of beetroot, eliciting greater reductions in blood pressure and improved blood flow. The vascular response to the beetroot will be greater with higher quantities in both populations.
3. More prolonged supplementation with beetroot will affect cognitive function in a group more at risk of cognitive impairment i.e. an ageing population. Prolonged supplementation may also affect functional capacity in an older population.

4. Prolonged beetroot supplementation will cause changes to the gut microbiota and have a positive effect on SCFA production and bacterial diversity.

The aims of this thesis were to test the above hypotheses by investigating the bioavailability of nitrate and its physiological effects following incremental whole beetroot doses in young and old adults. Furthermore, this thesis aimed to investigate the longer-term effects of whole beetroot consumption on cognitive function, functional capacity and gut health in older population.

The objectives of the study were:

1. To carry out a crossover trial using incremental portions of whole beetroot in addition to a positive control involving both young and old participants. This trial will investigate the pharmacokinetics of nitrate in both groups and the vascular effects of the doses.
2. To evaluate the acceptability and tolerance of beetroot portions to be used in a prolonged intervention study.
3. To carry out a prolonged free-living intervention trial using whole beetroot in older adults to test whether beetroot supplementation improves cognitive performance and functional capacity and affects the gut microbiome.

Chapter 2. The pharmacokinetics of nitrate and nitrite following incremental beetroot doses in young and old participants

2.1 Introduction

Fruit and vegetable intake is linked to protection against cardiovascular disease (CVD) and some cancers, and has been found to effectively lower blood pressure (Appel *et al.*, 2006). It has been postulated that dietary nitrate, found primarily in green leafy vegetables and beetroot, underpins this blood pressure-reducing effect (Larsen *et al.*, 2006). In light of this, research is focussing on nitrate-rich vegetables such as beetroot and the dietary nitrate pathway as a natural anti-hypertensive. Dietary nitrate, when sequentially broken down to nitrite and further to nitric oxide (NO) positively impacts the vascular system through the maintenance of blood pressure, vascular tone and endothelial integrity (Ignarro, 2002). Following ingestion, dietary nitrate mixes with endogenously produced nitrate and levels in plasma peak after around 60 minutes (McKnight *et al.*, 1997). Much of this nitrate is excreted through the urine but 25% of the absorbed nitrate is transported to the salivary glands where commensal bacteria begin the conversion of nitrate to nitrite. Nitrite is then reduced to NO by enzymes such as NO synthase and deoxymyoglobin (Shiva, 2013).

Nitrate has been found to be almost 100% bioavailable following the consumption of beetroot juice (Bescús *et al.*, 2011) and whole beetroot (van Velzen *et al.*, 2008) in young, healthy individuals. Bioavailability studies are required to accurately map the pharmacokinetic profile of nutrients following consumption to identify excretory pathways, bioconversion and the time course of physiological effects, and these studies are frequently carried out in young populations (Clifford *et al.*, 2017; van Velzen *et al.*, 2008). The ageing process has been found to have an impact on NO availability through lower levels of the precursor and co-factors involved in endogenous NO production (Delp *et al.*, 2008). The pharmacokinetic profile of dietary nitrate and the bioconversion to nitrite by bacterial species may therefore be altered in an older population, affecting ultimately the availability of NO and its downstream vasodilatory properties. There is a lack of pharmacokinetic data in older adults, particularly when directly compared to a younger population following a dose of

dietary nitrate. Furthermore, large, often impractical doses of beetroot are provided in such bioavailability studies, which make the applicability of the vegetable in a real world setting problematic. Data on the pharmacokinetic profile of smaller doses of beetroot, especially in older adults, would aid research into achievable and practical vegetable intakes and their impact on health parameters such as blood pressure.

Therefore, the aim of this study is assess if whole beetroot effectively raises NO levels in both young and old participants through the stepwise breakdown of nitrate to nitrite and finally to NO. To better understand the pharmacokinetic profile of nitrate and nitrite, this study will measure changes in plasma and salivary nitrate and nitrite levels over five hours, as well as urinary excretion in both the young and the old participants following incremental doses of whole cooked beetroot. It is hypothesised that the bioavailability of nitrate from whole beetroot would be less in older adults than in younger adults and that there would be a trend towards a dose-response to incremental portions i.e. higher amounts of beetroot would show an increased bioavailability of nitrate in both populations.

2.2 Methods

2.2.1 Participants

This study involved two age groups: 18-35 years and 60-75 years. Participants were recruited through the Newcastle university email system, poster dispersion and NHS intranet from November 2016 to October 2017 and provided informed written consent before beginning the study. Recruited participants had a body mass index (BMI) between 20.0 and 29.9kg/m² and were non-smoking, healthy and both male and female. The young group comprised six males and six females and the old group comprised of three males and nine females. The study was registered with the ISRCTN registry (ISRCTN86706442) and was approved by the Cambridge Research Ethical Committee (Appendix A). The sample size calculation was based on preliminary data from a previous, similar trial testing the acute effects of inorganic nitrate supplementation compared to placebo in young and older healthy individuals. The calculation can be seen in Appendix C.

2.2.2 Exclusion criteria

Healthy participants i.e. those without chronic illness and disease were recruited to take part in this study. Excluded medications included: corticosteroids, sildenafil, aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), anti-hypertensives (Ca²⁺ channel blockers, ACE inhibitors), diuretics, beta-blockers, antacids, anticoagulants, nitrate-derived agents, and anti-cholinergics. Each of these medications can have an effect on NO production. Participants were excluded if they were vegetarian, as they are likely to have a higher habitual nitrate intake, or self-reported to consume excessive volumes of alcohol (>30 units per week). Other exclusion criteria included a high blood pressure (>140/90mmHg), smoker, history of repetitive gastric reflux and an intolerance or allergy to beetroot.

2.2.3 Study design and randomisation

This study was a four-arm, randomised, crossover clinical trial. Randomisation was generated online using Research Randomizer, where 30 sets of numbers from one to four that represented the interventions were generated; participants were assigned to a set following screening. Participants were given 100g, 200g, and 300g of whole cooked beetroot and a 1000mg potassium nitrate (PN) solution in a random order as per their allocated set. A sample of the beetroot was analysed using ozone-based chemiluminescence and found to contain 272mg nitrate per 100g cooked weight. This is around the average content defined in the literature (Hord *et al.*, 2009), so this value was used to describe the dose provided in this study.

2.2.4 Study protocol

Participants who registered an interest in the study were contacted and sent the participant information sheet (Appendix B). Potential participants were given time to deliberate and ask questions and were then screened over the phone via a short questionnaire. During this telephone call, participants were asked questions about their medications, medical conditions, current height and weight and availability for the study. Eligible participants were invited to attend the Newcastle NIHR Clinical Research Facility (CRF) at the Royal Victoria Infirmary (RVI) for a full screening visit, where height, weight and blood pressure were measured to confirm eligibility. Four further visits were booked in, separated by seven to ten days to allow for the

complete return of nitrate and nitrite to pre-supplementation levels between trials. For 24 hours before the first visit, participants were asked to collect their total urine volume in a 2.5L urine container prepped with 5ml sodium hydroxide (1M NaOH) to preserve the nitrate in the sample.

Each of the four visits followed the same protocol, differing only in the intervention provided (Figure 2.1). Participants were asked to fast on the morning of the visit and arrived between 8:15am and 9:00am for testing. Body composition and weight were measured at the beginning of each visit using bioelectrical impedance (*TANITA Body Composition Analyser*). Participants removed their shoes before standing on the scales and 0.5kg was added to account for clothing weight. Waist circumference was measured with a tape measure at the narrowest part of the trunk. Baseline blood pressure was measured three times with the participant seated, with a one-minute rest in between each measurement. Blood flow was measured by laser Doppler at baseline using a standardised protocol; participants were lying supine and in a temperature-controlled room (22-24°C). A cannula was fitted in the antecubital fossa or other appropriate area and baseline blood samples were taken. A saliva sample was collected, as detailed in section 2.2.8. The final baseline measure was fractional exhaled nitric oxide (FeNO), measured using a handheld device (NIOX), which involved participants exhaling into a mouthpiece for around 10 seconds to provide a reading. Participants were then given a 15-minute window in which to consume the beetroot or PN that had been provided. Once the relevant dose had been taken, the five-hour intervention period began, and participants were fed a standardised breakfast of cereal with milk and natural yoghurt. This meal contained around 450kcal with 62% carbohydrate, 16% protein and 22% fat. Breakfast items were chosen from the list of low-nitrate foods described by Wang *et al.* (1997) and the nitrate content of the meal was around 2.8mg. Following breakfast, participants rested in the research facility and further measurements of blood flow were taken at 150 and 300 minutes. Blood pressure was measured every 30 minutes after the consumption of the intervention, with the final measurement at 300 minutes. Saliva and blood samples and FeNO were measured at 30, 60, 120, 180, 240 and 300 minutes. Urine samples were collected at 0-3 hours, 3-6 hours, 6-12 hours, and 12-24 hours to gain a full 24hr sampling period. Participants were given disposable jugs

to measure the total volume of each urine sample, which was noted, and 50ml was collected from the larger volume and stored for later analysis.

During their first visit, participants completed the international physical activity questionnaire (IPAQ) (Craig *et al.*, 2003), which estimated their activity levels in the previous seven days. Participants also completed a nitrate intake questionnaire for the previous seven days to estimate their habitual nitrate intake before study visits and a visual analogue scale (VAS) to assess their opinion of the beetroot intervention. Participants completed the IPAQ, nitrate intake questionnaire and VAS on each subsequent visit. On the final visit, participants were asked to complete a feedback questionnaire that included questions on their opinion of the beetroot dose and positive and negative aspects of the study. The nitrate intake questionnaire and VAS can be found in Appendices D and E. At the end of the five-hour intervention period and following the final blood sample, the cannula was removed and participants were provided with a standardised low-nitrate lunch of a chicken or tuna sandwich and a packet of ready salted crisps, which they consumed in the research facility. Participants were provided with sample pots to continue the urine collection for the remainder of the day, as described above, and a low-nitrate ready meal to consume at home. They were then free to leave the research facility.

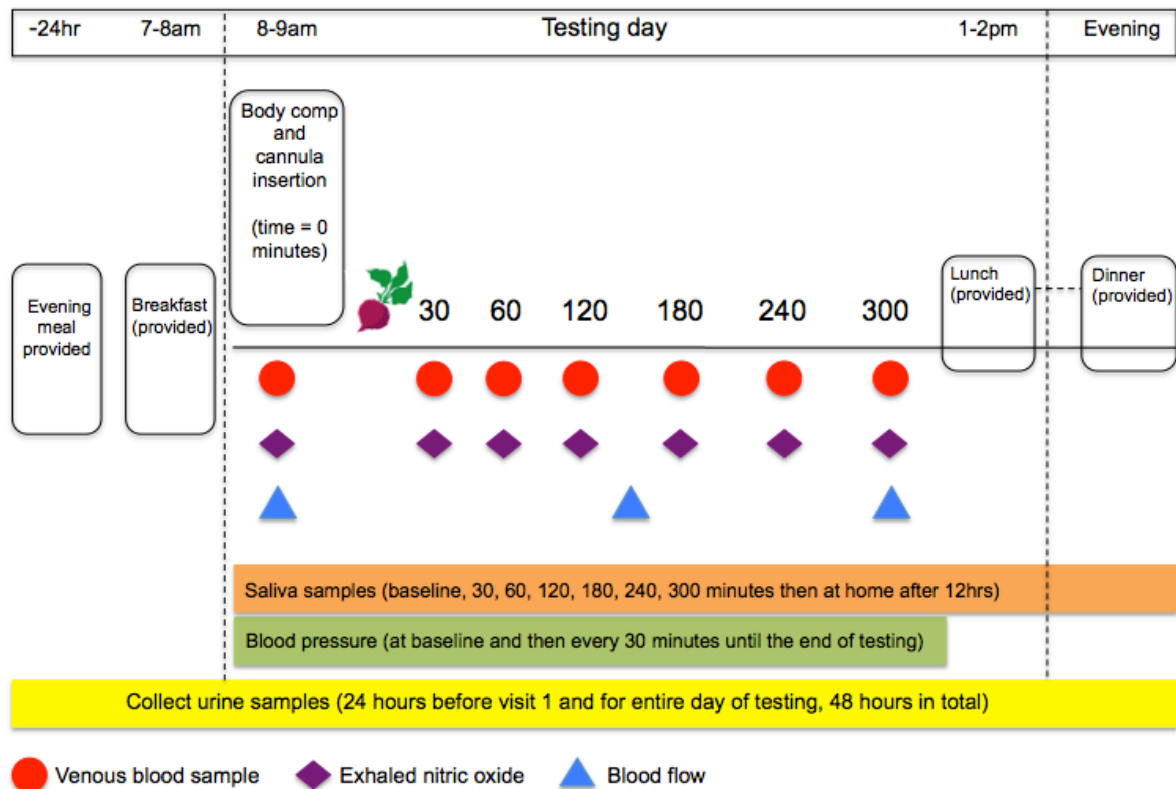


Figure 2.1: Study day protocol followed for each intervention. Low-nitrate meals provided at times shown and beetroot or potassium nitrate consumed within 15 minutes following baseline measurements and cannula insertion. Participants collected urine and saliva samples after leaving the research facility, which were frozen and returned to the research facility. 30-300 represents time points where blood and saliva samples were collected and exhaled nitric oxide (FeNO) was measured. Blood flow using laser Doppler measured at 0, 150 and 300 minutes.

2.2.5 Interventions

Participants were randomised to receive either 1) 100g of whole cooked beetroot (~272mg nitrate), 2) 200g beetroot (~544mg nitrate), 3) 300g beetroot (~816mg nitrate), 4) 200ml solution of potassium nitrate (1000mg nitrate). The cooked beetroot was grown and processed by G's Fresh Ltd in Cambridgeshire and purchased from Tesco. Beetroot was purchased on a per participant basis to adhere to the use by dates and was refrigerated until used. The beetroot was steamed and dipped in vinegar and represented what members of the public would buy as a ready-to-eat product. The potassium nitrate was produced by Newcastle Specials Pharmacy Production Unit in the Royal Victoria Infirmary with codes assigned to them at the time of preparation e.g. Y01 or OL01 for young and old participants, respectively. The intervention was given at the same time each visit, depending on cannulation, and

consumed within 15 minutes, with exception of three participants who took between 15 and 30 minutes to consume the beetroot due to an aversion to the larger amounts.

2.2.6 Dietary control

Participants were required to follow a low nitrate and phenolic diet in the two days before each trial, as well as the entirety of the testing day. This included avoidance of most vegetables, fruits, cured meats, strong cheese, chocolate, wholegrain breads and grains, tea, coffee and alcohol; a list of foods to avoid was provided alongside a food diary. Participants were also instructed to consume the same or similar meals in the days before each of the trials. To reduce the burden of the dietary restrictions, participants were provided with ready meals to consume on the night before and day of testing. Meals were chosen based on the content of low nitrate and phenolic compound products and included fish pie, mushroom risotto and macaroni cheese. The aim of these restrictions was to eliminate the potential influence that the consumption of nitrate- or phenolic-rich foods other than the intervention under investigation may have on study outcomes, particularly surrounding bioavailability. These restrictions have been implemented in other studies to more accurately establish the bioavailability of functional foods (Clifford *et al.*, 2017; Frank *et al.*, 2005; Keane *et al.*, 2016). Participants were given three-day food diaries (Appendix F) to record their dietary intake before and during the study days in order for compliance to the dietary restrictions to be assessed. All participants were also asked to refrain from using anti-bacterial mouthwash and gum throughout the study due to its potential influence on the conversion of nitrate to nitrite (Webb *et al.*, 2008).

2.2.7 Urinary output

The day before their first visit, participants collected a 24-hour urine sample in 2.5L urine containers prepped with 5ml of a 1M NaOH solution, prepared in the laboratory. This was stored out of direct sunlight, either in the fridge or in a provided cooler bag, and returned to the researcher on the morning of their first visit. During the study visit, total urinary output was measured over two time periods: 0-3 hours and 3-6 hours post-intervention. Total volume was measured using disposable measuring jugs and a small sample pot was filled. The sample pot was prepped with 100µl of

NaOH solution. Participants were given labelled sample pots and measuring jugs to repeat the collections at home between 6-12 hours and 12-24 hours post-intervention. The sample pots were refrigerated or frozen and returned to the researcher. The total volume of each time point was recorded and the urine was aliquoted and frozen at -20°C until analysis. Before analysis, samples were defrosted and diluted to 1:100 with deionized water.

2.2.8 Saliva sampling

Salivary nitrate was measured in the saliva following stimulation by chewing. Participants chewed a small cotton wool ball for approximately two minutes or until wet and this was deposited into a syringe. The cotton wool ball was then squeezed into a 1ml Eppendorf tube prepped with 3.7µL NaOH solution. Tubes were then frozen at -20°C until analysis. Before nitrate analysis, samples were defrosted and diluted to 1:100 with deionized water.

2.2.9 Blood sampling and processing

17ml of blood was collected at each time point in three vacutainers: two lithium heparin and one serum gel. One lithium heparin tube was spun immediately at 5000rpm for three minutes, aliquoted into dark-coloured Eppendorf tubes and frozen at -80°C to preserve the nitrite. Each Eppendorf tube was pre-treated with a stop solution of 10µl of diethylenetriaminepentaacetic acid (DTPA) and 6.5µl of N-ethylmaleimide (NEM), to prevent transition between compounds, as described by Nagababu and Rifkind (2007). To prepare the solutions, firstly a 1M NaOH solution was prepared by dissolving 40g NaOH pellets in 1L water. To make the DTPA solution, 39.3mg DTPA was suspended in 5ml distilled water. With the contents being constantly stirred, 1M NaOH was added drop wise until the DTPA was completely dissolved. The final volume was made up to 10ml with distilled water. This solution was stable at room temperature and was prepared twice over the course of the study. Before each visit the NEM solution was prepared by dissolving 12.5mg NEM in 10ml of distilled water. This was kept at 4°C and fresh reagent was prepared for each study visit. DTPA and NEM prevent the destruction of plasma S-nitrosothiols to nitrite (Nagababu & Rifkind, 2007) and samples were spun and frozen as quickly as possible in order to maintain stability in the samples. The remaining vacutainers

were kept cool and processed within 30 minutes, or after clotting had occurred in the serum tubes. They were spun at 3000rpm for 10 minutes at 4°C then aliquoted and frozen at -80°C. The plasma samples were used to measure nitrate and nitrite concentrations. Due to time constraints, not every time point was able to be analysed, so the most appropriate samples were chosen: baseline, 60 minutes, 180 minutes and 300 minutes.

2.2.10 Fractional exhaled nitric oxide (FeNO)

A handheld device called the NIOX VERO was developed as a tool for non-invasive measurement of FeNO as a marker of airway inflammation (Travers *et al.*, 2007; Ricciardolo, 2004), aiding the diagnosis and management of asthma. The NIOX VERO device (Circassia, USA) was used to collect data on FeNO in this study. This portable, non-invasive device provides a simple and quick method of data collection; set up takes around two minutes per trial. Each participant receives a new mouthpiece per trial and is trained on its use at the first visit. The participant is required to breathe steadily and consistently into the handheld sensor until a suitable measurement has been taken, as signalled by the device. The sensor then takes around one minute to produce a reading of FeNO in parts per billion (ppb). Exhaling too vigorously or too gently renders the measurement unusable and it must then be restarted. Measurements were taken at baseline and at 30, 60, 120, 180, 240 and 300 minutes following the intervention. Readings were recorded in ppb in a data sheet throughout the visit and inputted into a spreadsheet after completion. Each sensor supplied by the manufacturers takes 500 readings and the sensor was replaced and calibrated mid-way through the study. The breathing handle was also replaced, as guided by the software.

2.2.11 Sample analysis

Plasma samples were first deproteinised before analysis using cold ethanol precipitation. The required ethanol was first chilled to 0°C. 0.5ml of each sample was placed in a 1.5ml microcentrifuge tube and 1ml of cold ethanol was added. The tubes were then vortexed for 10 seconds and placed at 0°C for 30 minutes. Samples were centrifuged at 14,000rpm for five minutes. The supernatant was removed for the determination of nitrate and nitrite. The whole beetroot was juiced and diluted to

1:1000 in deionized water prior to analysis for nitrate content. The researcher conducted the deproteinisation and dilution, however ozone-based chemiluminescence was carried out by a trainer laboratory assistant who worked closely with a principal investigator in the research group.

The ozone-based chemiluminescence method was used to measure plasma and salivary nitrate and nitrite and urinary nitrate concentrations using the Sievers gas-phase chemiluminescence nitric oxide analyser (*NOA 280i, Analytix, UK*). Briefly, prior to analysis a standard curve was created for both nitrate and nitrite and an equation was derived to calculate nitrate and nitrite concentrations by fitting a linear regression line to the standard concentrations. R^2 was greater than 0.99. Diluted samples were injected into the purge vessel using a glass Hamilton (Fisher) syringe with an injection volume ranging from 10 μ l to 100 μ l. The injection volume was adjusted to maximise the sensitivity of the measurements and taken into account when calculating the final concentrations. The area under the curve of the peaks was calculated automatically by the Analytix software, which minimises the risk of between-operator differences in the calculation of results, and was used to calculate the concentrations of the samples using the standard regression equations. Samples were analysed in singlicate and quality of the peaks was checked visually based on height, fronting and tailing. Analyses were repeated for samples with low quality peaks. Calibration of the device is ensured by a maintenance contract with Analytix and a service is provided twice per year. The internal calibration of the analysis is performed by using serial dilutions of nitrate and nitrite standards with known concentrations. In addition, each run of the samples is preceded by the injection of a standard with known concentration to ensure the accuracy of the results. The assay performance of the nitrate and nitrite analysis is high, with a within-operator coefficient of variation of 91% for nitrate and 89% for nitrite after a series of ten consecutive manual injections of a standard solution with known concentration.

2.2.12 Statistical analysis

All statistical analyses were completed using IBM SPSS (*version 24.0*). Summary data are presented as means \pm SD with 95% confidence intervals (CI). Intraclass coefficient values (ICC) were used to calculate the reliability of the measurements

over the multiple baseline values (time 0) obtained at the beginning of each supplementation visit. FeNO values were amalgamated into baseline (0min), early (30-90min), mid (120-210min) and late (240-300min) measurement points to better capture changes in FeNO after supplementation and improve graphical presentation of the results across the time points. Independent t-tests were used to assess differences in baseline measures between the two age groups. Changes over time were analysed using a two-factor repeated measures analysis of variance (ANOVA) with time and intervention as within-subjects factors. Area under the curve (AUC) over the five-hour period was calculated using the trapezoid method to assess age-related differences, which were analysed using a mixed model repeated measures ANOVA with intervention as the within-subjects factor and age as the between-subjects factor. Data was assessed for normality using the Shapiro-Wilks test. Models were checked for sphericity using Mauchly's test and multivariate models were applied if these assumptions were violated. Where significances were found and to assess intervention or age specific outputs, *post hoc* tests with Bonferroni adjustment to control for multiple comparisons were run. Associations between changes in plasma nitrate and nitrite and changes in FeNO were analysed using Pearson product-moment correlation coefficient. Statistical significance was set at $P<0.05$.

2.3 Results

2.3.1 Baseline characteristics

A total of 24 participants were randomised to the interventions, 12 young (27.3 ± 3.8 years) and 12 old (64.3 ± 4.6 years) participants (Figure 2.2). The interventions were well tolerated and no adverse events were reported, although participants did describe beeturia and faecal discolouration following ingestion of the beetroot. Baseline characteristics (Table 2.1) illustrates that the young and old group were matched for BMI, height and weight but the old group had a significantly higher waist circumference ($P=0.04$) and body fat percentage ($P=0.04$) than the young group. Baseline systolic and diastolic blood pressures and FeNO were similar across the groups. Physical activity levels, measured in metabolic equivalent in minutes per week (MET-min/wk), were significantly lower in the old group than the young group ($P=0.02$) with values of 3546 MET-min/wk and 4260 MET-min/wk, respectively.

2.3.2 Dietary intake

Inspection of the food diaries revealed that participants adhered to the dietary restrictions and their food intake prior to each visit did not significantly differ between trials. Habitual daily nitrate intakes over the four-week period were similar for both groups but the mean for all participants was approximately 238mg/day, which is higher than the average daily intake of 108mg/day (Babateen *et al.*, 2018).

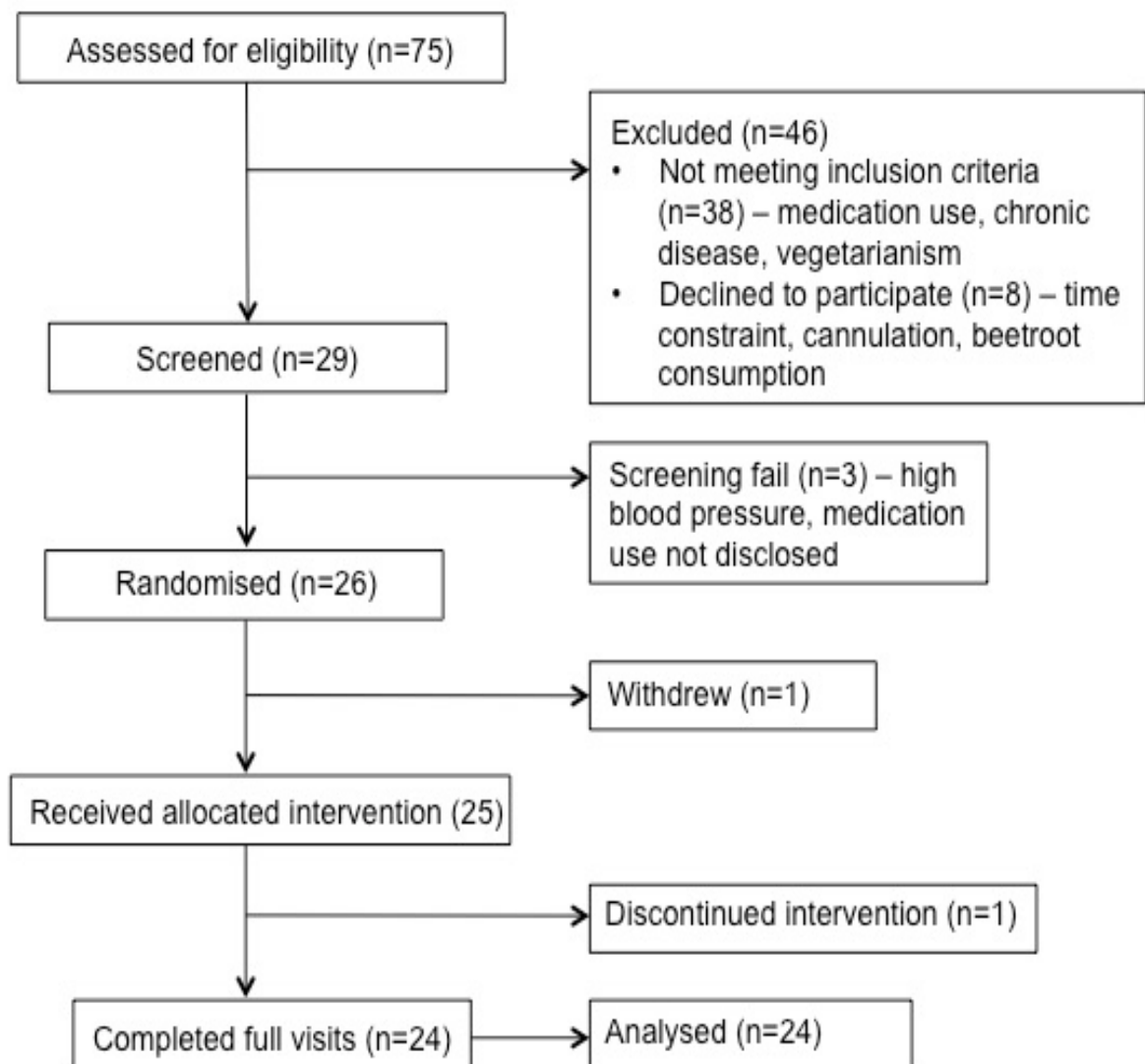


Figure 2.2: Recruitment flowchart, study 1.

Table 2.1: Mean (\pm SD) baseline characteristics for participants in study 1.

	<i>All</i>	<i>Young</i>	<i>Old</i>	<i>P value</i>
<i>Number</i>	24	12	12	
<i>Gender (male/female)</i>	9/15	6/6	3/9	
<i>Age (years)</i>	45.8 \pm 19.4	27.3 \pm 3.8	64.3 \pm 4.6	<0.001
<i>Height (cm)</i>	169.0 \pm 8.5	172.1 \pm 7.5	166.0 \pm 8.5	0.07
<i>Weight (kg)</i>	68.2 \pm 8.8	70.0 \pm 9.3	66.4 \pm 8.2	0.33
<i>Body mass index (kg/m²)</i>	23.9 \pm 2.5	23.6 \pm 2.1	24.2 \pm 2.9	0.59
<i>Waist circumference (cm)</i>	82.9 \pm 6.3	80.3 \pm 4.9	85.5 \pm 6.7	0.04
<i>Body fat (%)</i>	26.7 \pm 10.1	22.6 \pm 9.2	30.8 \pm 9.5	0.04
<i>SBP (mmHg)</i>	117.7 \pm 10.5	115.3 \pm 8.4	120.2 \pm 12.1	0.27
<i>DBP (mmHg)</i>	74.6 \pm 2.3	72.6 \pm 4.2	76.54 \pm 7.0	0.11
<i>FeNO (ppb)</i>	26.5 \pm 18.4	30.9 \pm 22.2	22.2 \pm 13.2	0.25
<i>Nitrate intake (mg/day)</i>	238.4 \pm 126.7	223.8 \pm 124.6	252.9 \pm 132.6	0.59
<i>Physical activity (MET-min/wk)</i>	3839.5 \pm 517.2	4260.4 \pm 2088.2	3456.7 \pm 2650.8	0.02

Statistical analysis using independent t-tests. SBP = systolic blood pressure, DBP = diastolic blood pressure.

2.3.3 Plasma nitrate

Young

There was a significant effect of time ($P<0.001$) and intervention ($P<0.001$) on plasma nitrate levels in the young and a significant interaction between the two factors ($P<0.001$), as illustrated in Figure 2.3. Pairwise comparisons show that plasma nitrate levels following PN were significantly higher than following 100g beetroot (+157.5 μ mol/L; $P=0.001$; 95%CI: 66.74, 248.22), 200g beetroot (+121.1 μ mol/L; $P=0.001$; 95%CI: 52.15, 190.04) and 300g beetroot (+85.6 μ mol/L; $P=0.04$; 95%CI: 3.87, 167.40). Levels following 300g beetroot were also higher than following 100g beetroot (+71.8 μ mol/L; $P=0.01$; 95%CI: 14.72, 128.96), which demonstrates a stepwise dose response to increasing nitrate intakes. Plasma nitrate was significantly higher at one, three and five hours than at baseline ($P<0.001$). Analyses show that plasma nitrate levels were significantly raised from baseline at one, three, and five hours post-ingestion of all nitrate doses but there was a stepwise increase alongside higher doses compared to 100g beetroot after one hour with +50.4 μ mol/L, +89.3 μ mol/L and +254.1 μ mol/L higher following 200g, 300g and PN,

respectively. Over the 300-minute period, plasma nitrate increased from baseline by 257%, 358%, 538% and 1027% on average following 100g, 200g, 300g beetroot and PN, respectively.

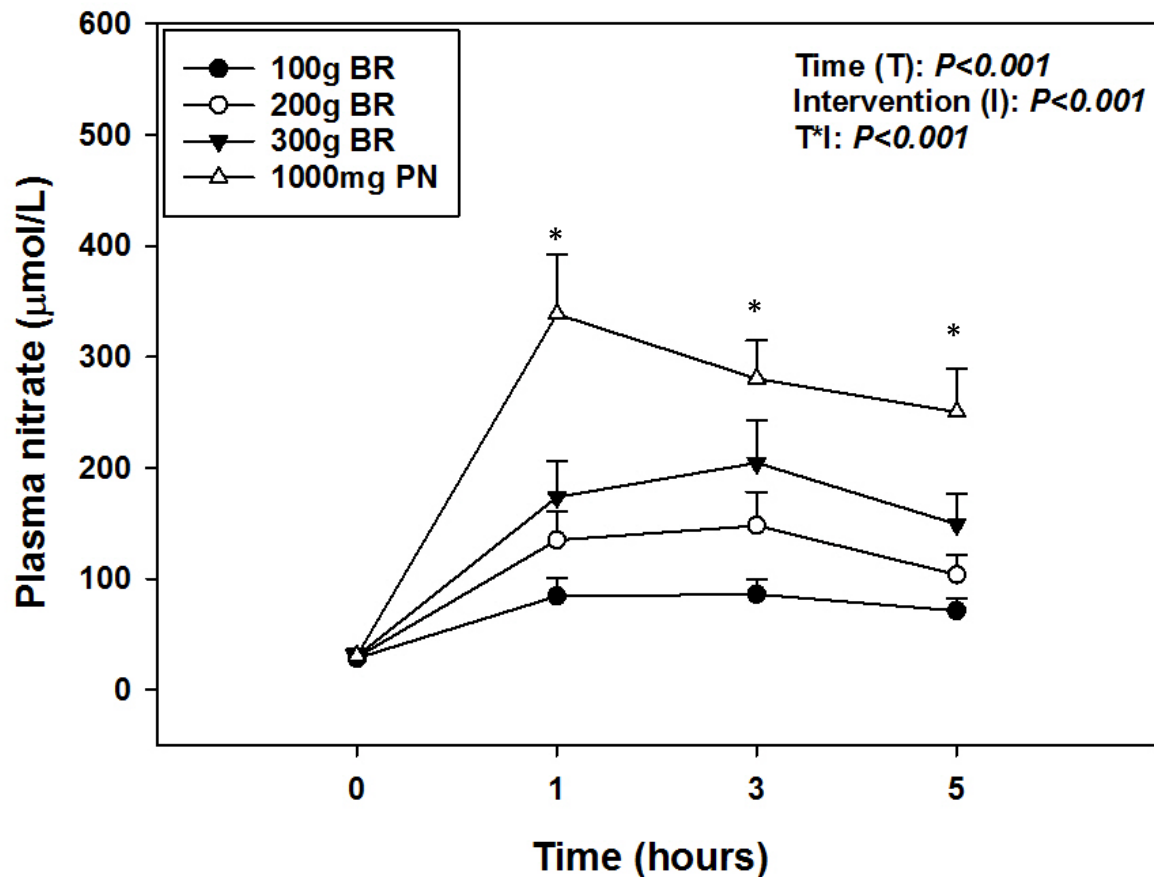


Figure 2.3: Mean (\pm SEM) plasma nitrate levels over five hours following incremental doses of nitrate in the young group; $n = 12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate positive control. * denotes significant increase from baseline across nitrate doses.

Old

There was a significant effect of time ($P < 0.001$) and intervention ($P < 0.001$) on plasma nitrate levels in the old and a significant interaction between the two factors ($P < 0.001$), as illustrated in Figure 2.4. Pairwise comparisons show that plasma nitrate levels following PN were significantly higher than following 100g beetroot ($+217.9\mu\text{mol/L}$; $P < 0.001$; 95%CI: 121.43, 314.46), 200g beetroot ($+169.9\mu\text{mol/L}$; $P = 0.02$; CI: 27.02, 312.80) and 300g beetroot ($+164.5\mu\text{mol/L}$, $P = 0.001$; 95%CI: 65.42, 263.61), demonstrating a stepwise dose response to increasing nitrate intakes. *Post hoc* analyses show that plasma nitrate levels were significantly raised from baseline at one, three, and five hours post-ingestion of all nitrate doses

($P<0.001$), although after one hour there was a larger increase following PN than 100g beetroot (+302.9 $\mu\text{mol/L}$; $P<0.001$; 95%CI: 165.2, 440.8), 200g beetroot (+248.8 $\mu\text{mol/L}$; $P=0.002$; 95%CI: 90.8, 406.7) and 300g beetroot (+249.3 $\mu\text{mol/L}$; $P=0.002$; 95%CI: 93.9, 404.7). This stepwise increase is evident in Figure 2.4. Over the 300-minute period, plasma nitrate levels were higher than baseline by 373%, 749%, 1015% and 1442% on average following 100g, 200g, 300g beetroot and PN, respectively.

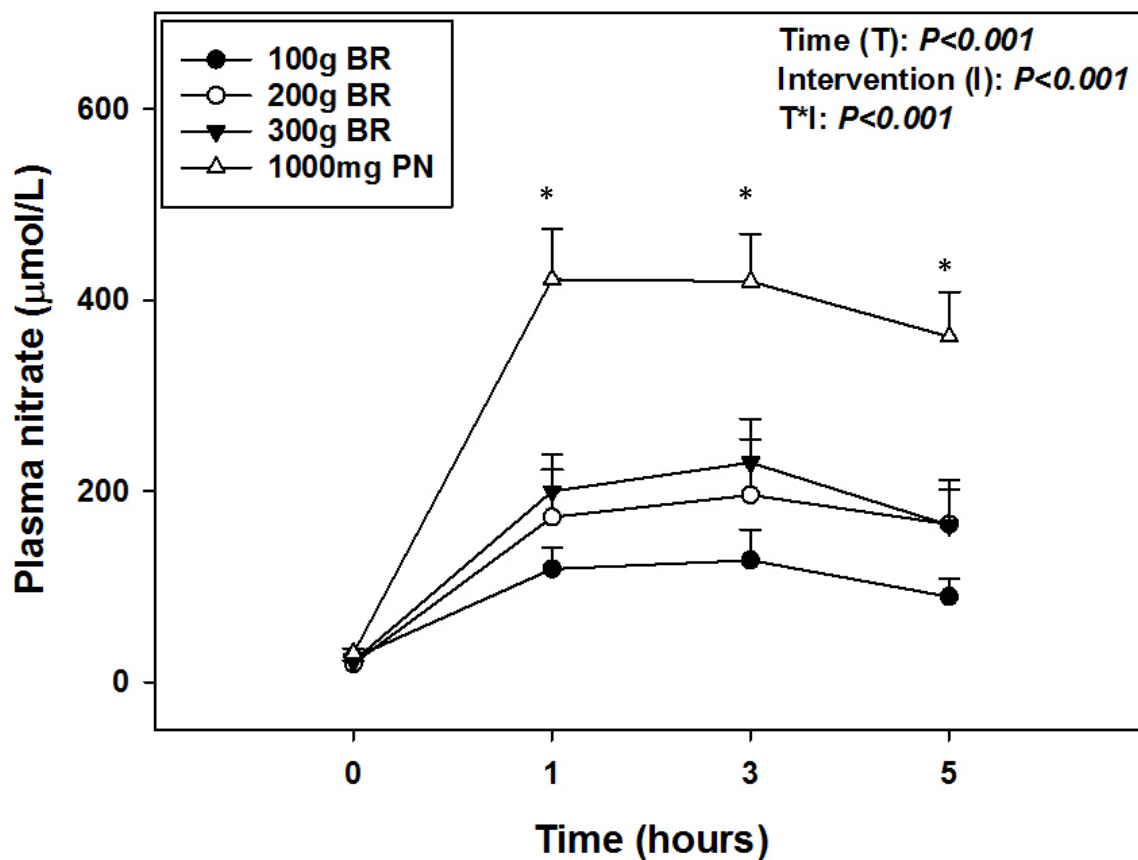


Figure 2.4: Mean ($\pm\text{SEM}$) plasma nitrate levels over five hours following incremental doses of nitrate in the old group; $n = 12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate positive control. * denotes significant increase from baseline across nitrate doses.

AUC

The AUC over the five-hour period for the young and the old groups demonstrates no age effect ($P=0.23$) but a significant effect of intervention ($P<0.001$) (Figure 2.5). There was no interaction between age and intervention ($P=0.24$). Pairwise comparisons indicate that there was no difference between responses to 100g and

200g beetroot but consumption of 300g beetroot produced a larger AUC than the consumption of 100g (+23424.3; $P=0.004$; 95%CI: 6300.5, 40548.2), as did PN (+67894.6; $P<0.001$; 95%CI: 46672.4, 89116.8). Analyses show that this occurs in both the young and the old group ($P<0.05$). There is also a notable trend towards a smaller AUC following PN in the young group than the old group (-30821.2; $P=0.08$; 95%CI: -66095.1, 4452.8).

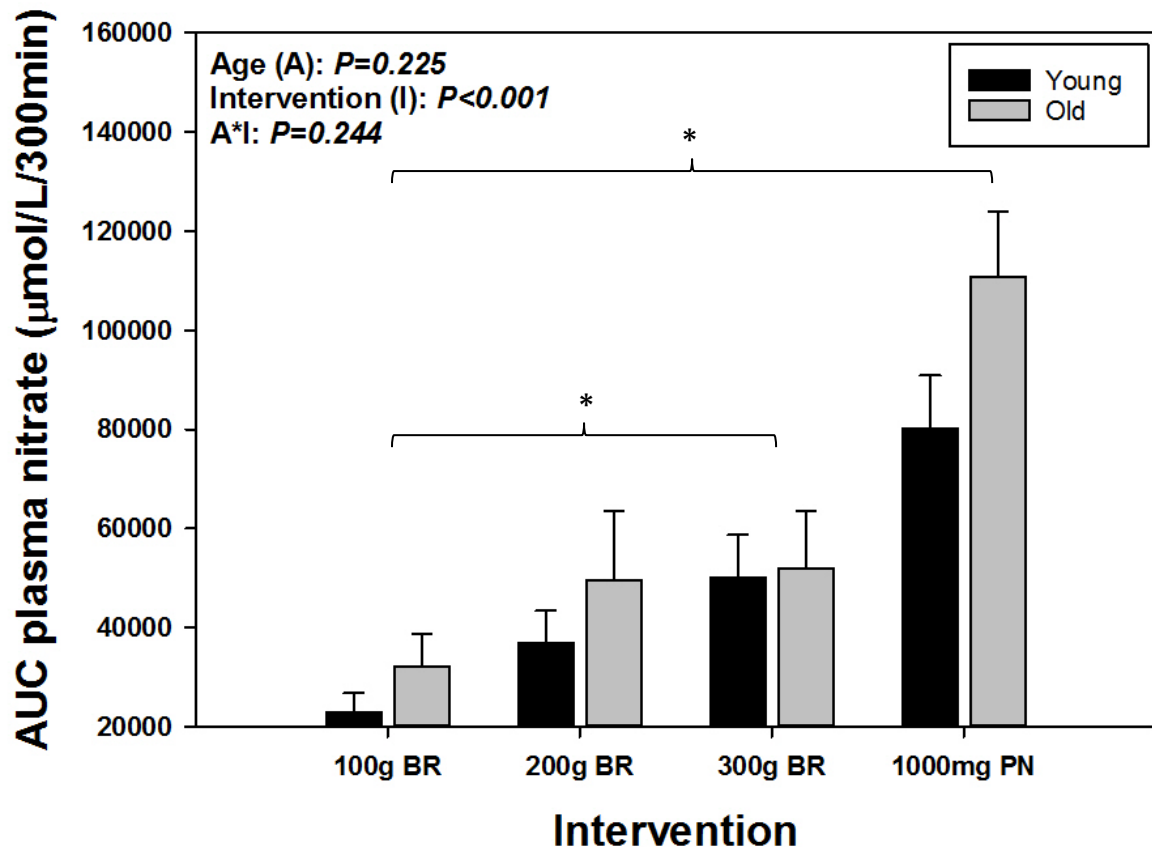


Figure 2.5: Mean (\pm SEM) area under the curve (AUC) for plasma nitrate levels in the young and the old groups; $n = 24$ (12 young and 12 old). Data analysed using mixed model repeated measures ANOVA (intervention as within-subjects factor and age as between-subjects factor). BR = beetroot, PN = potassium nitrate positive control. * denotes significant difference between interventions for both age groups.

2.3.4 Plasma nitrite

Young

There was a significant effect of time ($P<0.001$) and intervention ($P<0.001$) on plasma nitrite levels in the young group and a significant interaction between the two factors ($P<0.001$), as illustrated in Figure 2.6. Pairwise comparisons show that 100g

beetroot caused the lowest rise in nitrite levels, with larger values following 200g (+39.3nmol/L; $P=0.009$; 95%CI: 9.1, 69.5), 300g beetroot (+137.7nmol/L; $P<0.001$; 95%CI: 87.0, 188.3), and PN (+473.0nmol/L; $P<0.001$; 95%CI: 384.8, 561.2). As with plasma nitrate, this demonstrates a stepwise dose response in plasma nitrite to higher doses of nitrate. Analyses show that all doses of beetroot significantly increased plasma nitrite levels from baseline at all subsequent time points ($P<0.001$) but at five hours plasma nitrite levels were higher than 100g beetroot following 300g beetroot (+230.1nmol/L; $P<0.001$; 95%CI: 128.3, 331.8) and PN (+837.0nmol/L; $P<0.001$; 95%CI: 664.1, 1009.9). Over the 300-minute period, plasma nitrite increased from baseline by 93%, 144%, 179% and 495% on average following 100g, 200g, 300g beetroot and PN, respectively.

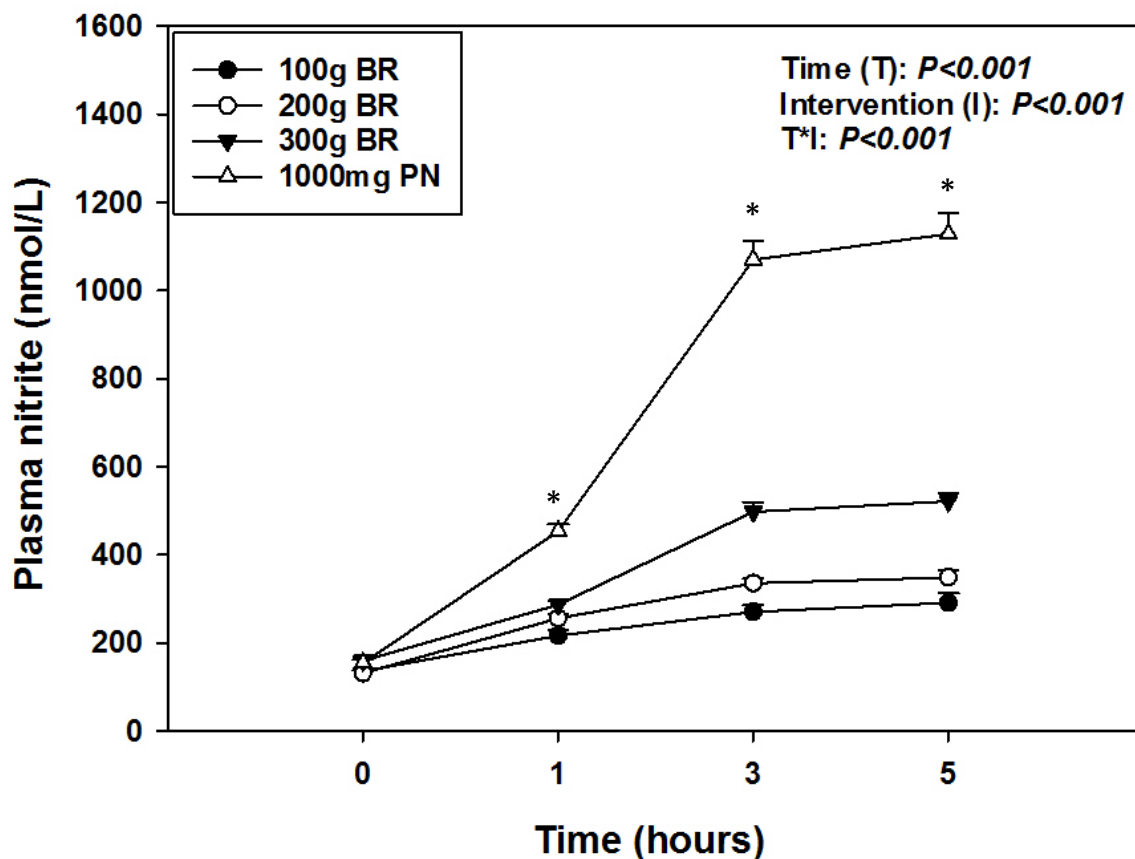


Figure 2.6: Mean (\pm SEM) plasma nitrite levels over five hours following incremental doses of nitrate in the young group; $n = 12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate positive control. * denotes significant increase from baseline across nitrate doses.

Old

As with the young group, there was a significant effect of time ($P<0.001$) and intervention ($P<0.001$) on plasma nitrite levels in the old group and a significant interaction between the two factors ($P<0.001$), as shown in Figure 2.7. Higher nitrate consumption raised plasma nitrite levels in an incremental fashion, with higher levels following 200g beetroot (+33.1nmol/L; $P=0.04$; 95%CI: 10.3, 55.8), 300g beetroot (+106.8nmol/L; $P<0.001$; 95%CI: 51.6, 161.9), and PN (+398.3nmol/L; $P<0.001$; 95%CI: 268.2, 527.9) compared to 100g beetroot ingestion. 100g beetroot itself did significantly raise plasma nitrite levels from baseline by 167.3nmol/L ($P<0.001$; 95%CI: 117.1, 217.6). Plasma nitrite levels were significantly higher than baseline at one, three and five hours following all doses of nitrate ($P<0.05$). Over the 300-minute period, plasma nitrite levels were higher than baseline by 121%, 141%, 239% and 528% on average following 100g, 200g, 300g beetroot and PN, respectively.

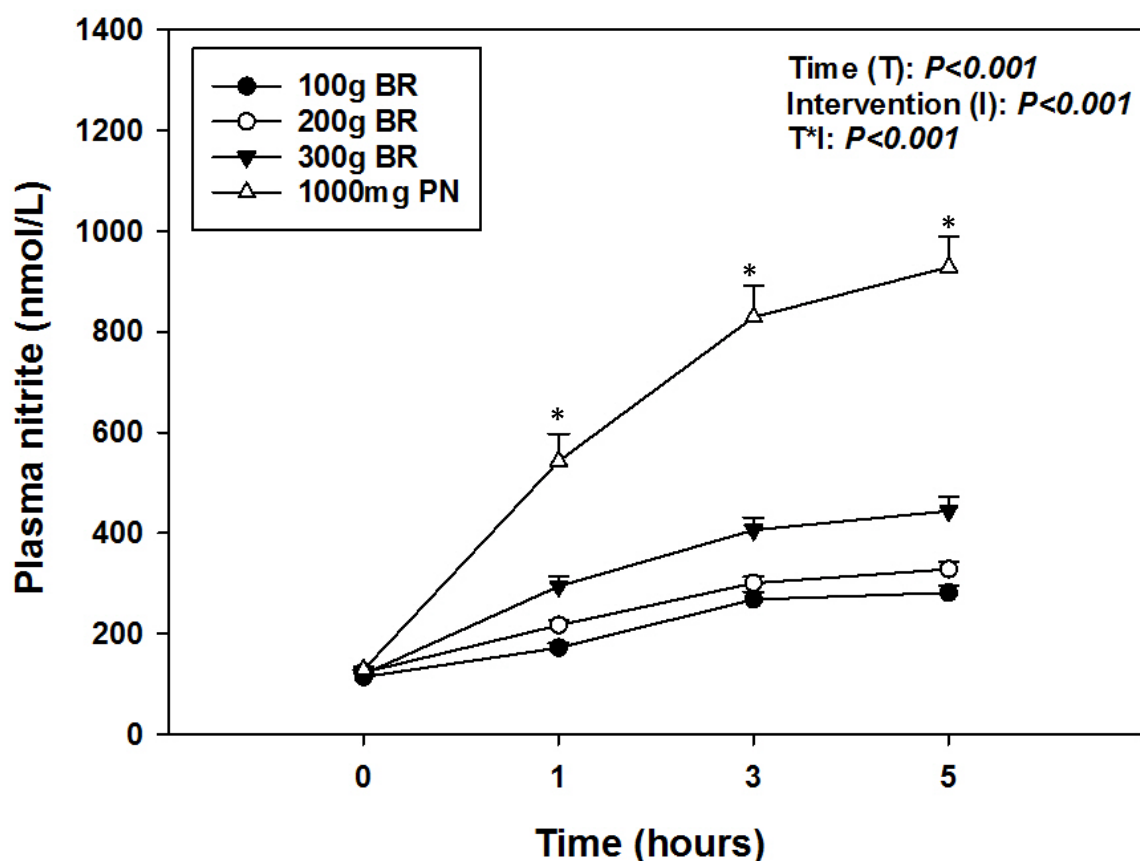


Figure 2.7: Mean (\pm SEM) plasma nitrite levels over five hours following incremental doses of nitrate in the old group; $n = 12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate positive control. * denotes significant increase from baseline across nitrate doses.

AUC

The AUC over the five-hour period for plasma nitrite in the young and the old groups shows a significant effect of age ($P=0.008$), as illustrated in Figure 2.8. There was also a significant effect of intervention ($P<0.001$) but no interaction between intervention and age ($P=0.17$). Pairwise comparisons show that there was a dose response to nitrate intake, with each incremental dose producing a larger AUC than the smaller doses (all $P<0.001$). Pairwise comparisons also demonstrate that the young had a significantly larger AUC than the old group (+16096.3; $P=0.008$; 95%CI: 4717.3, 27475.2) across the interventions. *Post hoc* analyses show that the young group had a significantly larger AUC than the old group following 200g beetroot (+9180; $P=0.03$; 95%CI: 1182.0, 17177.9) and 300g beetroot (+16147.5; $P=0.04$; 95%CI: 1264.8, 31030.2). There was also a notable trend towards a larger AUC following PN in the young group compared to the old (+33587; $P=0.07$; 95%CI: -

2436.3, 69611.3). The AUC following 100g beetroot was not significantly different in the young and the old groups ($P=0.29$).

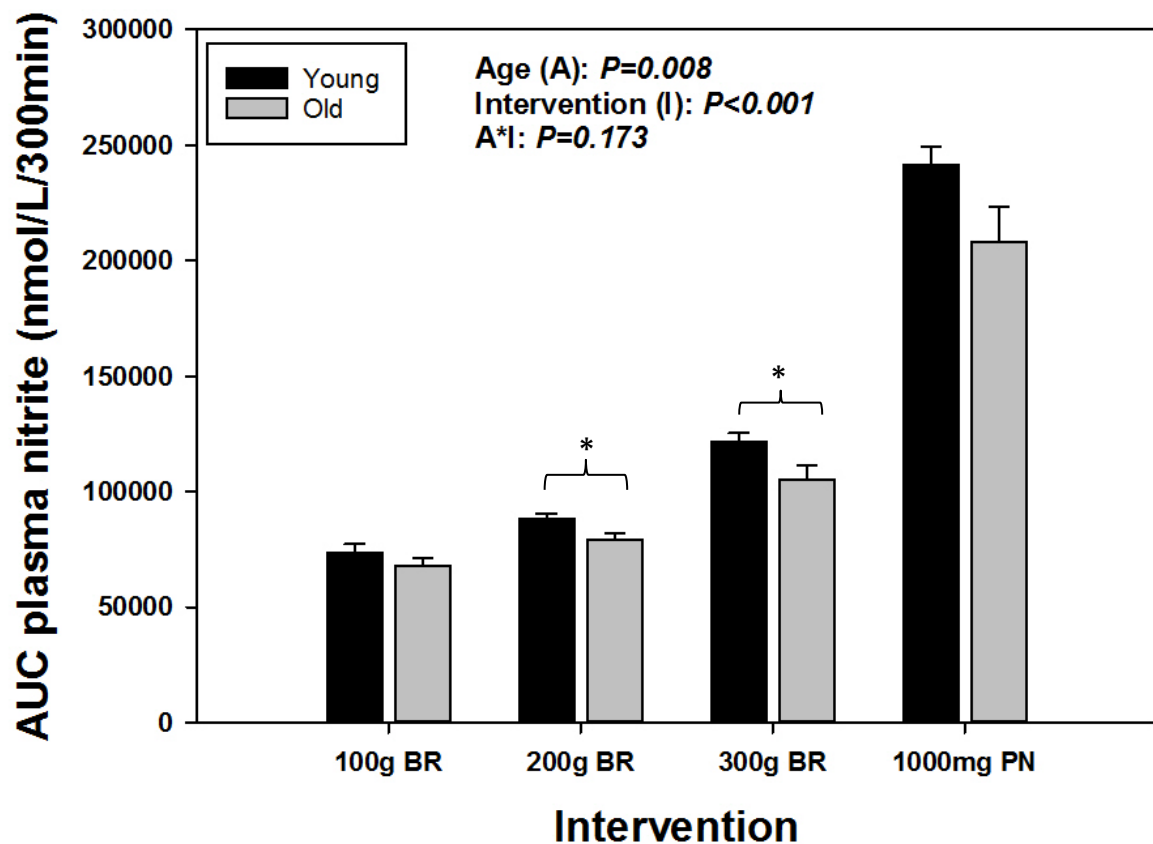


Figure 2.8: Mean (\pm SEM) area under the curve (AUC) for plasma nitrite levels in the young and the old groups; $n = 24$ (12 young and 12 old). Data analysed using mixed model repeated measures ANOVA (intervention as within-subjects factor and age as between-subjects factor). BR = beetroot, PN = potassium nitrate positive control. * denotes significant difference between age groups.

2.3.5 Salivary nitrate

Young

Analysis shows a significant effect of time ($P<0.001$) and intervention ($P<0.001$) on salivary nitrate in the young and an interaction between the two factors ($P=0.01$). This is illustrated in Figure 2.9. Pairwise comparisons show that there was no difference in nitrate levels between 100g and 200g beetroot ($P=0.51$) or 200g and 300g beetroot ($P=0.16$) but nitrate levels following 100g were significantly lower than following 300g (-1.7mmol/L ; $P=0.02$; 95%CI: $-3.2, -0.2$) and following PN (-4.4mmol/L ; $P=0.004$; 95%CI: $-7.4, -1.4$). Nitrate levels following the consumption of PN were also higher than following 300g beetroot ($+2.7\text{mmol/L}$; $P=0.02$; 95%CI: $0.5,$

5.0), demonstrating a dose response to higher amounts of nitrate. Salivary nitrate levels were higher than baseline at one and two hours following consumption of all nitrate doses but levels following 300g beetroot and PN remained elevated over the five-hour period; nitrate levels following 100g and 200g beetroot were not maintained at a significantly higher level. Nitrate levels at the 12-hour period were not different from baseline ($P=1.00$), demonstrating that salivary nitrate levels had returned to normal. Over the 300-minute period, salivary nitrate levels rose by on average 466%, 669%, 510% and 1089% from baseline following 100g, 200g, 300g beetroot and PN, respectively.

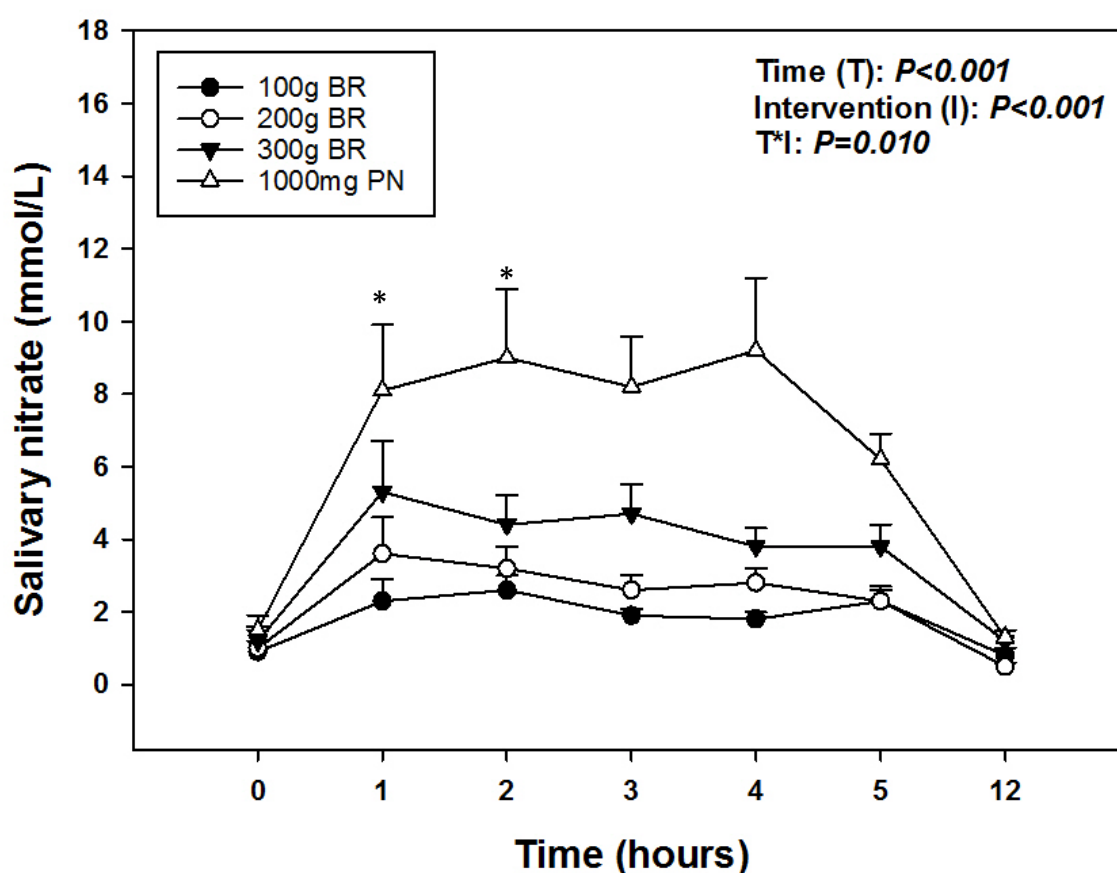


Figure 2.9: Mean (\pm SEM) salivary nitrate levels over 12 hours following incremental doses of nitrate in the young group; $n = 12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate. * denotes significant increase from baseline across nitrate doses.

Old

There was a significant effect of time ($P<0.001$) and intervention ($P<0.001$), as well as an interaction between the two factors ($P<0.001$), on salivary nitrate levels in the old group (Figure 2.10). Nitrate levels were incrementally higher compared to 100g

beetroot following 200g (+0.8mmol/L; $P=0.005$; 95%CI: 0.3, 1.3), 300g (+1.8mmol/L; $P=0.003$; 95%CI: 0.6, 2.9), and PN (+5.1mmol/L; $P<0.001$; 95%CI: 3.7, 6.3). Nitrate levels were elevated from baseline at all time points across the five-hour period following all nitrate doses (all $P<0.05$). Similar to the young group, nitrate levels at baseline and at 12 hours were not significantly different in any of the interventions ($P=1.00$). Over the 300-minute period, salivary nitrate levels rose by 649%, 1035%, 437% and 1129% from baseline following 100g, 200g, 300g beetroot and PN, respectively.

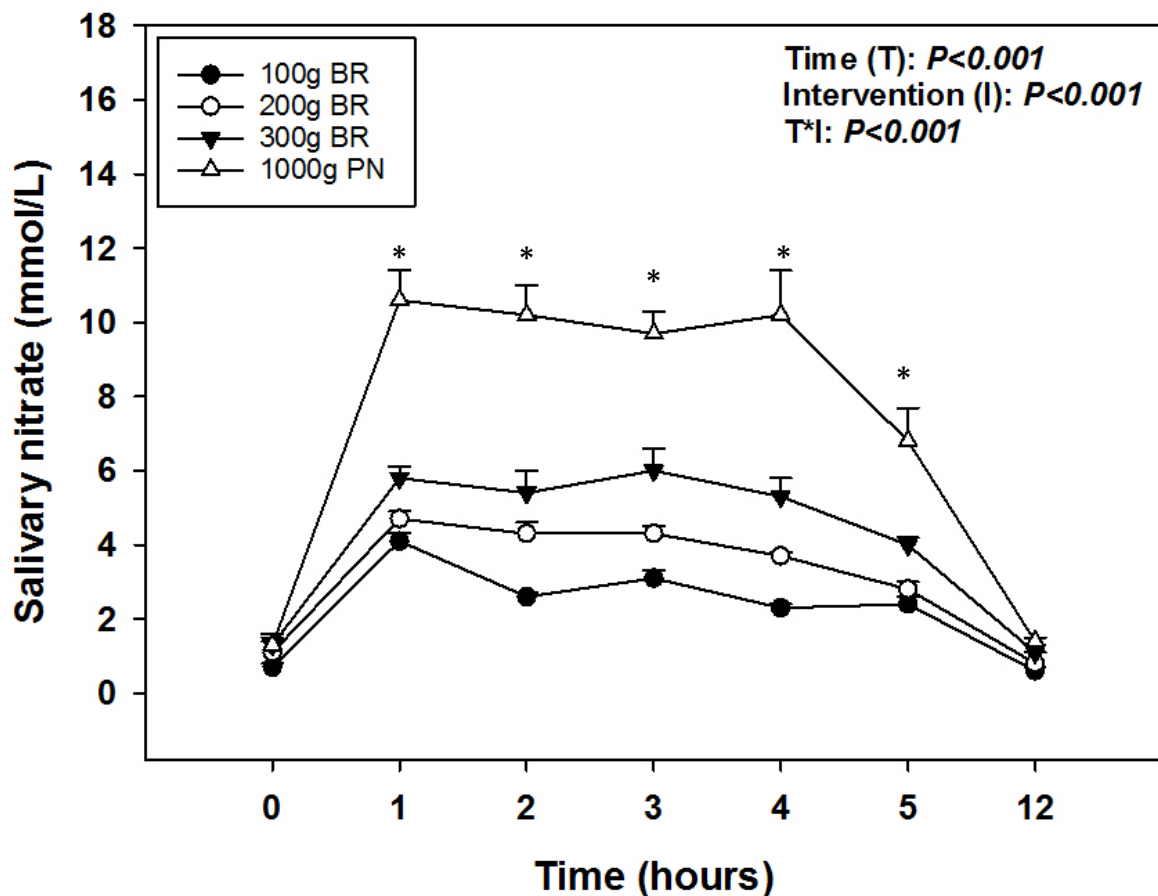


Figure 2.10: Mean (\pm SEM) salivary nitrate levels over 12 hours following incremental doses of nitrate in the old group; $n = 12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate. * denotes significant increase from baseline across nitrate doses.

AUC

There was no effect of age ($P=0.28$) but a significant effect of intervention ($P<0.001$) on AUC for salivary nitrate (Figure 2.11). There was no interaction between intervention and age ($P=0.86$). Pairwise comparisons show that the AUC was significantly larger following PN than all other interventions (all $P<0.001$) and AUC

rose incrementally, with higher values after 200g beetroot than 100g beetroot (+289.7; $P=0.009$; 95%CI: 59.3, 520.1) and higher values after 300g than 200g (+675.3; $P=0.001$; 95%CI: 232.9, 1117.8). Analyses show that there was no difference in AUC between the young and old groups following any of the interventions (all $P>0.05$) but there was a trend towards a higher AUC in the old than the young following 200g beetroot (+416.2; $P=0.09$; 95%CI: -70.9, 903.3).

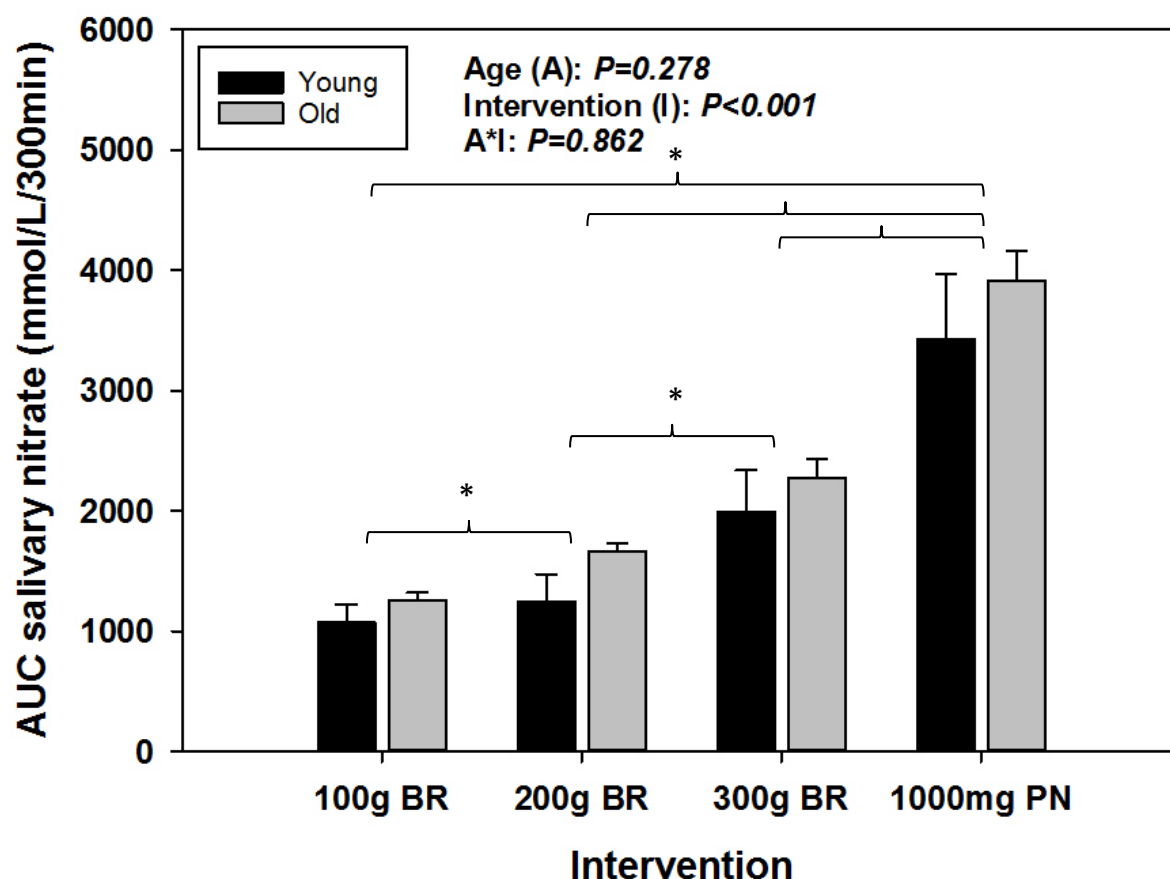


Figure 2.11: Mean (\pm SEM) area under the curve (AUC) for salivary nitrate levels in the young and the old groups; $n = 24$. Data analysed using mixed model repeated measures ANOVA (intervention as within-subjects factor and age as between-subjects factor). BR = beetroot, PN = potassium nitrate positive control. * denotes significant difference between interventions for both age groups.

2.3.6 Salivary nitrite

Young

There was a significant effect of time ($P<0.001$) and intervention ($P<0.001$) on salivary nitrite levels in the young and an interaction between the two factors ($P=0.04$), as shown in Figure 2.12. Pairwise comparisons identify higher levels following PN compared to 100g beetroot (+460.4 μ mol/L; $P=0.03$; 95%CI: 41.1, 879.7)

and compared to 200g beetroot ($+425.1\mu\text{mol/L}$; $P=0.005$; 95%CI: 122.2, 727.9). Nitrite levels were significantly higher at all time points over the five-hour period than baseline across interventions. As with salivary nitrate, there was no difference between salivary nitrite levels at baseline and at 12 hours. 100g beetroot did not significantly raise nitrite levels over time but 200g ($+422.9\mu\text{mol/L}$; $P=0.008$; 95%CI: 92.3, 753.5) and 300g beetroot ($+617.3\mu\text{mol/L}$; $P=0.01$; 95%CI: 116.4, 1118.1) did. Over the 300-minute period, salivary nitrite levels rose by 381%, 308%, 564%, and 573% from baseline following 100g, 200g, 300g beetroot and PN, respectively.

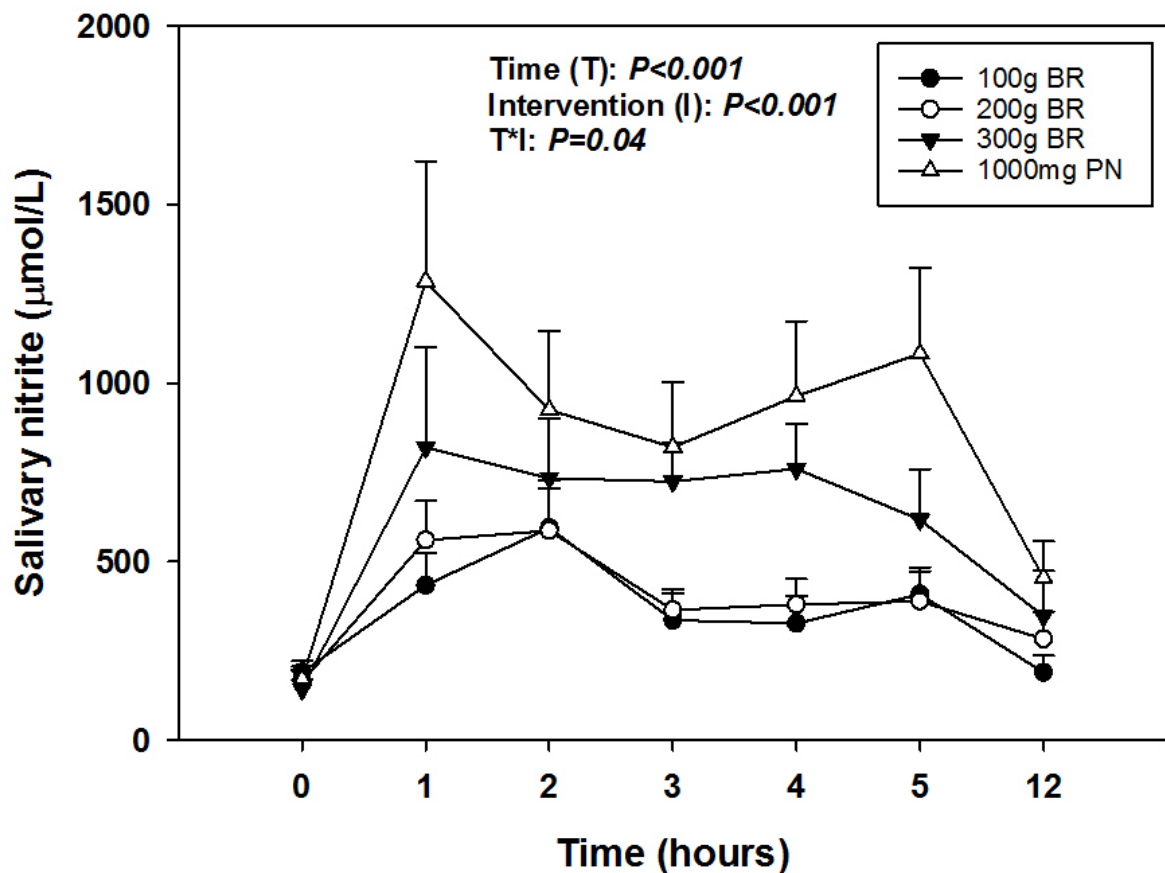


Figure 2.12: Mean (\pm SEM) salivary nitrite levels over 12 hours following incremental doses of nitrate in the young group; $n = 12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate positive control.

Old

There was a significant effect of time ($P<0.001$) and intervention ($P=0.004$) on salivary nitrite levels in the old group and an interaction between the two factors ($P=0.01$) (Figure 2.13). There was no difference between 100g and 200g beetroot but

levels following 100g were significantly lower than following 300g ($-215.4\mu\text{mol/L}$; $P=0.05$; 95%CI: $-433.6, 2.8$) and following PN ($-426.3\mu\text{mol/L}$; $P=0.008$; 95%CI: $-747.2, -105.5$). Contrary to what was seen in the young group, salivary nitrite levels did not return to baseline after 12 hours; levels were $289.5\mu\text{mol/L}$ higher than baseline ($P=0.003$; 95%CI: $93.1, 485.9$) for all interventions combined. 100g beetroot did not significantly raise nitrite levels over the five-hour period but there was a noteworthy trend towards an increase in nitrite following 200g beetroot ($+446.9\mu\text{mol/L}$; $P=0.06$; 95%CI: $-11.4, 905.3$). Levels were raised at multiple time points following 300g beetroot and at all time points following PN. Over the 300-minute period, salivary nitrite levels were 206%, 466%, 544%, and 860% higher than baseline following 100g, 200g, 300g beetroot and PN, respectively.

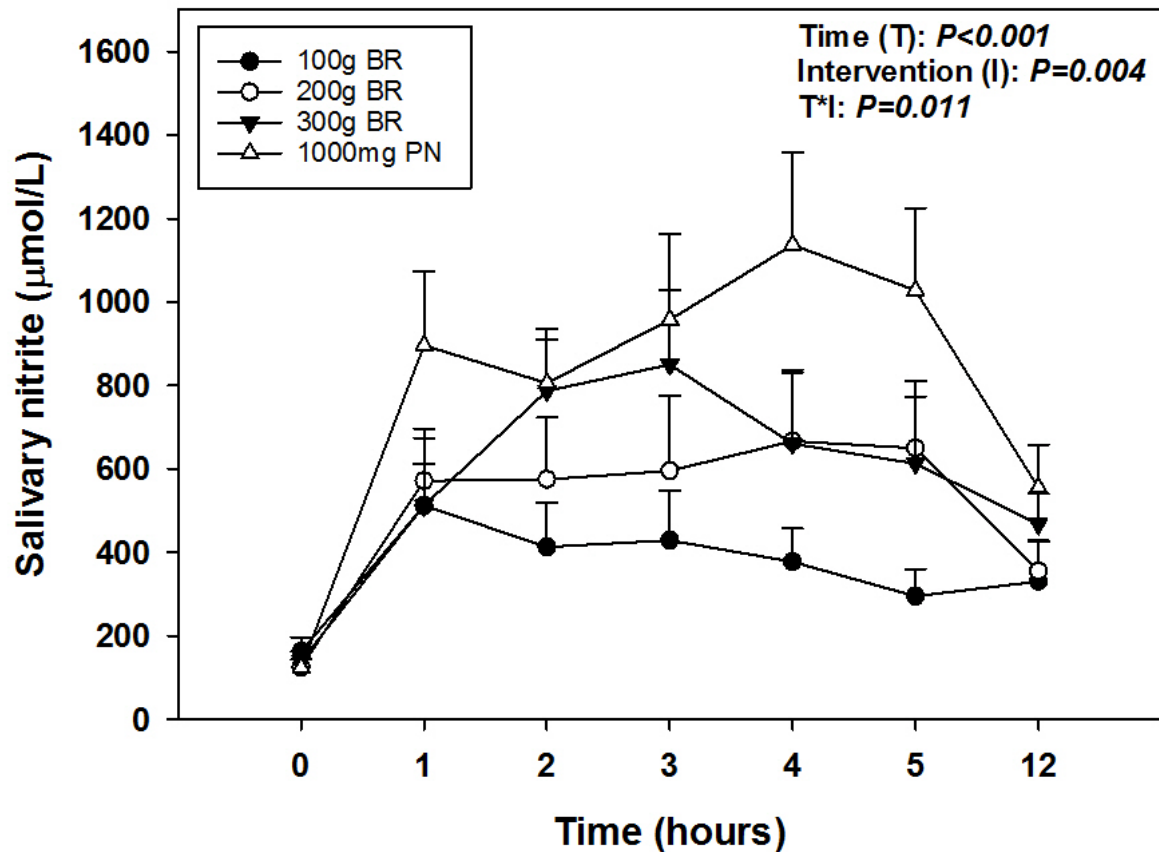


Figure 2.13: Mean (\pm SEM) salivary nitrite levels over 12 hours following incremental doses of nitrate in the old group; $n = 12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate positive control.

AUC

There was no effect of age ($P=0.74$) but a significant effect of intervention on AUC over the 300-minute period for salivary nitrite ($P<0.001$), as shown in Figure 2.14. There was no interaction between intervention and age ($P=0.74$). The AUC over the 300-minute period was significantly larger following PN than 100g beetroot (+293492.5; $P<0.001$; 95%CI: 137490.6, 449494.4), 200g beetroot (+229182.5; $P<0.001$; 95%CI: 92273.5, 366091.5) and 300g beetroot (+152418.8; $P=0.03$; 95%CI: 13240.5, 291596.9). The AUC following 300g was also significantly larger than following 100g beetroot (+141073.8; $P=0.01$; 95%CI: 22463.6, 259683.9).

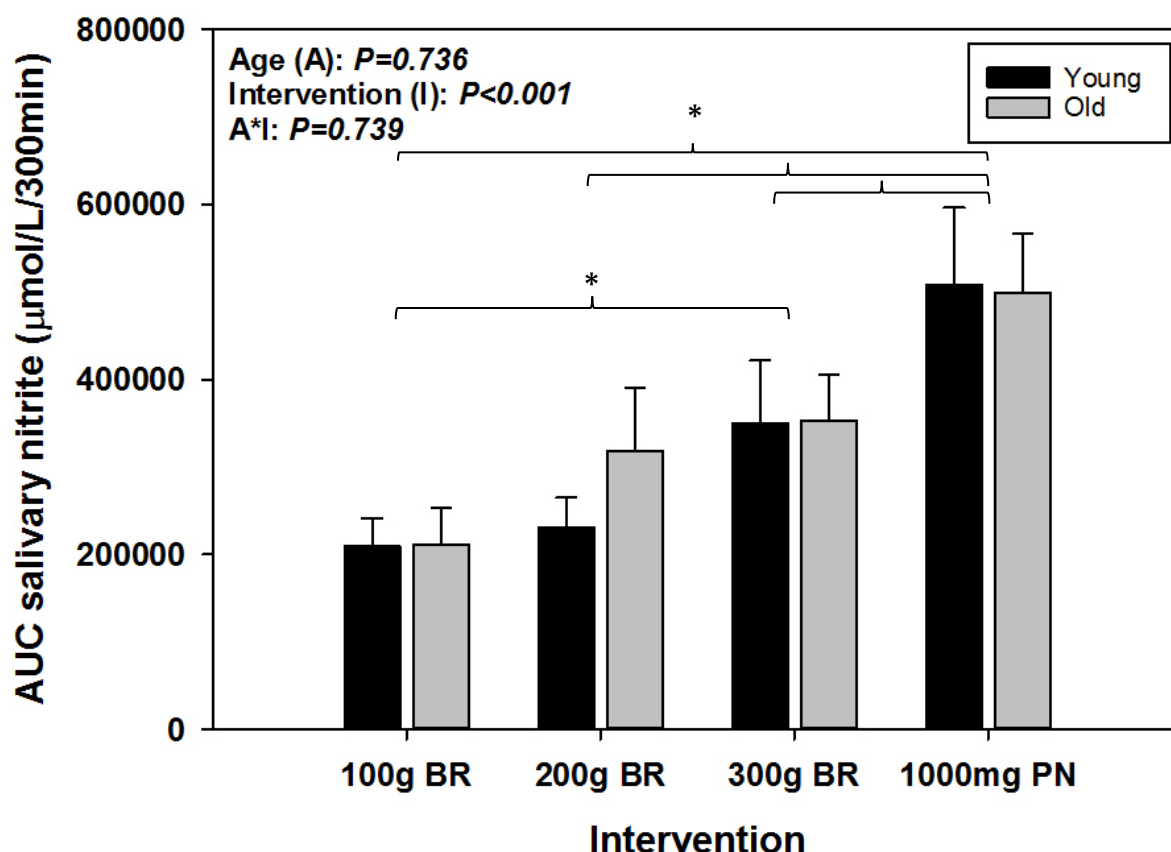


Figure 2.14: Mean (\pm SEM) area under the curve (AUC) for salivary nitrite levels in the young and the old groups; $n = 24$ (12 young and 12 old). Data analysed using mixed model repeated measures ANOVA (intervention as within-subjects factor and age as between-subjects factor). BR = beetroot, PN = potassium nitrate positive control. * denotes significant difference between interventions across both age groups.

2.3.7 Urinary nitrate

Presented here are the urinary nitrate concentrations and total nitrate excretion for both age groups. Nitrate excretion for both age groups following each intervention can be seen in Appendix H.

Concentrations - young

There was a significant effect of time ($P<0.001$) and intervention ($P<0.001$) on urinary nitrate concentrations in the young (Figure 2.15). There was also an interaction between the two factors ($P=0.003$). Pairwise comparisons indicate that there were lower concentrations compared to PN after 100g beetroot (-1.8mmol/L ; $P=0.002$; 95%CI: $-2.9, -0.6$) and after 200g beetroot (-1.3mmol/L ; $P=0.01$; 95%CI: $-2.4, -0.3$), demonstrating a dose response to larger amounts of nitrate. Urinary nitrate concentration was higher than baseline at each other time point measured, but there

was no difference between concentrations at 3-6, 6-12 or 12-24 hours. 0-3 hours post-nitrate ingestion, urinary nitrate concentrations were significantly higher following PN than following 100g beetroot (+1.3mmol/L; $P=0.003$; 95%CI: 0.5, 2.2) and following 200g beetroot (+1.0mmol/L; $P=0.02$; 95%CI: 0.1, 1.9). At 3-6 hours and 12-24 hours there were no differences between the interventions, but levels following PN were significantly higher than 100g beetroot at 6-12 hours post-ingestion (+3.3mmol/L; $P=0.008$; 95%CI: 0.8, 5.8).

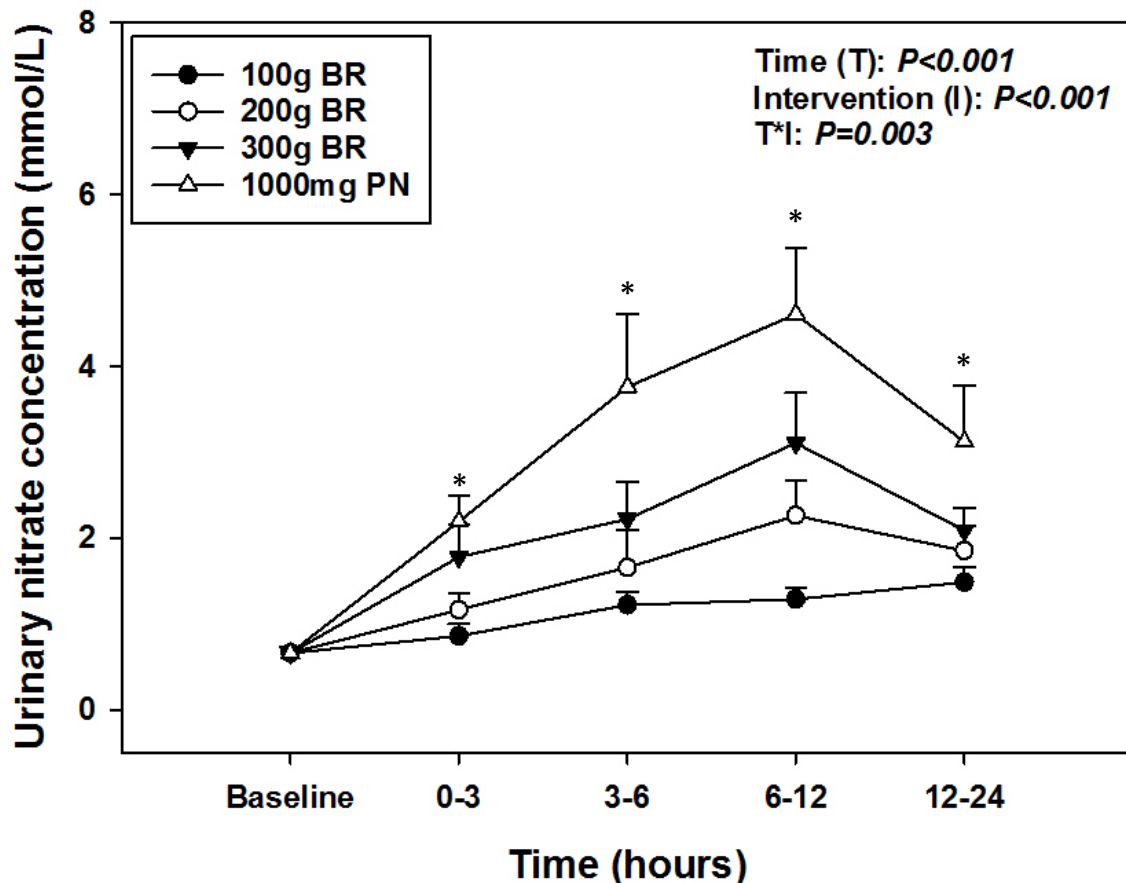


Figure 2.15: Mean (\pm SEM) urinary nitrate concentrations over 24 hours following incremental doses of nitrate in the young group; $n = 12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate positive control. * denotes significant difference from baseline across nitrate doses.

Old

There was a significant effect of time ($P<0.001$) and intervention ($P<0.001$) on urinary nitrate concentrations in the old group and a significant interaction between the two factors ($P<0.001$) (Figure 2.16). Similar to the young group, urinary nitrate concentrations in the old group were lower for 100g (-3.1mmol/L; $P<0.001$; 95%CI: -

4.5, -1.7) and 200g beetroot (-2.6mmol/L; $P=0.001$; 95%CI: -4.0, -1.1) than for PN. Nitrate levels were significantly higher than baseline for all other time points measured (all $P<0.05$).

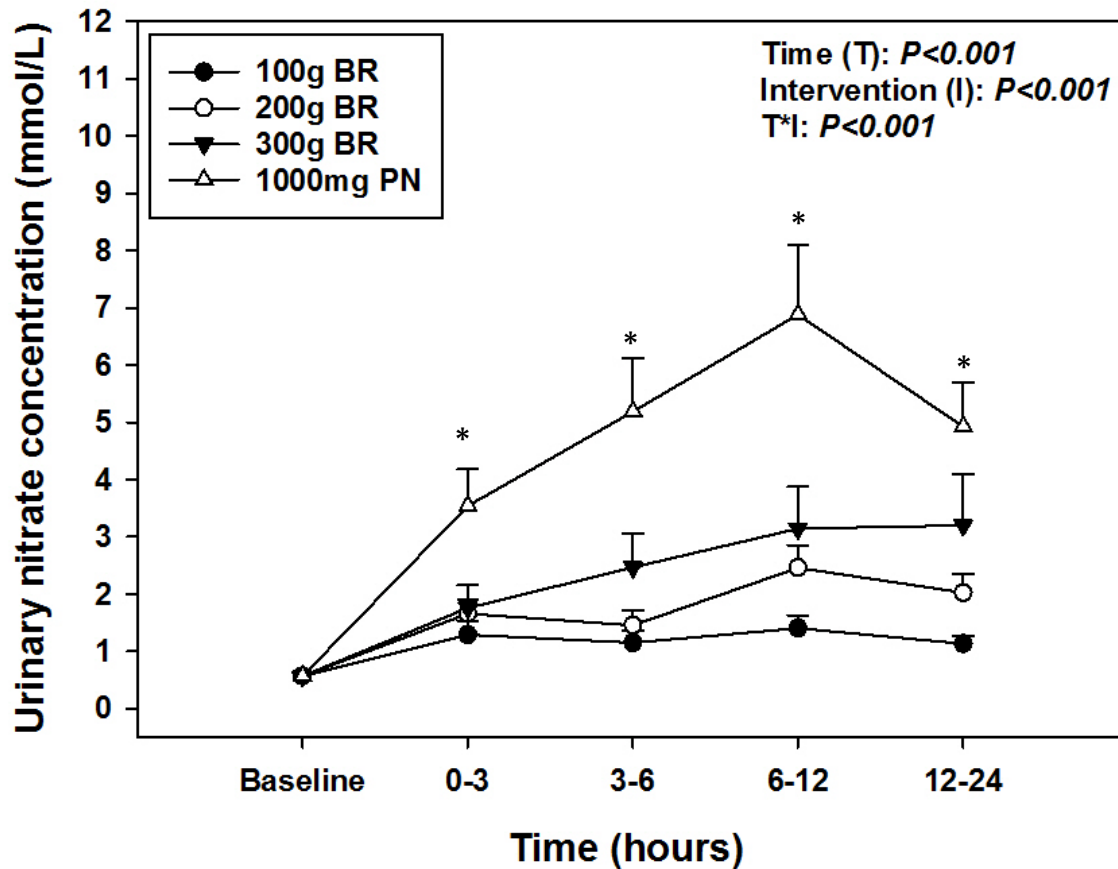


Figure 2.16: Mean (\pm SEM) urinary nitrate concentrations over 24 hours following incremental doses of nitrate in the old group; $n = 12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate positive control. * denotes significant difference from baseline across nitrate doses.

Total nitrate excretion and nitrate recovery

There was no effect of age ($P=0.74$) but a significant effect of intervention on total urinary nitrate excretion ($P<0.001$), as shown in Figure 2.17A. There was no interaction between age and intervention ($P=0.44$). Pairwise comparisons show that total nitrate excretion was significantly higher following PN than following 100g beetroot (+302.3mg; $P<0.001$; 95%CI: 203.9, 400.8), 200g beetroot (+256.6mg; $P<0.001$; 95%CI: 144.7, 368.5) and 300g beetroot (+162.2mg; $P=0.03$; 95%CI: 11.9, 312.4), demonstrating a dose response across both groups. Excretion was also higher following 300g beetroot than 100g (+140.2mg; $P=0.01$; 95%CI: 27.3, 253.1).

Urinary nitrate recovery was calculated as a percentage of the ingested nitrate excreted in 24 hours for each intervention. There was no effect of age ($P=0.35$) but a significant effect of intervention ($P=0.009$) on urinary nitrate recovery. There was no interaction between intervention and age ($P=0.21$) (Figure 2.17B). Pairwise comparisons show that recovery was significantly greater following 100g beetroot than 200g beetroot (+17.7%; $P=0.04$; 95%CI: 0.6, 34.7) but there were no other significant differences between interventions. Recovery after 100g beetroot was significantly greater than 200g beetroot in the young group (+22.9%; $P=0.05$; 95%CI: -0.1, 45.9) but there were no significant differences in the old group.

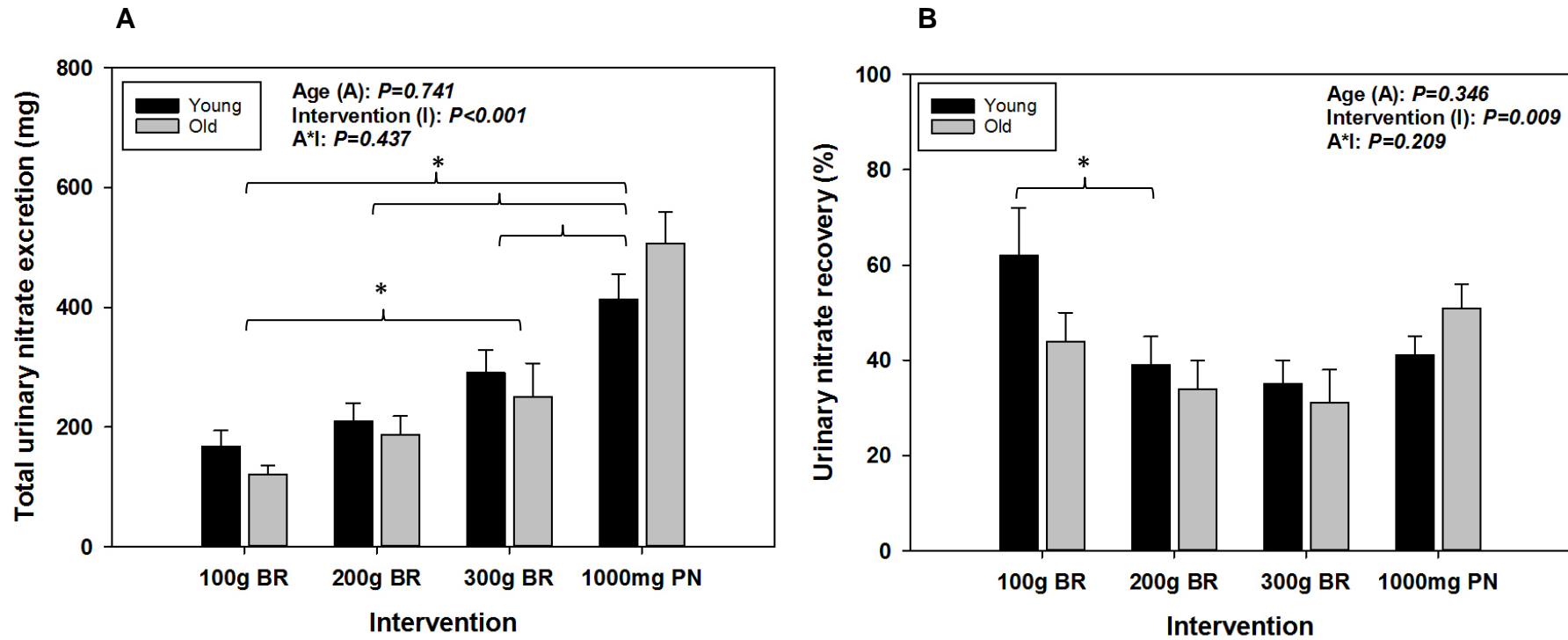


Figure 2.17: Mean (\pm SEM) total urinary nitrate excretion (A) and urinary nitrate recovery (B) over 24 hours following incremental doses of nitrate in the young and old groups; $n = 24$ (12 young and 12 old). Statistical analysis using mixed model repeated measures ANOVA (intervention as within-subjects factor and age as between-subjects factor). BR = beetroot, PN = potassium nitrate positive control. * denotes significant difference between interventions.

2.3.8 FeNO

Young and old participants had statistically similar FeNO values at baseline of 30.92ppb and 22.17ppb, respectively ($P=0.25$). The ICC values of 0.96 and 0.92 demonstrate that, for the young and the old respectively, there was consistency across baseline values for each intervention.

Young

There was a significant time effect ($P<0.001$) on FeNO levels but no difference between interventions ($P=0.51$), as shown in Figure 2.18. There was no interaction between time and intervention ($P=0.23$). Pairwise comparisons show that levels rose significantly from baseline levels at the early (+8.9ppb; $P=0.01$; 95%CI: 2.0, 15.8), mid (+9.6ppb; $P=0.001$; 95%CI: 3.9, 15.3), and late (+10.1ppb; $P<0.001$; 95%CI: 4.9, 15.1) time points, demonstrating increasing FeNO levels over time. Over the 300-minute period, FeNO levels rose by 32%, 58%, 54%, and 70% following 100g, 200g, 300g beetroot and PN, respectively.

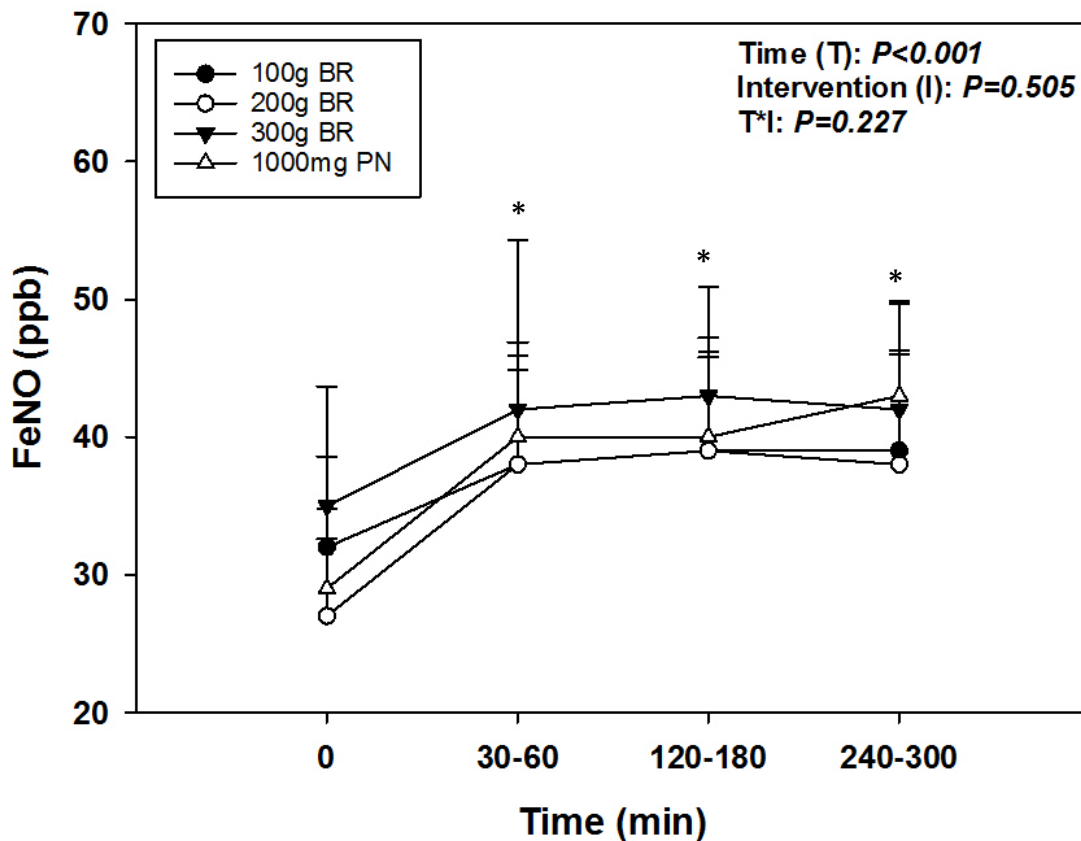


Figure 2.18: Mean (\pm SEM) exhaled nitric oxide (FeNO) levels over 300 minutes in young group given incremental doses of nitrate; $n = 12$. Data analysed with a two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate positive control. * denotes significant difference to baseline across nitrate doses.

Old

There was a significant time effect in the old group ($P < 0.001$) but no significant effect of intervention ($P = 0.11$) (Figure 2.19). Pairwise comparisons show that FeNO levels significantly rose from baseline at all time points (all $P < 0.05$). There was no significant interaction between time and intervention ($P = 0.18$). Over the 300-minute period FeNO levels rose by 36%, 46%, 77%, and 61% following 100g, 200g, 300g beetroot and PN, respectively.

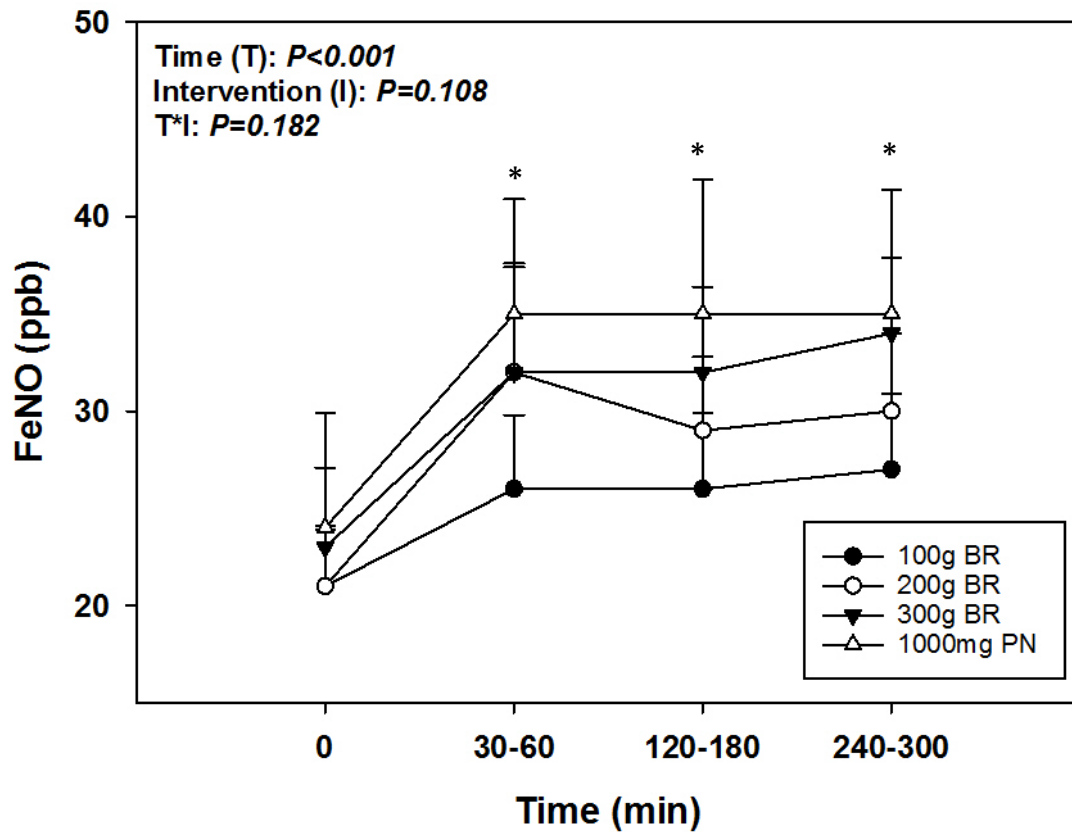


Figure 2.19: Mean (\pm SEM) exhaled nitric oxide (FeNO) levels over 300 minutes in old group given incremental doses of nitrate; $n = 12$. Data analysed with a two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate positive control. * denotes significant difference to baseline across nitrate doses.

AUC

The AUC over the 300-minute period shows a noteworthy trend for an effect of intervention ($P=0.07$) but no difference between age groups ($P=0.28$) (Figure 2.20). There was no interaction between intervention and age ($P=0.60$). *Post hoc* analyses identify only a trend towards a lower AUC following 100g beetroot than PN (+2426.3; $P=0.09$; 95%CI: -260.6, 5113.1) but no other differences between interventions.

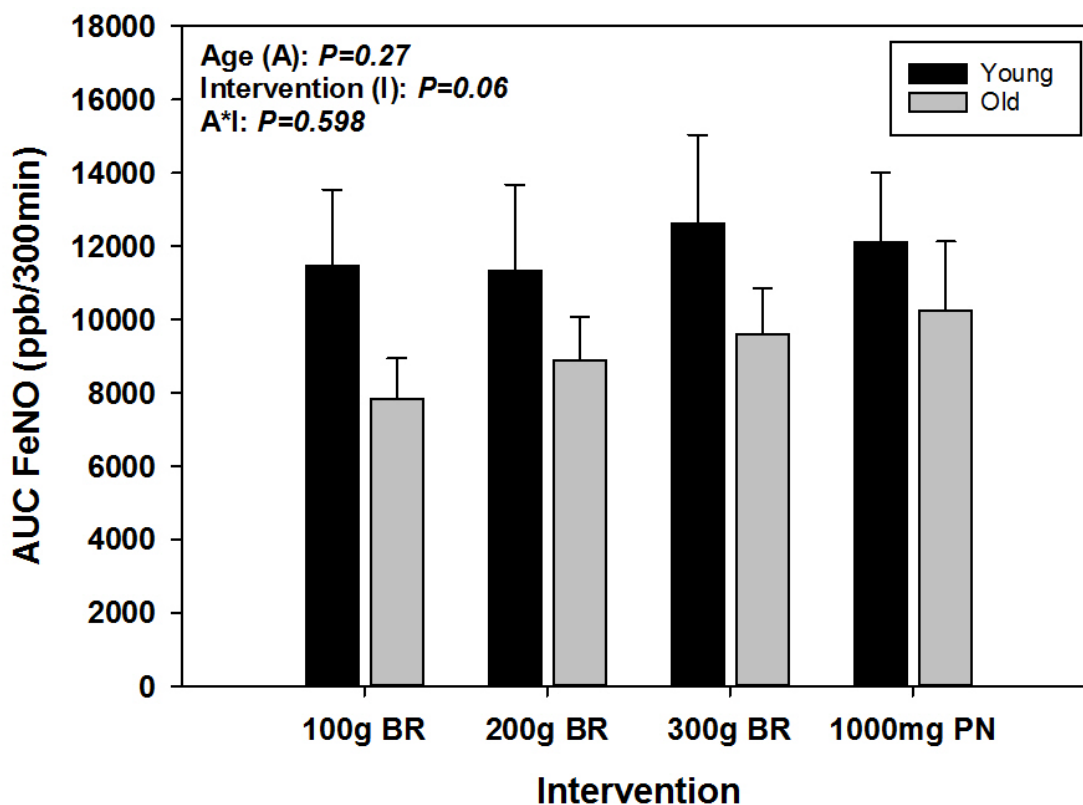


Figure 2.20: Mean (\pm SEM) area under the curve (AUC) over 300 minutes for exhaled nitric oxide (FeNO) in the young and old groups; $n = 24$ (12 young and 12 old). Data analysed using mixed model repeated measures ANOVA (intervention as within-subjects factor and age as between-subjects factor). BR = beetroot, PN = potassium nitrate positive control.

2.3.9 Correlation between plasma nitrate and nitrite and FeNO

A Pearson product-moment correlation coefficient was calculated to assess the relationship between changes in plasma nitrate and nitrite levels and changes in FeNO. Across both age groups, there was no correlation between changes in plasma nitrate and changes in FeNO ($r=0.17$; $n=288$; $P=0.005$) or between changes in plasma nitrite and changes in FeNO ($r=0.11$; $n=288$; $P=0.07$).

In the young group, there was no correlation between changes in plasma nitrate and changes in FeNO ($r=0.13$; $n=144$; $P=0.13$) or between plasma nitrite and FeNO ($r=0.08$; $n=144$; $P=0.33$), as demonstrated in Figure 2.21A. In the old group, there was a small, positive correlation between plasma nitrate levels and FeNO ($r=0.22$; $n=144$; $P=0.007$). There was no correlation between plasma nitrite and FeNO ($r=0.15$; $n=144$; $P=0.08$). This is shown in Figure 2.21B.

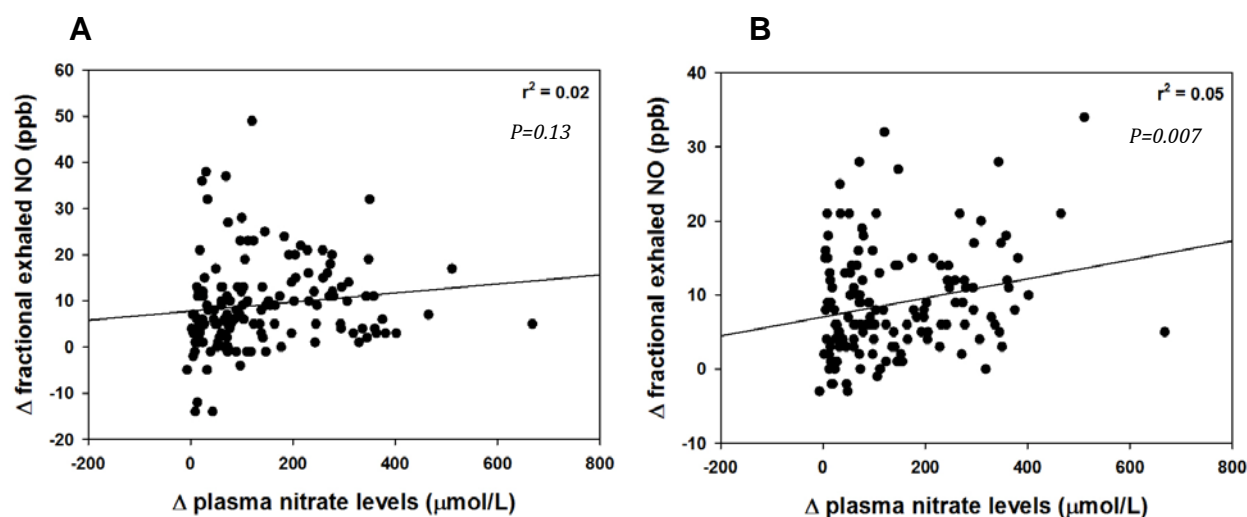


Figure 2.21: Scatterplot showing correlation between change in plasma nitrate levels and change in exhaled nitric oxide (FeNO) in the young group (A); $n = 144$ and old group (B); $n = 144$. Statistical analysis using Pearson's coefficient.

2.4 Discussion

2.4.1 Summary of the findings

This is the first study to investigate the pharmacokinetics of nitrate and nitrite following incremental doses of whole beetroot in both younger and older healthy participants. The main findings were: 1) whole beetroot can raise plasma nitrate and nitrite levels in both the young and the old; 2) despite comparable if not higher plasma nitrate levels to the young group, older adults had lower levels of plasma nitrite following nitrate loads; and 3) FeNO was raised following beetroot consumption and to comparable levels in the young and old participants. These results together suggest that a small dose of whole beetroot, just above what is considered a normal vegetable portion of 80g, is effective at raising plasma nitrite levels, implying that NO production can also be raised by this food. This response is more pronounced in a young population. What is unclear is whether these plasma nitrite levels are high enough to elicit physiological benefits such as a reduction in blood pressure and this is discussed in Chapter 3.

2.4.2 The pharmacokinetics of nitrate and nitrite from whole beetroot

Plasma

Baseline values for both nitrate and nitrite demonstrate adherence to the dietary restrictions in place in the two days before each visit. 100g of the whole beetroot used in this study was found to contain approximately 272mg of nitrate, which is close to the average 250mg described by Hord, Tang and Bryan in 2009 and the range described by van der Avoort *et al.* (2018) as 35 to 258mg/100g. As mentioned previously, the nitrate content of beetroot can vary during the growing season due to a number of environmental factors, however the above average values were used for analysis purposes in this study. Results show that the smallest dose of beetroot, 100g, significantly raised plasma nitrate levels in both the young and the old participants, from 28 to 86 μ mol/L and from 25 to 128 μ mol/L, respectively, demonstrating the efficient absorption of nitrate from the whole vegetable.

The pharmacokinetics of dietary nitrate through the nitrate-nitrite-NO process are well known in young adults but the age-related factors mentioned in Chapter 1, such as changes to the oral microbiome, are likely to alter the process in older adults. One of the first groups to assess the pharmacokinetics of dietary nitrate in older adults was Miller *et al.* (2012), who found that a high-nitrate diet providing a maximum of 77mg nitrate in a single bolus was not sufficient to increase plasma nitrate and nitrite levels. Their high nitrate diet included foods such as bacon, spinach, beetroot and green vegetables, totalling a daily nitrate intake of ~155mg spread throughout the day. Previously, Presley *et al.* (2011) demonstrated that a high-nitrate diet significantly increased plasma nitrate and nitrite levels in older adults. The diet itself contained 242mg nitrate but this was supplemented by 500ml beetroot juice containing 527mg nitrate, taking the overall nitrate content of the diet to 769mg. Results from the present study show that 300g beetroot elicited the highest rise in plasma nitrate levels compared to 100g by 2.3- and 1.7-fold in the young and the old, respectively. Furthermore, plasma nitrite levels following 200g and 300g beetroot were around 1.4- and 1.8-fold higher than following 100g. These data together suggest a dose-dependent response to increasing nitrate intakes on plasma nitrate and nitrite levels

It has previously been described that nitrate in the form of a potassium nitrate capsule, equivalent to 248mg, can significantly raise plasma nitrate and nitrite levels and this was deemed the minimum dose required to exert beneficial effects on blood pressure (Kapil *et al.*, 2010). The results of the plasma nitrate analysis in the present study demonstrate that levels were raised following a single bolus of 272mg whole beetroot in both a young and an old population. Plasma pharmacokinetic data for nitrate and nitrite following 300g beetroot in the present study mirror those of other studies supplementing with potassium nitrate capsules or beetroot juice (Kapil *et al.*, 2010; Shepherd *et al.*, 2015). Plasma nitrate levels peaked between two and three hours post-ingestion of all doses of beetroot, while PN ingestion caused an earlier peak around one hour. Although not all time points were analysed, these peaks are thought to accurately reflect the pharmacokinetic profile of nitrate based on previous studies using beetroot (Clifford *et al.*, 2017; van Velzen *et al.*, 2008). The difference in time to peak for PN most likely reflects the body's ability to process a nitrate solution faster than nitrate from a whole vegetable. Plasma nitrite peaked later at around five hours, demonstrating the utilization of the salivary pathway in the breakdown of nitrate to nitrite after first reaching the blood. There was no difference in peak time between beetroot and PN for plasma nitrite. This comparable pharmacokinetic profile reveals that beetroot in its whole form, and at amounts equivalent to a standard vegetable dose, is a rich source of available dietary nitrate. Furthermore, it is clear that the food matrix, including dietary fibre for example, is not a limiting factor in the availability of dietary nitrate from whole beetroot. The pharmacokinetic profile of nitrate and nitrite following beetroot consumption, including time to peak, is a useful tool in identifying the timing of physiological effects associated with the ingestion of these compounds.

Saliva

25% of the nitrate that reaches the blood from dietary nitrate ingestion is transported to the salivary glands, while 75% is excreted in the urine. Lundberg and Govoni (2004) describe that saliva is necessary for a rise in plasma nitrite, elegantly demonstrated through the interruption in swallowing saliva following nitrate ingestion. Salivary nitrate levels rose quickly in both age groups following 100g beetroot in the present study, peaking around one to two hours at 2.6mmol/L and 4.1mmol/L in the young and old, respectively. Levels peaked at 5.3mmol/L and 6.0mmol/L following

300g beetroot in the young and the old, respectively, again demonstrating the dose response to larger amounts of nitrate evident in plasma. Salivary nitrate levels appear higher in the older group than the younger group, although it was not found to be statistically different. Nitrate levels in the older group also remained significantly elevated following all beetroot doses, whereas levels in the young group fell below significance. This correlates with the higher plasma nitrate levels seen in the old compared to the young, although again this was not found to be a significant difference. This suggests heightened utilisation of dietary nitrate in older participants, potentially as a protective mechanism against the reduced ability to convert nitrate to nitrite.

It is evident from the results of nitrite analysis that 100g of beetroot i.e. a dose of 272mg nitrate is not sufficient to raise salivary nitrite levels in the young or the old, despite significantly raising salivary nitrate. This suggests that the small dose of nitrate does not sufficiently stimulate the nitrate-reducing bacteria located in the salivary glands to reduce salivary nitrate to nitrite. As early as 1976, it was determined that salivary nitrite levels were highly dependent on nitrate ingestion and there was a limit below 54mg in which salivary nitrate and nitrite levels did not change (Spiegelhalder *et al.*, 1976). Bacteria use nitrate and nitrite as final electron acceptors in respiration when it is available in the oral cavity (Spiegelhalder *et al.*, 1976), therefore a low availability from small nitrate doses may affect respiration pathways in the bacteria. It is estimated that 4-8% of the ingested nitrate is converted to nitrite in the oral cavity (van Velzen *et al.*, 2008). Higher doses of nitrate i.e. 200g beetroot and above were shown to increase salivary nitrite levels in both age groups, which suggests that these nitrate doses are able to stimulate nitrate to nitrite reduction in the saliva.

It has been shown that there is a continuous uptake of nitrate from the blood to the salivary glands and although the exact mechanism behind it is not known, the protein sialin may have a role (Qu *et al.*, 2016). Sialin has been found to be a nitrate transporter, mediating nitrate influx into the salivary glands where it can be processed by nitrate-reducing bacteria (Qu *et al.*, 2016). Although the analysis is beyond the scope of this thesis, saliva samples collected are being used for further

research into sialin and age-related differences in the uptake of nitrate to the salivary glands.

Urine

Nitrate from whole beetroot is absorbed from the gut into the systemic circulation and 75% of the consumed nitrate is excreted in the urine, unprocessed. Nitrate was found in the urine within three hours following consumption of beetroot in the present study and tended to peak between six and 12 hours, which correlates with evidence in the literature (Pannala *et al.*, 2003). Pannala *et al.* (2003) found that urinary nitrate levels peaked at four to six hours following the ingestion of ~222mg nitrate, which is similar to the dose provided by 100g beetroot in the present study. Nitrate concentrations remained elevated at 12-24 hours post-ingestion in the present study, demonstrating that dietary nitrate had not been cleared within 24 hours. Dietary nitrate has a half-life of around eight hours and 60% of ingested nitrate has been found to be excreted in the urine within 48 hours (Jajja *et al.*, 2014); a longer sampling period in the present study may therefore have identified the time when levels returned to baseline. The greater excretion and lower retention of nitrate following the consumption of larger doses of nitrate suggest a tightly regulated negative feedback loop to maintain nitrate levels within a specific range. It may be that nitrate reserves become saturated and the body excretes excess amounts through urine. This is the first study to investigate incremental doses of nitrate on nitrate excretion in urine and future studies are warranted in this area.

2.4.3 Bioavailability

The pharmacokinetic profile of nitrate following beetroot consumption demonstrates bioavailability of nitrate from this source. van Velzen *et al.* (2008) demonstrated a bioavailability of $106 \pm 15\%$ from cooked beetroot in young adults, which provided 643mg nitrate. Another study to include whole beetroot in its measurements was that by Clifford *et al.* (2017), who found that NO levels, measured through nitrate and nitrite concentrations, were raised following the consumption of 300g whole beetroot in young, healthy males. The present study shows that dietary nitrate from whole beetroot is available to the body in older adults using a direct comparison to a younger population.

Dietary nitrate exerts its beneficial effects on the body through its breakdown to nitrite and further to NO, which has a vasodilatory effect on the vascular system. The significant increase in plasma nitrite levels following doses of beetroot demonstrates that commensal bacteria in the saliva are converting nitrate to nitrite and raising plasma nitrite concentrations on swallowing. As nitrite levels are a marker for NO production in the body, this demonstrates that whole beetroot, even in small amounts, can increase NO production and therefore have a positive effect on the vascular system.

Clifford *et al.* (2017) found that NO concentration peaked at two hours post-ingestion of beetroot and returned to baseline within five hours. The present study found a later peak time of around five hours post-beetroot ingestion for nitrite levels. Furthermore, plasma nitrite levels remained elevated above baseline at the five-hour period following all doses of beetroot, which suggests that NO levels would also remain elevated for a longer period. The differences seen here may be due to the analysis techniques used, as Clifford *et al.* (2017) did not use a stop solution to preserve plasma nitrite, which may have underestimated the peak nitrite levels.

Although bioavailable to the body, dietary nitrate from smaller doses of beetroot may not have physiological effects. For example, Dejam *et al.* (2007) found that plasma nitrite levels of 350nm and above increased forearm blood flow and this level was not reached by beetroot amounts below 300g in the old group and below 200g in the young group. However, 300g beetroot was found to raise plasma nitrite to physiologically beneficial levels in both the young and the old group, at least for forearm blood flow. A blood pressure-lowering effect was seen with a nitrate intake of ~248mg in a study by Kapil *et al.* (2010b). This nitrate intake can be achieved through the consumption of 300g beetroot but not by smaller doses than this. The physiological responses to dietary nitrate from beetroot are described in Chapter 3.

2.4.4 Fractional exhaled nitric oxide (FeNO)

NO can be formed from nitrite in the oral cavity and stomach (Olin *et al.*, 2001) and elevated FeNO levels following a nitrate load demonstrate this pathway. There is large variation in normal baseline FeNO, with values described between 3 and 88ppb

(Dweik, 2011) and an average of 18ppb (Dressel *et al.*, 2008; Travers *et al.*, 2007). FeNO values in the present study can be described as normal, although there is not a reliable reference range for older adults. A number of studies have identified gender differences in FeNO (Taylor *et al.*, 2007; Travers *et al.*, 2007) but it is out with the scope of this thesis to assess FeNO at this level. Other factors that influence FeNO, such as smoking status, were controlled for during the screening process.

Each of the beetroot doses in the present study raised FeNO levels within one hour of consumption and these levels remained elevated over the five-hour intervention period. There was no difference between the interventions, showing that a small amount of beetroot, providing 272mg nitrate, has the ability to increase NO levels in exhaled air. Vints *et al.* (2005) tracked exhaled NO over 20 hours following a meal providing 230mg nitrate and demonstrated a 60% rise in exhaled NO after two hours in participants aged between 24 and 61 years. The percentage increase in FeNO between 32% and 36% following the consumption of 272mg nitrate in the present study was smaller than that seen by Vints *et al.* (2005) but levels were still above baseline five hours post-ingestion, so it is unclear how long levels remained elevated for and if they continued to rise. A longer intervention period is therefore required to show the full extent of the effects. Larger doses of beetroot and the PN led to larger percentage changes in FeNO in both the young and the old group, reaching a maximum of 70% following PN in the young and 77% following 300g beetroot in the old. Changes in FeNO following PN in both age groups are larger than those demonstrated by Olin *et al.* (2001) following a meal containing 1g nitrate; a 47% increase was seen. This is likely to be due to the different sources of nitrate i.e. potassium nitrate solution compared to a nitrate-rich vegetable meal.

Results from the present study demonstrate a small correlation between changes in plasma nitrate and FeNO levels in the old group. The trend towards higher plasma nitrate levels in the old group than the young group may explain this correlation and demonstrates an efficient breakdown of dietary nitrate to NO in the oral cavity. Furthermore, although there was no significant difference between young and old values for FeNO, there appears to be correlation between higher nitrate doses and smaller differences between the groups. The graph for 100g beetroot shows a large difference between the young and the old, whereas the PN graph shows the lines for

each group converging. The mean difference between FeNO values for the young and the old groups following 100g of beetroot is 12ppb, while the mean difference following PN is 6ppb. This suggests that larger doses of nitrate bring the FeNO of younger and older adults to a more comparable value, which may be more evident with a larger sample size.

2.4.5 The effect of age on nitrate and nitrite levels

There was a trend towards higher plasma nitrate levels in the old group than the young group following PN consumption but not following beetroot consumption. It could be that reduced functioning of the nitrate-nitrite-NO breakdown pathway stimulates a rise in plasma nitrate levels in a compensatory manner in older adults. Despite comparable plasma nitrate levels across the interventions, particularly following beetroot consumption, plasma nitrite levels were significantly lower in the older group than the younger group. The conversion of nitrate to nitrite occurs in the mouth and therefore plasma levels of nitrite are only increased following swallowing; this suggests that age differences lie in the oral conversion of nitrate to nitrite. However, salivary nitrate and nitrite levels are similar in the young and the old groups following the ingestion of nitrate, which contradicts the hypothesis of impaired nitrate-nitrite breakdown in the older group. 100g beetroot was not sufficient to raise salivary nitrite levels in the young or the old but the young responded to 200g beetroot more favourably than the old group. This implies that larger nitrate doses are required to elicit responses in an older group compared to a younger group. The majority of nitrite, ~70%, is produced in the body from endogenous NO synthase-derived NO and the lower plasma nitrite levels seen in the old group could be due to a reduced background nitrite production and storage pool. The oxidation of NO to nitrite is catalysed by ceruloplasmin in the plasma or cytochrome c oxidase in tissues (Shiva, 2013) but lower levels of NO, which is evident in older age, may result in lower circulating nitrite. An independent t-test demonstrates that baseline plasma nitrite levels were on average 24nmol/L lower in the old group than the young group ($P=0.08$), suggesting impairment in endogenous nitrite production. Although the pharmacokinetic profile shows that the time of peak plasma nitrite levels were the same in the young and the old, the lower baseline nitrite and lower amounts

produced from dietary nitrate in the old group in this study allude to an overall mechanistic impairment compared to younger adults.

Ultimately, plasma nitrite provides a marker for NO production and therefore lower levels of plasma nitrite in the old group suggests that NO production following ingestion of dietary nitrate is lower in the older group compared to the younger group. Although age-related differences in plasma nitrite levels have been demonstrated, there was no difference in FeNO between the age groups. Baseline levels were comparable, although the older group had an ~8ppb lower value than the young group; the sample size here may have been too small to identify significant effects. All doses of nitrate significantly raised FeNO levels over time in both age groups, demonstrating that dietary nitrate from whole beetroot is bioavailable and it can impact on NO levels in both the young and the old. More research is warranted into the production of nitrite from both dietary nitrate and endogenous NO into older age, as it is evident that there is a reduced efficiency in one or other of these pathways.

2.4.6 Strengths and limitations

This study investigated, for the first time, supplementation with practical amounts of whole beetroot and the bioavailability of dietary nitrate from this food source in both young and old adults. The dietary restrictions and seven-day washout period between trials ensured no carry-on effect of nitrate-rich foods consumed before testing or of previous trials. It has been demonstrated that concentrations of plasma nitrate and nitrite return to baseline levels around 24 hours following consumption of dietary nitrate (James *et al.*, 2015). Furthermore, the stringent screening process ensured that disease states or medications that may have confounded the effects of the interventions on the nitrate-nitrite-NO cycle were screened out. Kidney function was not assessed in relation to urinary excretion but exclusion criteria also ensured that participants had no history of kidney disorders.

The addition of salivary nitrate and nitrite and urinary nitrate measurement to measurement of plasma concentrations of these compounds benefits the body of research on the pharmacokinetics of dietary nitrate from whole beetroot in an older population. Furthermore, nitrite is unstable in whole blood, so rapid processing and

the addition of a stop-solution ensured that analysis was reliable for the measurement of nitrite. Previous studies have highlighted omission of this step as a limitation in their analysis (Ashor *et al.*, 2016; McIlvenna *et al.*, 2017). This study also utilised ozone-based chemiluminescence, which is acknowledged as the gold standard method and the most sensitive technique available for the measurement of NO and its metabolites (Pinder *et al.*, 2008).

The small sample size limits the application of this study and the conclusions that can be drawn from it. However, the crossover design and inclusion of both a young and an older participant group of equal size allowed for direct comparison and assessment of the impact of age on dietary nitrate processing. The nitrate content of commercially available beetroot varies and it was not possible to ensure that all beetroot given to participants was from one batch. Purchasing beetroot on a per-participant basis therefore meant that the nitrate content of the beetroot could have varied across participants. However, the beetroot purchased came from the same supplier and all trials were conducted within a small a timeframe as possible to reduce the impact of nitrate variability. Presenting the participants with beetroot meant that the trials were not blinded and measurements may have been unconsciously influenced by the allocation to the interventions. The crossover randomisation ensured a strict within-volunteer control of factors that may impact on outcomes, such as body composition and healthy status. Steps were taken to ensure that trials were replicated closely, bar the dose of nitrate provided, in an attempt to standardise the study visits. It is acknowledged that instructing participants to follow a restrictive diet throughout the study could have influenced the results. Therefore, future studies are needed to establish whether results can be replicated alongside the maintenance of habitual dietary intakes.

2.4.7 Conclusions

This study highlights age differences in utilisation of dietary nitrate from whole beetroot, evident from the lower levels of plasma nitrite observed in old participants compared to young. As plasma nitrite is a reliable marker for NO levels in the body, this suggests that NO production is lower in an older population following the consumption of beetroot when compared to a younger population. Despite this,

whole beetroot was found to be a source of bioavailable nitrate for both young and old participants but larger amounts, up to 300g, are required by older individuals to stimulate an increase in plasma nitrite. More research is warranted into the production of nitrite from both dietary nitrate and endogenous NO into older age, as it is evident that there is a reduced efficiency in one or other of these pathways. Subsequent research should now focus on whether whole beetroot, in practical amounts, has physiological effects on the body in both young and old participants.

Chapter 3. Physiological effects of acute beetroot supplementation in young and old participants

3.1 Introduction

Nitric oxide (NO) has a very short half-life but has a profound relaxant effect on vascular smooth muscle and has been found to inhibit platelet aggregation (Mellion *et al.*, 1981). Through its dilatory properties, NO regulates vascular tone and blood pressure (Ignarro, 2002) and this prompted investigation into NO availability and consequent impacts on blood pressure and blood flow. NO levels can be raised by the consumption of dietary nitrate, which is broken down to nitrite and further to NO in a stepwise process involving nitrate-reducing bacteria. The richest sources of dietary nitrate include beetroot, rocket and lettuce, and beetroot has received a lot of attention as an active intervention food. In 2008, Webb *et al.* first demonstrated blood pressure-lowering effects of beetroot ingestion in young, healthy volunteers, which led to further exploration into the manipulation of NO levels through the intake of nitrate-rich foods.

Beetroot juice, as a potent source of dietary nitrate, has been repeatedly found to reduce blood pressure and improve blood flow (Lara *et al.*, 2016; Siervo *et al.*, 2013). There has been, however, a wide variety of nitrate doses provided and a preponderance of research on young, healthy volunteers. The use of beetroot juice is widespread in dietary nitrate research, propelled by the recent development of a nitrate-free placebo juice. Young, healthy adults are at a low risk of cardiovascular disease (CVD) but are efficient in the breakdown of dietary nitrate to NO and have a greater endogenous production of NO than older adults (Ashor *et al.*, 2016). This population is therefore able to provide an elegant portrayal of the physiological effects of NO following dietary nitrate intake but their low risk of CVD make the practical application of supplementation less relevant. Older adults who are more at risk of CVD because of the ageing process provide a more suitable cohort in which to investigate the positive effects of dietary nitrate intake.

Although the majority of work on beetroot has focussed on juice form, there are many other ways to utilise the vegetable, such as in gel form (Morgado *et al.*, 2016),

incorporated into bread (Hobbs *et al.*, 2012), or as a whole vegetable. Beetroot in its whole form is underused and although nitrate levels vary depending on factors such as time of harvest, it provides a bioavailable source of nitrate, as well as dietary fibre. What is unclear to date is whether whole beetroot is an acceptable and practical form of dietary nitrate that can be consumed in an attempt to reduce blood pressure and improve blood flow in both young and older adults. This study explores the effects of incremental doses of whole beetroot on blood pressure and vascular function in young and old participants and investigates whether or not these are correlated with plasma biomarkers of NO production i.e. nitrate and nitrite. The acceptability of the beetroot doses is assessed in both groups in an effort to gain an understanding of compliance and applicability of whole beetroot consumption. It was hypothesised that the positive effect on blood pressure and microvascular function associated with nitrate would be reduced in the older population and that the response to the beetroot would be greater with higher quantities in both populations. To investigate these hypotheses a four-arm, crossover trial was set up, directly comparing the physiological responses to incremental doses of whole beetroot and a positive control in a young and an old group.

3.2 Methods

The participants, interventions, and study design and protocol for this chapter are the same as those described in Chapter 2.2. The study day protocol is illustrated in Figure 2.1 and involved the consumption of 100g, 200g, and 300g whole beetroot and a positive control in the form of potassium nitrate (PN). Two age groups were recruited for the direct comparison of physiological effects in a young and an old group; participants were aged 18-35 or 60-75 years. Participants followed a two-day low-nitrate diet before attending the research facility and were instructed to refrain from caffeine intake in these two days also. Participants were asked to avoid the use of anti-bacterial mouthwash and gum for the entirety of the study.

3.2.1 Blood pressure

Blood pressure and heart rate were measured using an automated pressure cuff (*Spot Vital Sign Device, Welch Allyn*). A cuff was placed on the opposite upper arm to the cannula used for blood sampling with the participant in a seated and comfortable position. Baseline measurements were taken three times with a one-

minute interval between each measurement, and one measurement was taken at each time point after that i.e. 30, 60, 90, 120, 180, 210, 240, 270, and 300 minutes. Measurements were concealed from the participants to ensure that their knowledge of their blood pressure readings did not influence later measurements. Time points were amalgamated into baseline (0min), early (30-90min), mid (120-210min) and late (240-300min) points to better capture changes in blood pressure after supplementation and improve graphical presentation of the results across the time points.

3.2.2 Post-occlusive reactive hyperaemia

A laser Doppler (*Moor LDF, Moor Instruments, Axminster, UK*) was used to assess cutaneous microvascular reactivity, which is based on the ability of the endothelium to release NO as a response to proximal arterial occlusion. After the end of occlusion, when the peripheral perfusion is restored, the immediate reduction of vascular resistance results in an increase in the blood flow i.e. post-occlusive reactive hyperaemia (PORH). This is closely linked to NO release and integrity of endothelial function (Carasca *et al.*, 2017). Reactive hyperaemia was chosen for its sensitivity to external stimuli and the gold standard, PWV, was deemed unsuitable due to the acute nature of the study; FMD was not available to the research team. Previous work has shown within-subjects coefficients of variation from 6-11% for reactive hyperaemia (Minson and Wong, 2004). The measurement lasted 12 minutes and took place with participants lying supine in a 22-24°C temperature-controlled room. A pressure cuff was placed on the participants' upper arm, on the opposite arm to the cannula when possible, and a laser Doppler probe was placed on the inner forearm of the same arm. A resting measurement lasting five minutes was taken, after which the cuff was inflated to 200mmHg to obstruct the blood flow in the brachial artery, and remained pressurized for three minutes. The pressure in the cuff was then released and the hyperaemic response, measured in perfusion units (PU), was recorded using MoorVMS V3.1 software. The measurement continued for a further four minutes to allow the blood flow to return to baseline. Participants were instructed to keep their arm still during the measurement and tape was used to reduce movement of the probe cable. PORH measurements were saved in participant folders on a PC and later analysed using the Moor VMS software. The

values used for analysis were: resting level (RL), maximum level (ML), time to recovery (TR), time to maximum (TM), and PORH index, as outlined in Table 3.1. Figure 3.1 illustrates a representation of PORH analysis by the MoorVMS software. The laser Doppler was recalibrated twice during the study, as instructed by the manufacturer. Researchers have defined a PORH index as the ratio of the post-area under the curve (AUC) one minute after the release of the pressure cuff, relative to the AUC of a one-minute period of pressure cuff inflation (Yamamoto-Suganuma & Aso, 2009).

Table 3.1: Cutaneous microvascular reactivity variables measured at baseline, 150 minutes and 300 minutes for each intervention.

<i>Variable</i>	<i>Definition</i>
<i>Resting flux (RL; PU)</i>	First three minutes of the procedure
<i>Maximum flux (ML; PU)</i>	Maximum level of the graph of the blood flow after the release of the cuff at 200mmHg
<i>Time to recovery (TR; s)</i>	First few seconds following release of the cuff
<i>Time to maximum flux (TM; s)</i>	Time taken to reach the peak of the graph of hyperaemia
<i>PORH index (PU)</i>	Area under the curve one minute after the deflation of the cuff and area under the curve one minute before inflation

PU = perfusion units; s = seconds; PORH = post-occlusive reactive hyperaemia.

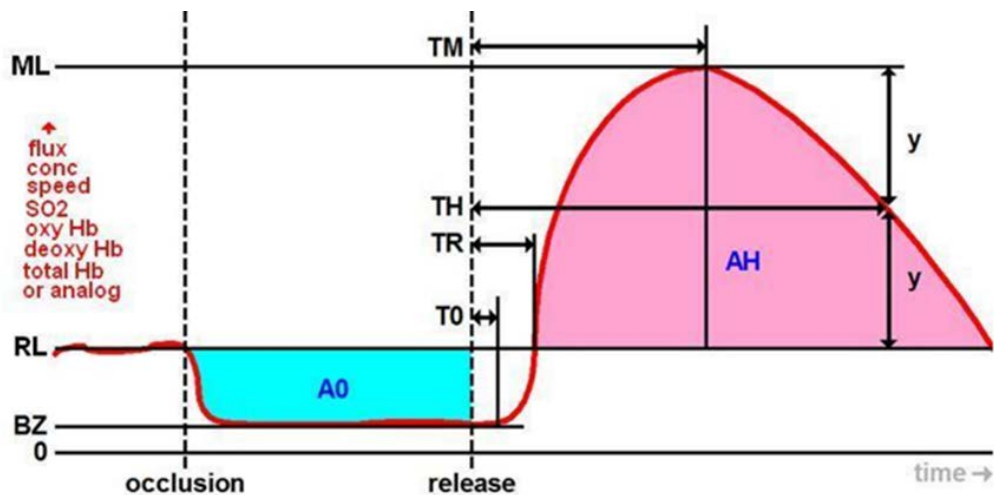


Figure 3.1: Graphical representation of PORH analysis. Taken from MoorVMS recording and analysis software, Vascular Monitoring System Software Manual.

3.2.3 Questionnaires

Habitual nitrate intake in the days between each visit was monitored using a nitrate intake questionnaire, which asked questions on the frequency and volume of common high-, medium-, and low-nitrate foods. Answers were put into a prepared spreadsheet, which provided an estimate of the total daily nitrate intake. The short version of the international physical activity questionnaire (IPAQ) was used to monitor physical activity over the seven days prior to each visit and consistency throughout the study (Craig *et al.*, 2003). A VAS was created to assess the palatability and acceptability of the beetroot given as an intervention, which participants completed immediately after the consumption of the beetroot. Participants answered questions on taste, texture, volume, aftertaste, smell, and visual appeal, giving their answer on a scale of 0 to 10. The scores were totalled for each intervention, giving a VAS score out of 60 for acceptability of the beetroot doses. These questionnaires can be seen in Appendices D and E.

3.2.4 Statistical analysis

All statistical analyses were completed using IBM SPSS (version 24.0). Summary data are presented as means \pm SD with 95% confidence intervals (CI). Intraclass coefficient values (ICC) were used to calculate the reliability of the measurements using the multiple baseline values (time 0) obtained at the beginning of each supplementation visit. Baseline differences were analysed using independent t tests

and changes were analysed using repeated measures analysis of variance (ANOVA) with time and intervention as within-subject factors and age as a between-subject factor. Where age groups were compared directly, the area under the curve (AUC) across the 300-minute period was calculated for each intervention using the trapezoid method. Models were checked for sphericity using Mauchly's test and multivariate models were applied if these assumptions were violated. Where significances were found and to assess intervention and age specific outputs, *post hoc* tests with Bonferroni adjustment to control for multiples comparisons were run. Associations between changes in plasma nitrite and changes in blood pressure were analysed using Pearson product-moment correlation coefficient. Statistical significance was set at $P<0.05$.

3.3 Results

3.3.1 Baseline characteristics

24 participants in total completed the crossover trial, with 12 younger adults aged 27.3 ± 3.8 years and 12 older adults aged 64.3 ± 4.6 years. No adverse events were reported and review of the food diaries revealed that participants adhered to the dietary restrictions stipulated. Average baseline systolic blood pressure (SBP) was 115.3 ± 8.4 mmHg and 120.2 ± 12.1 mmHg for the young and old group, respectively. These were not significantly different ($P=0.27$). Average baseline diastolic blood pressure (DBP) was 72.6 ± 4.2 mmHg and 76.5 ± 7.0 mmHg for the young and old group, respectively ($P=0.11$). Groups were matched for weight, with an average of 68.2 ± 8.8 kg across the young and old participants. Both groups also had a healthy BMI, averaging 23.9 ± 2.5 kg/m² across groups (Table 2.1).

3.3.2 Systolic blood pressure

Baseline values were taken at the beginning of each of the four trials, but the ICC shows strong, significant correlation (all $P<0.001$), with values of 0.91 for SBP and 0.90 for DBP in the young and 0.92 for SBP and 0.90 for DBP in the old. This indicates a comparable basal level for all trials for both age groups. At baseline, SBP and DBP did not differ between age groups ($P>0.05$).

Changes in systolic blood pressure in the young

There was a significant effect of time ($P<0.001$) but no significant difference between interventions for SBP ($P=0.55$), as shown in Figure 3.2. There was no interaction between time and intervention ($P=0.81$). Pairwise comparisons show that SBP at the mid and late time points was significantly lower than at baseline (-5.1mmHg; $P=0.004$; 95%CI: -8.6, -1.7 and -5.2mmHg; $P=0.008$; 95%CI: -9.2, -1.3, respectively) across interventions. Peak reduction occurred between four and five hours for all interventions combined. *Post hoc* analyses show that 100g beetroot reduced SBP at the mid time point (-6.4mmHg; $P=0.05$; 95%CI: -12.8, -0.6) compared to baseline and PN reduced SBP at the mid (-6.1mmHg, $P=0.004$; 95%CI: -10.2, -1.9) and late (-7.3mmHg, $P=0.004$; 95%CI: -12.4, -2.3) time points compared to baseline. SBP at the late time point was also significantly lower than SBP at the early time point following PN (-2.5mmHg; $P=0.02$; 95%CI: -4.6, -0.4). There were no significant effects of 200g or 300g beetroot on SBP in the young group.

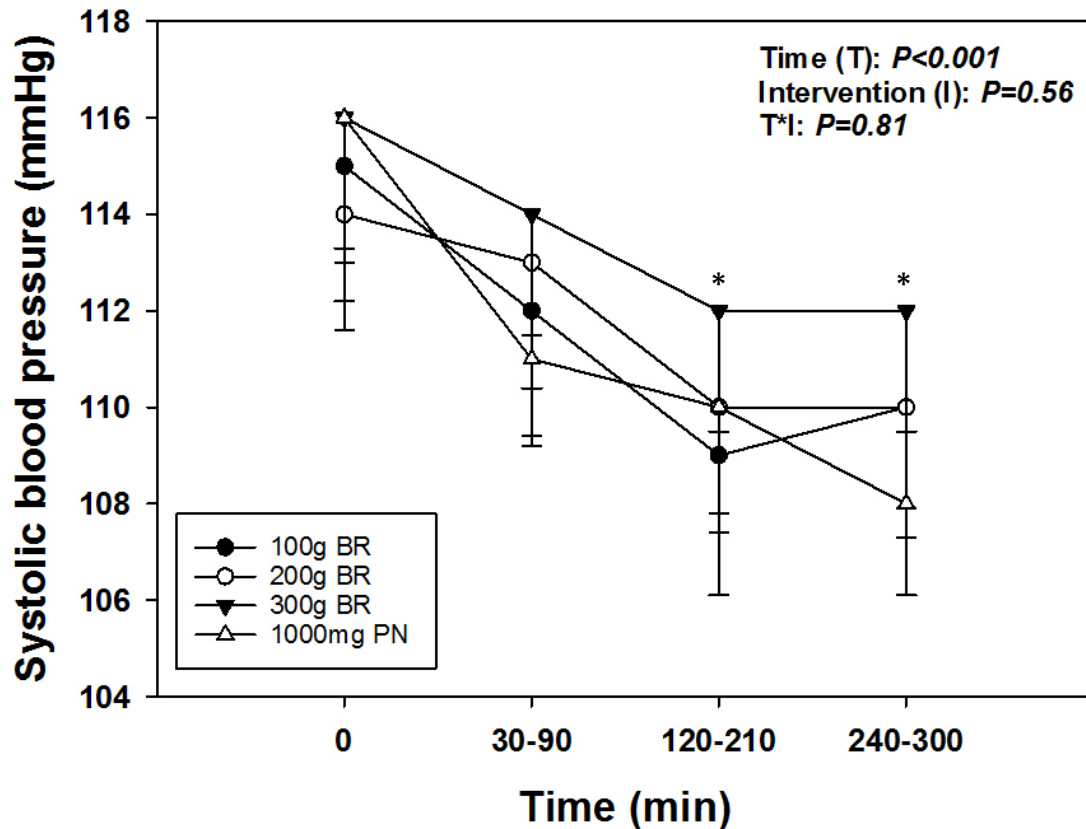


Figure 3.2: Mean (\pm SEM) baseline, early, mid and late systolic blood pressure (SBP) absolute values in the young group following incremental doses of nitrate; $n = 12$. Statistical analysis using a two-factor repeated measures ANOVA (intervention \times time). BR = whole beetroot, PN = potassium nitrate positive control. * denotes significant difference from baseline across nitrate doses.

Changes in systolic blood pressure in the old

There was no effect of time ($P=0.50$) or intervention ($P=0.31$) on SBP in the old group (Figure 3.3) but there was an interaction between time and intervention ($P=0.04$). However, further analyses did not reveal any significant differences between interventions at baseline, mid, late or end time points. Figure 3.3 illustrates that PN was the only trial to cause a reduction in SBP but statistical analysis does not identify this as a significant reduction from baseline or compared to the beetroot doses.

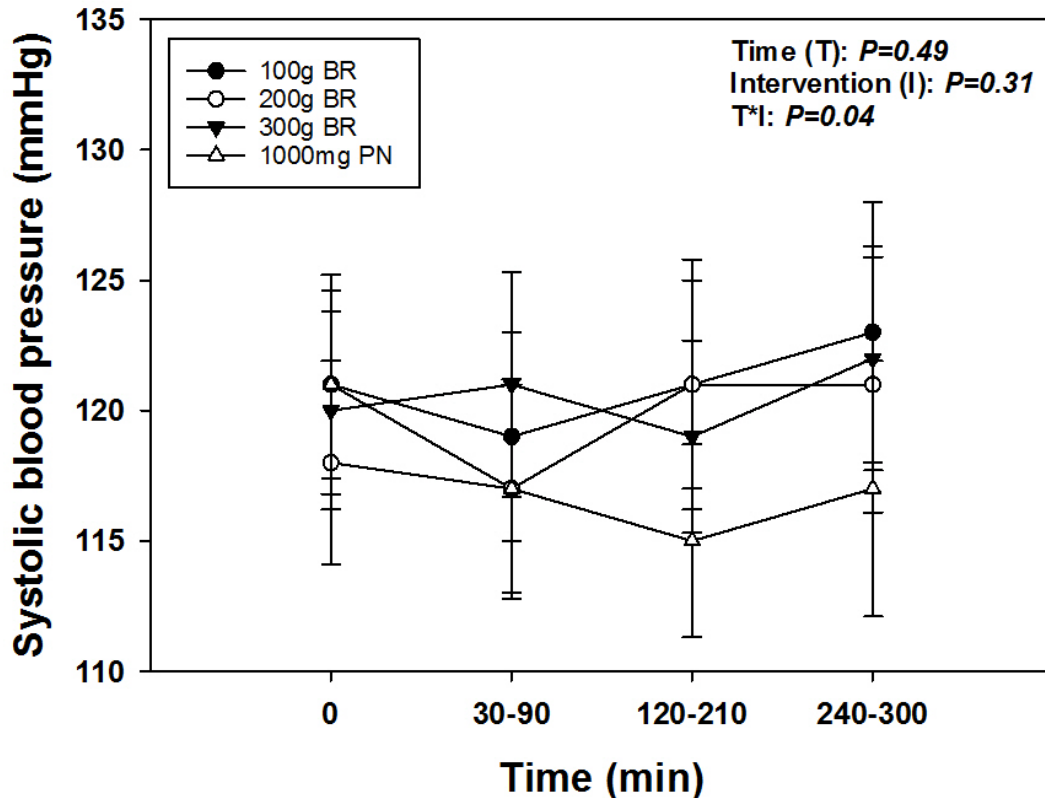


Figure 3.3: Mean (\pm SEM) baseline, early, mid and late systolic blood pressure (SBP) absolute values in the old group following incremental doses of nitrate; $n = 12$. Statistical analysis using a two-factor repeated measures ANOVA (intervention \times time). BR = whole beetroot, PN = potassium nitrate positive control.

AUC for systolic blood pressure in both age groups

The AUC of SBP is not statistically different between age groups but shows a trend ($P=0.08$). Similarly, there is no statistically significant difference between interventions but a noteworthy trend ($P=0.07$) (Figure 3.4). There is no interaction between age and intervention ($P=0.43$). There was no difference between interventions for the young group but AUC was significantly smaller following PN than 100g beetroot in the old group (-1403 , $P=0.05$; 95%CI: -2810 , 3). The young group had a significantly smaller AUC than the old only following 100g beetroot (-3111 ; $P=0.04$; 95%CI: -6138 , -83).

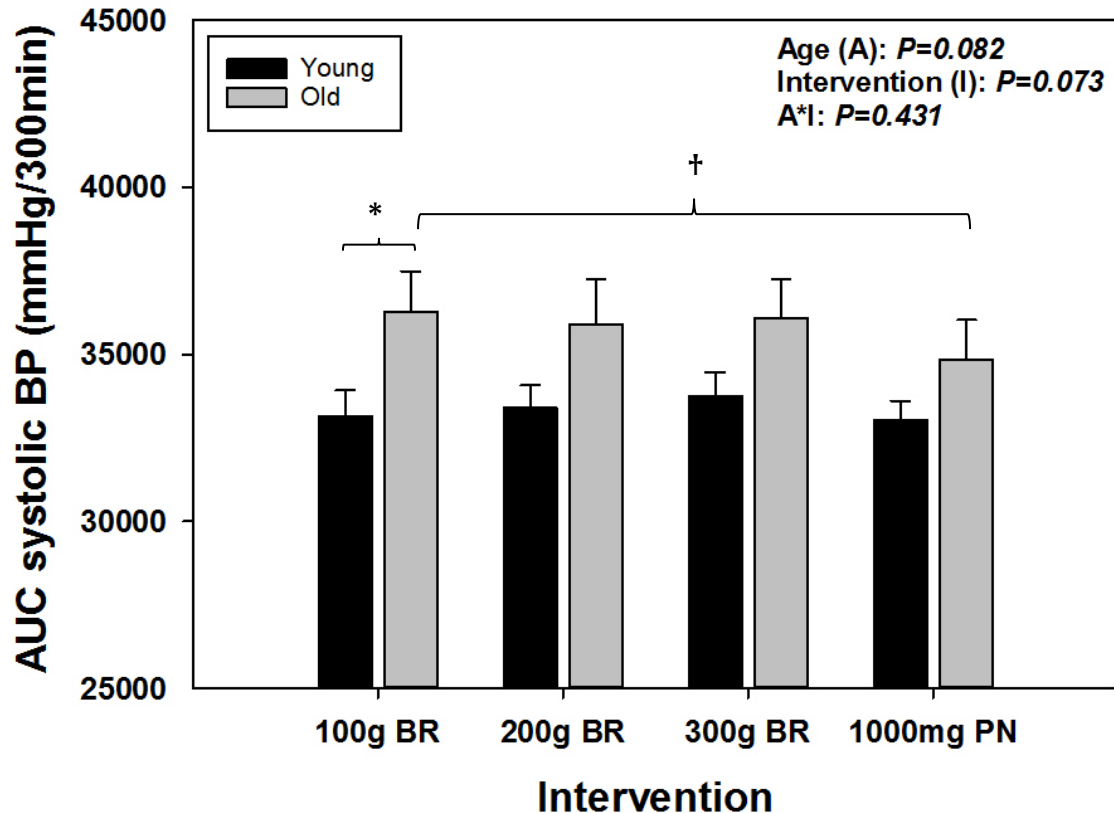


Figure 3.4: Mean (\pm SEM) area under the curve (AUC) of systolic blood pressure (SBP) values over 300 minutes in both the young and old groups; $n = 24$ (12 young and 12 old). Data analysed by mixed model repeated measures ANOVA (intervention as within subjects factor and age as between subjects factor). BR = whole beetroot, PN = potassium nitrate positive control. * denotes difference between age groups following 100g beetroot ($P=0.04$); † denotes difference between interventions in old group ($P=0.05$).

3.3.3 Diastolic blood pressure

Changes in diastolic blood pressure in the young

There was a significant effect of time ($P<0.001$) but no effect of intervention ($P=0.77$) on DBP in the young (Figure 3.5). There was no interaction between time and intervention ($P=0.35$). Pairwise comparisons show that DBP was significantly lower at the early (-3.9mmHg ; $P=0.01$; 95%CI: $-7.2, -0.6$), mid (-4.7mmHg ; $P<0.001$; 95%CI: $-7.2, -2.3$) and late time points (-4.1mmHg ; $P=0.004$; 95%CI: $-6.9, -1.3$) than at baseline, demonstrating a blood pressure-lowering effect for all interventions combined. Peak reduction occurred between two and 3.5 hours post-nitrate ingestion across the interventions. Analyses shows that 100g beetroot reduced DBP between baseline and mid (-5.0mmHg ; $P=0.05$; 95%CI: $-10.0, -0.0$) and late (-4.3mmHg ; $P=0.03$; 95%CI: $-8.1, -0.4$). 200g beetroot had no effect on DBP. The consumption of

300g beetroot reduced DBP between baseline and early (-5.1mmHg; $P=0.01$; 95%CI: -9.4, -0.7), mid (-4.3mmHg; $P=0.01$; 95%CI: -7.9, -0.6) and end (-5.1mmHg; $P=0.005$; 95%CI: -8.7, -1.5). PN reduced DBP between the baseline and mid (-6.4mmHg; $P=0.007$; 95%CI: -11.1, -1.7) time points.

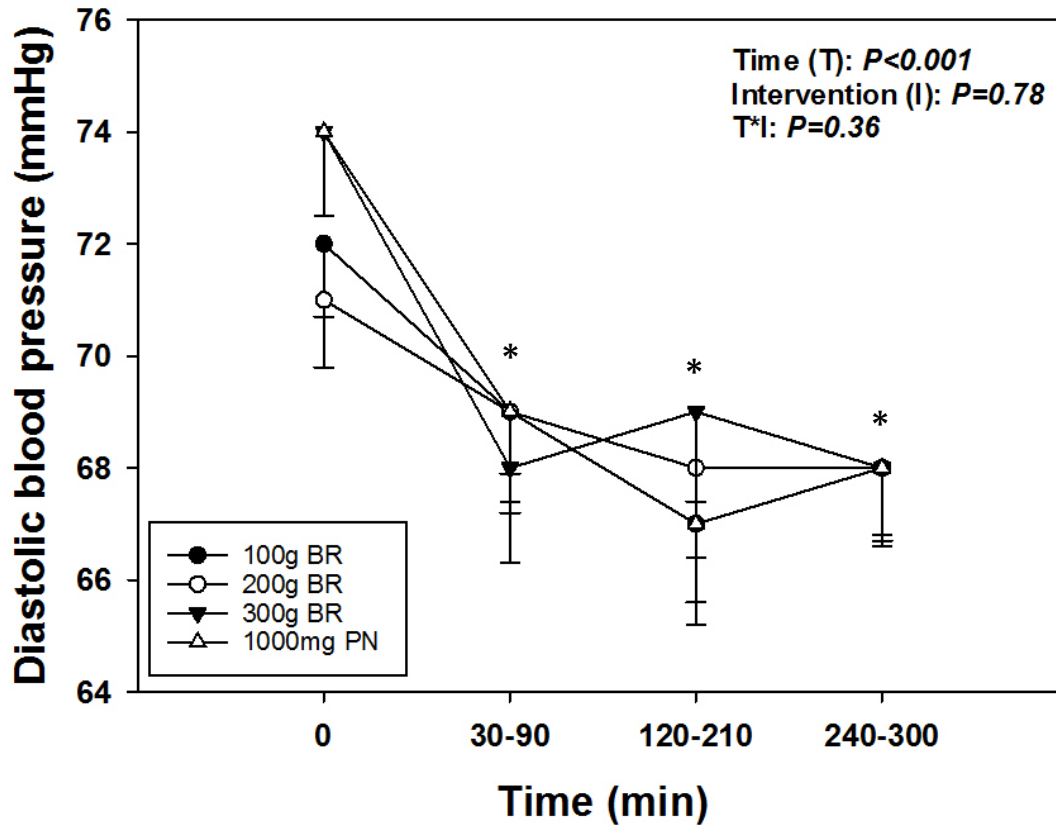


Figure 3.5: Mean (\pm SEM) baseline, early, mid and late diastolic blood pressure (DBP) absolute values in the young group following incremental doses of nitrate; $n = 12$. Statistical analysis using a two-factor repeated measures ANOVA (intervention \times time). BR = whole beetroot, PN = potassium nitrate positive control. * denotes significant difference compared to baseline.

Changes in diastolic blood pressure in the old

There was an observable trend towards an effect of time ($P=0.07$) and intervention ($P=0.07$) on DBP in the old group, as shown in Figure 3.6. There was no interaction between time and intervention ($P=0.18$). Pairwise comparisons indicate that PN caused a greater reduction in DBP than 100g beetroot (-2.8mmHg; $P=0.05$; 95%CI: -5.79, -0.79) but neither the whole beetroot nor PN significantly reduced DBP overall. At the mid time point, blood pressure was significantly lower after PN than 100g

beetroot (-4.9mmHg; $P=0.007$; 95%CI: -8.6, -1.3), demonstrating a dose response in the blood pressure-lowering effect.

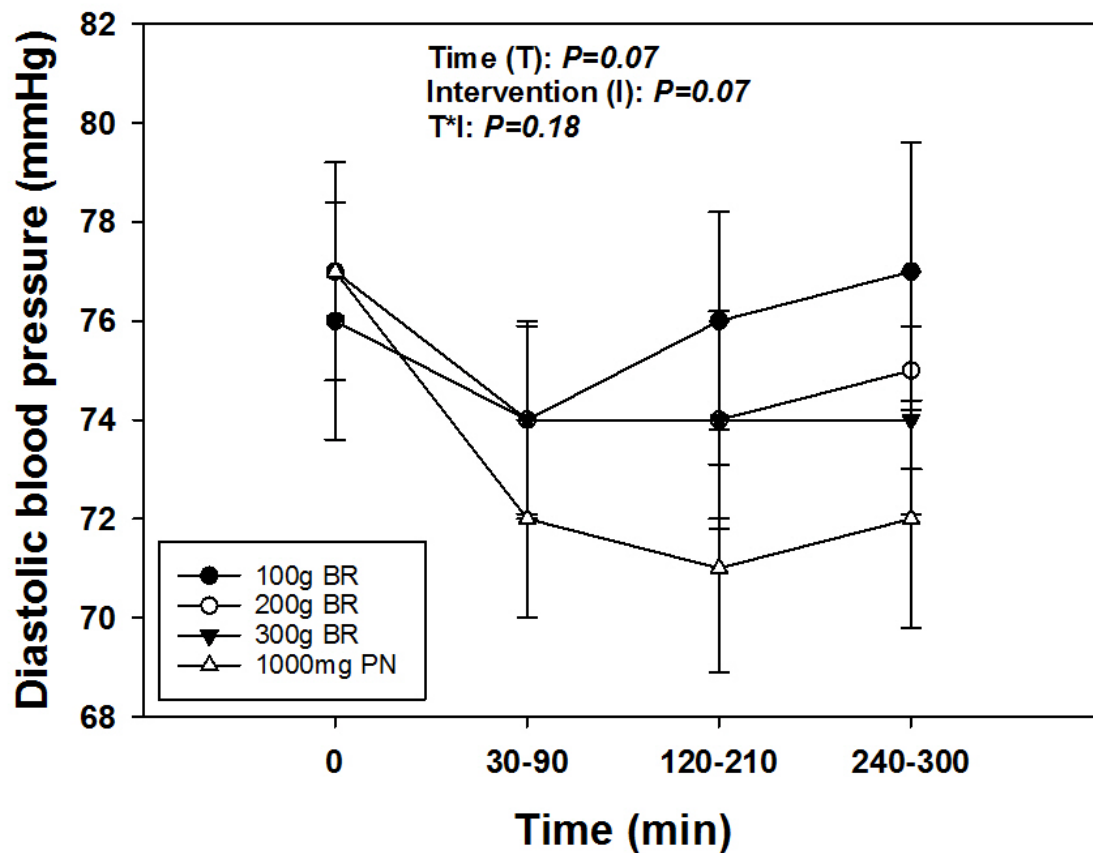


Figure 3.6: Mean (\pm SEM) baseline, early, mid and late diastolic blood pressure (DBP) absolute values in the old group following incremental doses of nitrate; $n = 12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = whole beetroot, PN = potassium nitrate positive control.

AUC for diastolic blood pressure in both age groups

There was a significant effect of age ($P=0.01$) but no effect of intervention on DBP ($P=0.16$) (Figure 3.7). There was no interaction between age and intervention ($P=0.12$). Analyses show that there was no significant difference between interventions for the young group but the AUC for PN beetroot was significantly smaller than for 100g beetroot in the old group (-1130; $P=0.002$; 95%CI: -1881.39, -378.62,). The AUC for the young group is significantly smaller than the old group following 100g (-2325; $P=0.005$; 95%CI: -3866, -783) and 200g (-1766; $P=0.02$; 95%CI: -3310, -222) and 300g (-1490; $P=0.05$; 95%CI: -2986, -6), demonstrating a greater blood pressure-lowering response following each of the interventions. There

is no difference between age groups following PN. This suggests that high dose of nitrate is required in the old to elicit the responses to nitrate seen in younger participants.

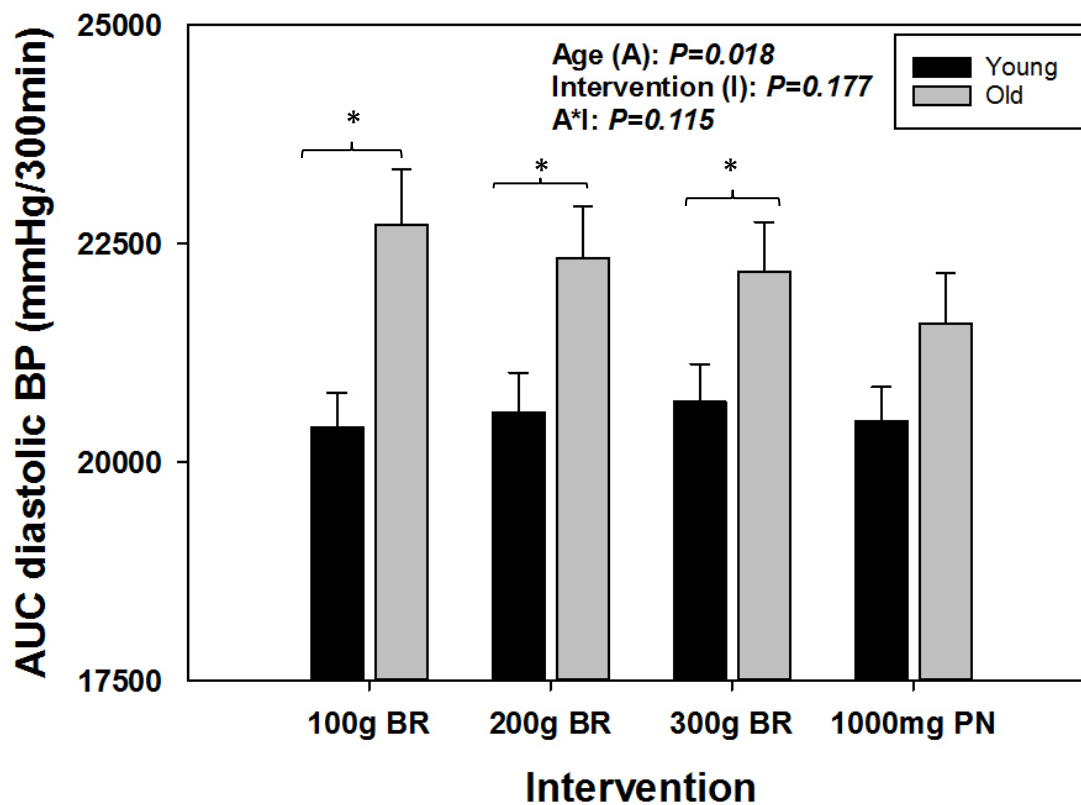


Figure 3.7: Mean (\pm SEM) area under the curve (AUC) over 300 minutes for diastolic blood pressure (DBP) in the young and old groups; $n = 24$ (12 young and 12 old). Data analysed by mixed model repeated measures ANOVA (intervention as within subjects factor and age as between subjects factor). BR = whole beetroot, PN = potassium nitrate positive control. * denotes significant difference between age groups.

3.3.4 Cutaneous microvascular activity

Resting flux (RL)

The ICC for baseline RL across trials shows that they were modestly correlated, with a value of 0.33 and 0.54 for the young and the old group, respectively. This shows that the repeatability of the measurements was overall moderate. There was no effect of intervention ($P=0.35$) or time ($P=0.24$) on RL in the young group, or an interaction effect ($P=0.87$). Similarly, there was no effect of intervention ($P=0.46$), time ($P=0.28$) or an interaction effect ($P=0.31$) on RL in the old group.

Time to maximum flux (TM)

There was no effect of intervention ($P=0.37$) or time ($P=0.56$) on TM in the young group, or an interaction effect ($P=0.23$). Similarly, there was no effect of intervention ($P=0.58$) or time ($P=0.85$), or an interaction effect ($P=0.36$) in the old group.

Time to resting flux (TR)

There was no effect of intervention ($P=0.76$) or time ($P=0.72$) or an interaction effect ($P=0.19$) on TR in the young group. There was also no effect of intervention ($P=0.15$), time ($P=0.74$) or an interaction effect ($P=0.45$) in the old group.

Maximum flux (ML)

There was no effect of intervention on ML in the young ($P=0.55$) but a significant effect of time ($P=0.05$). There was no interaction effect of intervention and time ($P=0.55$). Pairwise comparisons reveal that MF at 300 minutes was significantly higher than at 150 minutes (+19.1PU; $P=0.02$, 95%CI: 3.55, 34.59) for all interventions combined. This can be seen in Figure 3.8. There was no effect of intervention ($P=0.17$) or time ($P=0.16$) on MF in the old group, or an interaction between intervention and time ($P=0.54$) (Figure 3.9).

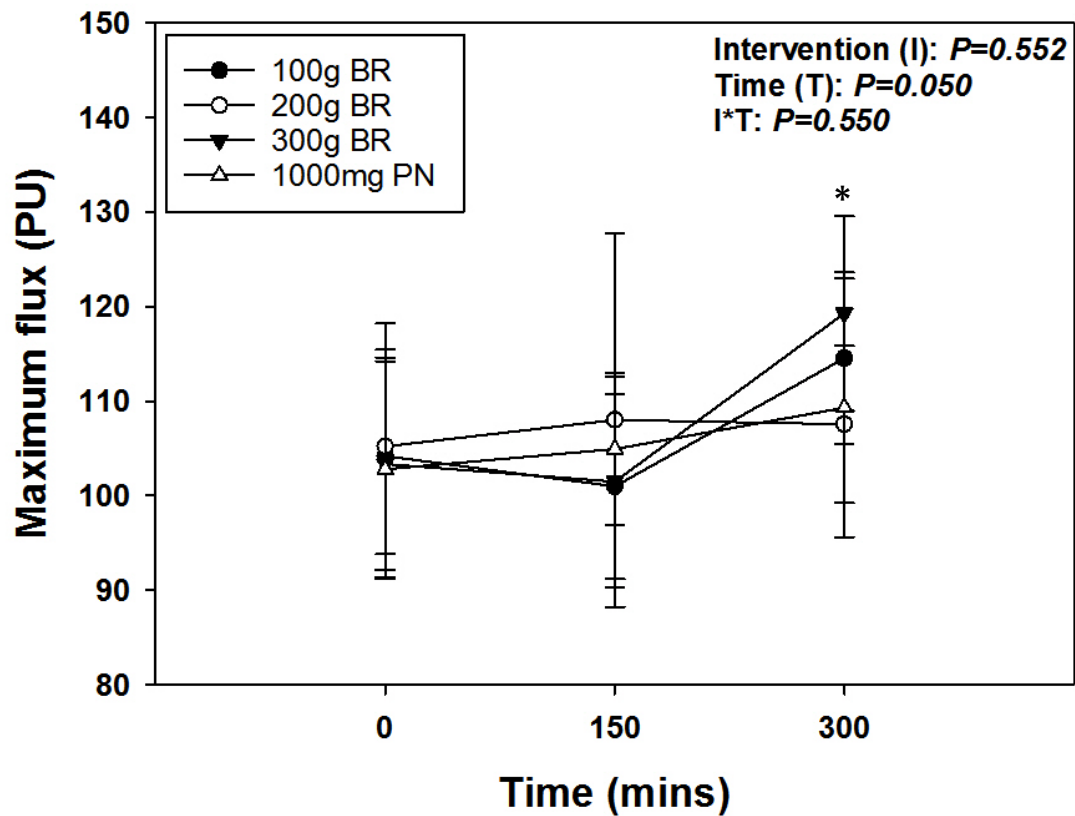


Figure 3.8: Mean (\pm SEM) maximum flux measured by cutaneous microvascular activity in the young group; $n = 12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = whole beetroot, PN = potassium nitrate positive control. * denotes significant difference compared to baseline.

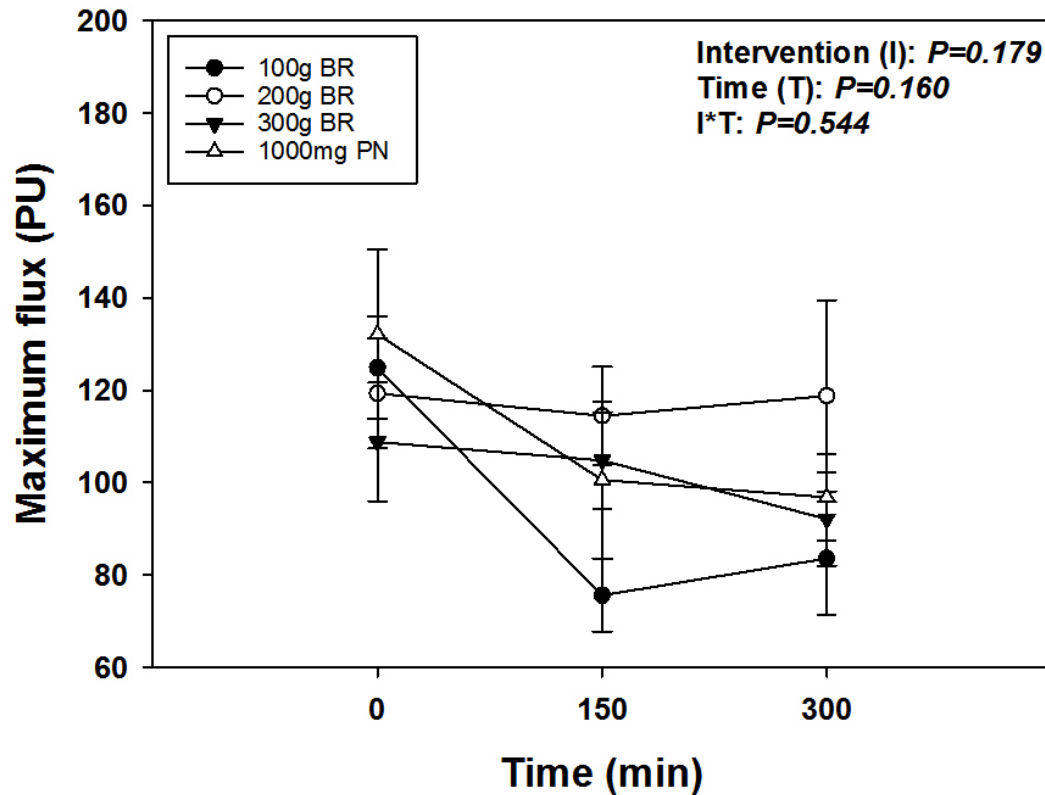


Figure 3.9: Mean (\pm SEM) maximum flux measured by cutaneous microvascular activity in the old group; $n = 12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = whole beetroot, PN = potassium nitrate positive control.

Post-occlusive reactive hyperaemia (PORH)

PORH was significantly lower in the older group than the younger group across each intervention and time period ($P=0.02$). Values were 3.53PU and 3.04PU for young and old, respectively. There was no effect of intervention ($P=0.53$) or time ($P=0.93$) on PORH in the young group, or an interaction between intervention and time ($P=0.67$). There was a noteworthy trend towards an effect of intervention on PORH in the old group ($P=0.06$) but no effect of time ($P=0.32$). There was no interaction between intervention and time ($P=0.32$). Pairwise comparisons show that PORH was significantly higher following 200g beetroot than 300g beetroot (+1.4PU; $P=0.04$, 95%CI: 0.07, 2.73) but values fluctuated over the time period (Table 3.2).

Table 3.2A: Vascular function variables in the young group; n = 12.

<i>Variable</i>	<i>100g BR</i>			<i>200g BR</i>			<i>300g BR</i>			<i>1000mg PN</i>			
<i>Minutes</i>	<i>0</i>	<i>150</i>	<i>300</i>	<i>0</i>	<i>150</i>	<i>300</i>	<i>0</i>	<i>150</i>	<i>300</i>	<i>0</i>	<i>150</i>	<i>300</i>	<i>P</i>
<i>RF</i>	12.44	12.75	13.58	14.18	14.07	13.48	14.84	14.06	15.50	12.87	13.81	15.83	0.24
<i>MF</i>	104.20	100.97	114.55	105.19	108.01	107.56	103.34	101.48	119.28	102.79	104.93	109.29	0.05
<i>PORH</i>	3.72	3.78	3.65	3.50	3.63	3.59	3.10	3.05	3.61	3.84	3.78	3.06	0.93
<i>TR</i>	0.65	0.94	1.14	0.78	0.90	0.87	1.24	0.79	0.98	0.67	2.01	2.28	0.72
<i>TM</i>	10.44	10.58	10.75	9.55	12.02	10.19	11.70	9.94	11.44	12.17	11.61	14.57	0.56

Table 3.2B: Vascular function variables in the old group; n = 12.

<i>Variable</i>	<i>100g BR</i>			<i>200g BR</i>			<i>300g BR</i>			<i>1000mg PN</i>			
<i>Minutes</i>	<i>0</i>	<i>150</i>	<i>300</i>	<i>0</i>	<i>150</i>	<i>300</i>	<i>0</i>	<i>150</i>	<i>300</i>	<i>0</i>	<i>150</i>	<i>300</i>	<i>P</i>
<i>RF</i>	18.17	14.77	11.94	17.28	14.43	13.35	17.22	16.55	13.39	19.63	16.03	12.47	0.28
<i>MF</i>	124.88	75.58	83.57	119.31	114.45	118.78	108.79	104.80	92.09	132.15	100.57	96.81	0.16
<i>PORH</i>	3.52	2.19	2.86	3.54	3.47	3.62	2.90	2.64	2.95	2.92	3.00	2.86	0.32
<i>TR</i>	0.78	0.65	0.45	0.65	0.80	0.73	0.90	0.61	1.56	0.87	0.76	0.79	0.74
<i>TM</i>	9.74	13.08	12.99	8.90	16.00	12.13	10.24	10.05	10.60	9.53	16.92	11.14	0.85

BR = whole beetroot, PN = potassium nitrate positive control, RF = resting flux, MF = maximal flux, PORH = post-occlusive reactive hyperaemia, TR = time to resting, TM = time to maximum. P values denote changes over the 300-minute period for all intervention combined.

3.3.5 Correlation between plasma biomarkers and blood pressure

A Pearson product-moment correlation coefficient was calculated to assess the relationship between changes in plasma nitrate and nitrite levels reported in Chapter 2 and changes in blood pressure. Across both age groups, there was no correlation between change in plasma nitrate levels and change in SBP over the 300-minute period ($r=-0.17$; $n=288$; $P=0.003$). There was no correlation between changes in plasma nitrate levels and changes in DBP over time ($r=-0.11$; $n=288$; $P=0.06$). In the young group, changes in plasma nitrate were not correlated with changes in SBP ($r=0.06$; $n=144$; $P=0.45$) or changes in DBP ($r=0.02$; $n=144$; $P=0.79$). In the old group, however, there was a small negative correlation between changes in plasma nitrate levels and SBP ($r=-0.35$; $n=144$; $P<0.001$). There was also a small negative correlation between changes in plasma nitrate levels and changes in DBP ($r=-0.21$; $n=144$; $P=0.01$).

Across both age groups, there was a small negative correlation between change in plasma nitrite levels and change in SBP over the 300-minute period ($r=-0.28$; $n=288$; $P<0.001$). There was also a small correlation between change in DBP and changes in plasma nitrite levels over 300 minutes ($r=-0.26$; $n=288$; $P<0.001$). These are summarised in Figures 3.10A and B.

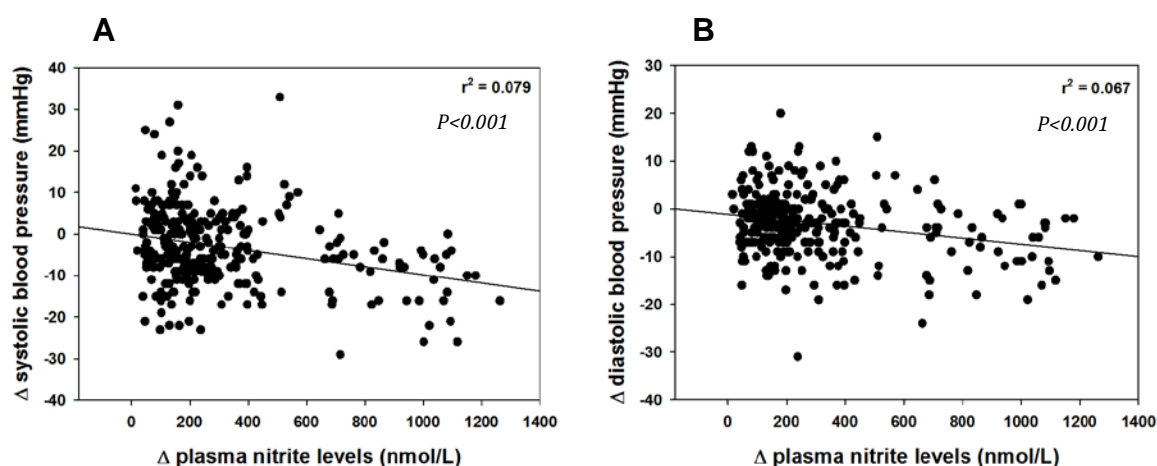
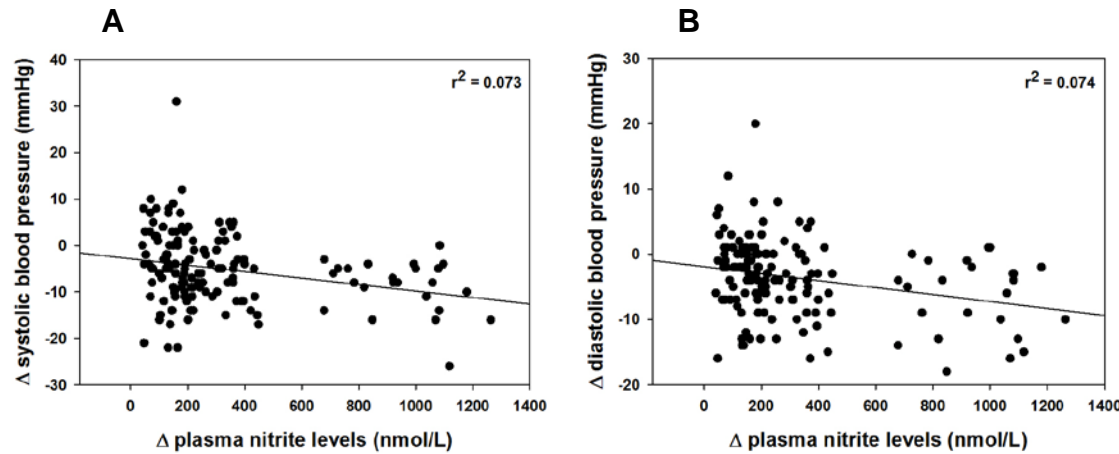


Figure 3.10: Changes in plasma nitrite levels compared to changes in systolic blood pressure (A) and diastolic blood pressure (B) across both age groups. Statistical analysis by Pearson's correlation coefficient.

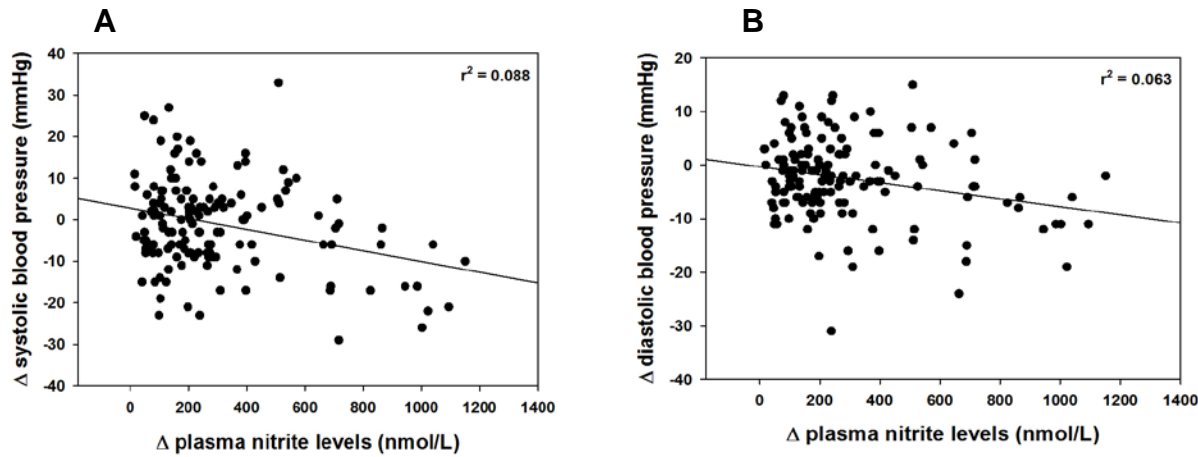
Taking each age group separately, the Pearson correlation coefficient shown that there was a small negative relationship between changes in plasma nitrite and changes in SBP ($r=-0.27$; $n=144$; $P=0.001$) and DBP ($r=-0.27$; $n=144$; $P=0.001$) in the young group (Figures 3.11A and B).



$P=0.001$

Figure 3.11: Changes in plasma nitrite levels compared to changes in systolic blood pressure (A) and diastolic blood pressure (B) in the young group. Statistical analysis by Pearson's correlation coefficient.

In the old group, there was also a small negative correlation between changes in plasma nitrite and changes in SBP ($r=-0.30$; $n=144$; $P<0.001$) and DBP ($r=-0.25$; $n=144$; $P=0.002$) (Figures 3.12A and B)



$P=0.002$

Figure 3.12: Changes in plasma nitrite levels compared to changes in systolic blood pressure (A) and diastolic blood pressure (B) in the old group. Statistical analysis by Pearson's correlation coefficient.

3.3.6 Questionnaires

Results from the seven-day nitrate intake questionnaire indicate that participants, on average, consumed 223mg/day in the young group and 252mg/day in the old group

($P=0.59$ between age groups). Across both groups, the average intake relative to body weight (BW) was 3.6 ± 2.0 mg nitrate/kg BW/day. This value is very close to the ADI of 3.7 mg/kg BW/day, demonstrating that participants maintained the recommended nitrate intake across the four-week study period.

The IPAQ results show that participants were very active and maintained activity levels for the duration of the study. Average activity levels were 3839 MET-min/week, which is significantly higher than the recommended 500 MET-min/week (Kaminsky & Montoye, 2014). This may have been skewed by a number of highly active individuals who took part in the study. Average IPAQ values across the four trials ranged from 1545 to 8405 MET-min/week in the young group and from 682 to 7911 MET-min/week in the old group. Activity levels were significantly higher in the younger group than the older group ($+804$ MET-min/week; $P=0.02$).

3.3.7 Acceptability of interventions

Visual analogue scale (VAS) scores reflect participants' preference to the dose of beetroot given and therefore the acceptability of the supplementation. There was a significant effect of intervention on VAS scores ($P<0.001$) and a significant effect of age group ($P=0.05$), but no interaction between intervention and age ($P=0.30$) (Figure 3.13). Pairwise comparisons show that the old group scored the interventions higher than the young group ($+8.9$; $P=0.04$; 95%CI: 0.2, 17.6). 300g scored significantly lower than 100g beetroot (-7.3 ; $P<0.001$; 95%CI: 4.1, 10.4) and 200g beetroot (-4.6 ; $P<0.001$; 95%CI: 2.1, 7.1), and although not statistically significant there was a trend towards higher scores for 200g than 300g beetroot ($+2.7$; $P=0.08$; -0.3, 5.6).

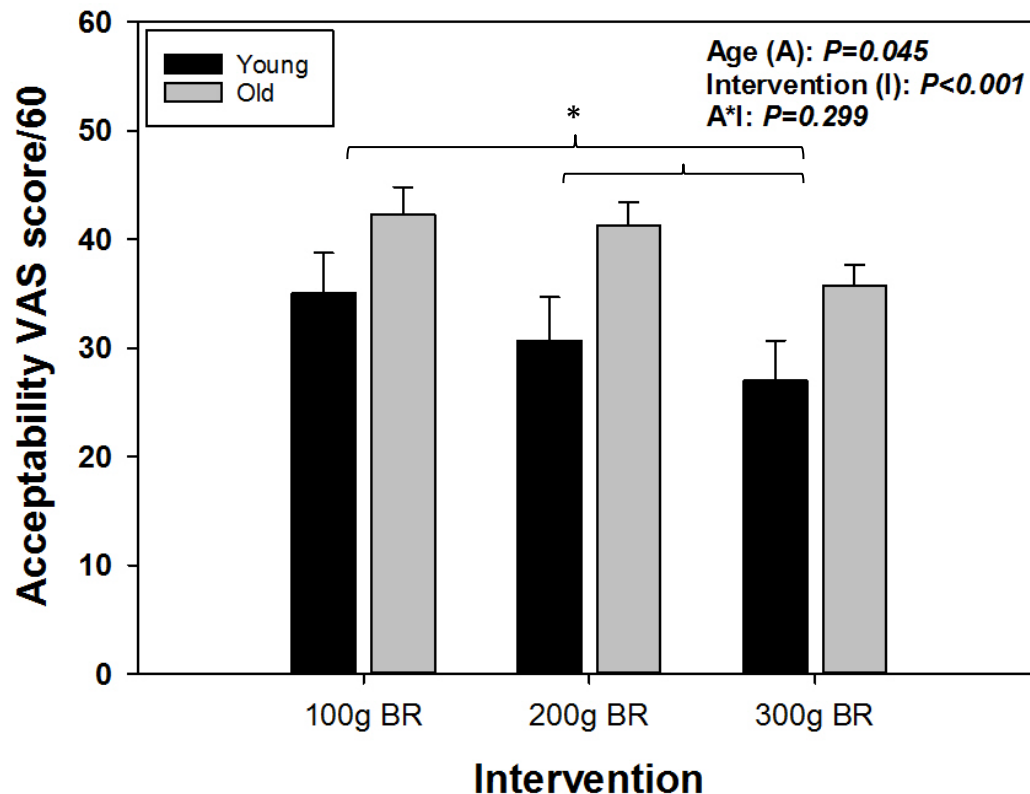


Figure 3.13: Mean (\pm SEM) visual analogue scale (VAS) scores out of 60 for each beetroot dose in the young and old groups; $n = 24$. Data analysed using a mixed model repeated measures ANOVA (intervention as within-subjects factor and age as between-subjects factor). BR = beetroot. * denotes significant difference between interventions across both age groups.

3.4 Discussion

3.4.1 Summary of findings

Results from this study reaffirm the suggestion of age-related differences in the effects of dietary nitrate. The main findings were: 1) a small dose of beetroot reduced SBP by 6.4mmHg during the five-hour period in young adults; 2) despite receiving the same doses over the time period, there was no effect of whole beetroot on blood pressure in the old group; 3) higher doses of nitrate in the form of PN had a blood-pressure lowering effect on the old group; 4) older adults are more tolerant of larger doses of beetroot and up to 200g beetroot is a suitable amount for supplementation in an acute dosage setting. Together these data suggest that whole beetroot can have positive acute effects on the vascular system but larger doses of nitrate are required to elicit beneficial effects in older adults. However, larger doses of nitrate in

the form of whole beetroot are well tolerated by older adults, which may result in good compliance rates with the application of this vegetable in future research.

3.4.2 The effect of beetroot on blood pressure

A recent systematic review reiterated the positive effect of acute nitrate intake on blood pressure, highlighting a reduction in SBP by 4.8mmHg and DBP by 1.7mmHg (Jackson *et al.*, 2018). To date, 248mg nitrate has been deemed the lowest amount found to have beneficial effects on a healthy blood pressure (Kapil *et al.*, 2010). In this study, SBP and DBP both fell by 2mmHg in participants ~25 years of age, following the consumption of potassium nitrate capsules. Kapil *et al.* (2010) also describe a dose-response to increasing amounts of nitrate and found that 341mg nitrate provided in the form of beetroot juice reduced SBP by 5mmHg, with no effect on DBP. Similarly, Webb *et al.* (2008) found that 1395mg nitrate in the form of beetroot juice reduced SBP by 10mmHg in participants aged 25 years and DBP was reduced by 8mmHg. Peak blood pressure reduction was seen between one and three hours post-nitrate ingestion in both studies. In the present study, 100g beetroot, providing ~272mg nitrate, reduced SBP by 6.4mmHg and DBP by 5.0mmHg between two and three and a half hours in the young group, which is a larger reduction than seen by Kapil *et al.* (2010) with a similar dose of nitrate. This also corresponds to the peak plasma nitrate levels seen between two and three hours post-ingestion of beetroot. The larger dose of nitrate provided by 300g of beetroot reduced DBP to the same extent as the smaller dose but 1000mg of nitrate elicited the largest reduction in DBP by 6.4mmHg between two and 3.5 hours post-ingestion. 1000mg also reduced SBP by 7.3mmHg between four and five hours, which corresponds with the peak plasma nitrite level. It is evident from this that there is a dose response and that whole beetroot is effective at reducing blood pressure in young, healthy adults. In an older population, however, beetroot or PN supplementation did not result in a significant reduction in either SBP or DBP. There was a trend towards an effect of nitrate on DBP in the old group and, although not significant, PN was found to cause a reduction in DBP by 4.9mmHg between two and 3.5 hours. It may be the case that the sample size here was too small to detect significant differences in the old group due to individual variations.

This is consistent with results seen in the literature, in which the effects of dietary nitrate, predominantly from beetroot juice, are irregular in an older population (Table 1.2). In a healthy cohort, 595mg nitrate from beetroot juice was enough to reduce both SBP and DBP in adults aged on average 64 years (Kelly *et al.*, 2013) but there was no effect on blood pressure in adults aged on average 73 years following a similar dose of nitrate (Miller *et al.*, 2012). Gilchrist *et al.* (2014) found no effect of beetroot juice on blood pressure in participants with Type 2 diabetes and Bondonno *et al.* (2015) similarly found no effect of the juice on blood pressure in older adults with high blood pressure and taking anti-hypertensive medication. Conversely, Kenjale *et al.* (2011) described a reduction in DBP following beetroot juice in participants with peripheral arterial disease (PAD). The difference in results may simply be due to the dose, as Kenjale *et al.* (2011) provided 1116mg nitrate, which is more than double that given by Gilchrist and Bondonno. However, patients with Type 2 diabetes have been found to generate less NO from L-arginine than healthy individuals, which may result in a reduced response to a nitrate load (Avogaro *et al.*, 2003). Work by Shepherd *et al.* (2015) found no effect of beetroot juice on blood pressure in patients with COPD despite rises in plasma nitrate following consumption of the nitrate load. It can be speculated that beneficial effects of beetroot juice on blood pressure are blunted in volunteers with chronic conditions such as COPD and diabetes, potentially due to elevated oxidative stress increasing the scavenging of NO (Shepherd *et al.*, 2015). A high concentration of free radicals such as superoxide can increase the breakdown of NO and thus reduce its effectiveness (Taddei *et al.*, 2001).

Furthermore, the study by Kelly *et al.* (2013) was the only one with a cohort with a healthy BMI, while the other cohorts were overweight or obese. The negative effects of a high BMI, such as low-grade inflammation, may therefore be blunting the positive effects of dietary nitrate, especially in an older population in which low-grade inflammation may already be extant. Participants in the present study were both a normal weight and overweight, with BMIs below 29.9kg/m². This could have produced an inconsistent result in blood pressure lowering.

Three weeks of supplementation with beetroot juice led to a progressive decline in daily SBP in older obese adults (Jajja *et al.*, 2014), who were given ~165mg

nitrate/day in concentrated beetroot juice for 21 days. The more prolonged intervention period here may account for the positive effects of the beetroot juice despite participants receiving a smaller dose than that provided by other researchers. The longer-term supplementation with nitrate- and antioxidant-rich beetroot here may have reduced the negative effects of oxidative stress and inflammation associated with ageing and obesity and allowed the bioactive nitrate to induce blood pressure reduction through NO. Additive effects of nitrate and/or nitrite may have also heightened this over the more prolonged time period but little is known about the relevance of this.

The present study contributes to the data on sources of dietary nitrate and their effects on blood pressure and demonstrates that the consumption of whole beetroot, in doses akin to a vegetable portion of 80g, can acutely reduce blood pressure in young individuals. It demonstrates that beetroot in forms other than juice can be used as effective supplements or additions to the diet. These same benefits do not translate to an older population, although large quantities of nitrate reduce the difference in blood pressure seen between young and old participants. Longer-term studies are required to further examine whether the effect of dietary nitrate on blood pressure is solely due to the immediate dose or whether there are benefits that accumulate over a more prolonged period. This is particularly relevant for an ageing population in which the acute blood pressure-lowering effects appear to be dampened.

3.4.3 Correlations between plasma nitrate and nitrite and blood pressure

In 2010 and 2018, Kapil *et al.* described a correlation between plasma nitrite but not nitrate and blood pressure following supplementation with 1488mg and 496mg potassium nitrate. Peak blood pressure reductions have also been associated with higher plasma cGMP levels around three hours following nitrate ingestion (Kapil *et al.*, 2015). This demonstrates increased NO availability following the nitrate load, which works to increase cGMP levels and subsequently dilate blood vessels (Snyder, 1992). Baseline plasma nitrite levels are also thought to be an accurate reflection of endogenous NO generation via L-arginine (Lauer *et al.*, 2001). In the present study, there was no correlation between plasma nitrate levels and blood pressure in the

young group but both SBP and DBP reductions were modestly correlated with higher plasma nitrate levels in the old group. Although not found to be statistically significant, plasma nitrate levels were observed to be higher in the old group than young group, which might explain the correlation with blood pressure and these higher levels. However, peak plasma nitrate levels were found between two and three hours in the old group but blood pressure was not significantly lower than baseline at this time. There was large inter-individual variation in blood pressure due to the small sample size, which may have influenced the correlation between NO metabolites and blood pressure. As discussed in Chapter 2, the association between nitrite and blood pressure has been elegantly demonstrated through the blocking of the nitrate-nitrite conversion by anti-bacterial mouthwash or spitting (Webb *et al.*, 2008), therefore more research is warranted in this area. It has been proposed that nitrite is involved in “setting” basal blood pressure (Kapil *et al.*, 2018), thus larger studies on older adults may further elucidate the correlation between plasma nitrite levels following beetroot consumption and consequent effects on blood pressure.

3.4.4 The effects of beetroot on vascular function

The expected observation of a lower baseline PORH value for the old compared to the young reiterates the deterioration in vascular function into older age. PORH is a sensitive marker of vascular function and lower values are associated with higher risk of CVD. Nitrate ingestion in the present study was found to have no notable influence on PORH in the young or the old groups but the variability in measurements between and within participants may have negated any positive effects. Kapil *et al.* (2015) saw no change in PORH following four weeks of daily consumption of beetroot juice containing 397mg in hypertensive older adults but demonstrated improvements in Alx and PWV over this time. However, de Oliveira *et al.* (2016) found reactive hyperaemia (RH) to be increased following a single bolus of 756mg from beetroot gel in older adults with cardiovascular risk factors. A recent systematic review identified that inorganic nitrate significantly reduced arterial stiffness, evident through a reduction in Alx and PWV (Jackson *et al.*, 2018). It may be that these markers are more sensitive to the effects of nitrate consumption and can provide a more accurate measure of vascular function. Indeed, Kapil *et al.* (2015) and Rammos *et al.* (2014) demonstrated a reduction in both Alx and PWV following four weeks intervention with

nitrate in the form of beetroot juice or sodium nitrate. These interventions may be of most benefit to those with CVD risk factors or elicit benefits due to their prolonged supplementation period, as Hughes *et al.* (2016) and Oggioni *et al.* (2018) found no effect of shorter-term consumption of dietary nitrate on AIx in healthy older adults.

Maximum flux was the only marker of microvascular function to be positively influenced by nitrate ingestion and this occurred in the young but not the old group. Ingestion of nitrate had no impact on resting flux, time to maximum or time to resting. No studies around nitrate ingestion have described the impact on these markers, so comparisons cannot be drawn at this time. The effect of nitrate intake on maximum flux in the young but not the old group may be reflected in the higher levels of plasma nitrite shown in the younger group, which provide evidence for increased NO production in this young group. The plasma nitrite levels seen in the old group may not have been elevated to the point in which to elicit physiological benefits to the vascular system. This has been also been shown to be true for blood pressure, with nitrate ingestion having a reduced impact on blood pressure in the old group compared to the young.

3.4.5 The acceptability of whole beetroot as a supplement to the diet

The VAS results on the acceptability of the beetroot intervention provide information about participants' preferences and attitudes towards the consumption of whole beetroot. Overall, the old group rated the beetroot higher than the young group, scoring it ~9 points higher out of 60 on the VAS. This could be due to familiarity and increased exposure to beetroot in older adults. 300g beetroot, which was used to provide a high dose of dietary nitrate, received the lowest VAS score, and this was also reflected in three participants' aversion to the consumption of the large dose. 100g and 200g beetroot received comparable scores; therefore these doses are deemed acceptable in an acute intervention study and elicited high compliance. There is no data on the compliance and tolerability of beetroot consumption over a more prolonged period of time but these results provide an insight into the acceptance of beetroot as an intervention food.

3.4.6 Strengths and limitations

This study investigated, for the first time, the effects of whole beetroot consumption on blood pressure and vascular function, making a direct comparison between young and old participants provided with the same dose. As with Chapter 2, the dietary restrictions and seven-day washout period between trials ensured no carry-on effect of nitrate-rich foods consumed before testing. The screening process also identified and excluded those on blood-pressure medication and with a high blood pressure, which may have confounded the effects of the interventions.

Blood pressure was assessed in clinic, which is the most commonly used method but may introduce measurement bias due to white-coat syndrome (Bloomfield & Park, 2017), operator bias and protocol variations. These were minimised by a single operator conducting the blood pressure measurement according to the written protocol, using standardised clinical equipment and concealing blood pressure readings from participants. Raubenheimer *et al.* (2017) note that circadian rhythms can affect blood pressure, which was minimised in the present study by participants attending the research facility at least one hour after waking and with all trials beginning around the same time, between 8am and 9am.

This study did not employ PWV or FMD to measure vascular function, which are considered the gold standards for measurement. However, RH is considered a sensitive marker of vascular function in healthy participants and responds to a given stimulus, making this a valuable tool in assessing microvascular activity. There was large variation in baseline measurements of RH markers, so it is likely that a larger sample size is required to reduce the noise associated with the readings and to detect a significant effect size.

3.5 Conclusions

Small, practical amounts of beetroot have a positive effect on blood pressure in young participants but larger doses are required to elicit comparable effects in an older population. This suggests mechanistic differences in the production of NO from dietary nitrate in young and old participants. Furthermore, whole beetroot, in amounts up to 200g, is a suitable supplement for dietary intervention and is well accepted in

acute settings. This data may be used to inform dietary nitrate dosages for longer-term intervention studies on beetroot, during which the complex of nutrients in the whole vegetable may elicit synergistic and additive effects not evident in acute trials.

Chapter 4. Physiological effects of prolonged beetroot ingestion in older adults

4.1 Introduction

Beetroot consumption, most often in juice form, has been found to elicit a range of positive effects on the human body, including blood pressure reduction (Siervo *et al.*, 2013), increased blood flow (Lara *et al.*, 2016) and improvements in cognitive performance (Wightman *et al.*, 2015). These effects have been attributed to the ingestion of dietary nitrate and the subsequent increase in nitric oxide (NO) production throughout the body following the stepwise breakdown of this nitrate. Increased NO levels have also been found to acutely improve muscle strength and endurance in young adults (Bailey *et al.*, 2010; Lansley *et al.*, 2011) but there is little known about the impact of prolonged consumption of dietary nitrate on NO levels and on physiological outcomes in other demographics such as older adults. Remaining healthy and active into older age is important for maintaining muscle mass, functional capacity and cognitive performance (Everard *et al.*, 2000; Hairi *et al.*, 2010), therefore supplementing or increasing the consumption of foods rich in nitrate may potentially improve activities of daily living (ADL) and quality of life in older adults (Gault & Willems, 2013).

Pre-clinical data has demonstrated that consuming sodium nitrite for eight weeks improves functional capacity, including grip strength and open-field distance covered (Justice *et al.*, 2015). Human data has also supported the use of longer-term sodium nitrate supplementation as a potential therapy for risk factors of CVD e.g. arterial stiffness and for improving cognitive function (DeVan *et al.*, 2016; Justice *et al.*, 2015). These studies highlight the potential longer-term benefits of nitrite, which can be produced in the oral cavity following the consumption of dietary nitrate. There are concerns, however, over the consumption of nitrite and the conversion to carcinogenic compounds in the body, so more natural sources of nitrate and nitrite, such as beetroot, may be more acceptable in long-term intervention studies.

Beetroot juice has been shown to lower systolic (SBP) and diastolic (DBP) blood pressure in hypertensive older adults (Kapil *et al.*, 2015) and improve vascular

function (Velmurugan *et al.*, 2016). The improvements in vascular function were closely correlated with the oral microbiome, with increases in nitrate-reducing bacteria, *Rothia Mucilaginosa* and *Neisseria Flavescens*. Research suggests similarities between the oral and gut microbiome (Kodukula *et al.*, 2017), which together form the majority of the human microbiome. The oral microbiome is generally considered stable in healthy adults (Hall *et al.*, 2017) but gut microbiota are heavily influenced by dietary intake.

Research into the gut microbiome has demonstrated the symbiotic relationship between the host and the microbes that reside within the GI tract, where they have been shown to be involved in immunity, inflammation and digestion (van der Beek *et al.*, 2017; Belkaid & Hand, 2014). The gut microbiome is therefore a significant contributor to health and has been implicated in obesity, diabetes and CVD (Korpela *et al.*, 2014; Tilg & Moschen, 2014; Tuohy *et al.*, 2014). Some of the main drivers of changes to the gut microbiome are age, genetics and dietary intake (Wu *et al.*, 2011). For example, older age is associated with a reduction in bacterial diversity, which can contribute to co-morbidities and poor quality of life (O'Toole & Claesson, 2010). In addition, the production of key metabolites such as short-chain fatty acids (SCFAs), which have been shown to have beneficial effects on the gut and in cardiovascular health (Marques, 2016), has been found to be reduced in older populations (Biagi *et al.*, 2017). The effect of dietary fibre has been studied in dietary interventions and gut microbiome studies, and has been found to be beneficial in the risk of CVD and some cancers (Dahl *et al.*, 2017). However, supplementation with whole beetroot, which may also offer benefits such as anti-inflammatory action (Noble *et al.*, 2017), increased mucosal blood flow and mucus thickness (Björne *et al.*, 2004), and increased species richness and diversity (Vandeputte *et al.*, 2016), is not well understood in human trials, specifically in older adults.

Therefore, this study investigates longer-term beetroot consumption and its effects on functional capacity, blood pressure and gut health in older adults. It was hypothesised that regular consumption of whole beetroot over an eight-week period would have a positive impact on functional capacity, blood pressure, SCFA production and gut microbial composition. To this end, participants consumed whole cooked beetroot and/or a control food consistently over an eight-week period

and attended the research facility on three occasions to assess physiological changes over this period.

4.2 Methods

4.2.1 Participants

Participants were recruited through Voice, a research participant database, the Newcastle Upon Tyne Hospitals intranet, poster dispersion and adverts in the local papers from January 2018 to April 2018. Each volunteer was given a participant information sheet and screened over the phone prior to attending the research facility. Participants provided informed written consent before beginning the study. The study was approved by the Newcastle university ethical committee (Appendix G). Recruited participants had a BMI below 30kg/m², were both male and female and were aged between 60 and 80 years. The total participant group consisted 14 males and 22 females.

4.2.2 Exclusion criteria

This study was based on healthy participants i.e. those with a BMI below 30kg/m² and free of chronic illness. Excluded medications included: beta-blockers, antacids, anticoagulants, nitrate-derived agents, anti-cholinergics, anti-hypertensives (Ca²⁺ channel blockers, ACE inhibitors), aspirin and diuretics. Each of these medications can have an effect on NO production via various mechanisms. The use of antibiotics in the past month was excluded due to the negative effect on the gut bacteria. Participants were excluded if they were vegetarian, as research points towards a higher habitual nitrate intake (Mitek *et al.*, 2013), or consumed excessive volumes of alcohol (classed as above 30 units/week). Other exclusion criteria included smoking, history of repetitive gastric reflux and an intolerance or allergy to the intervention foods.

4.2.3 Study design and randomisation

This study was a two-arm, parallel, open-label intervention trial that took place at the research facility NU-Food, Newcastle University. Randomisation was generated online using www.randomization.com, where the two treatments, beetroot and

control, were allocated treatment codes by an independent third party. Participants were then assigned a code following screening.

4.2.4 Study protocol

Eligible volunteers were invited to attend NU-Food for a training visit, where height, weight and blood pressure were measured. Informed written consent was received at this visit and participants were then booked in for their baseline visit four to seven days following training. Participants were asked to fast for two hours prior to their baseline and subsequent visits and to refrain from caffeine intake in this period. Those opting to provide stool samples for the assessment of gut health were given a collection kit and instructions to take home and asked to collect a sample in the three days prior to their visit.

Participants attended a baseline, mid-way, and end of study testing visit, each lasting up to one hour and 15 minutes. The visit started with the completion of a caffeine intake tool for the 24-hour period before their visit. Following this, blood pressure, body composition, grip strength, lung function and timed up and go were measured using standard protocols. A blood sample was taken from the antecubital fossa by a trained phlebotomist and a urine sample was deposited into a sample pot. Participants then completed a 24-hour recall with the researcher and a physical activity questionnaire as instructed. Each participant was provided with his or her intervention foods at the end of the visit and sample collection kits as required. An intervention foods calendar was created for ease with contact details should there be any problems. Midway and end of study visits were conducted during weeks five and nine, respectively. This can be seen in Figure 4.1.

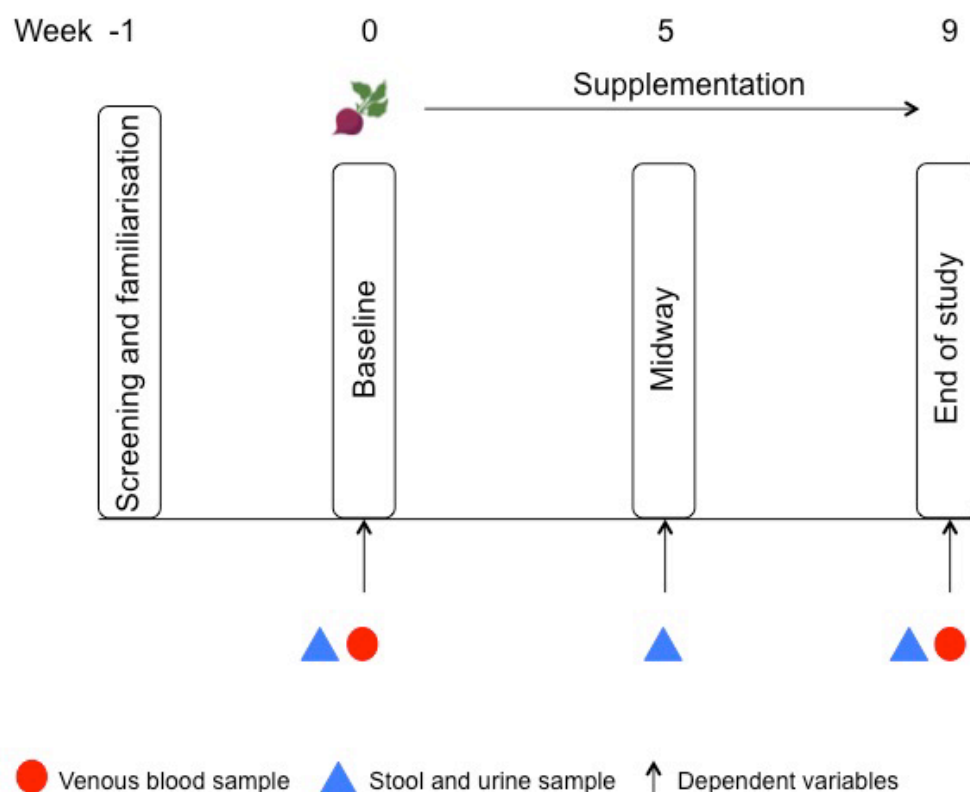


Figure 4.1: Study 2 protocol. Variables measured during study visits: blood pressure, functional capacity and cognitive function.

4.2.5 Interventions

Participants were randomised to consume either 150g of whole cooked beetroot plus one medium-sized banana every second day, or one medium-sized banana every second day. Beetroot was provided for the entirety of the study in pre-cooked vacuum packed portions of 250g provided by G's Fresh Ltd, Cambridgeshire. Bananas were provided on a per-participant basis and purchased at home in between study visits. Participants consumed the intervention foods at home during the eight-week period and were given instructions on the consumption and storage of the beetroot. As the beetroot was already cooked, participants were instructed not to cook the beetroot further or combine into meals that would be consumed by other people. Examples and recipes were provided to aid in appropriate consumption of the beetroot. Participants were instructed to maintain their normal diet, consuming the foods on top as “supplements”; the beetroot could be consumed at any time of the day. This has been utilised before to maximise the external validity of the outcomes (Coles & Clifton, 2012; Shannon *et al.*, 2016). The beetroot provided was

found to contain 390mg nitrate per 100g cooked weight. Therefore, participants consumed ~585mg nitrate every second day for eight weeks. Due to the difficulty in providing a control diet, it was deemed appropriate for both the intervention group and the control group to consume the control food. This resulted in a comparable background diet with beetroot consumption as an addition. Bananas were chosen as the control food because of their low nitrate content, similar fibre content and standardisation due to the lack of varieties available to purchase (Table 4.1). According to Public Health England's Composition of Foods, dietary fibre "is present in significant quantities but there is no reliable information on the amount" in beetroot; packet information from the beetroot suppliers was therefore used. Compliance to the interventions was self-reported and participants provided receipts for the purchase of the control food in order to claim expenses.

Table 4.1: Nutrient composition of intervention foods.

	<i>Beetroot/100g</i>	<i>Banana/100g</i>
<i>Energy (kcal)</i>	46	81
<i>Carbohydrate (g)</i>	9.5	20.3
<i>Protein (g)</i>	2.3	1.2
<i>Fat (g)</i>	0.1	0.1
<i>Sugar (g)</i>	8.8	14.4
<i>Fibre (g)</i>	2.3	1.4
<i>Nitrate (mg)</i>	390	5

Data from the McCance and Widdowson's Composition of Foods Integrated Dataset (Public Health England, 2015).

4.2.6 Blood pressure

Blood pressure was measured in triplicate at each visit using an automated device (OMRON M2). Participants were seated and the pressure cuff was placed on the upper arm. The arm was then placed in a comfortable and relaxed position and one-minute rest was given between measurements. Measurements were hidden from the participants so that their knowledge of their blood pressure reading did not influence future measurements.

4.2.7 Hand-grip strength

Hand-grip strength is used as an indicator of frailty in older adults, as it predicts increased risk of disability and functional limitations (Norman *et al.*, 2011; Syddall *et al.*, 2003). It is used as a simple and non-invasive marker of muscle strength in the upper extremities. Grip strength was measured using a handheld digital dynamometer (*Takei 5401*). Participants began with their dominant hand and grip strength in each hand was measured in duplicate. Participants remained standing and with their hands by their side for the assessment. Values for each hand were averaged and measured in kilograms (kg). Coldham, Lewis and Lee (2006) noted that one maximal trial is as reliable as, and less painful than, either the best of or mean of three. With an older population suffering from arthritis and other conditions, measurement of two maximal trials per hand was chosen. The most important aspect is that the same design and instrument is employed for each measurement (Mathiowetz, 1984).

4.2.8 Spirometry

Lung function was assessed using a portable, handheld spirometer at each visit (*Vitalograph Micro Handheld Spirometer, UK*). Spirometry is a quick and simple way to measure lung volume against time (Ranu *et al.*, 2011). Participants were sitting upright and in a comfortable position, with both feet on the floor and legs uncrossed. Participants were guided through the technique and a disposable, single-use mouthpiece was used for each participant. Tidal (normal) breaths were taken first, and then participants took a deep breath in before breathing out as hard and as fast as possible, until they had emptied their lungs. Forced expiratory volume in one second (FEV₁) and forced vital capacity (FVC) were measured in triplicate. The protocol used was that devised by Moore (2012). The ratio of the two volumes was then calculated using the equation:

$$FEV_1/FVC$$

4.2.9 Timed-up-and-go

The timed up-and-go (TUG) test assesses agility and dynamic balance, and this test was performed at each visit using a standardised protocol described by Barry *et al.* (2014). Participants were instructed to rise from a chair, walk three metres to a

marker at a comfortable pace, turn around, walk back to the chair and sit down. Each participant walked through the test once without being timed to become familiar with the task. A stopwatch was used to time the second trial, stopping when the participant sat back down on the chair. TUG test times have been found to increase with increasing age (Bohannon, 2006), and normative reference values for the test are 8.1s for those aged 60-69 years and 9.2s for those aged 70-79 years (Bohannon, 2006).

4.2.10 Questionnaires

Participants were asked to complete a stool frequency questionnaire in the three days prior to each of their visits. This questionnaire involved the use of the Bristol Stool Chart to identify the type of stool and the frequency of bowel movements during those days. During each of the visits, participants completed a 24-hour dietary recall with the researcher, to report their nitrate and dietary fibre intake the day before testing. Nitrate intake was then analysed using a nitrate specific database developed by researchers at the University of Queensland, Australia. Fibre intake was analysed using the McCance and Widdowson Composition of Foods Integrated Dataset. The short version of the IPAQ was used to monitor physical activity over the seven days prior to each visit and consistency throughout the study.

4.2.11 Blood sampling, processing and analysis

15ml of blood was collected at baseline and at the end of study visit, in three vacutainers: one lithium heparin, one serum gel and one EDTA. The lithium heparin and EDTA samples were processed within 30 minutes, while the serum gel was given at least 30 minutes to clot before processing. The samples were spun at 3000rpm for 10 minutes, aliquoted and frozen at -80°C for future analysis. Samples were prepared for analysis in the same way as in Chapter 2, using cold ethanol precipitation. Plasma nitrate was then analysed by ozone-based chemiluminescence, as described in Chapter 2. In order to measure the prolonged effects of beetroot ingestion rather than the acute effects, blood samples were taken at least 12 hours after the last beetroot dose was consumed.

4.2.12 Urine sampling

Spot urine samples were taken at each visit for the analysis of nitrate. Participants deposited a urine sample in a designated bottle and aliquots were drawn and frozen immediately at -20°C. Before analysis, urine samples were defrosted and diluted to 1:100 in deionized water (DH₂O). As described in Chapter 2, urinary nitrate was analysed using ozone-based chemiluminescence by the Sievers gas-phase chemiluminescence nitric oxide analyser (NOA). Human Tissues Act (HTA) training was completed by the researcher and samples were stored in an HTA licenced freezer and laboratory.

4.2.13 Stool sample, processing and analysis

17 participants collected stool samples throughout the study; eight from the intervention group and nine from the control group. One stool sample was collected prior to each visit on the day before or the morning of the visit day. Participants were instructed to freeze the sample immediately and bring it with them in a provided cooler bag with ice packs. The stool sample was then transferred to a -80°C freezer until analysis. Samples were stored in an HTA licenced freezer and laboratory. A Research Associate within the Mitochondrial Research Group at Newcastle University carried out the short chain fatty acid (SCFA) analysis. Bacterial analysis was conducted at Northumbria University.

SCFA

Short chain fatty acid (SCFA) content was determined by gas chromatography (GC). Firstly, a deproteinising solution (DPS) was prepared by dissolving metaphosphoric acid and 3-methyl valeric acid (3MV) in distilled water, giving a 50mM 3-MV solution, which was then stored at 4°C. Stock solutions of 0.2M were prepared for acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid. This was achieved by adding the appropriate amount of each acid to DH₂O as follows (Table 4.2):

Table 4.2: Stock solutions of short chain fatty acids made using deionised water.

	<i>Acid (μl)</i>	<i>Water (ml)</i>
<i>Acetic acid</i>	286	24.71
<i>Propionic acid</i>	375	24.63
<i>Isobutyric acid</i>	469	24.53
<i>Butyric acid</i>	461	24.54
<i>Isovaleric acid</i>	558	24.44
<i>Valeric acid</i>	545	24.45

Each of these SCFA standards was run individually on the GC (*Shimadzu GC-2014 with AutoInjector AOC-20i, Japan*) in duplicate to identify peaks and retention times. A SCFA “master mix” was then prepared using 10ml acetic acid, 5ml propionic acid, 1ml isobutyric acid, 2ml butyric acid, 1ml isovaleric acid and 1ml valeric acid. Standard SCFAs were prepared by combining 2ml of the master mix with 2ml distilled water and 1ml DPS, giving a final concentration of 40mM acetate, 20mM propionate, 4mM isobutyrate, 8mM butyrate, 4mM isovalerate, and 4mM valerate. The standard SCFA mix was then run on the GC to confirm peaks and retention times; this graph can be seen in Appendix I. To calculate the response factor (RF) the following formula was used (example for acetate):

$$\text{peak AUC 3MV} \times [\text{acetate}] / \text{peak AUC acetate} \times [3\text{MV}]$$

To calculate SCFA in the stool samples, 250mg of each stool sample was combined with DPS at half weight by volume (125μl) and mixed. 200μl DH₂O was then added, briefly mixed and then centrifuged at 15,000rpm for 20 minutes. 50μl of the supernatant was transferred to glass GC vials and sealed. The concentration of SCFAs in each sample was analysed by GC using a 2mm diameter column packed with a 10% SP-1200-1% phosphoric acid on 80/100 chromosorb in a PU 4550 gas liquid chromatograph. 1μL of sample was injected into the GC instrument using a glass-barrelled needle. Concentrations of SCFAs in samples were calculated relative to the standard master mixed of SCFA and internal standard (3MV), which is present

in the DPS. The following calculation was then used to calculate the concentration of each SCFA in the sample, using acetate as an example:

$$(RF \text{ for acetate} \times [3MV] \times \text{peak area for acetate}) / \text{peak area for 3MV}$$

DNA extraction and bacterial analysis

The methods used follow the same protocol as that described by Houghton *et al.* (2018). Briefly, DNA was extracted from 300mg of stool sample from each time point (baseline, midway and end of study) using the Powerlyzer Powersoil DNA isolation kit (MoBio, UK), following manufacturer's instructions. The 16s rDNA V4 region was amplified using PCR and then sequenced in the MiSeq platform (Illumina); the V4 region is one of the most well characterised regions of interest. 16s rRNA gene sequences were then clustered into operational taxonomic units (OTUs) based on sequence similarity, which categorise groups of bacteria that are closely related to a value of 97% similarity. OTUs were mapped to the SILVA database, which is an online alignment tool used to map the OTUs to up to date 16S sequence data for bacteria (Quast *et al.*, 2013). Abundances were reported by mapping demultiplexed reads to the OTUs. A custom script constructed a rarefied output table ensuring all samples had the same number of OTUs for downstream analysis of alpha- and beta-diversity, as well as phylogenetic trends.

4.2.14 Statistical analysis

All statistical analyses were completed using IBM SPSS (version 24.0). Summary data are presented as means \pm SD with 95% confidence intervals (CI). Independent t-tests were used to assess differences in baseline measures between the two intervention groups. Changes over time were analysed using a mixed model repeated measures ANOVA with time as the within-subjects factor and intervention as the between-subjects factor. Models were tested for sphericity using Mauchly's test and multivariate models were applied if these assumptions were violated. Where significances were found, *post hoc* tests with Bonferroni adjustment to control for multiple comparisons were run. R was used to analyse and visualise microbial communities using the phyloseq package, allowing alpha- and beta-diversity metrics to be calculated. Each sample was rarefied to 15,721 reads. The non-parametric test

Kruskal-Wallis was used to determine significance between categorical variables. Correlations between two continuous variables were determined with linear regression models with P values indicating the probability that the regression line is zero. Principal coordinate plots employed the Monte Carlo permutation test to estimate P values, and the principal component analysis (PCA) was used to highlight patterns and variation in the data. FDR algorithms were used to adjust P values when multiple comparisons were conducted. Statistical significance was set at $P < 0.05$.

4.3 Results

4.3.1 Baseline characteristics

A total of 36 participants were randomised to the interventions, with an age range of 60 to 79 years (Figure 4.2). The interventions were well tolerated and no adverse events were reported. Participants did not report beeturia (red-coloured urine) with the beetroot intervention. Baseline characteristics can be seen in Table 4.3. 17 participants were allocated into the control group and 19 into the intervention group. Participants in each intervention group were matched for height, weight, BMI, waist circumference and body fat ($P > 0.05$) but average age in the control group was 70.1 ± 5.6 years, compared to the intervention group of 65.1 ± 4.4 years ($P = 0.009$). Both groups had similar baseline systolic and diastolic blood pressure, and physical activity levels ($P > 0.05$). Nitrate intake on the day before baseline testing was similar for both groups, with values of 124.9 ± 66.1 mg and 103.4 ± 57.1 mg for the beetroot and control group, respectively ($P = 0.23$). Dietary fibre intakes were also similar across groups, with approximately 20.4 ± 3.1 g in the beetroot group and 17.8 ± 7.5 g in the control group ($P = 0.38$). The average across both groups of 19.1 ± 5.7 g matches the average population intake reported in the National Diet and Nutrition Survey (NDNS) of 19 g (Public Health England, 2018). Participants consisted of 14 males and 22 females overall, with a ratio of 6:13 and 8:9 male to female in the beetroot and control groups, respectively. One participant was withdrawn from the study due to beginning medication that may interfere with study outcomes during the intervention period.

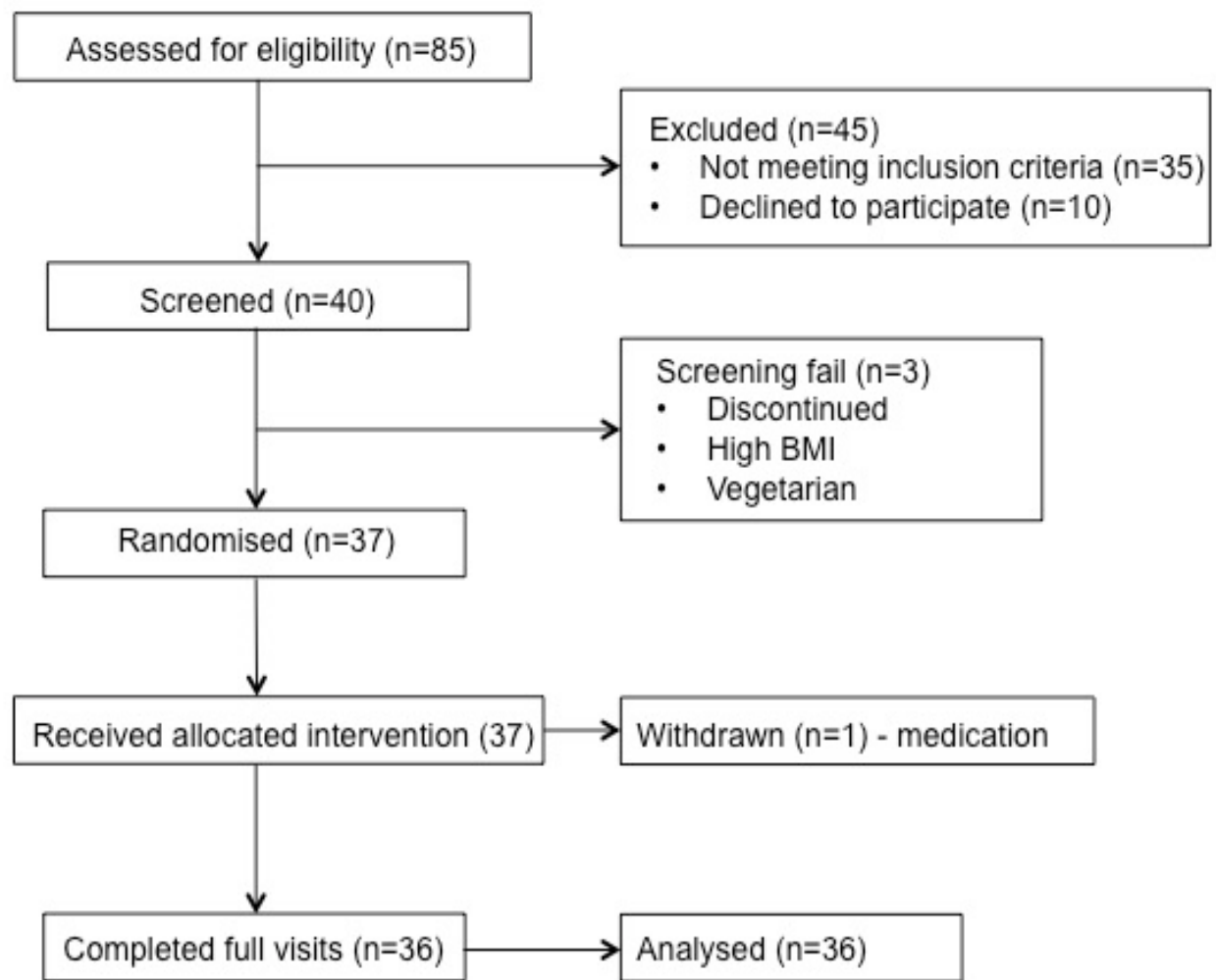


Figure 4.2: Recruitment flow diagram, study 2

Table 4.3: Mean (\pm SD) baseline characteristics, study 2; n=36.

	<i>All</i>	<i>Intervention</i>	<i>Control</i>	<i>P value</i>
<i>Number</i>	36	19	17	
<i>Gender (M/F)</i>	14/22	6/13	8/9	
<i>Age (years)</i>	67.4 \pm 5.6	65.1 \pm 4.4	70.1 \pm 5.6	0.005
<i>Height (cm)</i>	166.7 \pm 8.7	167.3 \pm 6.9	166.1 \pm 10.5	0.70
<i>Weight (kg)</i>	70.0 \pm 9.4	69.3 \pm 10.2	70.9 \pm 8.7	0.63
<i>BMI (kg/m²)</i>	25.1 \pm 2.4	24.6 \pm 2.7	25.6 \pm 1.9	0.23
<i>Waist (cm)</i>	85.9 \pm 9.9	83.9 \pm 10.2	88.3 \pm 9.3	0.19
<i>Body fat (%)</i>	27.9 \pm 8.6	28.8 \pm 8.7	26.8 \pm 8.6	0.52
<i>SBP (mmHg)</i>	137.9 \pm 16.7	135.5 \pm 19.6	140.3 \pm 13.8	0.40
<i>DBP (mmHg)</i>	81.6 \pm 10.5	86.0 \pm 12.8	86.4 \pm 6.8	0.89
<i>Heart rate (BPM)</i>	70.8 \pm 8.5	71.1 \pm 9.8	70.5 \pm 6.9	0.84
<i>Physical activity</i>	3376.0 \pm 2734.3	3866.0 \pm 3098.2	2828.4 \pm 2225.2	0.26

Statistical analysis using independent t-tests. SBP = systolic blood pressure, DBP = diastolic blood pressure.

4.3.2 Body composition, physical activity and dietary intake

Weight, waist circumference and body fat percentage did not change from baseline to end of study in either the beetroot group or control ($P>0.05$). Nitrate intakes on the day before testing were significantly higher at the midway and end of study visits than at baseline for the beetroot group, rising to 242.8 \pm 147.1mg at midway ($P=0.02$) and to 270.2 \pm 155.8mg at end of study ($P=0.002$). Nitrate intake the day before testing also rose in the control group to 203.3 \pm 162.8mg at midway and 154.7 \pm 105.5mg but these were not significantly different to baseline ($P>0.05$). Nitrate intakes the day before end of study testing were significantly higher in the beetroot group than the control group (+115.5mg; $P=0.02$; 95%CI: 19.7, 211.3), demonstrating compliance to the intervention and success at increasing nitrate intake. Dietary fibre intake on the day before testing across both groups rose to 23.5 \pm 5.4g, which is above the average daily intake in the UK. Fibre intake was significantly higher than baseline at the end of the study in the control group (+5.5g; $P=0.02$; CI: 0.8, 10.2) but there was no significant change in the beetroot group ($P=0.26$). There was, however, a large range of fibre intakes at baseline in the control group (8g – 32g) and a smaller range at end

of study (15g-32g), so results may have been skewed by individuals with particularly low or high intakes.

4.3.1 Plasma nitrate

There was no effect of time on plasma nitrate levels ($P=0.10$) but a noteworthy trend towards an effect of intervention ($P=0.06$) (Figure 4.3). There was no interaction between time and intervention ($P=0.16$). Plasma nitrate levels were found to be $7.05\mu\text{mol/L}$ lower in the control compared to the intervention group. *Post hoc* analyses show plasma nitrate levels were significantly higher at the end of study compared to baseline in the intervention group ($+11.9\mu\text{mol/L}$; $P=0.04$; 95%CI: 0.9, 22.9) but there was no significant difference in the control group ($P=0.86$). The beetroot given was found to contain 390mg nitrate per 100g cooked weight, meaning that if participants consumed their allocated amounts they would have consumed ~585mg nitrate every second day through the consumption of 150g beetroot. Based on the results from Chapter 2, this amount should have been enough to raise both plasma nitrate and nitrite levels in the participants. Although there was potentially a small additive effect evident through the higher plasma nitrate levels following only the beetroot intervention, nitrate levels were not raised from baseline to an extent deemed physiological. Levels remained similar to the baseline values observed in Chapter 2.

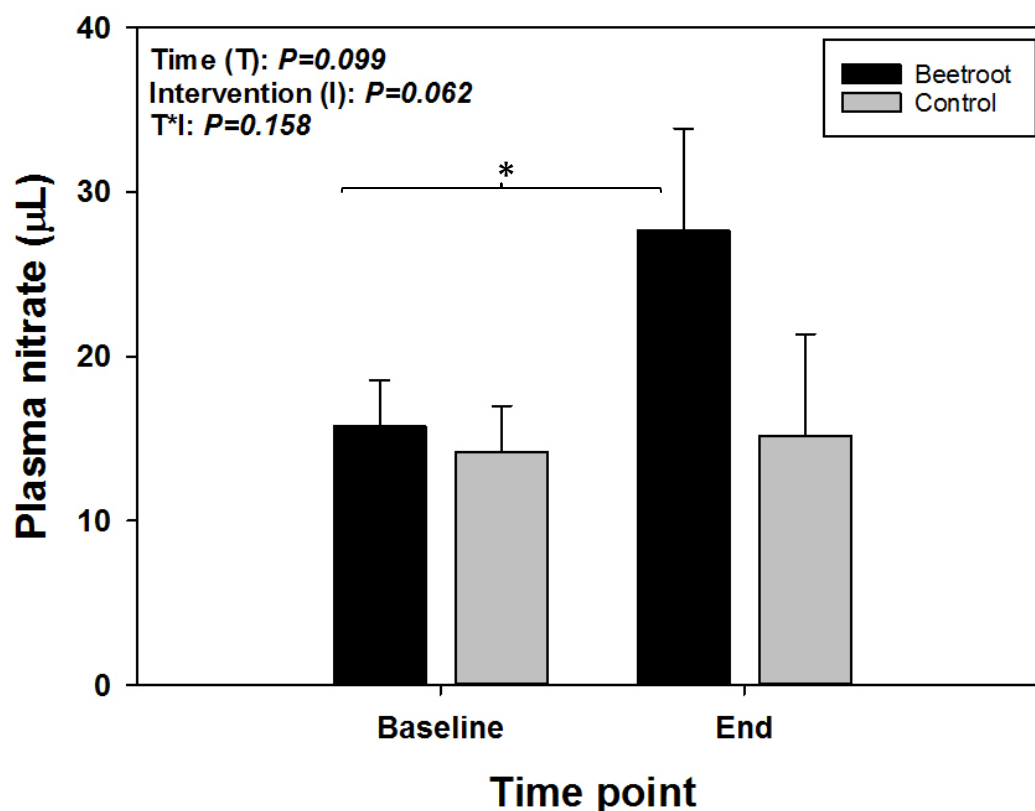


Figure 4.3: Mean (\pm SEM) plasma nitrate levels at baseline and after eight weeks of supplementation with beetroot or control; $n = 30$. Statistical analysis using mixed model repeated measures ANOVA (time as within-subjects factor and intervention as between-groups factor). * denotes a significant difference when compared to baseline in the beetroot group ($P < 0.05$).

4.3.2 Urinary nitrate

There was a significant effect of time on urinary nitrate levels ($P = 0.005$) but no effect of intervention ($P = 0.75$) (Figure 4.4). There was no interaction between time and intervention ($P = 0.47$). Pairwise comparisons show that urinary nitrate levels were higher than baseline at the mid point ($+0.39\text{mmol/L}$; $P = 0.006$; 95%CI: 0.09, 0.69) and end of study ($+0.33\text{mmol/L}$; $P = 0.003$; 95%CI: 0.09, 0.56) for both interventions combined.

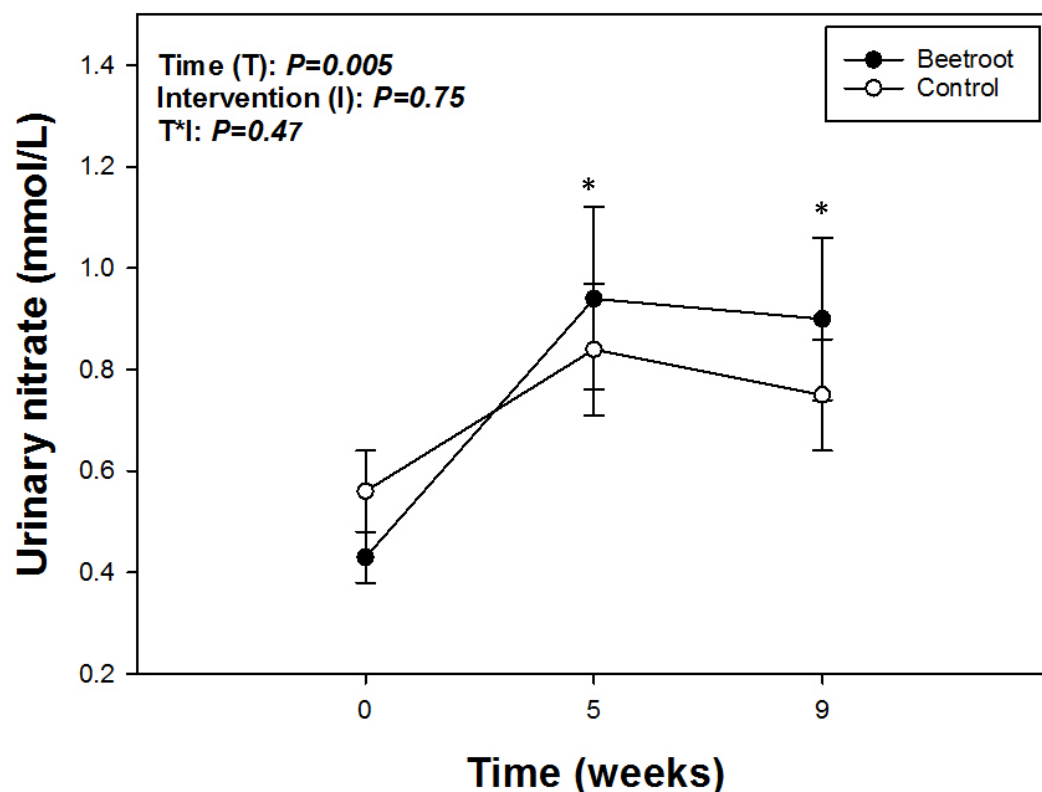


Figure 4.4: Mean (\pm SEM) urinary nitrate concentration at baseline (0), midway (5) and end of study (9) after beetroot supplementation and control; $n = 36$. Statistical analysis using mixed model repeated measures ANOVA (time as within-subjects factor and intervention as between-groups factor). * denotes a significant difference when compared to baseline across both groups ($P<0.05$).

4.3.3 Functional capacity

Baseline, midway and end of study values for functional capacity are shown in Table 4.4. There were no significant changes to functional capacity measures over the eight weeks following beetroot or the control food ($P>0.05$).

Table 4.4: Mean (\pm SD) functional capacity measurements; n = 36.

	<i>Intervention</i>				<i>Control</i>			
	Baseline	Midway	End	<i>P</i>	Baseline	Midway	End	<i>P</i>
<i>Left grip (kg)</i>	26.4 \pm 5.8	24.6 \pm 7.8	25.7 \pm 7.1	1.00	25.1 \pm 8.7	24.5 \pm 8.2	24.6 \pm 9.0	1.00
<i>Right grip (kg)</i>	26.5 \pm 6.6	24.9 \pm 8.5	25.4 \pm 8.8	1.00	27.2 \pm 8.6	26.1 \pm 10.4	26.5 \pm 9.3	1.00
<i>TUG (sec)</i>	6.2 \pm 0.9	6.1 \pm 1.0	6.2 \pm 1.1	1.00	6.7 \pm 1.5	6.5 \pm 1.4	6.8 \pm 1.3	1.00
<i>FVC</i>	3.4 \pm 0.8	3.3 \pm 0.8	3.2 \pm 0.7	0.29	3.3 \pm 1.2	3.3 \pm 1.0	3.2 \pm 1.0	1.00
<i>FEV1</i>	2.8 \pm 0.6	2.6 \pm 0.6	2.7 \pm 0.5	0.45	2.6 \pm 0.9	2.7 \pm 0.7	2.6 \pm 0.8	1.00
<i>FEV1%</i>	83.1 \pm 12.5	82.3 \pm 8.5	84.4 \pm 9.2	1.00	82.4 \pm 9.3	82.2 \pm 16.1	83.3 \pm 9.9	1.00

TUG = timed up-and-go; FVC = forced vital capacity; FEV1 = forced expiratory volume in one second. FEV1% = FVC/FEV1 ratio. P values represent change from baseline to end to study.

4.3.4 Blood pressure

Baseline SBP was similar between groups, with values of 140mmHg and 136mmHg for the control and intervention group, respectively. DBP was also matched across groups, with a mean of 86mmHg for both groups.

There was a significant effect of time on SBP ($P=0.003$) but no effect of intervention ($P=0.29$) (Figure 4.5). There was no interaction between time and intervention ($P=0.94$). Pairwise comparisons show that SBP at the end of study was significantly lower than at the mid point (-7.7mmHg ; $P=0.003$; 95%CI: $-13.1, -2.2$) for both interventions combined.

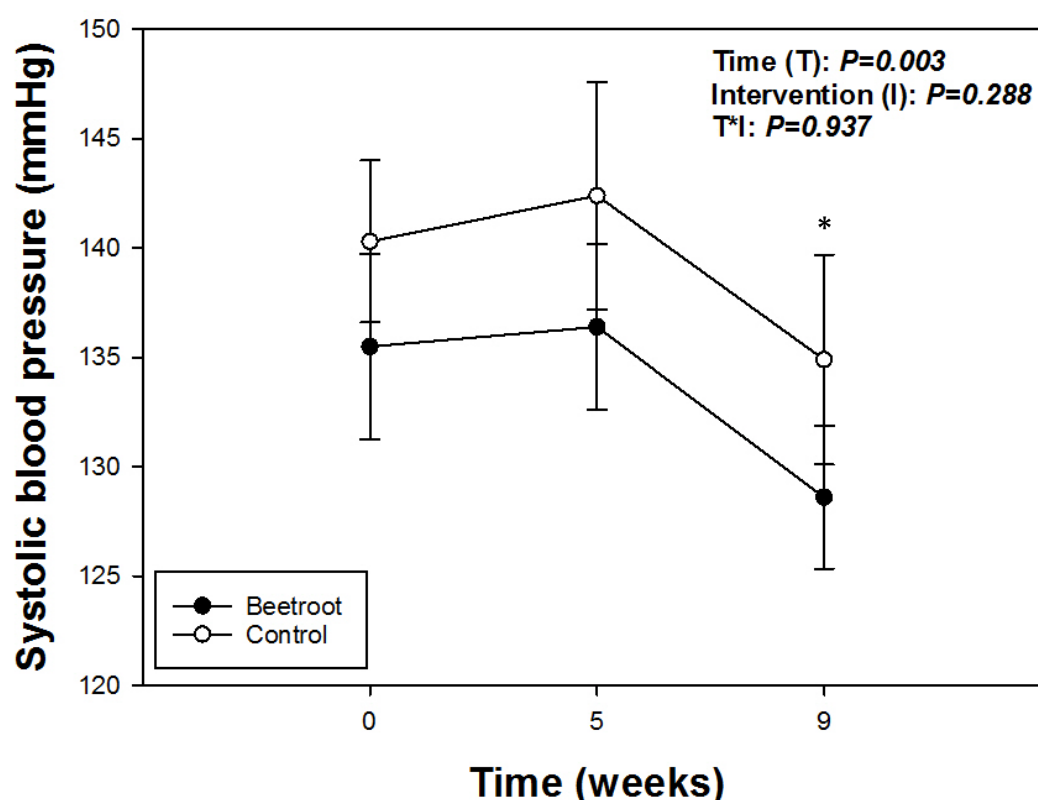


Figure 4.5: Mean (\pm SEM) systolic blood pressure (SBP) at baseline (0), midway (5) and end of study (9) in the beetroot and control groups; $n = 36$ (17 beetroot, 19 control). Statistical analysis using mixed model repeated measures ANOVA with time as the within-subjects factor and intervention group as the between-subjects factor. * denotes significant difference to midway across interventions.

Similarly, there was a significant effect of time ($P=0.04$) but no effect of intervention ($P=0.99$) on DBP and no interaction between time and intervention ($P=0.85$). Pairwise comparisons and *post hoc* analyses however do not identify any significant changes in DBP over time.

4.3.5 Gut microbiotal changes

Results from 16srRNA gene analysis of the 17 stool samples are described below, including bacterial abundance, alpha diversity and beta diversity. Alpha diversity describes the species richness, measured by both a count of the number of distinguishable OTUs in each sample (Goodrich *et al.*, 2014), and by species diversity, which measures evenness of distribution. Species diversity is measured by the Shannon Index, which examines how evenly bacterial species are distributed in a sample and if some species are more dominant than others. Beta diversity is a measure of microbial composition in one environment compared to another i.e. over time or between interventions. A Bray-Curtis dissimilarity profile, which was used in the present study, assesses abundance profiles from different samples and the overlap between them (Morgan & Huttenhower, 2012).

Bacterial abundance

Firmicutes and *Bacteroidetes* were the most dominant phyla in all samples, which is commonly reported in human studies (Duncan *et al.*, 2008; Karlsson *et al.*, 2013). *Firmicutes* was the most dominant phylum across intervention groups with $51.4\pm15.5\%$ relative abundance at baseline and $49.8\pm10.2\%$ at end of study. *Bacteroidetes* was also abundant across both groups showing $38.6\pm16.7\%$ and $39.3\pm12.0\%$ relative abundance at baseline and end of study, respectively. For the beetroot group, baseline abundance of *Firmicutes* was $52.7\pm17.2\%$, increasing to $54.1\pm8.0\%$ at end of study. In the control group, abundance of *Firmicutes* was $50.2\pm14.8\%$ and $46.2\pm10.8\%$ at baseline and end of study, respectively. A full list of the bacterial species analysed at phylum level for the beetroot and the control group is shown in Figure 4.6.

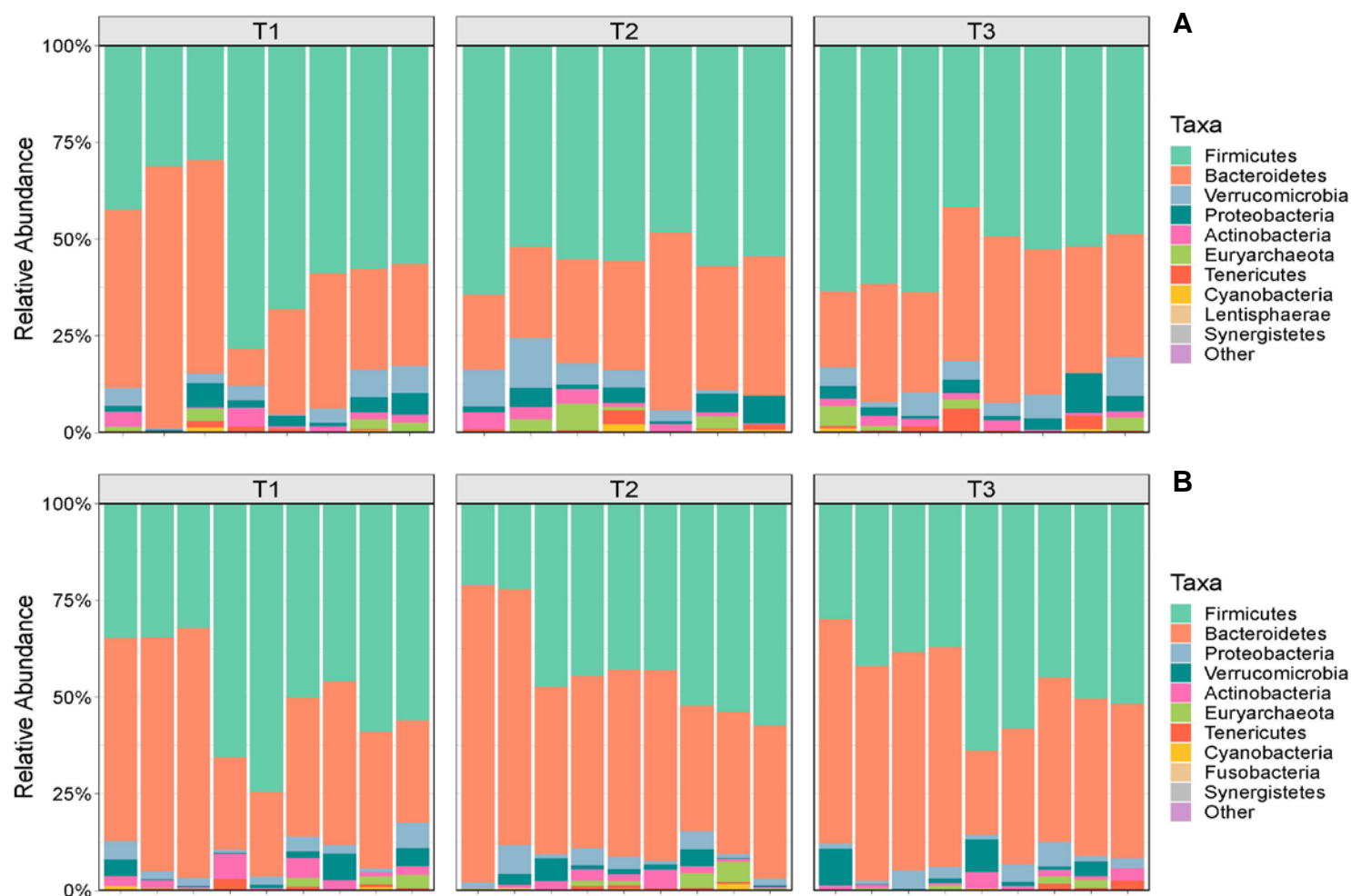


Figure 4.6: Stacked bar plots showing the relative abundance distribution of taxa at phylum level in the beetroot group (A) and control group (B) at baseline (T1), midway (T2) and end of study (T3); n = 17 (8 beetroot, 9 control). Each bar represents one sample.

At genus level, the most abundant bacteria across both groups was *Bacteroides*, which is commonly reported as the predominant genus within the lower intestinal tract in humans (Gibson, 1999). *Bacteroides* had $16.4 \pm 9.0\%$ relative abundance at baseline in the beetroot group, dropping to $14.2 \pm 7.0\%$ at end of study. In the control group, *Bacteroides* abundance was $19.4 \pm 13.0\%$ at baseline and $19.4 \pm 14.7\%$ at end of study. *Alistipes* abundance was similar across groups at baseline with $5.1 \pm 3.7\%$ and $5.3 \pm 2.6\%$ in the beetroot and control groups, respectively. Relative abundance of *Alistipes* rose to $9.5 \pm 4.3\%$ at end of study in the beetroot group and remained similar at $4.8 \pm 2.7\%$ in the control group. The most abundant bacteria at genus level in each intervention group are illustrated in Figure 4.7.

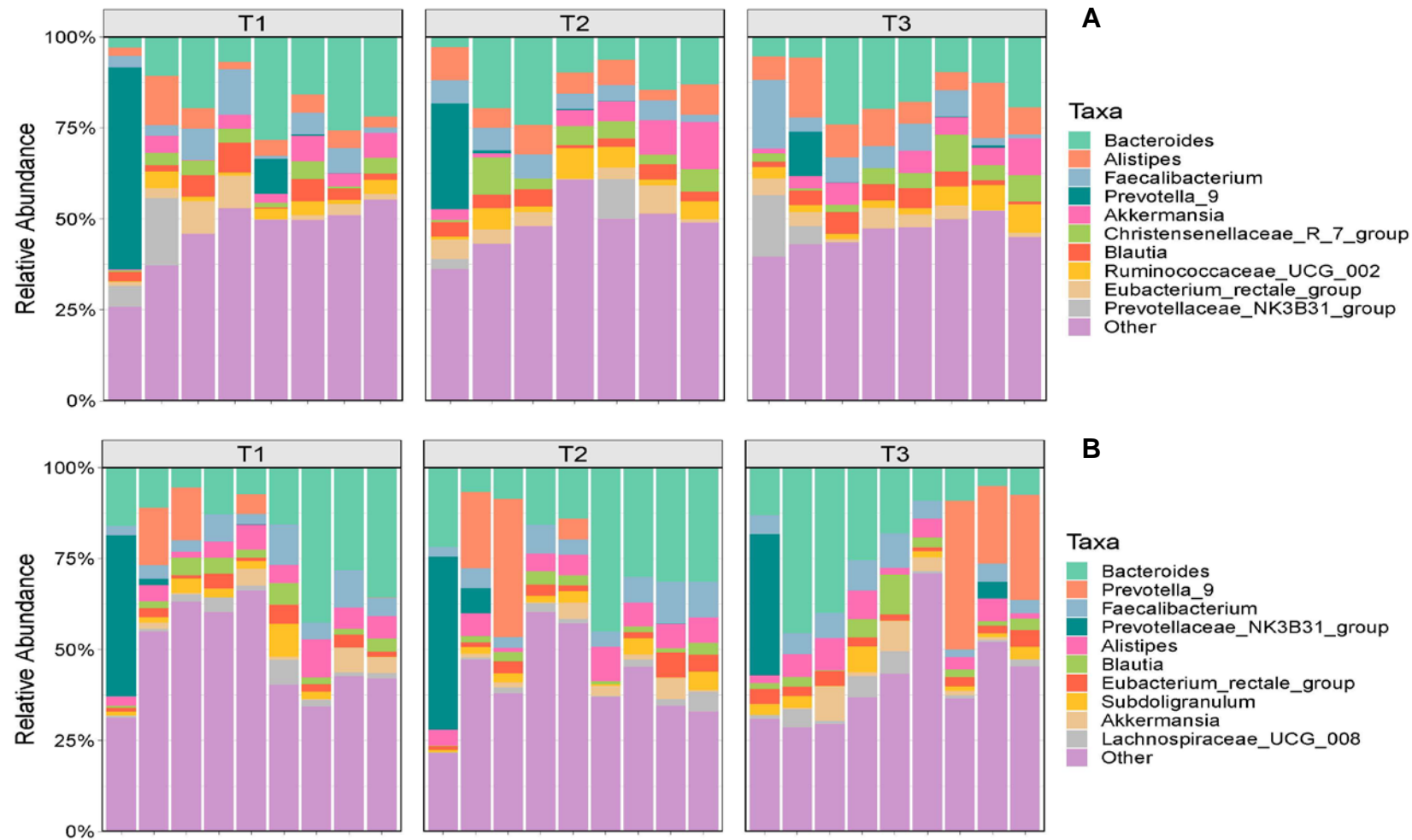
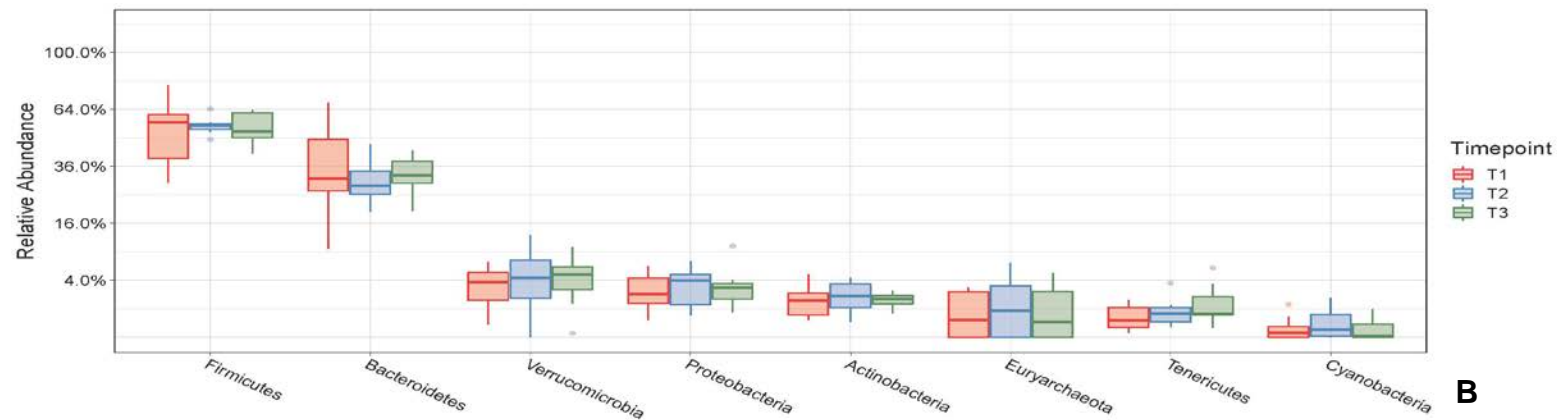


Figure 4.7: Stacked bar plots showing the distribution of taxa at genus level in the beetroot group (A) and control group (B) at baseline (T1), midway (T2) and end of study (T3); n = 17 (8 beetroot, 9 control).

Figures 4.8 and 4.9 below illustrate changes in bacteria at phylum and genus level over time in both intervention groups. Abundance of *Verrucomicrobia* and *Tenericutes* appears to rise in the beetroot group but not the control group, although these were not found to be significant ($P>0.05$). Figure 4.9 illustrates a rise in *Alistipes* and *Akkermansia* in the beetroot group but not the control group, but again these were not found to be significant changes ($P>0.05$). *Alistipes* abundance was found to rise by 88% in the beetroot group and fall by 8% in the control group between baseline and end of study. An increased abundance of *Alistipes* is indicative of the consumption of more plant-based foods. *Akkermansia* is a mucin-degrading bacterium that has been found to increase following the consumption of cranberry extract in mice, potentially due to the high content of polyphenols present (Anhê *et al.*, 2016). It has also been shown to be important for maintaining gut integrity through increased mucus thickness (Cani, 2018).

A



B

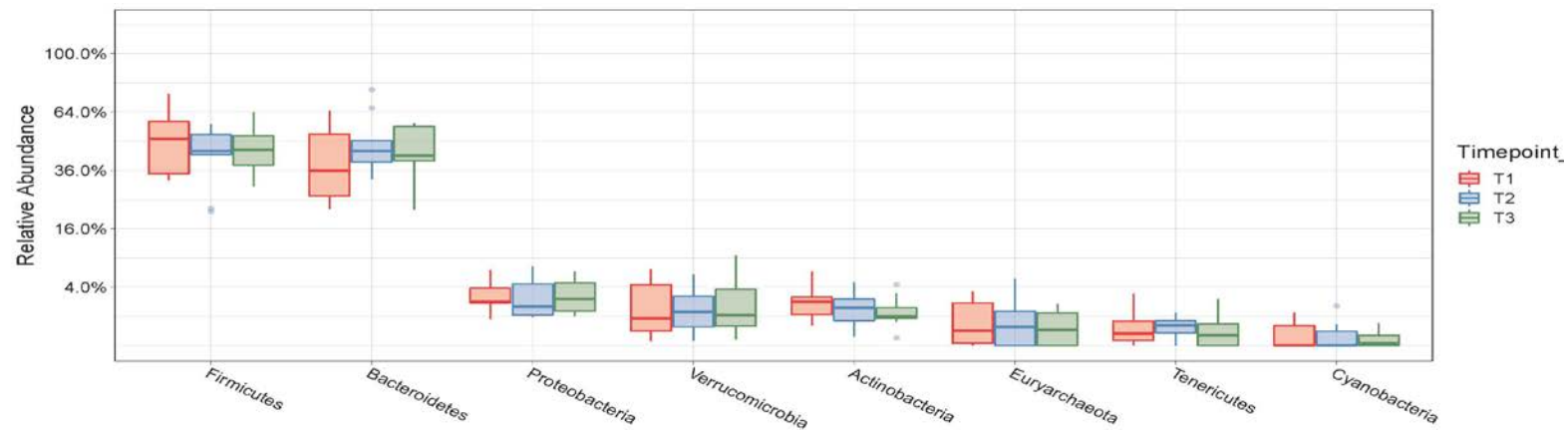


Figure 4.8: Boxplots showing relative abundance of bacteria at phylum level in the beetroot group (A) and the control group (B) at baseline (T1), midway (T2) and end of study (T3). Data presented as mean plus upper and lower quartile range. Statistical analysis using Kruskal-Wallis.

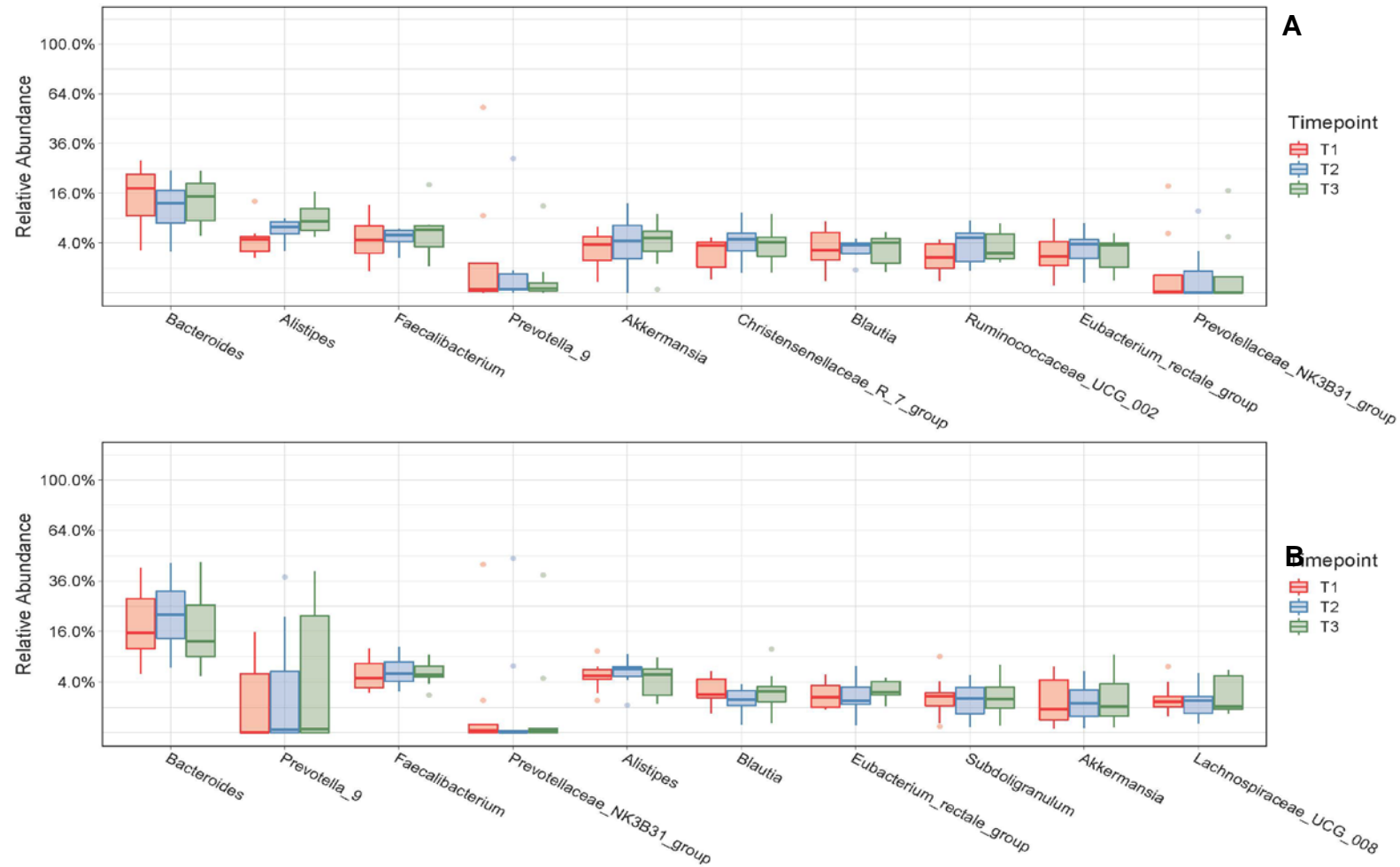


Figure 4.9: Boxplot showing relative abundance of bacteria at genus level in the beetroot group (A) and the control group (B) at baseline (T1), midway (T2) and end of study (T3). Data presented as mean plus upper and lower quartile range. Statistical analysis using Kruskal-Wallis.

Alpha diversity

Results from analysis of alpha diversity by OTU count and the Shannon Index is shown in Figure 4.10. There was no difference in observed OTU count over time in either intervention group ($P>0.05$). The Shannon Index was seen to rise over time in the beetroot group but not the control group, which signifies an increase in bacterial diversity following beetroot consumption over eight weeks. This was not found to be significant ($P>0.05$) but this is likely due to the small sample size in the present study.

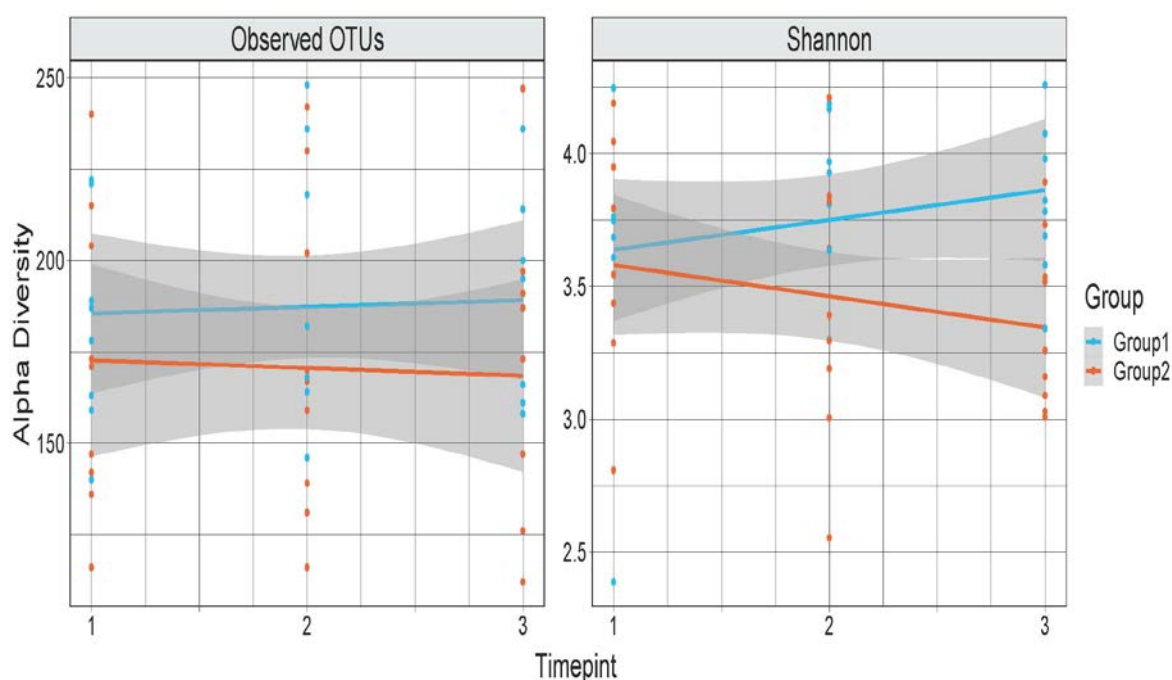


Figure 4.10: Alpha diversity regression analysis of the microbiome for observed operational taxonomic units (OTUs) and Shannon Index at baseline (time point 1), midway (time point 2) and end of study (time point 3). Group 1 = beetroot, group 2 = control. n=17 (8 beetroot, 9 control). The grey shading represents the 95% confidence interval of the mean. Statistical analysis conducted using Kruskal-Wallis one-way ANOVA.

Beta diversity

Principal component analysis across all time points shows a significant difference between the beetroot group and the control group ($P=0.03$). This is shown in Figure 4.11.

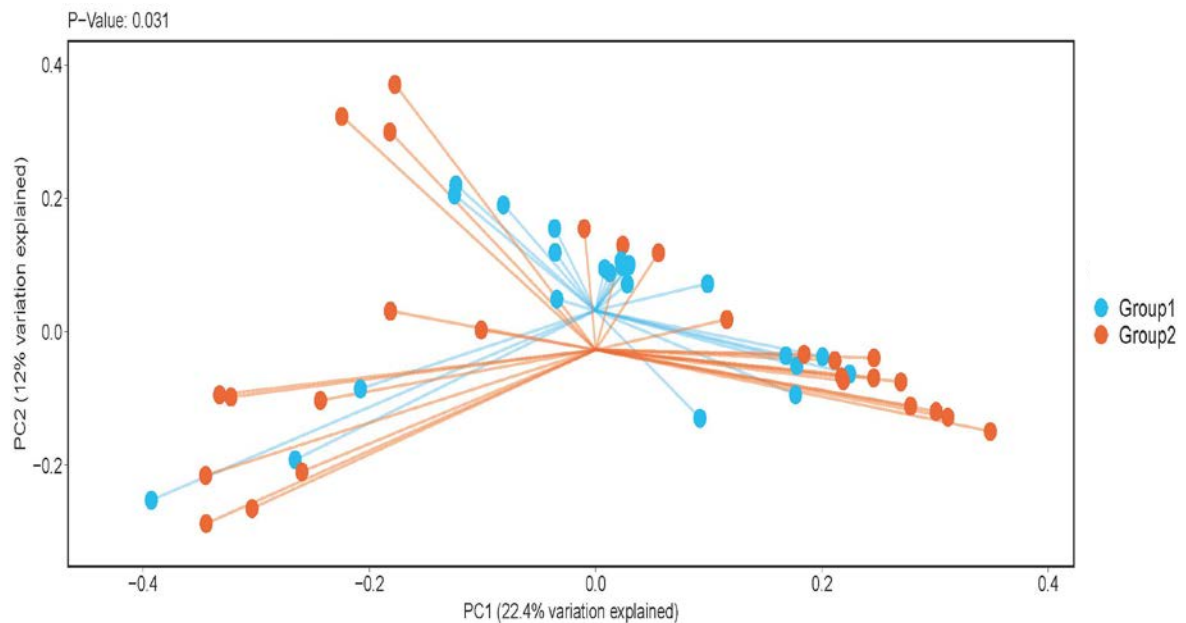


Figure 4.11: Principal component (PC) analysis using Bray Curtis dissimilarity across all time points (baseline, midway and end of study) in group 1 (beetroot) and group 2 (control).

Within the beetroot group, there were no significant changes in beta diversity over the eight-week period ($P=0.99$), however there is a shift at T2 and T3 when compared to T1 (baseline), indicating an alteration in bacterial composition. The PC analysis can be seen in Figure 4.12. Similarly, in the control group, there were also no changes in beta diversity over time ($P=1.0$) (Figure 4.12).

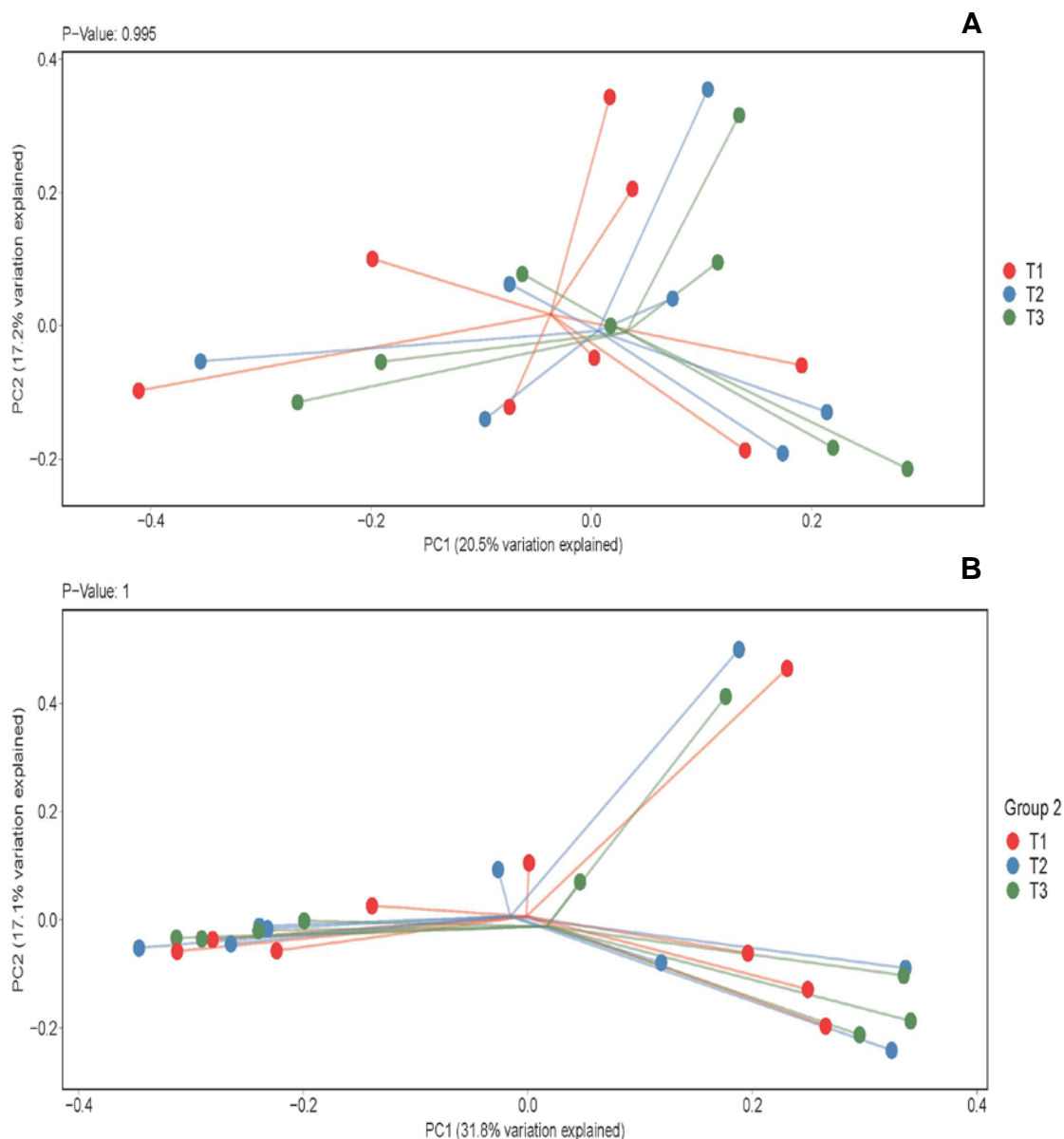


Figure 4.12: Bray Curtis beta diversity at baseline (T1), midway (T2) and end of study (T3) in the beetroot group (A); n = 8 and the control group (B); n = 9.

SCFA concentrations

The concentrations of the SCFAs acetate, propionate, isobutyrate, butyrate, isovalerate and valerate were assessed over time and across interventions in eight participants from the intervention group and nine from the control group. There were no significant differences between groups at baseline for total or individual concentrations of SCFA ($P>0.05$). Total SCFA concentration in the beetroot group rose from $22.7\pm0.4\text{mM}$ to $27.7\pm0.6\text{mM}$ after the eight-week intervention, which is a 22% increase from baseline to end of study. In the control group, SCFA

concentration rose from $27.9 \pm 1.1 \text{ mM}$ to $31.32 \pm 1.6 \text{ mM}$ over the eight weeks, which is an increase of 12% from baseline.

There was a significant effect of time ($P=0.01$) on total SCFA concentration but no effect of intervention ($P=0.16$) (Figure 4.13). There was no interaction between time and intervention ($P=0.58$). Pairwise comparisons show that total SCFA concentration was higher at the end of study than at baseline ($+5.1 \text{ mM}$; $P=0.009$; 95%CI: 1.2, 8.9).

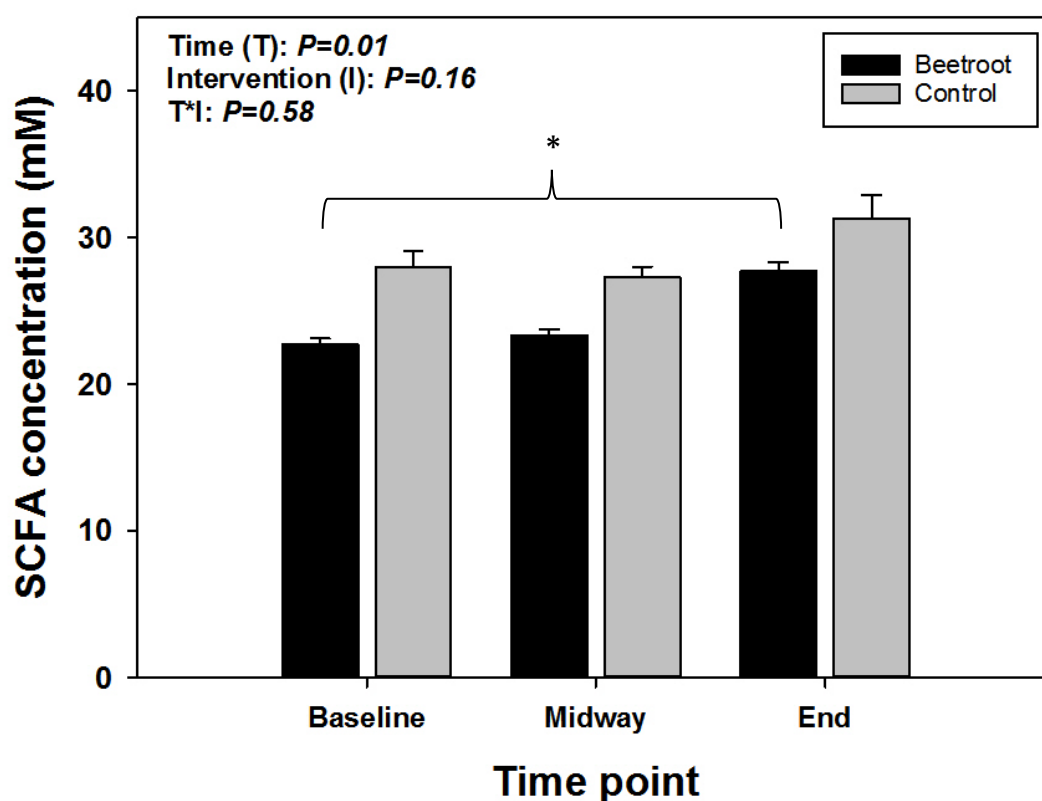


Figure 4.13: Mean (+SEM) total SCFA concentration at baseline, midway and end of study; n=17. Statistical analysis using mixed model repeated measures ANOVA with time as within-subjects factor and intervention as between-subjects factor. * denotes significant difference between time points across both groups.

Figure 4.14 illustrates the changes in individual SCFAs over time in both intervention groups. There is a significant effect of time ($P=0.03$) on acetate concentrations but no effect of intervention ($P=0.18$). There was no interaction between time and intervention ($P=0.36$). Pairwise comparisons show that acetate concentrations were higher at end of study compared to baseline across both groups ($+2.8 \text{ mM}$; $P=0.01$; 95%CI: 0.6, 5.0).

There was a significant effect of time ($P=0.01$) but no effect of intervention ($P=0.13$) on propionate concentration. There was no interaction between time and intervention ($P=0.51$). Pairwise comparisons show that the concentration of propionate was higher at the end of study than at midway ($+1.0\text{mM}$; $P=0.03$; 95%CI: 0.1, 1.9) and at baseline ($+0.9\text{mM}$; $P=0.04$; 95%CI: 0.03, 1.9) for both groups combined.

There was a trend towards an effect of time ($P=0.08$) but no effect of intervention ($P=0.34$) on concentrations of isobutyrate. There was no interaction between time and intervention ($P=0.49$).

There was a significant effect of time ($P<0.001$) but no effect of intervention ($P=0.16$) on concentrations of butyrate. There was no interaction between time and intervention ($P=0.58$). Pairwise comparisons show that concentrations are significantly higher at end of study compared to baseline ($+1.6\text{mM}$; $P<0.001$; 95%CI: 0.8, 2.4) across both groups combined. Butyrate concentrations increased by 99% in the beetroot group and 41% in the control group over the eight-week period.

There was no effect of time ($P=0.11$) or intervention ($P=0.49$) on concentrations of isovalerate. There was no interaction between time and intervention ($P=0.43$). There was also no effect of time ($P=0.51$) or intervention ($P=0.24$) on valerate concentrations, or an interaction between time and intervention ($P=0.71$).

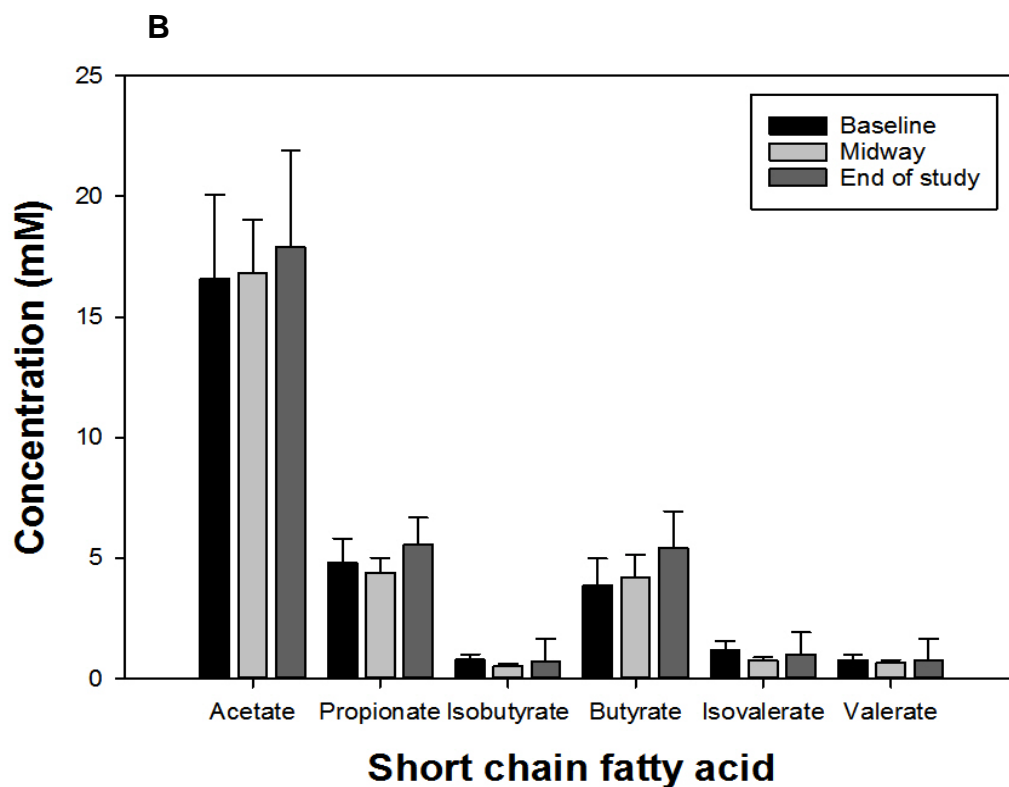
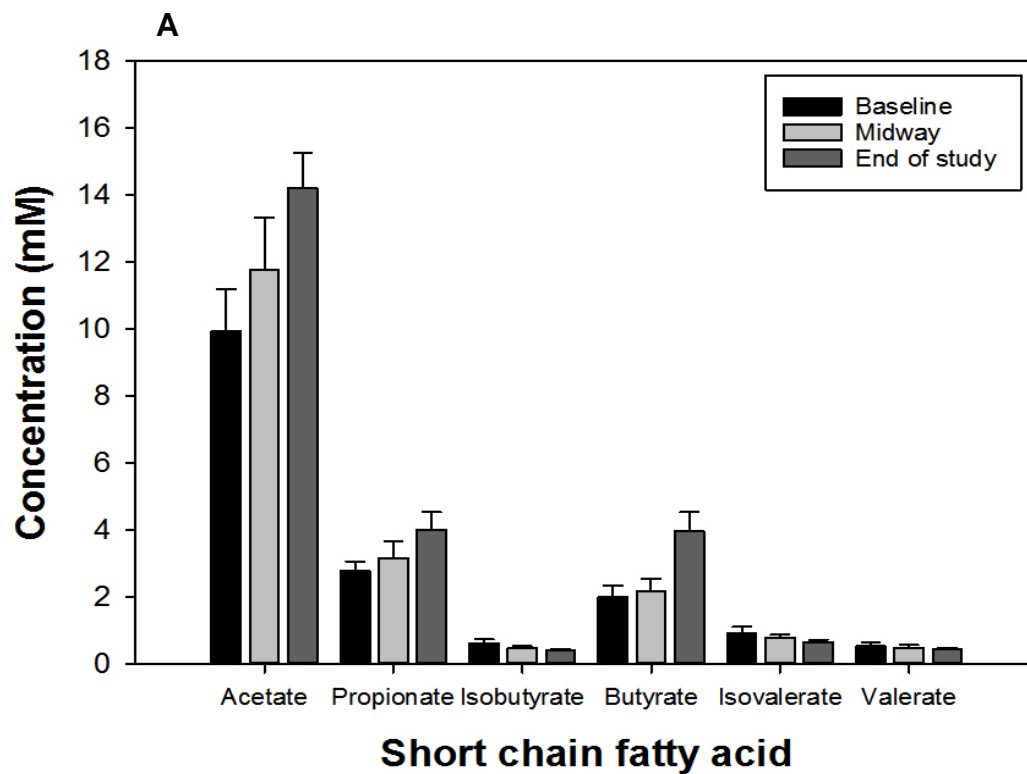


Figure 4.14: Mean (\pm SEM) concentration of SCFAs at baseline, midway and end of study in the beetroot group (A) and control group (B); n = 17.

4.4 Discussion

4.4.1 Summary of key findings

This is the first study to investigate the effects of prolonged dietary nitrate intake from whole beetroot on healthy older adults. The key findings are that eight weeks of dietary supplementation with beetroot: 1) significantly alters plasma nitrate levels; 2) significantly increased urinary nitrate; 3) had no negative GI tract symptoms; 4) was able to modulate the composition of the gut microbiome and 5) significantly increased SCFA concentrations. These data combined indicate that prolonged increased beetroot intake was well tolerated and may offer health benefits to those at increased risk of co-morbidities such as older adults.

4.4.2 Prolonged beetroot supplementation and functional capacity

Studies to date utilising beetroot supplementation have primarily focussed on acute interventions (\leq one week) in young adults. Bailey *et al.* (2010) and Lansley *et al.* (2011) have reported improvements in peak muscle contractile efficiency in knee-extensor exercise and time-to-exhaustion in high-intensity running following beetroot juice consumption in males under the age of 30. These authors attributed these improvements to an increase in NO availability and a reduction in energy and oxygen cost of muscle force production, but these may only be present at peak exercise. Although these studies do offer an insight into the benefits of beetroot supplementation, maximal functional capacity measures are generally not used in older populations. Instead, studies in older adults tend to focus on functional capacity measures such as walking distance, grip strength, balance and standing, which become more important to daily living in an ageing population.

In the present study there were no improvements in functional tests including grip strength and TUG, which were less challenging than the muscle contraction and exercise tests mentioned previously. Similarly, Kelly *et al.* (2013) and Shepherd *et al.* (2015) reported no significant differences in the six-minute walk test (6MWT) following two and 2.5 days of beetroot juice consumption in adults over the age of 60 years. Siervo *et al.* (2016) supplemented adults between 60 and 75 years with beetroot juice for seven days and utilised a more comprehensive battery of functional tests similar to the present study including handgrip strength, TUG, sit-to-stand and

10m walking speed in an attempt to detect improvements in functional capacity. However, despite additional functional measures, Siervo *et al.* (2016) did not report any significant improvements. The present study addresses the short duration intervention periods previously used but the lack of improvement in functional capacity suggests that the duration of beetroot supplementation is not the only factor to consider. It is more likely that the measures of functional capacity used here and in previous studies (Kelly *et al.*, 2013; Shepherd *et al.*, 2015; Siervo *et al.*, 2016) lack the sensitivity to detect small improvements or are not sufficient to stress the participant sufficiently to display physiological improvements. This may be further attenuated by the physically active cohorts used in the present study and in those by Kelly *et al.* (2013). However, longer-term intervention with sodium nitrite has been found to have positive effects on functional capacity measures, so it may be the case that seven days, which is the longest intervention period described in the literature, is not long enough to stimulate physiological benefits.

Other studies in this area employing a longer-term design were those by Justice *et al.* in 2015. Eight weeks of supplementation with sodium nitrite-enriched water improved forelimb grip strength and distance run in old mice, as well as reduced low-grade inflammation in muscle (Justice *et al.*, 2015). In human participants, 10 weeks of sodium nitrite supplementation in the form of daily capsules increased plasma nitrite levels, reduced balance errors in a step test and improved muscle strength measured by knee flexor and extensor rate of torque development, but did not affect TUG (Justice *et al.*, 2015). This was the first study to examine the longer-term effects of supplementation with nitrite and shows promise as a beneficial intervention on functional capacity in older adults. The improvements demonstrated may be due to the intake of nitrite, bypassing the requirement for the breakdown of nitrate in the body. As discussed in Chapter 1, this can be a limiting factor in the benefits of dietary nitrate to an older population, as changes to the oral microbiome or acidity of the stomach may reduce the conversion of nitrate to nitrite and further to NO. Furthermore, the reduction in low-grade inflammation in old mice shown by Justice *et al.* (2015) suggests that a more prolonged intake of nitrate or nitrite may be required to elicit improvements in functional capacity through the reduction of inflammation in the muscles. It is not clear whether nitrite intake has the same effect in humans as it does mice, and a review by Calder *et al.* (2011) suggests that the intake of a single

vegetable or fruit is not enough to elicit a reduction in the low-grade inflammation associated with ageing in humans. Instead, a high overall intake of vegetables and fruit has a beneficial effect on an inflammatory state. Beetroot alone, therefore, regardless of the duration of intervention, may not have a positive effect on inflammation status in older adults. A high intake of a combination of nitrate-rich vegetables, that contain both dietary nitrate and antioxidant compounds, may be more beneficial in this context.

4.4.3 The effects of beetroot intake on blood pressure

Dietary nitrate acutely lowers blood pressure due to the increased production of the vasodilator NO and consequent widening of the blood vessels (*Webb et al.*, 2008). However, little is known about the effects of prolonged dietary nitrate intake on blood pressure. The half-life of nitrate of around six hours suggests that the blood pressure-lowering effects will be acute and repeated intake is required to maintain the benefits. What is unclear is whether there are additive effects of prolonged dietary nitrate intake that may lead to a larger reduction in blood pressure over time. Conversely, it may be the case that familiarisation to a dietary nitrate load may reduce the blood pressure-lowering effects over time through desensitisation. The present study demonstrated a SBP reduction from 136mmHg to 128mmHg between baseline and end of study at week nine following repeated beetroot consumption. This reduction is consistent with a study by Rammos *et al.* (2014), who reported an 8mmHg reduction in SBP one day after the last intake of sodium nitrate, which was supplemented daily for four weeks. Similar to the study by Rammos *et al.* (2014), blood pressure was measured more than 12 hours after the nitrate dose in the present study, suggesting a chronic blood pressure-lowering effect of the prolonged beetroot consumption. Kapil *et al.* (2015) demonstrated a reduction in clinic blood pressure by 7.7mmHg following four weeks of supplementation with beetroot juice. Together these data suggest that the body is not becoming desensitised to the effects of dietary nitrate over a prolonged time period. In contrast, Jajja *et al.* (2014) demonstrated that a reduction in daily SBP found following 21 days of supplementation with beetroot was not maintained when the intervention was interrupted for seven days. If beetroot is able to offer physiological benefits such as reductions in blood pressure, patients at risk of CVD may be advised to increase beetroot intake on a regular basis.

Interestingly, there was no reduction in SBP at the midway point, which suggests that a longer intervention period is more beneficial when considering additive effects. However, a trend towards a reduction in SBP was also seen in the control group over the eight-week period. Although there was not a statistically significant difference between baseline SBP in the beetroot and the control group, the control group had a higher baseline SBP of 140mmHg, which is classed as hypertensive. It is conceivable that simply taking part in health-related research influenced lifestyle choices of participants over the study period, positively affecting their blood pressure. There were no changes in physical activity levels over the eight-week period but restrictions to caffeine and alcohol intake before study visits may have influenced the participants and were not picked up by the 24-hour dietary recall.

Reductions in blood pressure were not accompanied by increases in plasma nitrate levels at the time of testing, which represent the plasma levels of the more bioactive nitrite. Results from Chapter 3 suggest that higher levels of plasma nitrite are correlated with reductions in blood pressure. The plasma nitrate levels in the present study were not at a concentration that would stimulate an increase in plasma nitrite and subsequent NO production but the time of testing, which occurred at least 12 hours after the consumption of beetroot, may have missed the peak in plasma nitrate and nitrite levels. Chapter 2 demonstrates that plasma nitrite levels reach a peak after around three hours following the consumption of whole beetroot and levels rose following 100g and 200g beetroot by around 121% and 141%, respectively, in older adults. The dose of nitrate provided in the present study was larger than that provided in Chapter 2, so it is likely that plasma nitrite levels rose acutely to a greater extent. However, beetroot is also rich in other active compounds such as ascorbic acid and phenolic compounds, which have been shown to enhance NO synthase activity (Bondonno *et al.*, 2015). The blood pressure-lowering effect may therefore be due to an increase in NO production and subsequent vasodilatory action through the L-arginine pathway. Phenolic compounds such as anthocyanins, which closely resemble the betalains found in beetroot, are also considered to have prebiotic effects, as their consumption affects the composition of gut bacterial species (Faria *et al.*, 2014). The prolonged consumption of the phenolic compounds found in beetroot

may therefore have beneficial effects on bacterial species and the health and functioning of the gut.

4.4.4 The effects of beetroot supplementation on gut health

Bacterial colonisation of the human body is highly host-specific and recent developments have enhanced understanding of the relationship between host and microbiome and how the gut microbiome contributes towards metabolism, immune response, intestinal architecture and GI health (Fava *et al.*, 2018). Imbalance between the host and the gut microbiome could potentially contribute to a higher risk of co-morbidities and poor health, as previously identified with increased inter-individual variability and reduced diversity with age (Claesson *et al.*, 2012). Therefore, treatments aimed at modulating the gut microbiome by increasing diversity and re-balancing the symbiotic relationship between the host and gut microbiota could offer a beneficial therapeutic approach to healthy ageing.

One such potential therapy would be to modulate the gut microbiome through dietary supplementation, as the diet has been shown to play a significant role in shaping the composition of gut bacterial species (David *et al.*, 2014). In the present study, beetroot consumption resulted in an increase in abundance of *Alistipes* and *Akkermansia* over time, which are associated with increased plant-based foods (Jiang *et al.*, 2015) and improved gut integrity (Everard *et al.*, 2013). Although these changes were not significant, both of these bacteria are associated with a healthy gut and improved metabolic control (Zhou, 2017). In addition to individual alterations in specific changes at the genus level, diversity also has a crucial role in gut health (Claesson *et al.*, 2012). Species richness is associated with colonic transit time, which is assessed non-invasively using the Bristol Stool Chart (BSC) (Vandeputte *et al.*, 2016). The Western diet has led to more than 50% of stools passed belonging to categories 3 and 4 of the BSC, which lie in the medium to firmer end of the chart. Species richness has been found to significantly decline with increasing stool firmness (Vandeputte *et al.*, 2016). Samples in the present study were found to lie between three and four on the BSC, suggesting no effect of beetroot consumption on transit time. However, there was a significant difference in the Bray Curtis similarity when comparing the beetroot to the control group across time, demonstrating a shift

in bacterial composition. Furthermore, there was a slight increase in the number of OTUs and Shannon diversity index over time in the beetroot group, which was not observed in the control group. The lack of significance reported in these measures is likely to be due to the small sample size, especially given the variability commonly reported in microbiome studies. These data suggest that beetroot supplementation may offer a therapeutic approach to the ageing population.

Modulation of the gut microbiome will inevitably affect the metabolites produced. One of the main metabolites that has received considerable attention are the SCFAs, the most abundant and commonly reported of which are acetate, propionate and butyrate in a ratio of around 3:2:2 (Fava *et al.*, 2018). The present study demonstrated a significant increase in total SCFA, which was driven by increases in propionate, acetate and butyrate over the eight-week period. SCFAs have been shown to contribute to gut health through mechanisms such as maintaining integrity of the gut wall and regulating the inflammatory output of adipose tissue (Fava *et al.*, 2018; Natarajan & Pluznick, 2014). An increase in SCFA production, therefore, is a marker of gut health. The SCFA butyrate is thought to be one of the most important due to its ability to initiate apoptosis in colon cancer cells (Heerdt *et al.*, 1997). An increase in dietary fibre is known to stimulate the growth and/or activity of beneficial bacterial species such as *Faecalibacterium prausnitzii*, a known butyrate-producing species. Butyrate-producing bacteria are found within the *Firmicutes* and also include *Eubacterium rectale* and *Roseburia* (Scott *et al.*, 2008). In the present study the genus *Faecalibacterium* increased from baseline to end of study in the beetroot group. Although this was not a significant increase, which may be in part due to the small sample size here, these data suggest that the increases in SCFAs following beetroot consumption may be due to an increase in SCFA-producing bacteria.

Some dietary fibres, such as resistant starches (Schwartz *et al.*, 2002) stimulate the growth and/or activity of beneficial bacterial species such as *Roseburia* and are termed “prebiotic” (Fava *et al.*, 2018). The increase in butyrate production observed following the eight-week intervention in both groups might be due to the resistant starch present in the banana control food, as there was no greater affect following beetroot consumption in addition to the banana. The increase in total SCFAs following beetroot consumption, however, suggests that the dietary fibre, or other

compounds, present in whole beetroot, have a beneficial effect more generally on the gut. There is limited data available on the effects of the inclusion of foods on SCFA production and results are inconclusive. For example, the consumption of pinto beans, which are a source of resistant starch, was found to have no effect on the production of SCFAs in the colon of ten middle-aged men and women (Finley *et al.*, 2007). An *in vitro* study showed that gourds, which are a rich source of soluble and insoluble fibre, increased SCFA production in the colon and have significant prebiotic effects (Sergis *et al.*, 2013). The large variation in bacterial composition between individuals may be a significant limitation to human intervention studies on SCFA production and prebiotic abilities of vegetables. It is however, an important area of research given the low total dietary fibre intake in the UK population and the impact on diseases of the gastrointestinal tract.

4.4.2 Strengths and weaknesses

The present study is not without limitation. This feasibility study was the first to investigate the effects of more prolonged dietary nitrate intake from a fresh vegetable source on markers of functional capacity and gut health in an older population. The positive effects of beetroot consumption reported here provide a foundation for further research in this area and demonstrates that the addition of whole beetroot to the diet is feasible with excellent compliance rates.

The free-living design i.e. a two-hour fast prior to testing and no other dietary restrictions makes the study applicable to a real-world setting with an unregulated diet. However, the timing of blood collection and the consumption of beetroot every second day makes it difficult to accurately assess plasma nitrate and the peaks following consumption. Although this data does provide information relating to a prolonged effect of beetroot, a future study may combine the methods here with those used to ascertain the acute effects of dietary nitrate in conjunction.

The parallel design of this study enabled a smaller sample size but giving participants the option of providing a stool sample resulted in a very small sample size for the analysis of gut microbiota. However, including stool sample collection as a mandatory aspect of the study may have reduced compliance in participants, leading

to dropout or lower rates of recruitment. There were no dropouts in this study, which demonstrates that, at least over the eight-week period, the amounts of beetroot consumed were not a significant burden to the participants. Furthermore, the vacuum-packed cooked beetroot provided came from the same batch, ensuring consistency in nitrate levels across the eight-week intervention. This product is also available to the public and represents a widely-used form of beetroot, which increases the applicability of this study.

Participants were not matched for age at baseline due to the randomisation effect, which would potentially have been controlled for in a larger sample size. There was no placebo control in this study but the impacts of this were reduced by the inclusion of the control food in both the intervention arms. Any additional effects of beetroot consumption on top of the control food could then be identified.

4.4.3 Conclusions

The repeated consumption of whole beetroot for eight weeks did not have a positive impact on functional capacity in older adults, potentially due to a lack of stimulus to initiate a response in plasma nitrate levels at time of testing. There was, however, a reduction in blood pressure despite low plasma nitrate levels, which suggests an additive effect of nitrite on the vascular system. Beetroot consumption led to improvements in gut bacterial diversity and the production of SCFAs in the colon but the sample size was too small to identify significant changes in a number of markers of gut health. These data suggest that the addition of whole beetroot to the diet, over periods more than eight weeks, may improve overall gut health in an older population and promote a blood pressure-lowering effect. More research is warranted into the effects of beetroot on markers of inflammation, oxidative stress and gut health, and the effects of these on functional capacity measures that contribute to activities of daily living in the ageing population.

Chapter 5. Cognitive function in older adults following an eight-week intervention with whole beetroot

5.1 Introduction

The UK's population is ageing and it is estimated that there will be an additional 8.6 million people over the age of 65 in 50 years time (Storey, 2018). An ageing population puts more strain on the National Health Service due to age-related diseases such as CVD and dementia. Deary *et al.* (2009) describe that, in the UK, 40% of admissions to care institutions are due to cognitive failure. Cognitive decline has been linked to a reduced blood flow to the brain and this can contribute to the development of dementia (Rabbitt *et al.*, 2006). Normal cognitive ageing results in abnormal blood vessels, which can lead to chronic ischaemia in the brain. This ischaemic state can cause degeneration in the white matter of the brain, which is linked to executive functioning, for example working memory and task switching. Although dementia is not preventable, delaying brain ischaemia or modulating the low-grade inflammation that is associated with brain ageing is a viable target of nutritional intervention. Furthermore, a reduction in nitric oxide (NO) availability is once again implicated in vascular ageing in the brain, as disrupted neurovascular coupling occurs when blood flow is not matched to the demand in a specific brain region (Girouard & Iadecola, 2006).

NO is a potent vasodilator with wide diffusibility in the human body. It has a half-life of less than one second but the intake of dietary nitrate and the subsequent reduction to nitrite enables a more stable reservoir for the production of NO. NO is synthesised continually by endothelial NO synthase (eNOS), which converts the precursor L-arginine to citrulline and then to NO (Palmer *et al.*, 1988). NO then diffuses into the smooth muscle and stimulates the production of cyclic guanosine monophosphate (cGMP), leading to relaxation of the vessel wall (Lyons *et al.*, 1997). To date, much of the research around nitrate and vascular function focuses on vessels of the cardiovascular system and shows positive effects on blood flow, especially in a young, healthy population. Although effects on blood flow in older adults is equivocal, there appears to be a greater and more consistent benefit following a more prolonged intervention period with dietary nitrate i.e. over weeks rather than days or

hours. It is also emerging that dietary nitrate, in part through the vasodilatory effects of NO, can have a positive impact on cerebrovascular blood flow and consequently on cognitive function. Indeed, beetroot juice consumption has been found to acutely improve cerebral blood flow (CBF) in older adults (Presley *et al.*, 2011) and increase prefrontal cortex blood flow and improve cognitive function in young participants (Wightman *et al.*, 2015). Although these studies highlight the potential for cognitive function improvements following beetroot consumption, there is a significant gap in the research on more prolonged intervention with dietary nitrate, particularly with an older population who may already be showing signs of cognitive ageing.

Consequently, the present study explores the effects of prolonged beetroot consumption on cognitive function in older adults. This study investigated baseline, mid way and end of study cognitive test scores to assess the effects of repeated consumption of whole beetroot on domain specific cognitive functioning. The effects of an eight-week supplementation with whole beetroot have not been investigated before, so this pilot study aimed to assess not only the cognitive effects but also the compliance of the participants to a longer-term intervention with a whole vegetable. It was hypothesised that supplementing the diet with repeat beetroot consumption would have a positive impact on cognitive function in an older age group. This hypothesis was tested using an online cognitive-testing platform performed at baseline, after four weeks and after eight weeks of the consumption of 150g whole beetroot every second day compared to a control food.

5.2 Methods

The participants, exclusion criteria, interventions, and study design and protocol were the same as those used in Chapter 4. This study was approved by the Newcastle University ethical committee (Appendix G). Participants consumed 150g whole cooked beetroot every second day and/or a medium-sized banana for eight weeks. Participants were cognitively healthy with no diagnosis of clinical cognitive decline. They were also not taking psychoactive medication and fasted for at least two hours prior to attending the research facility; caffeine intake was also restricted.

5.2.1 Cognitive function

Participants were briefed on the cognitive tasks and given two full practice runs to reduce learning effects during the study. An online cognitive battery was provided by CogTrack™ and consisted of nine cognitive tasks: immediate word recall, pattern separation, simple reaction time, digit vigilance, choice reaction time, spatial working memory, numeric working memory, delayed word recall, and word recognition. The tasks took a total of approximately 20 minutes to complete. Scores from each test were combined to produce the following cognitive indices: Attentional Intensity Index, Sustained Attention Index, Working Memory Capacity Index, Cognitive Reaction Time, Attentional Fluctuation Index, and Memory Retrieval Speed Index. Each of these six factors combines the same task measures as used in the Cognitive Drug Research (CDR) system to form the factor scores named power of attention, continuity of attention, quality of memory and speed of memory. Participants performed the CogTrack™ system on two consecutive occasions at the screening visit to ensure that any effects of practice, familiarity or test anxiety were overcome prior to the first testing day. This method is known as pre-baseline massed practice and has been described by Goldberg *et al.* (2015) and Bell *et al.* (2018) as a solution to the issue of practice effects in cognitive testing. Participants then completed the nine tasks at baseline, midway and end of study testing visits, in a fasted state and in an individual booth in the research facility. They also completed the tasks on two occasions at home, during weeks three and seven, if they had access to a computer.

5.2.2 Cognitive indices

The cognitive indices described above were derived from variables such as number of false alarms, average speed and accuracy. Examples of these are shown in Figure 5.1.

IRCL	Immediate Word Recall - Number of words correctly recalled	#
IRCLACC	Immediate Word Recall - Percentage of words correctly recalled	%
IRCLERR	Immediate Word Recall - Number of words incorrectly recalled (errors)	#
SRT	Simple Reaction Time - Average Speed	msec
SRTM	Simple Reaction Time: Median	msec
SRTSD	Simple Reaction Time - Standard Deviation	msec
SRTC	Simple Reaction Time - Coefficient of Variance	CV%
VIGACC	Digit Vigilance - Targets Detected	%
VIGRT	Digit Vigilance - Average Speed	msec
VIGFA	Digit Vigilance - False Alarms	#
VIGSD	Digit Vigilance - Standard Deviation	msec
VIGCV	Digit Vigilance - Coefficient of Variance	CV%
CRTACC	Choice Reaction Time - Accuracy	%
CRT	Choice Reaction Time - Average Speed	msec
CRTM	Choice Reaction Time: Median	msec
CRTSD	Choice Reaction Time - Standard Deviation	msec
CRTC	Choice Reaction Time - Coefficient of Variance	CV%
SPMOACC	Spatial Working Memory Original Stimuli - Accuracy	%
SPMNACC	Spatial Working Memory New Stimuli - Accuracy	%
SPMORT	Spatial Working Memory Original Stimuli - Average Speed	msec
SPMNRT	Spatial Working Memory New Stimuli - Average Speed	msec
SPMRT	Spatial Working Memory -- Average Speed	msec

Figure 5.1: Example of the variables used to calculate indices of cognitive function. Column 1 represents the acronym used, column 2 represents the name and source of the variable and column 3 represents the units used for measurement.

The indices were then calculated using the equations in Figure 5.2 below.

COMPOSITES	VARIABLE NAME	CALCULATION
Attentional Intensity Index	POW_ATT	SRTM+CRTM+VIGRT
Sustained Attention Index	CONT_ATT	$((crtacc-50)*2 + (vigacc*.45 - vigfa)*100/45)/2$
Attentional Fluctuation Index	POW_CV	SRTCv+CRTCv+VIGCV
Memory Retrieval Speed Index	SPEEDMEMM	SPMRTM+NWMRTM+DRECRTM+DPICRTM
Cognitive Reaction Time	COGRTM	CRTM-SRTM
Working Memory Capacity index	QL_WORK	$SPMOACC+SPMNACC+NWMOACC+NWMNACC-200/2$

Figure 5.2: Indices of cognitive function, calculated using the variables shown in Figure 5.1.

5.2.3 Statistical analysis

Statistical analyses were completed using IBM SPSS (version 24.0). Summary data are presented as means \pm SD with 95% confidence intervals (CI). Two-tailed independent t-tests were used to assess differences in baseline measures between the two intervention groups. Changes over time were analysed using a mixed model repeated measures ANOVA with time as the within-subjects factor and intervention as the between-subjects factor. Models were tested for sphericity using Mauchly's test and multivariate models were applied if these assumptions were violated. Where significances were found, *post hoc* tests with Bonferroni adjustment to control for multiple comparisons were run. Statistical significance was set at $P < 0.05$.

5.3 Results

5.3.1 Baseline characteristics

A total of 36 participants were randomised to the interventions and completed the trial, with an average age of 67.4 ± 5.6 years (Table 4.3). 19 participants were randomised to the intervention group and 17 to the control group. Participants in both groups were matched for height, weight, BMI, waist circumference and body fat ($P > 0.05$) but the control group's average age was 70 years compared to the intervention group's average of 65 years, which were statistically different ($P = 0.009$). Interim cognitive test scores were not included in analysis due to the inability to control for the effects of dietary intake before testing at these time points. There were no significant differences between the cognitive scores in the beetroot and the control group at baseline (all $P > 0.05$). Number of years in education were similar across groups with a mean of 16.4 ± 3.3 ($P = 0.12$).

5.3.2 Dropout and body composition

The interventions were well tolerated and no adverse events were reported. One participant was withdrawn before the midway point due to starting a course of medication. Weight, BMI, waist circumference and body fat did not change over the course of the intervention period ($P > 0.05$).

Table 5.1: Mean cognitive indices over the eight-week intervention period following beetroot consumption and control; n=36.

	<i>Beetroot</i>			<i>Control</i>		
	<i>Baseline</i>	<i>Mid way</i>	<i>End</i>	<i>Baseline</i>	<i>Mid way</i>	<i>End</i>
<i>AI (ms)</i>	1372.7	1370.7	1391.4	1375.9	1366.3	1368.6
<i>SA (%)</i>	95.7	94.9	95.6	93.4	95.0	95.6
<i>WMC (%)</i>	93.2	91.9	91.4	86.2	93.5*	90.8
<i>CRT (ms)</i>	186.1	175.0	152.1 ^a	171.4	188.4	186.8 ^a
<i>AF</i>	62.3	55.2	50.8*	56.8	58.5	56.4
<i>MRS (ms)</i>	4202.1	3908.8	3872.6*	4157.9	3922.1	3856.2

AI= attentional intensity, SA= sustained attention, WMC= working memory capacity, CRT= cognitive reaction time, AF= attentional fluctuation, MRS= memory retrieval speed. * denotes significant difference to baseline. a denotes significant difference between beetroot and control groups at end of study.

Attentional Intensity Index (AI)

There was no effect of time ($P=0.63$) or intervention ($P=0.86$) on AI, or an interaction between time and intervention ($P=0.53$).

Sustained Attention Index (SA)

There was no effect of time ($P=0.31$) or intervention ($P=0.45$) on SA. There was no interaction between time and intervention ($P=0.14$).

Working Memory Capacity Index (WMC)

There was no effect of time ($P=0.28$) or intervention ($P=0.53$) on WMC following beetroot or the control. There was a notable trend towards an interaction between time and intervention ($P=0.07$). *Post hoc* analyses show indicate that there was no difference over time in the beetroot group but WMC was higher at midway than baseline in the control group (+7.2%; $P=0.01$; 95%CI: 1.3, 13.1).

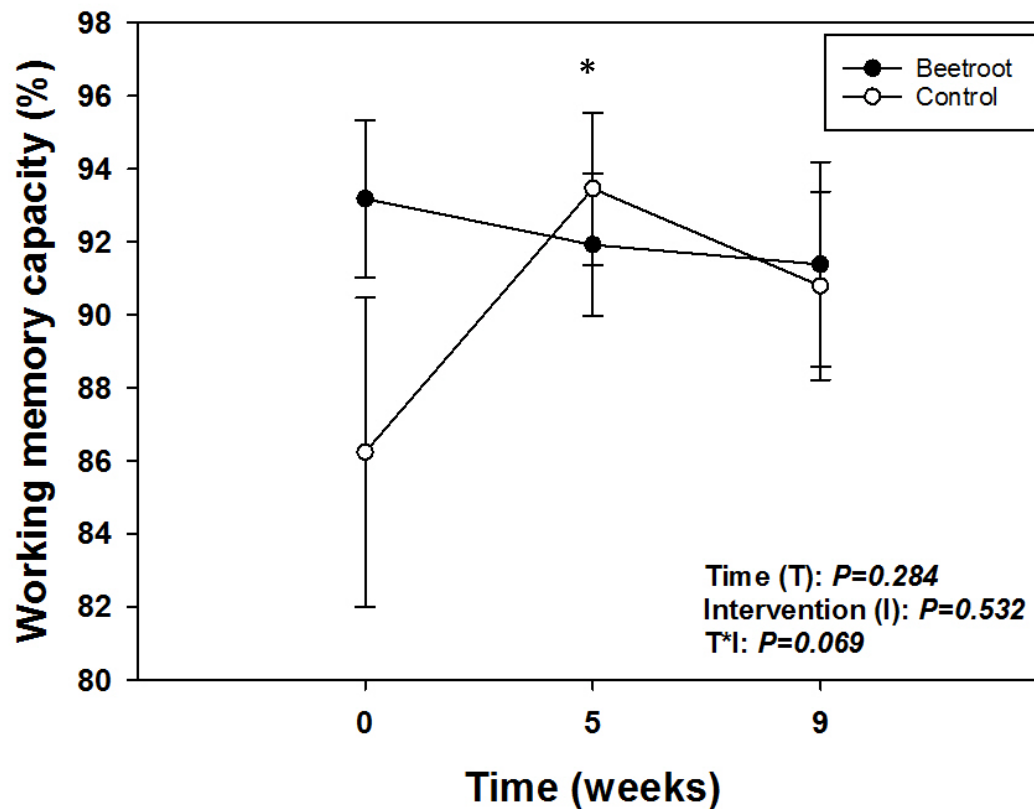


Figure 5.3: Mean (\pm SEM) working memory capacity over the course of eight-week supplementation with beetroot or a banana control; $n=36$. Statistical analysis using mixed model repeated measures ANOVA (time as within-subjects factor, intervention as between-subjects factor). * denotes significant difference to baseline in control group.

Cognitive Reaction Time Index (CRT)

There was no effect of time ($P=0.41$) or intervention ($P=0.46$) on CRT but there was an interaction between time and intervention ($P=0.04$), as shown in Figure 5.4. Analyses show that CRT at the end of study was significantly faster in the beetroot group than the control group (-34.7ms ; $P=0.02$; 95%CI: $-63.4, -6.1$). CRT at the end of study was also significantly faster than at baseline (-34ms ; $P=0.04$; 95%CI: $-67.1, -0.9$) in the beetroot group but not in the control group ($P=0.83$). There was no difference between CRT at baseline and mid way in either group ($P>0.05$).

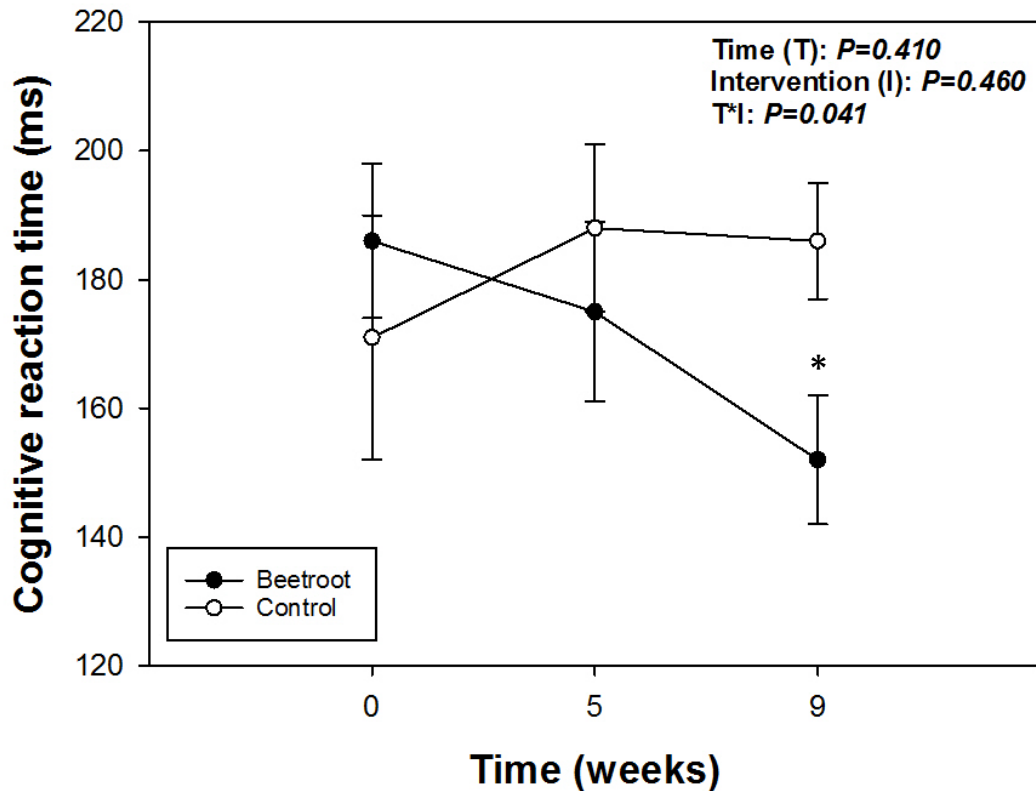


Figure 5.4: Mean (\pm SEM) cognitive reaction time over the course of eight-week supplementation with beetroot or a banana control; $n=36$. Statistical analysis using mixed model repeated measures ANOVA (time as within-subjects factor, intervention as between-subjects factor). * denotes significant difference to baseline.

Attentional Fluctuation Index (AF)

There was no effect of time ($P=0.14$) or intervention ($P=0.76$), or an interaction between time and intervention ($P=0.14$), on AF following eight weeks supplementation with beetroot or a control.

Memory Retrieval Speed Index (MRS)

There was a significant effect of time ($P=0.001$) on MRS but no effect of intervention ($P=0.93$) (Figure 5.5). There was no interaction between time and intervention ($P=0.93$). Pairwise comparisons show that MRS was significantly faster than baseline at mid way (-265.6ms ; $P=0.009$; 95%CI: -476.0 , -55.1) and at end of study (-316.7ms ; $P=0.003$; 95%CI: -540.9 , -92.4) across both groups.

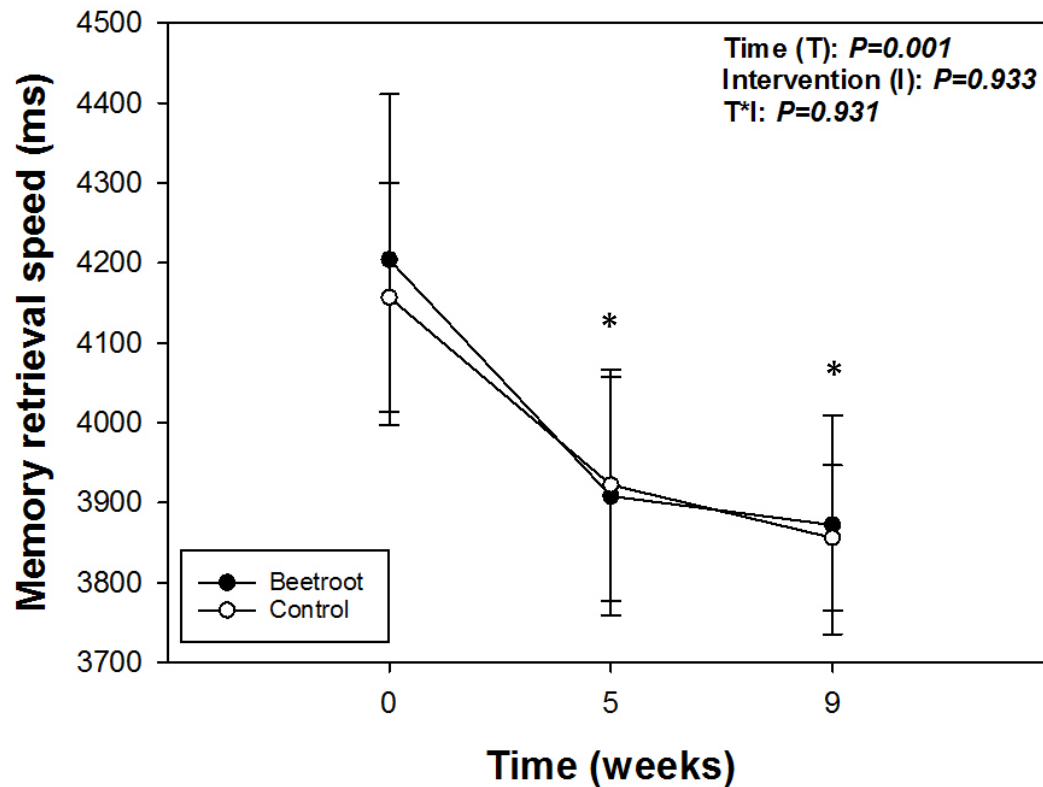


Figure 5.5: Mean (\pm SEM) memory retrieval speed over the eight-week intervention period following beetroot consumption or banana control; $n=36$. Statistical analysis using mixed model repeated measures ANOVA with time as within-subjects factor and intervention as between-subjects factor. * denotes significant difference to baseline across both groups.

5.4 Discussion

5.4.1 Summary of findings

This study is the first investigation into the effects of prolonged beetroot ingestion on cognitive function. Results show that eight weeks of supplementation with whole beetroot had positive effects on cognitive reaction time and memory retrieval speed in older adults. Other areas of cognition, such as attention and working memory were unaffected by the intervention, which suggests a specific benefit of beetroot on time-related indices or on certain sub-sets of cognitive tests. The beetroot intervention was well tolerated and results on reaction time mirror those demonstrated by other authors, showing that whole beetroot is an effective source of dietary nitrate that can be introduced in larger amounts to the diet to infer benefits to cognitive performance.

5.4.2 Prolonged beetroot consumption and cognitive function indices

Whole beetroot provides a source of nitrate, which is sequentially reduced to nitric oxide (NO) following ingestion. NO has a role in the regulation of cerebral blood flow (CBF), neurotransmission and blood flow-metabolism coupling (Szabo, 1996). The ageing process is associated with endothelial dysfunction, which can impair cerebral haemodynamics by reducing the availability of NO and increasing ROS production (Toda *et al.*, 2009). The ageing brain is therefore subject to diminished levels of NO and a consequent reduction in blood flow. These modulations can contribute to a decline in cognitive performance evident into older age. This is the first trial showing that the inclusion of whole beetroot in the diet may have positive effects on cognition in an older population. Improvements were seen specifically in cognitive reaction time and memory retrieval speed, although other indices, such as sustained attention and working memory capacity, were unaffected by the intervention. This suggests specific task-related improvements following beetroot consumption. These results reiterate those described by Gilchrist *et al.* (2014), who were the first to demonstrate a benefit of beetroot juice consumption on cognitive performance in older adults with Type 2 diabetes. These authors found improvements in simple reaction time but no effect on memory or rapid visual information processing (RVIP) following a 14-day intervention. Patients with Type 2 diabetes have been found to have deficits in cognitive function compared to healthy participants (Gilchrist *et al.*, 2014), which may heighten their sensitivity to cognitive testing. Indeed, Kelly *et al.* (2013) found no improvement in serial subtractions, RVIP and number recall following 2.5 days of supplementation with beetroot juice in older adults. The healthy cohort used by Kelly *et al.* (2013) may have had a reduced sensitivity to the effects of dietary nitrate. The positive results shown in the present study on healthy volunteers, however, suggest that this cohort was sensitive to the cognitive testing and intervention used and therefore other factors may be involved. For example, the more prolonged intervention period used in the present study and by Gilchrist *et al.* (2014) may have led to improvements in cognitive function not evident after an acute intervention period, such as the 2.5 days used by Kelly *et al.* (2013). Furthermore, Justice *et al.* (2015) supplemented with sodium nitrite for 10-weeks and demonstrated a 14% and 18% improvement in the trail making task (TMT) B following 80mg/d and 160mg/d,

respectively, in older adults. This again highlights that longer-term supplementation may be more beneficial to cognitive performance.

There was no improvement in TMT-A in the study by Justice *et al.* (2015), which further suggests task-specific improvements following nitrate supplementation. The more complicated TMT-B is used as an index of executive function. Task switching, working memory and executive function are all regulated by the frontal cortex in the brain, which has been found to be preferentially influenced by dietary nitrate supplementation (Presley *et al.*, 2011). Presley *et al.* (2011) demonstrated increased perfusion in the frontal cortex following high doses of beetroot juice incorporated into a nitrate-rich diet over two days in adults aged 75 years on average. Together these data suggest that heightened blood perfusion to specific regions of the brain following dietary nitrate supplementation may dictate alterations in cognitive performance.

Wightman *et al.* (2015) demonstrated that dietary nitrate in the form of beetroot juice modulated the CBF response to task performance in young volunteers. These authors found concomitant improvements in serial 3s subtractions, a task that relies on working memory and psychomotor speed, but not in serial 7s subtractions or RVIP 90-150 minutes after the ingestion of beetroot juice. Interestingly, the RVIP task gave rise to a lower CBF than the serial 3s subtractions task, which may explain the difference in performance. Wightman *et al.* (2015) timed outcome measures to coincide with the peak in plasma nitrite that occurs post-ingestion of nitrate, as described in Chapter 2, which suggest a peak in NO production and thus vasodilatory action. In the present study, cognitive testing took place out with the peak in plasma nitrite that would have occurred following the consumption of beetroot and the breakdown of dietary nitrate. The effects, therefore, may be underestimated if CBF is a key mediator in cognitive enhancements. This is mirrored by the study by Gilchrist *et al.* (2014), in which cognitive testing occurred more than 12 hours post-ingestion of dietary nitrate. The improvements in reaction time evident in this and the present study may be due to an additive effect of a longer-term supplementation period. Investigation into CBF and cognitive performance following prolonged ingestion of dietary nitrate is therefore warranted.

Beetroot is also rich in a number of potentially active phytochemicals such as betalains, which may work synergistically to scavenge ROS in the brain and reduce the negative effects of oxidative stress, and enhance the availability of NO as described in Chapter 1. A more prolonged intervention with foods containing these phytochemicals may afford more time in which to enhance vascular structure and function in the brain through an increase in NO. Due to the low bioavailability of betalains and the lack of available standards for analysis, it was beyond the scope of this thesis to investigate the effects of betalains in this study. However, this remains a promising avenue of research for future long-term interventions with beetroot.

A high vegetable intake is proposed to be associated with a slower rate of cognitive decline into older age (Kang *et al.*, 2005; Loefer & Walach, 2012; Morris *et al.*, 2006). Increasing vegetable consumption from 0.9 servings/day to 2.8 servings/day has been found to reduce the rate of decline by 40% (Morris *et al.*, 2006). Specifically, green leafy vegetables are identified as the most beneficial in maintaining normal cognitive functioning (Kang *et al.*, 2005; Loefer & Walach, 2012). Although the mechanism behind this is unclear and may be due to other lifestyle choices associated with an increased intake of vegetables, the preferential benefit of green leafy vegetables in the DASH may be involved. As mentioned in Chapter 1, Larsen *et al.* (2006) proposed that nitrate is responsible for the blood pressure-lowering effect of the DASH through the vasodilatory properties of NO. The role of NO in the regulation of CBF therefore puts this compound, and dietary nitrate from which it can be produced, in an important position regarding cognitive decline. An increase in nitrate-rich vegetables such as beetroot, therefore, may be particularly beneficial in slowing cognitive decline in older adults.

5.4.3 Strengths and weaknesses

This study investigated, for the first time, the effects of prolonged intake of dietary nitrate through whole beetroot on cognitive function. Using a cohort of adults over the age of 60 increases the applicability of the research, as older adults are more at risk of cognitive impairment than younger populations. Furthermore, the free-living design of the trial with minimal dietary restriction strengthens the external validity of the results.

Although the CogTrack™ cognition programme provided a comprehensive battery of tests for use in healthy adults, tasks more specifically directed at assessing executive function, task switching and working memory may have aided the interpretation of results in relation to CBF and dietary nitrate. This study did not assess CBF, which again would have allowed for further investigation into the effects of dietary nitrate. Whilst the results show promise, further investigation into longer-term supplementation with beetroot is warranted.

5.5 Conclusions

The addition of small amounts of whole beetroot to the habitual diet of older adults shows promise in improving aspects of cognitive performance over a prolonged period of time. It is likely that there is an additive effect of dietary nitrate following repeated consumption of beetroot, which works towards increasing NO in the vascular system in a population whose NO levels are diminished. The acceptability of whole beetroot as an intervention food over an extended time period allows for future research to investigate the beneficial effects of this vegetable more fully.

Chapter 6. General discussion and conclusions

6.1 Overview

With a growing concern over the lack of fruit and vegetables consumed in the UK on a daily basis and the negative effects that this can have on the health of the population, it is imperative that research continues to investigate the positive effects of whole vegetables. Although vegetable juices, gels and powders are a concentrated and practical source of nutrients, the applicability of these products long-term remains uncertain. Beetroot is one such vegetable that has undergone processing into numerous forms, primarily due to attention on its nitrate content, which is a bioactive compound that can be broken down into the vasodilator NO. Older adults have a lower endogenous production of NO, which puts them at risk of vascular dysfunction in both the cardiovascular and cerebrovascular systems. Therefore, an older population should benefit most from the potential effects of whole beetroot consumption. Consequently, the purpose of this thesis was, firstly to assess the availability of nitrate from whole beetroot in older adults and the dose required to elicit physiological effects while maintaining compliance, and secondly to investigate the effects of more prolonged and repeated consumption of whole beetroot in this population, principally on cognitive function and gut health.

The initial investigation into the acute effects of beetroot (Chapters 2 and 3) demonstrated the bioavailability of nitrate from the whole vegetable but a lack of physiological effects in older adults compared to younger participants. Despite this, whole beetroot, in amounts up to 200g, was found to be an acceptable supplement for an older population and one that could be applied to a longer-term study. Subsequently, whole beetroot was used as a supplement for an eight-week intervention in older adults (Chapters 4 and 5) and showed promise as a beneficial dietary component for gut health and cognitive function. Collectively, the results presented in this thesis suggest a benefit of whole beetroot beyond the widely studied effects on blood pressure, particularly when consumed over a prolonged period of time.

6.2 Acute effects of whole beetroot consumption

The acute effects of dietary nitrate are due to an increase in NO, which has vasodilatory properties in the vascular system (Ignarro, 2002). Whilst the pharmacokinetics and bioavailability of dietary nitrate from beetroot juice and in young adults are well known, age-related and source differences are still unclear. Furthermore, studies on dietary nitrate have involved large variation in doses, making direct comparison between age groups difficult. The present study showed that whole beetroot was bioavailable in both young and old adults following a direct comparison in two age groups. The pharmacokinetic profile of the nitrate was also similar across age groups, showing that the timing of physiological responses is comparable. Plasma nitrate and nitrite levels were found to rise significantly following the consumption of whole beetroot in a dose-dependent manner. It appears, however, that a tightly regulated excretory system comes into play to maintain nitrate levels within a certain range. Indeed, urinary nitrate retention was found to be lower with larger doses of nitrate in both age groups. It may be that beyond a certain threshold of dietary intake the body excretes unnecessary nitrate through urine and, although not tested in this study, excretion may occur through faeces also. A dose response was also evident in NO levels in exhaled breath (FeNO). Both age groups responded similarly to whole beetroot with FeNO increasing by up to 77% from baseline. FeNO was modestly correlated with plasma nitrate levels, which explains the dose response and demonstrates the increase in NO levels from dietary nitrate in whole beetroot.

There were a number of age-related differences to the beetroot doses and the physiological effects of the dietary nitrate. Firstly, older adults showed lower levels of plasma nitrite despite receiving the same doses of beetroot and with comparable plasma nitrate levels to the young group. This suggests inefficiency in the breakdown of plasma nitrate to nitrite. This breakdown occurs in the oral cavity by commensal nitrate-reducing bacteria and some aspects of ageing such as changes to the microbiome may hinder this pathway (Miller *et al.*, 2012). These lower levels of plasma nitrite may be responsible for the lack of physiological effects following beetroot consumption in the older group. Acute ingestion of beetroot did not lower blood pressure in the old but had a significant hypotensive effect on the young. Even

the smallest dose of beetroot, 100g, reduced both SBP and DBP in the young group in a matter of hours. This disparity, despite the age groups receiving equal doses again suggests age-related differences in the processing of dietary nitrate. It is pertinent that these mechanistic discrepancies are elucidated, in order for older adults to be able to yield the potent beneficial effects of dietary nitrate from natural sources such as whole beetroot. One promising aspect of the dose response is that larger amounts of nitrate, albeit from a potassium nitrate solution, stimulated a decrease in blood pressure in the old group and larger doses appeared to reduce the disparity in blood pressure response between the age groups. It may be the case, therefore, that larger doses of dietary nitrate are required by older populations in order to receive the same benefits of those experienced by younger age group. Advantageously, qualitative results from this study show that older adults are more tolerant of larger doses of beetroot than younger adults, which may allow for more age-specific supplementation of beetroot in longer-term trials.

6.3 Longer-term effects of whole beetroot consumption

A limited number of studies have investigated the effects of prolonged ingestion of nitrate, leaving a significant gap in the research. Chapters 4 and 5 describe the effects of eight-weeks of supplementation with beetroot on functional capacity, blood pressure, gut health and cognitive function, the latter of which have not previously been investigated in a longer-term setting.

There are equivocal results on dietary nitrate and functional capacity in a longer-term setting. Siervo *et al.* (2016) found no effects of a seven-day intervention with beetroot juice on multiple measures of functional capacity, while Justice *et al.* (2015) found modest improvements in grip strength and balance over 10 weeks but not in TUG. It was hypothesised that longer-term supplementation with beetroot would improve measures of functional capacity in the present study but there was no effect on grip strength, TUG or lung function. It is speculated that, despite a potential chronic increase in circulating nitrate, the time of testing fell out with the peak in plasma nitrite and therefore NO and consequently had no effect on functional capacity in a healthy cohort.

A chronic increase in circulating nitrate or nitrite would be of great benefit to the vascular system in older adults, who have lower levels of endogenously produced NO due to deficits in the precursor L-arginine and the NO synthase cofactor tetrahydrobiopterin. Lower availability of NO leads to vascular dysfunction and can cause hypertension. In the present study, SBP was found to be lower following the eight-week intervention period, which may be a reflection of the increase in circulating nitrate. Natural foods that can chronically increase nitrate and have a consequent blood pressure-lowering effect would be a substantial development in treatments to reduce hypertension. Furthermore, there was good compliance with the beetroot supplement provided, which is important for the application of this food to longer-term intervention studies.

Longer-term supplementation with beetroot also shows promise as a beneficial dietary component for gut and cognitive health, which recent evidence suggests may be linked (Leung & Thuret, 2015). Beetroot consumption was found to increase total SCFA production, which is a marker for overall gut health due to the effect of SCFAs such as butyrate and acetate on inflammation, gut wall integrity and cancer cell apoptosis. Butyrate specifically has anti-inflammatory actions on both the gut and the brain (Noble *et al.*, 2017) and concentrations were found to rise by 99% following the eight-week intervention period. This is particularly important in older adults, as this populations has lower levels of butyrate due to a reduction in the bacteria that produce it (Biagi *et al.*, 2013). Beetroot consumption had an additional effect on gut bacterial with analysis displaying an increase in diversity compared with the control food. More diversity in gut bacteria may increase numbers of SCFA-producing bacteria and therefore improve gut health. Overall, these benefits to gut health are likely to due to the prolonged intervention period and would not be evident in an acute setting. Similarly, any improvements to cognitive function that are linked to the gut microbiome would manifest after long-term supplementation. However, improvements in cognition function with dietary nitrate have been linked to increased CBF due to a rise in NO production (Presley *et al.*, 2011; Wightman *et al.*, 2015). In this thesis, cognitive function was not assessed acutely, so it is unclear as to the effects of whole beetroot in this setting. However, the eight-week intervention with beetroot was found to improve indices of cognitive function related to speed i.e. cognitive reaction time and memory retrieval speed. This may be related to the

chronic rise in plasma nitrate seen in Chapter 4 or the increase in SCFA production and bacterial diversity in the gut. Whilst cognitive decline into old age cannot be prevented, it may be possible to slow the rate of decline and improvements in cognitive reaction time could prove beneficial to ADLs and independence in later life.

6.4 Public health perspectives

One major benefit of the research conducted during this thesis is the applicability of results in a real-world setting. Although beetroot has been reformulated into a number of products such as gels and juices, the acceptability and benefit of these supplements long-term is debatable. These products are a more concentrated form of dietary nitrate and other potentially active compounds but they lack fibre, are higher in sugar and are costlier than the whole vegetable. This study found that whole beetroot was well tolerated both acutely and over a more prolonged period, with high compliance with the interventions in both studies. The consistent consumption of whole beetroot for longer than eight weeks may then be tolerable both for future research interventions and in the everyday diet of the population.

In young participants, the consumption of whole beetroot acutely lowered SBP by up to 7.3mmHg and DBP by up to 5.1mmHg, which, if maintained, would significantly lower the risk of stroke and CHD (Collins *et al.*, 1990). The longer-term study in Chapter 4 demonstrated a reduction in SBP by 7.8mmHg in an older population. As between 45% and 66.5% of adults over the age of 55 are class as hypertensive, a reduction in SBP to the extent demonstrated here could reduce some of the burden and risk of hypertension in the UK. More long-term studies are required to fully elucidate the chronic blood pressure-lowering effects of dietary nitrate.

Another public health concern is the lack of fibre intake in the general population and the negative effects of this on the GI system (Kendall *et al.*, 2010; Marlett *et al.*, 2002). As mentioned, processing of beetroot, and many other vegetables and fruit, into juices and other concentrated forms removes fibre and limits the scope of the benefits of consuming these products. Vegetables in their whole form offer benefits beyond the activity of an isolated compound, combining fibre with synergistic effects of antioxidants, phenolic compounds and vitamins and minerals. The study in Chapter 4 was the first to examine the effects of whole beetroot on the gut

microbiome but the promising results suggest an improvement in markers of gut health. With research on the importance of the gut to overall health in its early stages, the preliminary results shown here may lead to significant developments in the research surrounding population health.

6.5 Future research

While a number of interesting findings emerged from the studies in this thesis, there are also areas that warrant further investigation and replication. As mentioned, the lack of effect on blood pressure in the old group compared to the young merits exploration into the mechanisms behind age-related differences in the nitrate-nitrite-NO pathway. One such mechanism is the uptake of nitrate into the salivary system, which is regulated by the nitrate-transporter *sialin*. While it was outside the scope of this thesis to investigate this transporter specifically, future research on the saliva samples taken may begin to elucidate the mechanisms behind the lower plasma nitrite levels evident in an ageing population.

Despite *in vitro* and rodent studies illustrating the potent effects of betalains from beetroot and other foods such as the protection of red blood cells and LDLs from oxidation (Esatbeyoglu *et al.*, 2015; Sawicki *et al.*, 2016), their low bioavailability and the lack of standards for analysis have limited the scope of research in human intervention studies. The antioxidant capacity of betalains may be important in the cerebrovascular system, which is particularly vulnerable to oxidative stress, and in older populations in which oxidative stress is concomitant with the ageing process. Therefore, specific analysis into plasma levels of betalains or the accumulation of this compound in areas susceptible to oxidative stress may benefit the body of research on beetroot. Furthermore, on a similar note, research into the low-grade inflammation associated with ageing and therapeutic approaches to reducing it may affect markers of functional capacity. Justice *et al.* (2015) suggest that improvements seen in motor function in old mice may be related to a reduction in low-grade inflammation in the muscles; this analysis could be replicated in human volunteers.

The investigation into cognition in this study could have benefitted from the assessment of cerebral blood flow (CBF) and further investigation into the correlation between CBF and cognitive function following beetroot supplementation. Wightman

et al. (2015) for example, found heightened CBF acutely following beetroot juice consumption and subsequent improvements in cognitive function. It is unclear as to whether chronic improvements in cognition, potentially related to a persistent increase in circulating nitrate, would be correlated with CBF but it is worthy of further examination.

A limit to the application of this series of investigations in public health, and one that was mentioned in relation to functional capacity, is the use of healthy participants throughout. A healthy cohort allowed for an accurate representation of the age-related differences in pharmacokinetics and bioavailability in this thesis but limits the application in diseased populations. Continuing the investigations from this thesis into populations with hypertension or cognitive impairment may demonstrate additional benefits of beetroot consumption or highlight further deficits in the nitrate-nitrite-NO pathway with age-related disease. Either way, studies including more at-risk populations would increase the applicability of the work on beetroot and physiological outcomes.

6.6 Conclusion

In conclusion, the series of investigations in this thesis have provided evidence that whole beetroot is a well-accepted intervention food that can effectively raise nitrite and NO levels in both young and old populations. Furthermore, prolonged consumption of this vegetable can have a positive effect on blood pressure, gut bacterial diversity and cognitive function, making it a beneficial and under-used whole food and dietary staple. The mechanisms behind the nitrate-nitrite-NO pathway that lead to physiological benefits following beetroot consumption need to be further explored in an ageing population and in those more at risk of the deleterious effects of low NO availability such as cognitively impaired and hypertensive patients.

Reference list

- Anhê, F. F., Pilon, G., Roy, D., Desjardins, Y., Levy, E. & Marette, A. (2016) Triggering Akkermansia with Dietary Polyphenols: A New Weapon to Combat the Metabolic Syndrome? *Gut microbes* [Online], 7 (2), pp. 146–153.
- Appel, L. J., Brands, M. W., Daniels, S. R., Karanja, N., Elmer, P. J. & Sacks, F. M. (2006) Dietary Approaches to Prevent and Treat Hypertension: A Scientific Statement from the American Heart Association. *Hypertension*, 47 (2), pp. 296–308.
- Asgary, S., Afshani, M. R., Sahebkar, A., Keshvari, M., Taheri, M., Jahanian, E., Rafieian-Kopaei, M., Malekian, F. & Sarrafzadegan, N. (2016) Improvement of Hypertension, Endothelial Function and Systemic Inflammation Following Short-Term Supplementation with Red Beet (*Beta Vulgaris* L.) Juice: A Randomized Crossover Pilot Study. *Journal of Human Hypertension*, 30 (10), pp. 627–632.
- Asgary, S., Rastqar, A. & Keshvari, M. (2018) Functional Food and Cardiovascular Disease Prevention and Treatment: A Review. *Journal of the American College of Nutrition* [Online], 37 (5), pp. 429–455.
- Ashor, A. W., Chowdhury, S., Oggioni, C., Qadir, O., Brandt, K., Ishaq, A., Mathers, J. C., Saretzki, G. & Siervo, M. (2016) Inorganic Nitrate Supplementation in Young and Old Obese Adults Does Not Affect Acute Glucose and Insulin Responses but Lowers Oxidative Stress. *Journal of Nutrition* [Online], 146 (11), pp. 2224–2232.
- Ashworth, A., Mitchell, K., Blackwell, J. R., Vanhatalo, A. & Jones, A. M. (2015) High-Nitrate Vegetable Diet Increases Plasma Nitrate and Nitrite Concentrations and Reduces Blood Pressure in Healthy Women. *Public Health Nutrition*, 18 (14), pp. 2669–2678.
- Avogaro, A., Toffolo, G., Kiwanuka, E., Kreutzenberg, S. V. De, Tessari, P. & Cobelli, C. (2003) L-Arginine-Nitric Oxide Kinetics in Normal and Type 2 Diabetic Subjects: A Stable-Labelled ¹⁵N Arginine Approach. *Diabetes*, 52 (3), pp. 795–802.
- Avoort, C. M. T. van der, Loon, L. J. C. van, Hopman, M. T. E. & Verdijk, L. B. (2018) Increasing Vegetable Intake to Obtain the Health Promoting and Ergogenic Effects of Dietary Nitrate. *European Journal of Clinical Nutrition* [Online], pp. 1–5.

- Babateen, A. M., Fornelli, G., Donini, L. M., Mathers, J. C. & Siervo, M. (2018) Assessment of Dietary Nitrate Intake in Humans: A Systematic Review. *The American Journal of Clinical Nutrition* [Online], 108 (4), pp. 878–888.
- Bailey, S. J., Fulford, J., Vanhatalo, A., Winyard, P. G., Blackwell, J. R., Dimenna, F. J., Wilkerson, D. P., Benjamin, N. & Jones, A. M. (2010) Dietary Nitrate Supplementation Enhances Muscle Contractile Efficiency during Knee-Extensor Exercise in Humans. *J Appl Physiol*, 109, pp. 135–148.
- Bar-Shai, M., Carmeli, E., Ljubuncic, P. & Reznick, A. Z. (2008) Exercise and Immobilization in Aging Animals: The Involvement of Oxidative Stress and NF-KB Activation.
- Barry, E., Galvin, R., Keogh, C., Horgan, F. & Fahey, T. (2014) Is the Timed Up and Go Test a Useful Predictor of Risk of Falls in Community Dwelling Older Adults: A Systematic Review and Meta- Analysis. *BMC Geriatrics*, 14 (1).
- Bartsch, H., Ohshima, H. & Pignatelli, B. (1988) Inhibitors of Endogenous Nitrosation Mechanisms and Implications in Human Cancer Prevention. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, 202 (2), pp. 307–324.
- Basu, A., Rhone, M. & Lyons, T. J. (2010) Berries: Emerging Impact on Cardiovascular Health Arpita. *Nutrition Reviews* [Online], 68 (3), pp. 168–177.
- Bazzano, L. A., He, J., Ogden, L. G., Loria, C. M., Vupputuri, S., Myers, L. & Whelton, P. K. (2002) Fruit and Vegetable Intake and Risk of Cardiovascular Disease in US Adults : The First National Health and Nutrition Examination Survey Epidemiologic Follow-up Study 1 – 3.
- Beek, C. M. van der, Dejong, C. H. C., Troost, F. J., Masclee, A. A. M. & Lenaerts, K. (2017) Role of Short-Chain Fatty Acids in Colonic Inflammation, Carcinogenesis, and Mucosal Protection and Healing. *Nutrition Reviews*, 75 (4), pp. 286–305.
- Beghetti, M., Puigdefabregas, J. W. B. & Merali, S. (2014) Sildenafil for the Treatment of Pulmonary Hypertension in Children. *Expert Review of Cardiovascular Therapy*, 12 (10), pp. 1157–1184.
- Belkaid, Y. & Hand, T. (2014) Role of Microbiota in Immunity and Inflammation. *Cell*, 157 (1), pp. 121–141.
- Bell, L., Lamport, D. J., Field, D. T., Butler, L. T. & Williams, C. M. (2018) Practice Effects in Nutrition Intervention Studies with Repeated Cognitive Testing. *Nutrition and Healthy Aging*, 4 (4), pp. 309–322.

- Benetou, V., Orfanos, P., Lagiou, P., Trichopoulos, D., Boffetta, P. & Trichopoulou, A. (2008) Vegetables and Fruits in Relation to Cancer Risk: Evidence from the Greek EPIC Cohort Study. *Cancer Epidemiology Biomarkers and Prevention*, 17 (2), pp. 387–392.
- Benjamin, N., O'Driscoll, F., Dougall, H., Duncan, C., Smith, L., Golden, M. & McKenzie, H. (1994) Stomach NO Synthesis. *Nature*, 368, p. 502.
- Bescús, R., Rodríguez, F. A., Iglesias, X., Ferrer, M. D., Iborra, E. & Pons, A. (2011) Acute Administration of Inorganic Nitrate Reduces $\dot{V}O_2$ peak in Endurance Athletes. *Medicine and Science in Sports and Exercise*, 43 (10), pp. 1979–1986.
- Biagi, E., Candela, M., Turrioni, S., Garagnani, P., Franceschi, C. & Brigidi, P. (2013) Ageing and Gut Microbes: Perspectives for Health Maintenance and Longevity. *Pharmacological Research* [Online], 69 (1), pp. 11–20.
- Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., Pini, E., Nikkila, J., Monti, D., Satokari, R., Franceschi, C., Brigidi, P. & Vos, W. de (2010) Through Ageing, and beyond: Gut Microbiota and Inflammatory Status in Seniors and Centenarians. *PLoS ONE*, 5 (5).
- Biagi, E., Rampelli, S., Turrioni, S., Quercia, S., Candela, M. & Brigidi, P. (2017) The Gut Microbiota of Centenarians: Signatures of Longevity in the Gut Microbiota Profile. *Mechanisms of Ageing and Development* [Online], 165, pp. 180–184.
- Björne, H., Petersson, J., Phillipson, M., Weitzberg, E., Holm, L. & Lundberg, J. O. (2004) Nitrite in Saliva Increases Gastric Mucosal Blood Flow. *Journal of Clinical Investigation*, 113 (1), pp. 106–114.
- Blacher, J., Asmar, R., Djane, S., London, G. M. & Safar, M. E. (1999) Aortic Pulse Wave Velocity as a Marker of Cardiovascular Risk in Hypertensive Patients. *Hypertension* [Online], 33 (5), pp. 1111–1117.
- Blekkenhorst, L. C., Lewis, J. R., Prince, R. L., Devine, A., Bondonno, N. P., Bondonno, C. P., Wood, L. G., Puddey, I. B., Ward, N. C., Croft, K. D., Woodman, R. J., Beilin, L. J. & Hodgson, J. M. (2018) Nitrate-Rich Vegetables Do Not Lower Blood Pressure in Individuals with Mildly Elevated Blood Pressure: A 4-Wk Randomized Controlled Crossover Trial. [Online], 9 (June), pp. 894–908.
- Bloomfield, D. A. & Park, A. (2017) Decoding White Coat Hypertension. *World Journal of Clinical Cases* [Online], 5 (3), p. 82.
- Bogdan, C. (2001) Nitric Oxide and the Immune Response. *Nature Immunology*, 2 (10), pp. 907–916.

- Bohannon, R. W. (2006) Reference Values for the Timed Up and Go Test. *Journal of Geriatric Physical Therapy* [Online], 29 (2), pp. 64–68.
- Bondonno, C. P., Croft, K. D., Ward, N., Considine, M. J. & Hodgson, J. M. (2015) Dietary Flavonoids and Nitrate: Effects on Nitric Oxide and Vascular Function. *Nutrition Reviews*, 73 (4), pp. 216–235.
- Bondonno, C.P., Croft, K.D., Hodgson, J.M. (2015). Dietary nitrate, nitric oxide, and cardiovascular health. *Critical Reviews in Food Science and Nutrition*, 56 (12), pp. 2036-2052.
- 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 (2), pp. 368–375.
- Britton, E. & McLaughlin, J. T. (2013) Ageing and the Gut. *Proceedings of the Nutrition Society*, 72 (1), pp. 173–177.
- Brown, G., Rumsey, J., Worth, I., Lee, D. & Scaife, A. (2016) Food Statistics Pocketbook 2016. *National Statistics*, pp. 1–62.
- Bryan, N. S. (2015) The Potential Use of Salivary Nitrite as a Marker of NO Status in Humans. *Nitric Oxide - Biology and Chemistry*, 45, pp. 4–6.
- Bryan, N. S. & Ivy, J. L. (2015) Inorganic Nitrite and Nitrate: Evidence to Support Consideration as Dietary Nutrients. *Nutrition Research* [Online], 35 (8), pp. 643–654.
- Buckner, R. L. (2004) Memory and Executive Function in Aging and AD: Multiple Factors That Cause Decline and Reserve Factors That Compensate. *Neuron*, 44 (1), pp. 195–208.
- Bult, H., Boechxstaens, G., Pelckmans, P., Jordaens, F., Maercke, Y. Van & Herman, A. (1990) Nitric Oxide as an Inhibitory Non-Adrenergic Non-Cholinergic Neurotransmitter. *Nature*, 345 (6273), pp. 346–347.
- Butler, A. R. & Feelisch, M. (2008) Therapeutic Uses of Inorganic Nitrite and Nitrate: From the Past to the Future. *Circulation*, 117 (16), pp. 2151–2159.
- Cai, Y. Z., Sun, M. & Corke, H. (2003) Antioxidant Activity of Betalains from Plants of the Amaranthaceae. *J Agric Food Chem*, 51 (8), pp. 2288–2294.
- Calder, P. C., Ahluwalia, N., Brouns, F., Buetler, T., Clement, K., Cunningham, K., Esposito, K., Jönsson, L. S., Kolb, H., Lansink, M., Marcos, A., Margioris, A.,

- Matusheski, N., Nordmann, H., O'Brien, J., Pugliese, G., Rizkalla, S., Schalkwijk, C., Tuomilehto, J., Wärnberg, J., Watzl, B. & Winklhofer-Roob, B. M. (2011) Dietary Factors and Low-Grade Inflammation in Relation to Overweight and Obesity. *British Journal of Nutrition* [Online], 106 (S3), pp. S5–S78.
- Caldwell, K., Charlton, K. E., Roodenrys, S. & Jenner, A. (2016) Anthocyanin-Rich Cherry Juice Does Not Improve Acute Cognitive Performance on RAVLT. *Nutritional Neuroscience* [Online], 19 (9), pp. 423–424.
- Campanella, B., Onor, M., Pagliano, E. (2017). Rapid determination of nitrate in vegetables by gas chromatography mass spectrometry. *Analytica Chimica, Acta*, 980, pp 33-40.
- Cani, P. D. (2018) Human Gut Microbiome: Hopes, Threats and Promises. *Gut*, 67 (9), pp. 1716–1725.
- Canizzo, E., Clement, C., Sahu, R., Follo, C. & Santambrogio, L. (2011) Oxidative Stress, Inflammaging, and Immunosenesence. *Journal of Proteonomics* [Online], 74, pp. 2313–2323.
- Cao, G., Russell, R., Lischner, N. & Prior, R. (1998) Serum Antioxidant Capacity Is Increased by Consumption of Strawberries, Spinach, Red Wine or Vitamin C in Elderly Women. *The Journal of nutrition*, 128 (12), pp. 2383–2390.
- Carasca, C., Magdas, A., Tilea, I. & Incze, A. (2017) Assessment of Post-Occlusive Reactive Hyperaemia in the Evaluation of Endothelial Function in Patients with Lower Extremity Artery Disease. *Acta Medica Marisiensis* [Online], 63 (3), pp. 129–132.
- Carberry, P. a, Shepherd, A. M. M. & Johnson, J. M. (1992) Resting and Maximal Forearm Skin Blood Flows Are Reduced in Hypertension. *Hypertension*, 20 (3), pp. 349–355.
- Carosio, S., Berardinelli, M. G., Aucello, M. & Musarò, A. (2011) Impact of Ageing on Muscle Cell Regeneration. *Ageing Research Reviews*, 10 (1), pp. 35–42.
- Casey, D. P., Treichler, D. P., Ganger, C. T., Schneider, A. C. & Ueda, K. (2015) Acute Dietary Nitrate Supplementation Enhances Compensatory Vasodilation during Hypoxic Exercise in Older Adults. *Journal of Applied Physiology* [Online], 118 (2), pp. 178–186.
- Celidoni, M., Dal Bianco, C. & Weber, G. (2017) Retirement and Cognitive Decline. A Longitudinal Analysis Using SHARE Data. *Journal of Health Economics* [Online], 56 December, pp. 113–125.

- Chapman, T. H., Wilde, M., Sheth, A. & Madden, B. P. (2009) Sildenafil Therapy in Secondary Pulmonary Hypertension: Is There Benefit in Prolonged Use? *Vascular Pharmacology*, 51 (2–3), pp. 90–95.
- Cheng, H. M., Cheng, H. K. & George, T. W. (2015) The Effect of Red and Yellow Beetroot Juices on Blood Pressure in Free-Living Normotensive Males: A Pilot Study. *Proceedings of the Nutrition Society* [Online], 74 (OCE5), p. E333.
- Chobanian, A. V., Bakris, G. L., Black, H. R., Cushman, W. C., Green, L. A., Izzo, J. L., Jones, D. W., Materson, B. J., Oparil, S., Wright, J. T. & Roccella, E. J. (2003) Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Hypertension*, 42 (6), pp. 1206–1252.
- Claesson, M. J., Jeffery, I. B. & O'Toole, P. W. (2012) Gut Microbiota Composition Correlates with Diet and Health in the Elderly. *Nature*, 488, pp. 178–184.
- Clifford, T., Babateen, A., Shannon, O. M., Capper, T., Ashor, A., Stephan, B., Robinson, L., O'Hara, J. P., Mathers, J. C., Stevenson, E. & Siervo, M. (2018) Effects Of Inorganic Nitrate And Nitrite Consumption On Cognitive Function And Cerebral Blood Flow: A Systematic Review And Meta-Analysis Of Randomised Clinical Trials. *Critical Reviews in Food Science and Nutrition* [Online], 4 (0), pp. 1–11.
- Clifford, T., Constantinou, C. M., Keane, K. M., West, D. J., Howatson, G. & Stevenson, E. J. (2017) The Plasma Bioavailability of Nitrate and Betanin from Beta Vulgaris Rubra in Humans. *European Journal of Nutrition*, 56 (3), pp. 1245–1254.
- Close, G. L., Kayani, A., Vasilaki, A. & McArdle, A. (2005) Skeletal Muscle Damage with Exercise and Aging. *Sports Medicine*, 35 (5), pp. 413–427.
- Coldham, F., Lewis, J. & Lee, H. (2006) The Reliability of One vs. Three Grip Trials in Symptomatic and Asymptomatic Subjects. *Journal of Hand Therapy*, 19 (3), pp. 318–327.
- 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* [Online], 11 (1), p. 1.
- Collerton, J., Collerton, D., Arai, Y., Barrass, K., Eccles, M., Jagger, C., McKeith, I., Saxby, B. K., Kirkwood, T., Bond, J., James, O., Robinson, L. & Zglinicki, T. Von (2007) A Comparison of Computerized and Pencil-and-Paper Tasks in

- Assessing Cognitive Function in Community-Dwelling Older People in the Newcastle 85+ Pilot Study. *Journal of the American Geriatrics Society*, 55 (10), pp. 1630–1635.
- Collins, R., Peto, R., MacMahon, S., Godwin, J., Qizilbash, N., Collins, R., MacMahon, S., Hebert, P., Eberlein, K. A., Taylor, J. O., Hennekens, C. H., Fiebach, N. H., Qizilbash, N. & Hennekens, C. H. (1990) Blood Pressure, Stroke, and Coronary Heart Disease. *The Lancet*, 335 (8693), pp. 827–838.
- Corner, L. & Bond, J. (2004) Being at Risk of Dementia: Fears and Anxieties of Older Adults. *Journal of Aging Studies*, 18 (2), pp. 143–155.
- Cosby, K., Partovi, K., Crawford, J., Patel, R., Reiter, C., Martyr, S., Yang, B., Waclawiw, M., Zalos, G., Xu, X., Huang, K., Shields, H., Kim-Shapiro, D. B., Schechter, A. N., Cannon, R. O. & Gladwin, M. T. (2003) Nitrite Reduction to Nitric Oxide by Deoxyhemoglobin Vasodilates the Human Circulation. *Nature medicine*, 9 (12), pp. 1498–1505.
- Cracowski, J. L., Minson, C. T., Salvat-Melis, M. & Halliwill, J. R. (2006) Methodological Issues in the Assessment of Skin Microvascular Endothelial Function in Humans. *Trends in Pharmacological Sciences*, 27 (9), pp. 503–508.
- Craig, C., Marshall, A., Sjostrom, A., Bauman, A., Booth, M., Ainsworth, B., Pratt, M., Ekelund, U., Yngve, A., Sallis, J. & Oja, P. (2003) International Physical Activity Questionnaire: 12-Country Reliability and Validity. *Medicine and Science in Sports and Exercise*, 35 (8), pp. 1381–1395.
- Dahl, W., Agro, N., Eliasson, A., Mialki, K., Olivera, J., Rusch, C. & Young, C. (2017) Health Benefits of Fiber Fermentation. *J Am Coll Nutr*, 36 (2), pp. 127–136.
- David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J. & Turnbaugh, P. J. (2014) Diet Rapidly and Reproducibly Alters the Human Gut Microbiome. *Nature* [Online], 505 (7484), pp. 559–563.
- Deary, I. J., Corley, J., Gow, A. J., Harris, S. E., Houlihan, L. M., Marioni, R. E., Penke, L., Rafnsson, S. B. & Starr, J. M. (2009) Age-Associated Cognitive Decline. *British Medical Bulletin*, 92 (1), pp. 135–152.
- Dede, D. S., Yavuz, B., Yavuz, B. B., Cankurtaran, M., Halil, M., Ulger, Z., Cankurtaran, E. S., Aytemir, K., Kabakci, G. & Ariogul, S. (2007) Assessment of Endothelial Function in Alzheimer's Disease: Is Alzheimer's Disease a Vascular Disease? *Journal of the American Geriatrics Society*, 55 (10), pp. 1613–1617.

- Dejam, A., Hunter, C. J., Tremonti, C., Pluta, R. M., Hon, Y. Y., Grimes, G., Partovi, K., Pelletier, M. M., Oldfield, E. H., Cannon, R. O., Schechter, A. N. & Gladwin, M. T. (2007) Nitrite Infusion in Humans and Nonhuman Primates: Endocrine Effects, Pharmacokinetics, and Tolerance Formation. *Circulation*, 116 (16), pp. 1821–1831.
- Delp, M. D., Behnke, B. J., Spier, S. A., Wu, G. & Muller-Delp, J. M. (2008) Ageing Diminishes Endothelium-Dependent Vasodilatation and Tetrahydrobiopterin Content in Rat Skeletal Muscle Arterioles. *Journal of Physiology*, 586 (4), pp. 1161–1168.
- 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., Chonchol, M. B., Bassett, C. J., Sindler, A. L., Giordano, T. & Seals, D. R. (2016) Effects of Sodium Nitrite Supplementation on Vascular Function and Related Small Metabolite Signatures in Middle-Aged and Older Adults. *Journal of Applied Physiology* [Online], 120 (4), pp. 416–425.
- Dressel, H., la Motte, D. de, Reichert, J., Ochmann, U., Petru, R., Angerer, P., Holz, O., Nowak, D. & Jörres, R. A. (2008) Exhaled Nitric Oxide: Independent Effects of Atopy, Smoking, Respiratory Tract Infection, Gender and Height. *Respiratory Medicine*, 102 (7), pp. 962–969.
- Duncan, C., Dougall, H., Johnston, P., Green, S., Brogan, R., Leifert, C., Smith, L., Golden, M. & Benjamin, N. (1995) Chemical Generation of Nitric Oxide in the Mouth from the Enterosalivary Circulation of Dietary Nitrate. *Nature medicine*, 1 (6), pp. 546–551.
- Duncan, C., Li, H., Dykhuizen, R., Frazer, R., Johnston, P., MacKnight, G., Smith, L., Lamza, K., McKenzie, H., Batt, L., Kelly, D., Golden, M., Benjamin, N. & Leifert, C. (1997) Protection against Oral and Gastrointestinal Diseases: Importance of Dietary Nitrate Intake, Oral Nitrate Reduction and Enterosalivary Nitrate Circulation. *Comparative Biochemistry and Physiology - A Physiology*, 118 (4), pp. 939–948.
- Duncan, S. H. & Flint, H. J. (2013) Probiotics and Prebiotics and Health in Ageing Populations. *Maturitas* [Online], 75 (1), pp. 44–50.
- Duncan, S. H., Lobley, G. E., Holtrop, G., Ince, J., Johnstone, A. M., Louis, P. & Flint, H. J. (2008) Human Colonic Microbiota Associated with Diet, Obesity and Weight Loss. *International Journal of Obesity*, 32 (11), pp. 1720–1724.

- Dweik, R. A., Boggs, P. B., Erzurum, S. C., Irvin, C. G., Leigh, M. W., Lundberg, J. O., Olin, A., Plummer, A. L., Taylor, D. R., Thoracic, A. & Committee, S. (2011) An Official ATS Clinical Practice Guideline: Interpretation of Exhaled Nitric Oxide Levels (FENO) for Clinical Applications. *Am J Respir Crit Care Med*, 184, pp. 602–615.
- Ecobici, M. & Stoicescu, C. (2017) Arterial Stiffness and Hypertension - Which Comes First? *Maedica* [Online], 12 (3), pp. 184–190.
- Egashira, K., Inou, T., Hirooka, Y., Kai, H., Sugimachi, M., Suzuki, S., Kuga, T., Urabe, Y. & Takeshita, A. (1993) Effects of Age on Endothelium-Dependent Vasodilation of Resistance Coronary Artery by Acetylcholine in Humans. *Circulation*, 88 (1), pp. 77–81.
- Eggebeen, J., Kim-Shapiro, D. B., Haykowsky, M., Morgan, T. M., Basu, S., Brubaker, P., Rejeski, J. & Kitzman, D. W. (2016) One Week of Daily Dosing With Beetroot Juice Improves Submaximal Endurance and Blood Pressure in Older Patients With Heart Failure and Preserved Ejection Fraction. *JACC: Heart Failure*, 4 (6), pp. 428–437.
- Esatbeyoglu, T., Wagner, A. E., Schini-Kerth, V. B. & Rimbach, G. (2015) Betanin-A Food Colorant with Biological Activity. *Molecular Nutrition and Food Research*, 59, pp. 36–47.
- Ettehad, D., Emdin, C. A., Kiran, A., Anderson, S. G., Callender, T., Emberson, J., Chalmers, J., Rodgers, A. & Rahimi, K. (2016) Blood Pressure Lowering for Prevention of Cardiovascular Disease and Death: A Systematic Review and Meta-Analysis. *The Lancet* [Online], 387 (10022), pp. 957–967.
- Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J. P., Druart, C., Bindels, L. B., Guiot, Y., Derrien, M., Muccioli, G. G., Delzenne, N. M., Vos, W. M. de & Cani, P. D. (2013) Cross-Talk between Akkermansia Muciniphila and Intestinal Epithelium Controls Diet-Induced Obesity. *Proceedings of the National Academy of Sciences* [Online], 110 (22), pp. 9066–9071.
- Everard, K. M., Lach, H. W., Fisher, E. B. & Baum, M. C. (2000) Relationship of Activity and Social Support to the Functional Health of Older Adults. *The Journals of Gerontology Series B: Psychological Sciences and Social Sciences* [Online], 55 (4), pp. S208–S212.
- Fang, J. (2014) Bioavailability of Anthocyanins. *Drug Metabolism Reviews*, 46 (4), pp. 508–520.

- Faria, A., Fernandes, I., Norberto, S., Mateus, N. & Calhau, C. (2014) Interplay between Anthocyanins and Gut Microbiota. *Journal of Agricultural and Food Chemistry*, 62 (29), pp. 6898–6902.
- Fava, F., Rizzetto, L. & Tuohy, K. M. (2018) Gut Microbiota and Health: Connecting Actors across the Metabolic System. *Proceedings of the Nutrition Society* [Online], (June), pp. 1–12.
- Ferrari, A. U., Radaelli, A. & Centola, M. (2003) Aging and the Cardiovascular System. *Journal of Applied Physiology* [Online], 95 (6), pp. 2591–2597. Available from: <<http://jap.physiology.org/lookup/doi/10.1152/japplphysiol.00601.2003>>.
- Ferrucci, L., Harris, T., Guralnik, J., Tracy, R., Corti, M., Cohen, H., Penninx, B., Pahor, M., Wallace, R. & Havlik, R. (1999) Serum IL-6 Level and the Development of Disability in Older Persons. *Journal of the American Geriatrics Society*, 47 (6), pp. 639–646.
- Finley, J. W., Burrell, J. B. & Reeves, P. G. (2007) Pinto Bean Consumption Changes SCFA Profiles in Fecal Fermentations, Bacterial Populations of the Lower Bowel, and Lipid Profiles in Blood of Humans. *The Journal of nutrition* [Online], 137 (11), pp. 2391–2398.
- Forman, J.P., Rimm, E.B., Stampfer, M.J., Curham, G.C. (2005). Folate intake and the risk of incident hypertension among US women. *JAMA*, 293 (3), pp. 320-329.
- Franceschi, C., Capri, M., Monti, D., Giunta, S., Olivieri, F., Sevini, F., Panourgia, M. P., Invidia, L., Celani, L., Scurti, M., Cevenini, E., Castellani, G. C. & Salvioli, S. (2007) Inflammaging and Anti-Inflammaging: A Systemic Perspective on Aging and Longevity Emerged from Studies in Humans. *Mechanisms of Ageing and Development*, 128 (1), pp. 92–105.
- Frank, D. N., Amand, A. L. S., Feldman, R. A., Boedeker, E. C., Harpaz, N. & Pace, N. R. (2007) Molecular-Phylogenetic Characterisation of Microbial Community Imbalances in Human Inflammatory Bowel Diseases. *Proc Natl Acad Sci U S A*, 104 (34), pp. 13780–13785.
- Frank, T., Stintzing, F. C., Carle, R., Bitsch, I., Quaas, D., Straß, G., Bitsch, R. & Netzel, M. (2005) Urinary Pharmacokinetics of Betalains Following Consumption of Red Beet Juice in Healthy Humans. *Pharmacological Research*, 52 (4), pp. 290–297.
- Garaschuk, O., Semchyshyn, H. M. & Lushchak, V. I. (2018) Healthy Brain Aging: Interplay between Reactive Species, Inflammation and Energy Supply. *Ageing*

- Research Reviews*, 43, pp. 26–45.
- Gates, P. E., Boucher, M. L., Silver, A. E., Monahan, K. D. & Seals, D. R. (2007) Impaired Flow-Mediated Dilation with Age Is Not Explained by L-Arginine Bioavailability or Endothelial Asymmetric Dimethylarginine Protein Expression. *Journal of Applied Physiology* [Online], 102 (1), pp. 63–71.
- Gault, M. L. & Willems, M. E. T. (2013) Aging, Functional Capacity and Eccentric Exercise Training. *Aging and Disease* [Online], 4 (6), pp. 351–363.
- Gee, L. C. & Ahluwalia, A. (2016) Dietary Nitrate Lowers Blood Pressure: Epidemiological, Pre-Clinical Experimental and Clinical Trial Evidence. vol. 18.
- Georgiev, V. G., Weber, J., Kneschke, E. M., Denev, P. N., Bley, T. & Pavlov, A. I. (2010) Antioxidant Activity and Phenolic Content of Betalain Extracts from Intact Plants and Hairy Root Cultures of the Red Beetroot *Beta Vulgaris* Cv. Detroit Dark Red. *Plant Foods for Human Nutrition*, 65 (2), pp. 105–111.
- Gibson, G. R. (1999) Dietary Modulation of the Human Gut Microflora Using the Prebiotics Oligofructose and Inulin. *The Journal of Nutrition* [Online], 129 (7), p. 1438S–1441S.
- Gilchrist, M., Winyard, P. G., Aizawa, K., Anning, C., Shore, A. & Benjamin, N. (2013) Effect of Dietary Nitrate on Blood Pressure, Endothelial Function, and Insulin Sensitivity in Type 2 Diabetes. *Free Radical Biology and Medicine* [Online], 60, pp. 89–97.
- Gilchrist, M., Winyard, P. G., Fulford, J., Anning, C., Shore, A. C. & Benjamin, N. (2014) Dietary Nitrate Supplementation Improves Reaction Time in Type 2 Diabetes: Development and Application of a Novel Nitrate-Depleted Beetroot Juice Placebo. *Nitric Oxide - Biology and Chemistry* [Online], 40, pp. 67–74.
- Girouard, H. & Iadecola, C. (2006) Neurovascular Coupling in the Normal Brain and in Hypertension, Stroke, and Alzheimer Disease. *J Appl Physiol*, 100, pp. 328–335.
- Goldberg, T. E., Harvey, P. D., Wesnes, K. A., Snyder, P. J. & Schneider, L. S. (2015) Practice Effects Due to Serial Cognitive Assessment: Implications for Preclinical Alzheimer's Disease Randomized Controlled Trials. *Alzheimer's and Dementia: Diagnosis, Assessment and Disease Monitoring* [Online], 1, pp. 103–111.
- González, C. A., Pera, G., Agudo, A., Bueno-De-Mesquita, H. B., Ceroti, M., Boeing, H., Schulz, M., Giudice, G. Del, Plebani, M., Carneiro, F., Berrino, F., Sacerdote,

- C., Tumino, R., Panico, S., Berglund, G., Simán, H., Hallmans, G., Stenling, R., Martinez, C., Dorronsoro, M., Barricarte, A., Navarro, C., Quiros, J. R., Allen, N., Key, T. J., Bingham, S., Day, N. E., Linseisen, J., Nagel, G., Overvad, K., Jensen, M. K., Olsen, A., Tjønneland, A., Büchner, F. L., Peeters, P. H. M., Numans, M. E., Clavel-Chapelon, F., Boutron-Ruault, M. C., Roukos, D., Trichopoulou, A., Psaltopoulou, T., Lund, E., Casagrande, C., Slimani, N., Jenab, M. & Riboli, E. (2006) Fruit and Vegetable Intake and the Risk of Stomach and Oesophagus Adenocarcinoma in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST). *International Journal of Cancer*, 118 (10), pp. 2559–2566.
- Goodrich, J. K., Rienzi, S. C. Di, Poole, A. C., Koren, O., Walters, W. A., Caporaso, J. G., Knight, R. & Ley, R. E. (2014) Conducting a Microbiome Study. *Cell* [Online], 158 (2), pp. 250–262.
- Govoni, M., Jansson, E. A., Weitzberg, E. & Lundberg, J. O. (2008) The Increase in Plasma Nitrite after a Dietary Nitrate Load Is Markedly Attenuated by an Antibacterial Mouthwash. *Nitric Oxide - Biology and Chemistry* [Online], 19 (4), pp. 333–337.
- Guo, F. H., Comhair, S. A. A., Zheng, S., Dweik, R. A., Eissa, N. T., Thomassen, M. J., Calhoun, W. & Erzurum, S. C. (2000) Molecular Mechanisms of Increased Nitric Oxide (NO) in Asthma: Evidence for Transcriptional and Post-Translational Regulation of NO Synthesis. *The Journal of Immunology* [Online], 164 (11), pp. 5970–5980.
- Gupta, A., Butts, B., Kwei, K., Dvorakova, K., Stratton, S., Briehl, M. & Bowden, G. (2001) Attenuation of Catalase Activity in the Malignant Phenotype Plays a Functional Role in an in Vitro Model for Tumor Progression. *Cancer Letters*, 173 (2), pp. 115–125.
- Gutterman, D. D., Chabowski, D. S., Kadlec, A. O., Durand, M. J., Freed, J. K., Ait-Aissa, K. & Beyer, A. M. (2016) The Human Microcirculation: Regulation of Flow and Beyond. *Circulation Research*, 118 (1), pp. 157–172.
- Haber, G., Heaton, K., Murphy, D. & Burroughs, L. (1977) Depletion and Disruption of Dietary Fibre: Effects on Satiety, Plasma-Glucose, and Serum-Insulin. *Lancet*, 2 (8040), pp. 679–682.
- Hairi, N., Cumming, R., Naganathan, V., Handelsman, D., Couteur, D. Le, Creasey, H., Waite, L., Seibel, M. & Sambrook, P. (2010) Loss of Muscle Strength, Mass

- (Sarcopenia), and Quality (Specific Force) and Its Relationship with Functional Limitation and Physical Disability: The Concord Health and Ageing in Men Project. *The American Geriatrics Society*, 58, pp. 2055–2062.
- Hall, M. W., Singh, N., Ng, K. F., Lam, D. K., Goldberg, M. B., Tenenbaum, H. C., Neufeld, J. D., Beiko, R. G. & Senadheera, D. B. (2017) Inter-Personal Diversity and Temporal Dynamics of Dental, Tongue, and Salivary Microbiota in the Healthy Oral Cavity. *npj Biofilms and Microbiomes* [Online], 3 (1), pp. 0–1.
- Hallberg, L., Brune, M. & Rossander-Hulthen, L. (1989) Is There a Physiological Role of Vitamin C in Iron Absorption? *Annals of the New York Academy of Sciences*, 498 (1), pp. 324–332.
- Harman, D. (1956) Aging: A Theory Based on Free Radical and Radiation Chemistry. *Journals of Gerontology*, 11, pp. 298–300.
- Hasler, C. M. (2002) Functional Foods: Benefits, Concerns and Challenges-a Position Paper from the American Council on Science and Health. *The Journal of nutrition* [Online], 132 (12), pp. 3772–3781.
- Heerdt, B. G., Houston, H. & Augenlicht, L. H. (1997) Fatty Acid-Initiated Apoptosis Cell Cycle Arrest and Cells Is Linked to Mitochondrial Function. *Cell growth & differentiation*, 8 (May), pp. 523–532.
- Heijtz, R. D., Wang, S., Anuar, F., Qian, Y., Bjorkholm, B., Samuelsson, A., Hibberd, M. L., Forssberg, H. & Pettersson, S. (2011) Normal Gut Microbiota Modulates Brain Development and Behavior. *Proceedings of the National Academy of Sciences* [Online], 108 (7), pp. 3047–3052.
- Heiss, C., Dejam, A. & Kleinbongard, P. (2003) Vascular Effects of Cocoa Rich in Flavan-3-Ols. *Jama*, 290 (8), pp. 1030–1031.
- Hinojosa, E., Boyd, A. R. & Orihuela, C. J. (2009) Age-Associated Inflammation and Toll-Like Receptor Dysfunction Prime the Lungs for Pneumococcal Pneumonia. *The Journal of Infectious Diseases*, 200 (4), pp. 546–554.
- 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 (11), pp. 2066–2074.
- Holland, C., Smith, E., Csapo, I., Gurol, M., Brylka, D., Killiany, R., Blacker, D., Albert, M., Guttman, C. & Greenberg, S. (2008) Spatial Distribution of White-Matter Hyperintensities in Alzheimer Disease, Cerebral Amyloid Angiopathy, and

- Healthy Aging. *Stroke*, 39 (4), pp. 1127–1133.
- 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, pp. 1–10.
- Houghton, D., Stewart, C. J., Stamp, C., Nelson, A., Aj Ami, N. J., Petrosino, J. F., Wipat, A., Trenell, M. I., Turnbull, D. M. & Greaves, L. C. (2018) Impact of Age-Related Mitochondrial Dysfunction and Exercise on Intestinal Microbiota Composition. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 73 (5), pp. 571–578.
- Howdon, D. & Rice, N. (2018) Health Care Expenditures, Age, Proximity to Death and Morbidity: Implications for an Ageing Population. *Journal of Health Economics*, 57, pp. 60–74.
- Hughes, W. E., Ueda, K., Treichler, D. P. & Casey, D. P. (2016) Effects of Acute Dietary Nitrate Supplementation on Aortic Blood Pressure and Aortic Augmentation Index in Young and Older Adults. *Nitric Oxide - Biology and Chemistry* [Online], 59, pp. 21–27.
- Ignarro, L. J. (2002) Nitric Oxide as a Unique Signaling Molecule in the Vascular System: A Historical Overview. *Journal of Physiology and Pharmacology*, 53 (4 I), pp. 503–514.
- Imms, F. J., Lee, W. Sen & Ludlow, P. G. (1988) Reactive Hyperaemia in the Human Forearm. *Quarterly Journal of Experimental Physiology*, 73 (2), pp. 203–215.
- Inaba, Y., Chen, J. A. & Bergmann, S. R. (2010) Prediction of Future Cardiovascular Outcomes by Flow-Mediated Vasodilatation of Brachial Artery: A Meta-Analysis. *International Journal of Cardiovascular Imaging*, 26 (6), pp. 631–640.
- Jackson, J. K., Patterson, A. J., MacDonald-Wicks, L. K., Oldmeadow, C. & McEvoy, M. A. (2018) The Role of Inorganic Nitrate and Nitrite in Cardiovascular Disease Risk Factors: A Systematic Review and Meta-Analysis of Human Evidence. *Nutrition Reviews*, 76 (5), pp. 348–371.
- 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* [Online], 34 (10), pp. 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 - Biology and Chemistry* [Online], 48 (3), pp. 44–50.

- Jeffery, I. B., O'Toole, P. W., Ohman, L., Claesson, M. J., Deane, J., Quigley, E. & Simren, M. (2012) An Irritable Bowel Syndrome Subtype Defined by Species-Specific Alterations in Faecal Microbiota. *Gut*, 61 (7), pp. 997–1006.
- Jiang, W., Wu, N., Wang, X., Chi, Y., Zhang, Y., Qiu, X., Hu, Y., Li, J. & Liu, Y. (2015) Dysbiosis Gut Microbiota Associated with Inflammation and Impaired Mucosal Immune Function in Intestine of Humans with Non-Alcoholic Fatty Liver Disease. *Scientific Reports*, 5, pp. 1–7.
- Jin, K. (2010) Modern Biological Theories of Aging. *Aging and Disease*, 1 (2), pp. 72–74.
- Jobgen, W.S., Jobgen, S.C., Li, H., Meininger, C.J., Wu, G. (2007). Analysis of nitrite and nitrate in biological samples using high-performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci*, 851 (1), pp. 71-82.
- Jonvik, K. L., Nyakayiru, J., Pinckaers, P. J., Senden, J. M., Loon, L. J. van & Verdijk, L. B. (2016) Nitrate-Rich Vegetables Increase Plasma Nitrate and Nitrite Concentrations and Lower Blood Pressure in Healthy Adults. *The Journal of nutrition*, 146 (5), pp. 986–993.
- Joseph, J. A., Denisova, N., Fisher, D., Bickford, P., Prior, R. & Cao, G. (1998) Age-Related Neurodegeneration and Oxidative Stress: Putative Nutritional Intervention. *Neurol Clin*, 16 (3), pp. 747–755.
- Joshipura, K. J., Ascherio, A., Manson, J., Stampfer, M., Rimm, E., Speizer, F., Hennekens, C., Spiegelman, D. & Willett, W. (1999) Fruit and Vegetable Intake in Relation to Risk of Ischemic Stroke. *Jama* [Online], 282 (13), pp. 1233–1239.
- Justice, J. N., Gioscia-Ryan, R. A., Johnson, L. C., Battson, M. L., Picciotto, N. E. de, Beck, H. J., Jiang, H., Sindler, A. L., Bryan, N. S., Enoka, R. M. & Seals, D. R. (2015) Sodium Nitrite Supplementation Improves Motor Function and Skeletal Muscle Inflammatory Profile in Old Male Mice. *Journal of Applied Physiology* [Online], 118 (2), pp. 163–169.
- Justice, J. N., Johnson, L. C., DeVan, A. E., Cruickshank-Quinn, C., Reisdorph, N., Bassett, C. J., Evans, T. D., Brooks, F. A., Bryan, N. S., Chonchol, M. B., Giordano, T., McQueen, M. B. & Seals, D. R. (2015) Improved Motor and Cognitive Performance with Sodium Nitrite Supplementation Is Related to Small Metabolite Signatures: A Pilot Trial in Middle-Aged and Older Adults. *Aging*, 7 (11), pp. 1004–1021.
- Kaminsky, L. A. & Montoye, A. H. K. (2014) Physical Activity and Health: What Is the

- Best Dose? *Journal of the American Heart Association*, 3 (5), pp. 5–8.
- Kang, J. H., Ascherio, A. & Grodstein, F. (2005) Fruit and Vegetable Consumption and Cognitive Decline in Aging Women. *Ann Neurol*, 57, pp. 713–720.
- Kannel, W. B. (1996) Blood Pressure as a Cardiovascular Risk Factor. *JAMA*, 275 (20), pp. 1571–1576.
- Kanner, J., Harel, S. & Granit, R. (2001) Betalains - A New Class of Dietary Cationized Antioxidants. *Journal of Agricultural and Food Chemistry*, 49 (11), pp. 5178–5185.
- 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 (2), pp. 320–327.
- Kapil, V., Milsom, A. B., Okorie, M., Maleki-Toyserkani, S., Akram, F., Rehman, F., Arghandawi, S., Pearl, V., Benjamin, N., Loukogeorgakis, S., MacAllister, R., Hobbs, A. J., Webb, A. J. & Ahluwalia, A. (2010) Inorganic Nitrate Supplementation Lowers Blood Pressure in Humans: Role for Nitrite-Derived NO. *Hypertension*, 56 (2), pp. 274–281.
- Kapil, V., Rathod, K. S., Khambata, R. S., Bahra, M., Velmurugan, S., Purba, A., S. Watson, D., 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 (April), pp. 113–121.
- Karlsson, F., Tremaroli, V., Nielsen, J. & Bäckhed, F. (2013) Assessing the Human Gut Microbiota in Metabolic Diseases. *Diabetes*, 62 (10), pp. 3341–3349.
- Kazimierczak, R., Hallmann, E., Lipowski, J., Drela, N., Kowalik, A., Püssa, T., Matt, D., Luik, A., Gozdowski, D. & Rembiałkowska, E. (2014) Beetroot (*Beta Vulgaris* L.) and Naturally Fermented Beetroot Juices from Organic and Conventional Production: Metabolomics, Antioxidant Levels and Anticancer Activity. *Journal of the Science of Food and Agriculture*, 94 (13), pp. 2618–2629.
- Keane, K. M., George, T. W., Constantinou, C. L., Brown, M. A., Clifford, T. & Howatson, G. (2016) Effects of Montmorency Tart Cherry (*Prunus Cerasus* L.) Consumption on Vascular Function in Men with Early Hypertension. *American Journal of Clinical Nutrition*, 103, pp. 1531–1539.
- Kelly, J., Fulford, J., Vanhatalo, A., Blackwell, J. R., French, O., Bailey, S. J., Gilchrist, M., Winyard, P. G. & Jones, A. M. (2013) Effects of Short-Term Dietary

- Nitrate Supplementation on Blood Pressure, O₂ Uptake Kinetics, and Muscle and Cognitive Function in Older Adults. *AJP: Regulatory, Integrative and Comparative Physiology* [Online], 304 (2), pp. R73–R83.
- Kelm, M. & Schrader, J. (1990) Control of Coronary Vascular Tone by Nitric Oxide. *Circulation Research*, 66 (6), pp. 1561–1575.
- Kendall, C. W. ., Esfahani, A. & Jenkins, D. J. (2010) The Link between Dietary Fibre and Human Health. *Food Hydrocolloids* [Online], 24, pp. 42–48.
- Kenjale, A. A., Ham, K. L., Stabler, T., Robbins, J. L., Johnson, J. L., VanBruggen, M., Privette, G., Yim, E., Kraus, W. E. & Allen, J. D. (2011) Dietary Nitrate Supplementation Enhances Exercise Performance in Peripheral Arterial Disease. *Journal of Applied Physiology* [Online], 110 (6), pp. 1582–1591.
- Kent, K., Charlton, K., Roodenrys, S., Batterham, M., Potter, J., Traynor, V., Gilbert, H., Morgan, O. & Richards, R. (2017) Consumption of Anthocyanin-Rich Cherry Juice for 12 Weeks Improves Memory and Cognition in Older Adults with Mild-to-Moderate Dementia. *European Journal of Nutrition*, 56 (1), pp. 333–341.
- Kharitonov, S., Chung, K., Evans, D., O'Connor, B. & Barnes, P. (1996) Increased Exhaled Nitric Oxide in Asthma Is Mainly Derived From the Lower Respiratory Tract. *American Journal of Respiratory and Critical Care Medicine*, 153 (6), pp. 1773–1780.
- Kingston, A., Collerton, J., Davies, K., Bond, J., Robinson, L., Jagger, C. (2012) Losing the ability in activities of daily living in the oldest old: a hierarchic disability scale from the Newcastle 85+ Study. *PLoS One*, 7 (2).
- Kodukula, K., Faller, D. V., Harpp, D. N., Kanara, I., Pernokas, J., Pernokas, M., Powers, W. R., Soukos, N. S., Steliou, K. & Moos, W. H. (2017) Gut Microbiota and Salivary Diagnostics: The Mouth Is Salivating to Tell Us Something. *BioResearch Open Access* [Online], 6 (1), pp. 123–132.
- Korpela, K., Flint, H. J., Johnstone, A. M., Lappi, J., Poutanen, K., Dewulf, E., Delzenne, N., Vos, W. M. De & Salonen, A. (2014) Gut Microbiota Signatures Predict Host and Microbiota Responses to Dietary Interventions in Obese Individuals. *PLoS ONE*, 9 (3).
- Krikorian, R., Shidler, M. D., Nash, T. A., Kalt, W., Vinqvist-Tymchuk, M. R., Shukitt-Hale, B. & Joseph, J. A. (2010) Blueberry Supplementation Improves Memory in Older Adults. *Journal of Agricultural and Food Chemistry*, 58 (7), pp. 3996–4000.
- Kujala, T. S., Vienola, M. S., Klika, K. D., Loponen, J. M. & Pihlaja, K. (2002) Betalain

- and Phenolic Compositions of Four Beetroot (*Beta Vulgaris*) Cultivars. *European Food Research and Technology*, 214 (6), pp. 505–510.
- Kung, C. & Luscher, T. (1995) Different Mechanisms of Endothelial Dysfunction with Aging and Hypertension in Rat Aorta. *Hypertension*, 25 (2), pp. 194–200.
- Kunz, J. (2000) Initial Lesions of Vascular Aging Disease (Arteriosclerosis). *Gerontology*, 46 (6), pp. 295–299.
- la Fuente, M. De, Hernanz, A., Guayerbas, N., Alvarez, P. & Alvarado, C. (2004) Changes with Age in Peritoneal Macrophage Functions. Implication Ofleukocytes in the Oxidative Stress of Senescence. *Cellular and Molecular Biology*, 50, pp. 83–90.
- Lansley, K. E., Winyard, P. G., Fulford, J., Vanhatalo, A., Bailey, S. J., Blackwell, J. R., DiMenna, F. J., Gilchrist, M., Benjamin, N. & Jones, A. M. (2011) Dietary Nitrate Supplementation Reduces the O₂ Cost of Walking and Running: A Placebo-Controlled Study. *Journal of Applied Physiology* [Online], 110 (3), pp. 591–600.
- Lanza, G. & Crea, F. (2010) Primary Coronary Microvascular Dysfunction: Clinical Presentation, Pathophysiology, and Management. *Circulation*, 121 (21), pp. 2317–2325.
- Lara, J., Ashor, A. W., Oggioni, C., Ahluwalia, A., Mathers, J. C. & Siervo, M. (2016) Effects of Inorganic Nitrate and Beetroot Supplementation on Endothelial Function: A Systematic Review and Meta-Analysis. *European Journal of Nutrition*, 55 (2), pp. 451–459.
- LaRoche, D. P., Greenleaf, B. L., Croce, R. V. & McGaughy, J. A. (2014) Interaction of Age, Cognitive Function, and Gait Performance in 50-80-Year-Olds. *Age*, 36 (4).
- Larsen, F. J., Ekblom, B., Sahlin, K., Lundberg, J. O. & Weitzberg, E. (2006) Effects of Dietary Nitrate on Blood Pressure in Healthy Volunteers. *The New England journal of medicine* [Online], 355 (26), pp. 2792–2793.
- Lauer, T., Preik, M., Rassaf, T., Strauer, B. E., Deussen, A., Feelisch, M. & Kelm, M. (2001) Plasma Nitrite Rather than Nitrate Reflects Regional Endothelial Nitric Oxide Synthase Activity but Lacks Intrinsic Vasodilator Action. *Proc Natl Acad Sci U S A*, 98 (23), pp. 12814–12819.
- Lee, E. J., An, D., Nguyen, C. T. T., Patil, B. S., Kim, J. & Yoo, K. S. (2014) Betalain and Betaine Composition of Greenhouse- or Field-Produced Beetroot (*Beta*

- Vulgaris L.) and Inhibition of HepG2 Cell Proliferation. *Journal of Agricultural and Food Chemistry*, 62 (6), pp. 1324–1331.
- Legrand, D., Adriaensen, W., Vaes, B., Matheï, C., Wallemacq, P. & Degryse, J. (2013) The Relationship between Grip Strength and Muscle Mass (MM), Inflammatory Biomarkers and Physical Performance in Community-Dwelling Very Old Persons. *Archives of Gerontology and Geriatrics*, 57 (3), pp. 345–351.
- Leung, K. & Thuret, S. (2015) Gut Microbiota: A Modulator of Brain Plasticity and Cognitive Function in Ageing. *Healthcare* [Online], 3 (4), pp. 898–916.
- Li, J., O, W., Li, W., Jiang, Z.-G. & Ghanbari, H. (2013) Oxidative Stress and Neurodegenerative Disorders. *International Journal of Molecular Sciences* [Online], 14 (12), pp. 24438–24475.
- 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 (3), pp. 677–696.
- Loef, M. & Walach, H. (2012) Fruit, Vegetables and Prevention of Cognitive Decline or Dementia: A Systematic Review of Cohort Studies. *Journal of Nutrition, Health and Aging*, 16 (7), pp. 626–630.
- Luciana, M. (2003) Practitioner Review: Computerized Assessment of Neuropsychological Function in Children: Clinical and Research Applications of the Cambridge Neuropsychological Testing Automated Battery (CANTAB). *Journal of Child Psychology and Psychiatry and Allied Disciplines*, 44 (5), pp. 649–663.
- Lundberg, J. O., Feelisch, M., Björne, H., Jansson, E. Å. & Weitzberg, E. (2006) Cardioprotective Effects of Vegetables: Is Nitrate the Answer? *Nitric Oxide - Biology and Chemistry*, 15 (4), pp. 359–362.
- Lundberg, J. O. & Govoni, M. (2004) Inorganic Nitrate Is a Possible Source for Systemic Generation of Nitric Oxide. *Free Radical Biology and Medicine*, 37 (3), pp. 395–400.
- Lundberg, J. O. N., Weitzberg, E., Lundberg, J. M. & Alving, K. (1994) Intragastric Nitric Oxide Production in Humans: Measurements in Expelled Air. *Gut*, 35 (11), pp. 1543–1546.
- Lundberg, J. O., Weitzberg, E., Cole, J. A. & Benjamin, N. (2004) Nitrate, Bacteria and Human Health. *Nature reviews. Microbiology*, 2 (7), pp. 593–602.
- Lyons, D., Roy, S., Patel, M., Benjamin, N. & Swift, C. G. (1997) Impaired Nitric

- Oxide-Mediated Vasodilatation and Total Body Nitric Oxide Production in Healthy Old Age. *Clinical Science* [Online], 93 (6), pp. 519–525.
- Manassaram, D. M., Backer, L. C., Messing, R., Fleming, L. E., Luke, B. & Monteilh, C. P. (2010) Nitrates in Drinking Water and Methemoglobin Levels in Pregnancy: A Longitudinal Study. *Environmental Health: A Global Access Science Source*, 9 (1), pp. 1–12.
- Marcell, T. J. (2003) Sarcopenia : Causes , Consequences , and Preventions. *Journal of Gerontology*, 58 (10), pp. 911–916.
- Marlett, J. A., McBurney, M. I. & Slavin, J. L. (2002) Health Implications of Dietary Fiber [Online]. vol. 102. pp. 993–1000.
- Marshall, R.S., Lazar, R.M., Pile-Spellman, J., Young, W.L., Duong, D.H., Joshi, S., Ostapkovich, N. (2001) Recovery of brain function during induced cerebral hypoperfusion. *Brain*, 124 (6), pp. 1208-1217.
- Mathiowetz, V. (1984) Grip and Pinch Strength: Normative Data for Adults.
- 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 - Biology and Chemistry*, 64, pp. 61–67.
- McKnight, G. M., Smith, L. M., Drummond, R. S., Duncan, C. W., Golden, M. & Benjamin, N. (1997) Chemical Synthesis of Nitric Oxide in the Stomach from Dietary Nitrate in Humans. *Gut*, 40 (2), pp. 211–214.
- McRae, M. P. (2013) Betaine Supplementation Decreases Plasma Homocysteine in Healthy Adult Participants: A Meta-Analysis. *Journal of Chiropractic Medicine* [Online], 12 (1), pp. 20–25.
- Mellion, T. B., Ignarro, L. J., Ohlstein, E. H., Pontecorvo, E. G., Hyman, A. L. & Kadowitz, P. J. (1981) Evidence for the Inhibitory Role of Guanosine 3',5'-Monophosphate in ADP-Induced Human Platelet Aggregation in the Presence of Nitric Oxide and Related Vasodilators. *Blood*, 57 (3), pp. 946–955.
- Miller, G. D., Marsh, A. P., Dove, R. W., Beavers, D., Presley, T., Helms, C., Bechtold, E., King, S. B. & Kim-Shapiro, D. (2012) Plasma Nitrate and Nitrite Are Increased by a High-Nitrate Supplement but Not by High-Nitrate Foods in Older Adults. *Nutrition Research*, 32 (3), pp. 160–168.
- Minson, C.T., Woong, B.J. (2004). Reactive hyperemia as a test of endothelial or microvascular function. *Journal of the American College of Cardiology*, 43 (11).

- Mitek, M., Anyzewska, A. & Wawrzyniak, A. (2013) Estimated Dietary Intakes of Nitrates in Vegetarians Compared to a Traditional Diet in Poland and Acceptable Daily Intakes: Is There a Risk? *Roczniki Panstwowego Zakladu Higieny* [Online], 64 (2), pp. 105–109.
- Modin, A., Björne, H., Herulf, M., Alving, K., Weitzberg, E. & Lundberg, J. O. N. (2001) Nitrite-Derived Nitric Oxide: A Possible Mediator of ‘acidic-Metabolic’ Vasodilation. *Acta Physiologica Scandinavica*, 171 (1), pp. 9–16.
- Moncada, S. & Higgs, A. (1993) The L-Arginine-Nitric Oxide Pathway. *N Engl J Med*, 329 (27), pp. 2002–2012.
- Moorcroft, M.J., Davis, J., Compton, R.G. (2001). Detection and determination of nitrate and nitrite: a review. *Talanta*, 54 (5), pp 785-803.
- Moore, V. C. (2012) Spirometry: Step by Step. *Breathe*, 8 (3), pp. 233–240.
- Morgado, M., Oliveira, G. V. De, Vasconcellos, J., Monteiro, M. L., Conte-Junior, C., Pierucci, A. P. T. R. & Alvares, T. S. (2016) Development of a Beetroot-Based Nutritional Gel Containing High Content of Bioaccessible Dietary Nitrate and Antioxidants. *International Journal of Food Sciences and Nutrition*, 67 (2), pp. 153–160.
- Morgan, X. C. & Huttenhower, C. (2012) Human Microbiome Analysis. *PLoS Computational Biology*, 8 (12).
- Morris, M. C., Evans, D. A., Tangney, C. C., Bienias, J. L. & Wilson, R. S. (2006) Associations of Vegetable and Fruit Consumption with Age-Related Cognitive Change. *Neurology*, 67 (8), pp. 1370–1376.
- Mosconi, L. (2013) Glucose Metabolism in Normal Aging and Alzheimer’s Disease: Methodological and Physiological Considerations for PET Studies. *Clinical and Translational Imaging*, 1 (4).
- Nagababu, E. & Rifkind, J. M. (2007) Measurement of Plasma Nitrite by Chemiluminescence without Interference of S-, N-Nitroso and Nitrated Species. *Free Radical Biology and Medicine*.
- Natarajan, N. & Pluznick, J. L. (2014) From Microbe to Man: The Role of Microbial Short Chain Fatty Acid Metabolites in Host Cell Biology. *AJP: Cell Physiology* [Online], 307 (11), pp. C979–C985.
- Nathan, C. & Xie, Q. wen (1994) Nitric Oxide Synthases: Roles, Tolls, and Controls. *Cell*, 78 (6), pp. 915–918.
- Navaratnarajah, A. & Jackson, S. (2017) The Physiology of Ageing. *Medicine in*

Older Adults, 45 (1), pp. 6–10.

- Netzel, M., Stintzing, F. C., Quaas, D., Straß, G., Carle, R., Bitsch, R., Bitsch, I. & Frank, T. (2005) Renal Excretion of Antioxidative Constituents from Red Beet in Humans. In: *Food Research International*, 2005.
- Newman, A. B., Arnold, A. M., Naydeck, B. L., Fried, L. P., Burke, G. L., Enright, P., Gottdiener, J., Hirsch, C. & Leary, D. O. (2003) “Successful Aging”. *Archives of Internal Medicine*, 163, pp. 2315–2322.
- NICE (2011) *Hypertension in Adults: Diagnosis and Management* [Online].
- Ninfali, P. & Angelino, D. (2013) Nutritional and Functional Potential of Beta Vulgaris Cicla and Rubra. *Fitoterapia* [Online], 89 (1), pp. 188–199.
- Noble, E. E., Hsu, T. M. & Kanoski, S. E. (2017) Gut to Brain Dysbiosis: Mechanisms Linking Western Diet Consumption, the Microbiome, and Cognitive Impairment. *Frontiers in Behavioral Neuroscience* [Online], 11.
- Norman, K., Stobäus, N., Gonzalez, M. C., Schulzke, J. D. & Pirlich, M. (2011) Hand Grip Strength: Outcome Predictor and Marker of Nutritional Status. *Clinical Nutrition* [Online], 30 (2), pp. 135–142.
- O’Sullivan, M., Jones, D., Summers, P., Morris, R., Williams, S. & Markus, H. (2001) Evidence for Cortical “Disconnection” as a Mechanism of Age-Related Cognitive Decline. *Neurology*, 57 (4), pp. 632–638.
- O’Toole, P. W. & Claesson, M. J. (2010) Gut Microbiota: Changes throughout the Lifespan from Infancy to Elderly. *International Dairy Journal* [Online], 20 (4), pp. 281–291.
- Oggioni, C., Jakovljevic, D. G., Klonizakis, M., Ashor, A. W., 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* [Online], 69 (1), pp. 74–83.
- Ogra, P. L. (2010) Ageing and Its Possible Impact on Mucosal Immune Responses. *Ageing Research Reviews* [Online], 9 (2), pp. 101–106.
- Olin, A. C., Aldenbratt, A., Ekman, A., Ljungkvist, G., Jungersten, L., Alving, K. & Torén, K. (2001) Increased Nitric Oxide in Exhaled Air after Intake of a Nitrate-Rich Meal. *Respiratory Medicine*, 95 (2), pp. 153–158.
- Oliveira, G. V. de, Morgado, M., Pierucci, A. P. & Alvares, T. S. (2016) A Single Dose of a Beetroot-Based Nutritional Gel Improves Endothelial Function in the Elderly

- with Cardiovascular Risk Factors. *Journal of Functional Foods* [Online], 26, pp. 301–308.
- Omar, S. A., Artime, E. & Webb, A. J. (2012) A Comparison of Organic and Inorganic Nitrates/Nitrites. *Nitric Oxide - Biology and Chemistry* [Online], 26 (4), pp. 229–240.
- ONS (2017) National Population Projections: 2016-Based Statistical Bulletin - Office for National Statistics. [Online], pp. 1–10.
- Oosterman, J., Harten, B. Van, Weinstein, H., Scheltens, P., Sergeant, J. & Scherder, E. (2008) White Matter Hyperintensities and Working Memory: An Explorative Study. *Neuropsychol Dev Cogn*, 15 (3), pp. 384–399.
- Pacher, P., Beckman, J. S. & Liaudet, L. (2007) Nitric Oxide and Peroxynitrite in Health and Disease. *Physiological Reviews*, 87 (1), pp. 315–424.
- Padayatty, S., Katz, A., Wang, Y., Eck, P. & Kwon, O. (2003) Vitamin C as an Antioxidant: Evaluation of Its Role in Disease Prevention. *Journal of the American College of Cardiology*, 22 (1), pp. 18–35.
- Palmer, R. M. J., Rees, D. D., Ashton, D. S. & Moncada, S. (1988) L-Arginine Is the Physiological Precursor for the Formation of Nitric Oxide in Endothelium-Dependent Relaxation. *Biochemical and Biophysical Research Communications*, 153 (3), pp. 1251–1256.
- Pandey, K. B. & Rizvi, S. I. (2009) Plant Polyphenols as Dietary Antioxidants in Human Health and Disease. *Oxidative Medicine and Cellular Longevity*, 2 (5), pp. 270–278.
- Pannala, A. S., Mani, A. R., Spencer, J. P. E., Skinner, V., Bruckdorfer, K. R., Moore, K. P. & Rice-Evans, C. A. (2003) The Effect of Dietary Nitrate on Salivary, Plasma, and Urinary Nitrate Metabolism in Humans. *Free Radical Biology and Medicine*, 34 (5), pp. 576–584.
- Parkar, S. G., Trower, T. M. & Stevenson, D. E. (2013) Fecal Microbial Metabolism of Polyphenols and Its Effects on Human Gut Microbiota. *Anaerobe* [Online], 23, pp. 12–19.
- Patel, R. S., Mheid, I. Al, Morris, A. A., Ahmed, Y., Kavtaradze, N., Ali, S., Dabhadkar, K., Brigham, K., Hooper, W. C., Alexander, R. W., Jones, D. P. & Quyyumi, A. A. (2011) Oxidative Stress Is Associated with Impaired Arterial Elasticity. *Atherosclerosis* [Online], 218 (1), pp. 90–95.
- Penninx, B., Ferrucci, L., Leveille, S., Rantanen, T., Pahor, M. & Guralnik, J. (2000)

- Lower Extremity Performance in Nondisabled Older Persons as a Predictor of Subsequent Hospitalization. *J Gerontol A Biol Sci Med Sci*, 55 (11), pp. M691-7.
- Percival, R. S., Challacombe, S. J. & Marsh, P. D. (1991) Age-Related Microbiological Changes in the Salivary and Plaque Microflora of Healthy Adults. *Journal of Medical Microbiology*, 35 (1), pp. 5–11.
- Peters, R. (2006) Ageing and the Brain. *Postgraduate Medical Journal*, 82, pp. 84–88.
- PHE (2017) *Salt Reduction Targets for 2017 About Public Health England*.
- Pinder, A. G., Rogers, S. C., Khalatbari, A., Ingram, T. E. & James, P. E. (2008) The Measurement of Nitric Oxide and Its Metabolites in Biological Samples by Ozone-Based Chemiluminescence [Online]. In: *Methods in Molecular Biology*. vol. 476.
- Powlson, D. S., Addiscott, T. M., Benjamin, N., Cassman, K. G., Kok, T. M. de, Grinsven, H. van, L'hirondel, J.-L., Avery, A. A. & Kessel, C. van (2008) When Does Nitrate Become a Risk for Humans? *Journal of Environment Quality* [Online], 37 (2), p. 291.
- Presley, T. D., Morgan, A. R., Bechtold, E., Clodfelter, W., Dove, R. W., Jennings, J. M., Kraft, R. A., Bruce King, S., Laurienti, P. J., Jack Rejeski, W., Burdette, J. H., Kim-Shapiro, D. B. & Miller, G. D. (2011) Acute Effect of a High Nitrate Diet on Brain Perfusion in Older Adults. *Nitric Oxide - Biology and Chemistry* [Online], 24 (1), pp. 34–42.
- Pribis, P. & Shukitt-Hale, B. (2014) Cognition: The New Frontier for Nuts and Berries. *American Journal of Clinical Nutrition*, 100 (SUPPL. 1).
- Public Health England (2015) McCance and Widdowson's Composition of Foods Integrated Dataset. [Online].
- Public Health England (2018) National Diet and Nutrition Survey: Results from Years 7 and 8 (Combined) of the Rolling Programme (2014/2015 to 2015/2016). *PHE Publications* [Online], 8.
- Qu, X. M., Wu, Z. F., Pang, B. X., Jin, L. Y., Qin, L. Z. & Wang, S. L. (2016) From Nitrate to Nitric Oxide: The Role of Salivary Glands and Oral Bacteria. *Journal of Dental Research*, 95 (13), pp. 1452–1456.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. & Glockner, F. (2013) The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools. *Nucleic Acids Research*, 41,

- pp. D5990–D596.
- Rabbitt, P., Scott, M., Thacker, N., Lowe, C., Jackson, A., Horan, M. & Pendleton, N. (2006) Losses in Gross Brain Volume and Cerebral Blood Flow Account for Age-Related Differences in Speed but Not in Fluid Intelligence. *Neuropsychology*, 20 (5), pp. 549–557.
- Raitakari, O. T. & Celermajer, D. S. (2000) Flow-Mediated Dilatation. *Br J Clin Pharmacol*, 50, pp. 397–404.
- Rajendran, P., Rengarajan, T., Thangavel, J., Nishigaki, Y., Sakthisekaran, D., Sethi, G. & Nishigaki, I. (2013) The Vascular Endothelium and Human Diseases. *International Journal of Biological Sciences*, 9 (10), pp. 1057–1069.
- Rammos, C., Hendgen-Cotta, U. B., Sobierajski, J., Bernard, A., Kelm, M. & Rassaf, T. (2014) Dietary Nitrate Reverses Vascular Dysfunction in Older Adults with Moderately Increased Cardiovascular Risk. *Journal of the American College of Cardiology* [Online], 63 (15), pp. 1584–1585.
- Ranu, H., Wilde, M. & Madden, B. (2011) Pulmonary Function Tests. *The Ulster medical journal* [Online], 80 (2), pp. 84–90.
- Raubenheimer, K., Hickey, D., Leveritt, M., Fassett, R., Munoz, J. O. D. Z., Allen, J. D., Briskey, D., Parker, T. J., Kerr, G., Peake, J. M., Pecheniuk, N. M. & Neubauer, O. (2017) Acute Effects of Nitrate-Rich Beetroot Juice on Blood Pressure, Hemostasis and Vascular Inflammation Markers in Healthy Older Adults: A Randomized, Placebo-Controlled Crossover Study. *Nutrients*, 9 (11).
- Rein, M. J., Renouf, M., Cruz-Hernandez, C., Actis-Goretta, L., Thakkar, S. K. & Silva Pinto, M. da (2013) Bioavailability of Bioactive Food Compounds: A Challenging Journey to Bioefficacy. *British Journal of Clinical Pharmacology*, 75 (3), pp. 588–602.
- Ricciardolo, F. L. M. (2004) Nitric Oxide in Health and Disease of the Respiratory System. *Physiological Reviews* [Online], 84 (3), pp. 731–765.
- Rink, S. M., Mendola, P., Mumford, S. L., Poudrier, J. K., Browne, R. W., Wactawski-Wende, J., Perkins, N. J. & Schisterman, E. F. (2013) Self-Report of Fruit and Vegetable Intake That Meets the 5 a Day Recommendation Is Associated with Reduced Levels of Oxidative Stress Biomarkers and Increased Levels of Antioxidant Defense in Premenopausal Women. *Journal of the Academy of Nutrition and Dietetics* [Online], 113 (6), pp. 776–785.
- Robinson, K., Arheart, K., Refsum, H., Brattström, L., Ueland, P., Rubba, P., Palma-

- reis, R., Meleady, R., Witteman, J., Graham, I., Robinson, K., Arheart, K., Refsum, H. & Brattstro, L. (1998) Low Circulating Folate and Vitamin B 6 Concentrations Risk Factors for Stroke , Peripheral Vascular Disease , and Coronary.
- Ruitenber, A., Heijer, T. den, Bakker, S., Swieten, J. van, Koudstaal, P., Hofman, A. & Breteler, M. (2005) Cerebral Hypoperfusion and Clinical Onset of Dementia: The Rotterdam Study. *Ann Neurol*, 57 (6), pp. 789–794.
- Rutherford, T. (2012) Population Ageing: Statistics. *House of Commons library* [Online], (February), pp. 1–8.
- Saffrey, M. J. (2014) Aging of the Mammalian Gastrointestinal Tract: A Complex Organ System. *Age*, 36 (3), pp. 1019–1032.
- Salles, N. (2007) Basic Mechanisms of the Aging Gastrointestinal Tract. *Dig Dis* [Online], 25, pp. 112–117.
- Salminen, A., Huuskonen, J., Ojala, J., Kauppinen, A., Kaarniranta, K. & Suuronen, T. (2008) Activation of Innate Immunity System during Aging: NF-KB Signaling Is the Molecular Culprit of Inflamm-Aging. *Ageing Research Reviews*, 7 (2), pp. 83–105.
- Saul, R. L., Kabir, S. H., Bruce, W. R., Archer, M. C. & Cohen, Z. (1981) Reevaluation of Nitrate and Nitrite Levels in the Human Intestine. *Cancer Research*, 41 (6), pp. 2280–2283.
- Sawicki, T., Bączek, N. & Wiczowski, W. (2016) Betalain Profile, Content and Antioxidant Capacity of Red Beetroot Dependent on the Genotype and Root Part. *Journal of Functional Foods*, 27, pp. 249–261.
- Scahill, R., Frost, C., Jenkins, R., Whitwell, J., Rossor, M. & Fox, N. (2003) A Longitudinal Study of Brain Volume Changes in Normal Aging Using Serial Registered Magnetic Resonance Imaging. *Archives of Neurology*, 60 (7), pp. 989–994.
- Scalbert, A., Manach, C., Morand, C., Rémésy, C. & Jiménez, L. (2005) Dietary Polyphenols and the Prevention of Diseases. *Critical Reviews in Food Science and Nutrition*, 45 (4), pp. 287–306.
- Schini-Kerth, V. B., Auger, C., Kim, J. H., Etienne-Selloum, N. & Chataigneau, T. (2010) Nutritional Improvement of the Endothelial Control of Vascular Tone by Polyphenols: Role of NO and EDHF. *European Journal of Physiology*, 459 (6), pp. 853–862.

- Schini-Kerth, V. B., Auger, C., Kim, J. H., Étienne-Selloum, N. & Chataigneau, T. (2010) Nutritional Improvement of the Endothelial Control of Vascular Tone by Polyphenols: Role of NO and EDHF. *Pflugers Archiv European Journal of Physiology*, 459 (6), pp. 853–862.
- Schoental, R. (1963) Induction of Tumours of Stomach in Rats and Mice by N-Nitroso- N-Alkylurethanes. *Nature*, 13 (199), p. 190.
- Scholey, A. (2018) Nutrients for Neurocognition in Health and Disease: Measures, Methodologies and Mechanisms. *Proceedings of the Nutrition Society*, 77 (1), pp. 73–83.
- Schulz, E., Anter, E. & Keaney, J. F. (2004) Oxidative Stress, Antioxidants, and Endothelial Function. *Current Medicinal Chemistry*, 11 (9), pp. 1093–1104.
- Schwartz, A., Lehmann, U., Jacobasch, G. & Blaut, M. (2002) Influence of Resistant Starch on the SCFA Production and Cell Counts of Butyrate-Producing Eubacterium Spp. in the Human Intestine. *Journal of Applied Microbiology*, 93 (1), pp. 157–162.
- Scott-Warren, V. & Maguire, S. (2017) Physiology of Ageing. *Anaesthesia and Intensive Care Medicine* [Online], 18 (1), pp. 52–54.
- Scott, K. P., Duncan, S. H. & Flint, H. J. (2008) Dietary Fibre and Gut Microbiota. *Nutrition Bulletin*, 33 (3), pp. 201–211.
- Sepúlveda, C., Palomo, I. & Fuentes, E. (2015) Primary and Secondary Haemostasis Changes Related to Aging. *Mechanisms of Ageing and Development* [Online], 150, pp. 46–54.
- Sergis, N., Jackman, C. M., Masters, A., Krimigis, S. M., Thomsen, M. F., Hamilton, D. C., Mitchell, D. G., Dougherty, M. K. & Coates, A. J. (2013) Particle and Magnetic Field Properties of the Saturnian Magnetosheath: Presence and Upstream Escape of Hot Magnetospheric Plasma. *Journal of Geophysical Research: Space Physics* [Online], 118 (4), pp. 1620–1634.
- Sessa, W. C. (2004) ENOS at a Glance. *Journal of Cell Science*, 117 (12), pp. 2427–2429.
- Shannon, O. M., Duckworth, L., Barlow, M. J., Woods, D., Lara, J., Siervo, M. & O'Hara, J. P. (2016) Dietary Nitrate Supplementation Enhances High-Intensity Running Performance in Moderate Normobaric Hypoxia, Independent of Aerobic Fitness. *Nitric Oxide - Biology and Chemistry* [Online], 59, pp. 63–70.
- Shannon, O.M., McGawley, K., Nyback, L., Duckworth, L., Barlow, M.J., Woods, D.,

- Siervo, M., O'Hara, J.P. (2017). "Beet-ing" the mountain: a review of the physiological and performance effects of dietary nitrate supplementation at simulated and terrestrial altitude. *Sports Medicine*, 47 (11), pp. 2155-2169.
- Shepherd, A. I., Wilkerson, D. P., Dobson, L., Kelly, J., Winyard, P. G., Jones, A. M., Benjamin, N., Shore, A. C. & Gilchrist, M. (2015) The Effect of Dietary Nitrate Supplementation on the Oxygen Cost of Cycling, Walking Performance and Resting Blood Pressure in Individuals with Chronic Obstructive Pulmonary Disease: A Double Blind Placebo Controlled, Randomised Control Trial. *Nitric Oxide - Biology and Chemistry* [Online], 48, pp. 31–37.
- Shiva, S. (2013) Nitrite: A Physiological Store of Nitric Oxide and Modulator of Mitochondrial Function. *Redox Biology* [Online], 1 (1), pp. 40–44.
- Shukitt-Hale, B. (1999) The Effects of Aging and Oxidative Stress on Psychomotor and Cognitive Behavior. *Age (Omaha)*, 22 (1), pp. 9–17.
- Shukitt-Hale, B., Bielinski, D. F., Lau, F. C., Willis, L. M., Carey, A. N. & Joseph, J. A. (2015) The Beneficial Effects of Berries on Cognition, Motor Behaviour and Neuronal Function in Ageing. *British Journal of Nutrition*, 114 (10), pp. 1542–1549.
- Siervo, M., Lara, J., Ogbonmwan, I. & Mathers, J. C. (2013) Inorganic Nitrate and Beetroot Juice Supplementation Reduces Blood Pressure in Adults: A Systematic Review and Meta-Analysis. *The Journal of nutrition* [Online], 143 (6), pp. 818–826.
- Siervo, M., Oggioni, C., Jakovljevic, D. G., Trenell, M., Mathers, J. C., Houghton, D., Celis-morales, C., Ashor, A. W., Ruddock, A., Ranchordas, M., Klonizakis, M. & Williams, E. A. (2016) ScienceDirect Dietary Nitrate Does Not Affect Physical Activity Outcomes in Health Older Adults in a Randomized , Crossover Trial. *Nutrition Research* [Online].
- Siervo, M., Oggioni, C., Jakovljevic, D. G., Trenell, M., Mathers, J. C., Houghton, D., Celis-Morales, C., Ashor, A. W., Ruddock, A., Ranchordas, M., Klonizakis, M. & Williams, E. A. (2016) Dietary Nitrate Does Not Affect Physical Activity Outcomes in Health Older Adults in a Randomized, Crossover Trial. *Nutrition Research* [Online], 36 (12), pp. 1361–1369.
- Siervo, M., Oggioni, C., Lara, J., Celis-Morales, C., Mathers, J. C., Battezzati, A., Leone, A., Tagliabue, A., Spadafranca, A. & Bertoli, S. (2015) Age-Related Changes in Resting Energy Expenditure in Normal Weight, Overweight and

- Obese Men and Women. *Maturitas* [Online], 80 (4), pp. 406–413.
- Snyder, S. H. (1992) Nitric Oxide: First in a New Class of Neurotransmitters? *Science*, 257 (5069), pp. 494–496.
- Sobko, T., Marcus, C., Govoni, M. & Kamiya, S. (2010) Dietary Nitrate in Japanese Traditional Foods Lowers Diastolic Blood Pressure in Healthy Volunteers. *Nitric Oxide - Biology and Chemistry* [Online], 22 (2), pp. 136–140.
- Som, S., Raha, C. & Chatterjee, I. . (1983) Ascorbic Acid: A Scavenger of Superoxide Radical. *Acta Vitaminol Enzymol*, 5 (4), pp. 243–250.
- Spiegelhalder, B., Eisenbrand, G. & Preussmann, R. (1976) Influence of Dietary Nitrate on Nitrite Content of Human Saliva: Possible Relevance to in Vivo Formation of N-Nitroso Compounds. *Food Cosmet Toxicol.*, 14 (6), pp. 545–548.
- Stamler, J. S. & Meissner, G. (2001) Physiology of Nitric Oxide in Skeletal Muscle. *Physiological Reviews*, 81 (1), pp. 209–237.
- Steffen, T., Hacker, T. & Mollinger, L. (2002) Age- and Gender-Related Test Performance in Community-Dwelling Elderly People: Six-Minute Walk Test, Berg Balance Scale, Timed up and Go Test, and Gait Speeds. *Physical Therapy*, 82 (2), pp. 128–137.
- Storey, A. (2018) *Living Longer: How Our Population Is Changing and Why It Matters*.
- Sutton-Tyrrell, K., Najjar, S. S., Boudreau, R. M., Venkitachalam, L., Kupelian, V., Simonsick, E. M., Havlik, R., Lakatta, E. G., Spurgeon, H., Kritchevsky, S., Pahor, M., Bauer, D. & Newman, A. (2005) Elevated Aortic Pulse Wave Velocity, a Marker of Arterial Stiffness, Predicts Cardiovascular Events in Well-Functioning Older Adults. *Circulation*, 111 (25), pp. 3384–3390.
- Syddall, H., Cooper, C., Martin, F., Briggs, R. & Sayer, A. A. (2003) Is Grip Strength a Useful Single Marker of Frailty? *Age and Ageing*, 32 (6), pp. 650–656.
- Szabo, C. (1996) Physiological and Pathophysiological Roles of Nitric Oxide in the Central Nervous System. *Brain Research Bulletin*, 41 (3), pp. 131–141.
- Szelenyl, J. (2001) Cytokines and the Central Nervous System. *Brain Research Bulletin*, 54 (4), pp. 329–338.
- Taddei, S., Virdis, A., Ghiadoni, L., Salvetti, G., Magagna, A., Salvetti, A. & Bernini, G. (2001) Age-Related Reduction of NO Availability And. *Circulation*, pp. 274–279.
- Tagawa, T., Imaizumi, T., Endo, T. & et al (1994) Tole of Nitric Oxide in Reactive

- Hyperemia Inhuman Forearm Vessels. *Circulation*, 90, pp. 2285–2290.
- Tannenbaum, R., Wishnok, S. & Leaf, C. (1991) Inhibition of Nitrosamine by Ascorbic Acid. *The American Journal of Clinical Nutrition*, 53, p. 247S–250S.
- Taylor, D. R., Mandhane, P., Greene, J. M., Hancox, R. J., Filsell, S., McLachlan, C. R., Williamson, A. J., Cowan, J. O., Smith, A. D. & Sears, M. R. (2007) Factors Affecting Exhaled Nitric Oxide Measurements: The Effect of Sex. *Respiratory Research*, 8, pp. 1–9.
- Tesoriere, L., Gentile, C., Angileri, F., Attanzio, A., Tutone, M., Allegra, M. & Livrea, M. A. (2013) Trans-Epithelial Transport of the Betalain Pigments Indicaxanthin and Betanin across Caco-2 Cell Monolayers and Influence of Food Matrix. *European Journal of Nutrition*, 52 (3), pp. 1077–1087.
- Tesoriere, L., Tesoriere, L., Allegra, M., Butera, D. & Livrea, M. A. (2004) Absorption , Excretion , and Distribution of Dietary Antioxidant Betalains in LDLs : Potential Health Effects of Betalains in Humans Betalains in LDLs : Potential Health Effects of Betalains in Humans 1 – 3. (February 2016), pp. 941–945.
- Thijssen, D. H. J., Black, M. A., Pyke, K. E., Padilla, J., Atkinson, G., Harris, R. A., Parker, B., Widlansky, M. E., Tschakovsky, M. E. & Green, D. J. (2011) Reply to “Letter to the Editor: ‘Assessment of Flow-Mediated Dilation in Humans: A Methodological and Physiological Guideline’””. *American Journal of Physiology-Heart and Circulatory Physiology* [Online], 300 (2), pp. H713–H713.
- Thomas, F., Hehemann, J. H., Rebuffet, E., Czejek, M. & Michel, G. (2011) Environmental and Gut Bacteroidetes: The Food Connection. *Frontiers in Microbiology*, 2 (MAY), pp. 1–16.
- Tiihonen, K., Ouwehand, A. C. & Rautonen, N. (2010) Human Intestinal Microbiota and Healthy Ageing. *Ageing Research Reviews* [Online], 9 (2), pp. 107–116.
- Tilg, H. & Moschen, A. (2014) Microbiota and Diabetes: An Evolving Relationship. *Gut*, 63 (9), pp. 1513–1521.
- Ting, Y., Jiang, Y., Ho, C. T. & Huang, Q. (2014) Common Delivery Systems for Enhancing in Vivo Bioavailability and Biological Efficacy of Nutraceuticals. *Journal of Functional Foods* [Online], 7 (1), pp. 112–128.
- Toda, N., Ayajiki, K. & Okamura, T. (2009) Cerebral Blood Flow Regulation by Nitric Oxide: Recent Advances. *Pharmacological Research*, 61 (1), pp. 62–97.
- Torel, J., Cillard, J. & Cillard, P. (1986) Antioxidant Activity of Flavonoids and Reactivity with Peroxy Radical. *Phytochemistry*, 25 (2), pp. 383–385.

- Travers, J., Marsh, S., Aldington, S., Williams, M., Shirtcliffe, P., Pritchard, A., Weatherall, M. & Beasley, R. (2007) Reference Ranges for Exhaled Nitric Oxide Derived from a Random Community Survey of Adults. *Am J Respir Crit Care Med*, 176, pp. 238–242.
- Tuohy, K., Fava, F. & Viola, R. (2014) 'The Way to a Man's Heart Is through His Gut Microbiota' - Dietary pro- and Prebiotics for the Management of Cardiovascular Risk. *Proc Nutr Soc*, 73 (2), pp. 172–185.
- Umar, A. & Iqbal, M. (2007) Nitrate Accumulation in Plants, Factors Affecting the Process, and Human Health Implications. A Review. *Agronomy for Sustainable Development*, 27, pp. 45–57.
- Vandeputte, D., Falony, G., Vieira-Silva, S., Tito, R. Y., Joossens, M. & Raes, J. (2016) Stool Consistency Is Strongly Associated with Gut Microbiota Richness and Composition, Enterotypes and Bacterial Growth Rates. *Gut*, 65 (1), pp. 57–62.
- Vanhatalo, A., Bailey, S. J., Blackwell, J. R., DiMenna, F. J., Pavey, T., 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 . *Am J Physiol Regul Integr Comp Physiol*. [Online], 299 (4), pp. R1121-31.
- Vanin, A., Bevers, L., Slama-Schwok, A. & Faassen, E. van (2007) Nitric Oxide Synthase Reduces Nitrite to NO under Anoxia. *Cell Mol Life Sci*, 64 (1), pp. 96–103.
- Velmurugan, S., Gan, J. M., Rathod, K. S., Khambata, R. S., Ghosh, S. M., Hartley, A., Eijl, S. Van, Sagi-kiss, V., Chowdhury, T. A., Curtis, M., Kuhnle, G. G. C. & Wade, W. G. (2016) Dietary Nitrate Improves Vascular Function in Patients with Hypercholesterolemia : A Randomized , Double-Blind , Placebo-Controlled Study. *Am J Clin Nutr*, 103 (July), pp. 25–38.
- Velzen, A. G. van, Sips, A. J. A. M., Schothorst, R. C., Lambers, A. C. & Meulenbelt, J. (2008) The Oral Bioavailability of Nitrate from Nitrate-Rich Vegetables in Humans. *Toxicology Letters*, 181 (3), pp. 177–181.
- Vints, A. M., Oostveen, E., Eeckhaut, G., Smolders, M. & Backer, W. A. De (2005) Time-Dependent Effect of Nitrate-Rich Meals on Exhaled Nitric Oxide in Healthy Subjects. *Chest* [Online], 128 (4), pp. 2465–2470.
- Vuilleumier, P., Decosterd, D., Maillard, M., Burnier, M. & Hayoz, D. (2002)

- Postischemic Forearm Skin Reactive Hyperemia Is Related to Cardiovascular Risk Factors in a Healthy Female Population. *Journal of Hypertension*, 20 (9), pp. 1753–1757.
- Wang, J., Brown, M. A., Tam, S. H., Chan, M. C. & Whitworth, J. A. (1997) Effects of Diet on Measurement of Nitric Oxide Metabolites. *Clinical and Experimental Pharmacology and Physiology*, 24 (6), pp. 418–420.
- Wang, Y., Branicky, R., Noë, A. & Hekimi, S. (2018) Superoxide Dismutases: Dual Roles in Controlling ROS Damage and Regulating ROS Signaling. *Journal of Cell Biology*, 217 (6), pp. 1915–1928.
- Webb, A. J., Patel, N., Loukogeorgakis, S., Okorie, M., Aboud, Z., Misra, S., Rashid, R., Miall, P., Deanfield, J., Benjamin, N., MacAllister, R., Hobbs, A. J. & Ahluwalia, A. (2008) Acute Blood Pressure Lowering, Vasoprotective, and Antiplatelet Properties of Dietary Nitrate via Bioconversion to Nitrite. *Hypertension*, 51 (3), pp. 784–790.
- Weitzberg, E. & Lundberg, J. O. (2013) Novel Aspects of Dietary Nitrate and Human Health. *Annual Review of Nutrition* [Online], 33 (1), pp. 129–159.
- Weller, R., Pattullo, S., Smith, L., Golden, M., Ormerod, A. & Benjamin, N. (1996) Nitric Oxide Is Generated on the Skin Surface by Reduction of Sweat Nitrate. *Journal of Investigative Dermatology*, 107 (3), pp. 327–331.
- Werner, P. & Korczyn, A. D. (2008) Mild Cognitive Impairment: Conceptual, Assessment, Ethical, and Social Issues. *Clinical Interventions in Aging*, 3 (3), pp. 413–420.
- Wightman, E. L., Haskell-Ramsay, C. F., Thompson, K. G., Blackwell, J. R., Winyard, P. G., Forster, J., Jones, A. M. & Kennedy, D. O. (2015) Dietary Nitrate Modulates Cerebral Blood Flow Parameters and Cognitive Performance in Humans: A Double-Blind, Placebo-Controlled, Crossover Investigation. *Physiology and Behavior* [Online], 149, pp. 149–158.
- Wild, K., Howieson, D., Webbe, F., Seelye, A. & Kaye, J. (2008) The Status of Computerized Cognitive Testing in Aging: A Systematic Review. *Alzheimer's and Dementia: Diagnosis, Assessment and Disease Monitoring*, 4 (6), pp. 428–437.
- Winquist, R., Bunting, P., Baskin, E. & Wallace, A. (1984) Decreased Endothelium-Dependent Relaxation in New Zealand Genetic Hypertensive Rats. *Journal of Hypertension*, 2 (5), pp. 541–545.
- Woodmansey, E. J. (2007) Intestinal Bacteria and Ageing. *Journal of Applied*

- Microbiology*, 102, pp. 1178–1186.
- Wootton-Beard, P. C., Brandt, K., Fell, D., Warner, S. & Ryan, L. (2014) Effects of a Beetroot Juice with High Neobetanin Content on the Early-Phase Insulin Response in Healthy Volunteers. *Journal of Nutritional Science* [Online], 3, p. e9.
- Wootton-Beard, P. C. & Ryan, L. (2011) A Beetroot Juice Shot Is a Significant and Convenient Source of Bioaccessible Antioxidants. *Journal of Functional Foods* [Online], 3 (4), pp. 329–334.
- Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y., Keilbaugh, S. A., Bewtra, M., Knights, D., Walters, W. A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F. & Lewis, J. (2011) Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. 334 (October), pp. 105–109.
- Yamamoto-Suganuma, R. & Aso, Y. (2009) Relationship between Post-Occlusive Forearm Skin Reactive Hyperaemia and Vascular Disease in Patients with Type 2 Diabetes - A Novel Index for Detecting Micro- and Macrovascular Dysfunction Using Laser Doppler Flowmetry. *Diabetic Medicine*, 26, pp. 83–88.
- Yeboah, J., Crouse, J. R., Hsu, F. C., Burke, G. L. & Herrington, D. M. (2007) Brachial Flow-Mediated Dilation Predicts Incident Cardiovascular Events in Older Adults: The Cardiovascular Health Study. *Circulation*, 115 (18), pp. 2390–2397.
- Yusuf, S., Reddy, S., Ôunpuu, S. & Anand, S. (2001) Clinical Cardiology : New Frontiers Global Burden of Cardiovascular Diseases. *Clinical Cardiology*, 104, pp. 2746–2753.
- Zhang, Z., Naughton, D., Winyard, P. G., Benjamin, N., Blake, D. R. & Symons, M. C. R. (1998) Generation of Nitric Oxide by a Nitrite Reductase Activity of Xanthine Oxidase: A Potential Pathway for Nitric Oxide Formation in the Absence of Nitric Oxide Synthase Activity. *Biochemical and Biophysical Research Communications*, 249 (3), pp. 767–772.
- Zhou, K. (2017) Strategies to Promote Abundance of Akkermansia Muciniphila, an Emerging Probiotics in the Gut, Evidence from Dietary Intervention Studies. *Journal of Functional Foods* [Online], 33, pp. 194–201.
- Zielińska-Przyjemska, M., Olejnik, A., Kostrzewa, A., Łuczak, M., Jagodziński, P. P. & Baer-Dubowska, W. (2012) The Beetroot Component Betanin Modulates ROS Production, DNA Damage and Apoptosis in Human Polymorphonuclear

Neutrophils. *Phytotherapy research : PTR* [Online], 26 (6), pp. 845–852.

Appendix A: Confirmation of NHS ethical approval



Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

26 September 2016

Dr Daniel West
Institute of Cellular Medicine
Room M4.079, William Leech Building
Newcastle University
NE2 4HH

Dear Dr West,

Study title:	Bioavailability of phenolic compounds, betalain and inorganic nitrate following incremental portions of whole beetroot in older and younger adults.
REC reference:	16/EE/0376
IRAS project ID:	202902

Thank you for your letter of September 2016, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager Rebecca Morledge, NRESCcommittee.EastofEngland-CambridgeCentral@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Appendix B: Participant information sheet

Participant Information Sheet

Title of project: Bioavailability of phenolic compounds, betalain and inorganic nitrate following incremental portions of whole beetroot in older and younger adults.

You are invited to participate in this dietary intervention study. Please take time to read the following information carefully. It explains why the research is being done and what it involves. If you have any questions about the information, please just ask for further explanation. Thank you for reading this. Discuss with others if you wish and please take time to decide whether you would like to take part.

PART 1

What is the purpose of the research project?

Ageing has an effect on many systems in the body, including the ability to breakdown and utilise compounds within food. Beetroot is rich in nitrate and polyphenols, and these can have beneficial effects on the body. Younger and older adults however, may process these compounds differently and therefore their beneficial effects can be attenuated. The aim of this study is: 1) to assess the availability of compounds following consumption of beetroot or a dose of inorganic nitrate 2) to assess the effect of these compounds on vascular function

Why have I been chosen?

You have been chosen because you are healthy and of a suitable age to take part in this study. The project will involve 30 participants in total.

Do I have to take part?

Your participation is entirely voluntary and all results will be strictly anonymous. If you decide to take part you are still free to withdraw at any time without reason and without your medical care being affected. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form.

What will the research project involve?

We will contact you by phone to answer any questions you may have about the study. We will then ask you some questions about your medical history to check whether you can participate. You will not be included in the study if you have any medical conditions or are taking medications that will affect the measurements in the study. If you are a suitable participant, you will be invited

to attend the Clinical Research Facility (CRF) at the Royal Victoria Infirmary where the study will take place.

Initial screening: You will arrive at the CRF for an initial screening visit, which should take no longer than 45 minutes. We will go through the participant information sheet and answer any questions. After having had time to make a decision, you will be asked to sign a consent form stating that you would like to take part in this research study. We will go through a short medical history questionnaire, measure your height and weight to calculate your BMI, and will take a resting blood pressure measurement. These results will be needed to decide whether you can be included in the study or not. If your BMI and blood pressure readings are outside a specific range, you will not be able to take part in the study. The visit will then conclude by taking a blood sample, which will amount to about two teaspoons in volume. We will provide you with all the information and materials necessary to continue the study. You will be asked to attend the CRF on a further four occasions, separated by around seven days, for further testing. Before each of the four visits, you will be provided with an evening meal to consume the day before. You will be asked to follow a restricted diet in the two days leading up to each visit and on the day before the first visit we will ask you to collect urine samples. You will also be asked to refrain from using anti-bacterial gum and mouthwash for the duration of the study.

Study visits: You will attend the CRF in the morning and your blood pressure, blood flow and anthropometric measurements will be taken, along with a blood, urine and saliva sample. You will be given breakfast and will then be asked to consume a set amount of beetroot (100g, 200g or 300g) or will be given a dose of potassium nitrate in a solution. Over the next 5 hours, blood pressure, blood flow, and blood, urine and saliva samples will be taken at regular intervals. You will be fitted with a cannula for blood samples, which may cause slight discomfort when initially inserted but should be painless once in place. After the last sample has been taken, you will receive lunch and will then be free to leave. You will be provided with your evening meal to consume for that day and some sample pots to measure your urine and saliva over the following 12 hours. This protocol will be repeated on the following three visits. During the study you will be asked to complete a food diary, physical activity questionnaire and complete a survey to assess your enjoyment of the beetroot provided.

Measurements and samples taken

10ml of blood (about two teaspoons) will be taken at seven time points during the visit, to analyse the compounds in the blood following beetroot or potassium nitrate consumption. 70ml of blood will be taken in total for each visit. Blood samples will be taken using a cannula that will be inserted into your arm for the entirety of the visit. You will also be asked to provide urine and saliva samples at similar time points, which will involve simply depositing a sample into a designated container. Your blood pressure will be measured using an inflatable cuff similar to the one used in GP surgeries. We will measure your blood flow at the start, middle and end of the testing period,

using a non-invasive technique called post-occlusive reactive hyperaemia. This will assess whether the beetroot or potassium nitrate that you consume has any impact on the width of your blood vessels and how much blood flows through them. The final measurement will be the amount of nitric oxide that you exhale in your breath, called fractional exhaled nitric oxide (FeNO). Everyone exhales nitric oxide normally but we know that the amount you exhale can be influenced by diet and the amount of nitrate consumed. We will measure your FeNO at seven time points throughout the visit by asking you to breath into a handheld sensor.

Expenses and payments

You will receive a one-off voucher worth £100 on completion of the study, as long as you have completed all four visits, as compensation for your time. In addition, some meals will be provided the day before and day of each visit. Reasonable travel costs to and from the CRF will also be covered.

What do I have to do?

You will be asked to follow a low-nitrate and low-polyphenol diet for the two days leading up to each study visit and your evening meal will be provided on the day before each visit. A list of foods and beverages to avoid will be provided. You will also be given breakfast and lunch when attending the CRF on each day and will be provided with a meal to consume at the end of the visit day.

What data do I need to supply?

We will require your name and address or email for correspondence and for transport. Your details will not be linked to that data collected during the study. We will require your age to determine your eligibility for the study and will ask you for details of your medical history during a telephone screening call.

What are the side effects of the treatment when taking part?

Consumption of beetroot can cause beeturia, turning your urine and/or stool a red or pink colour. This is harmless and completely normal.

Are there any other possible disadvantages of taking part?

Giving up time to participate must be considered. We will be taking blood from a vein in your arm by inserting a cannula and it is possible that you might experience slight discomfort when it is being placed in the arm and/or bruising when it is taken out. You will be asked to follow a restricted diet in the run up to testing, which may be very different to your normal diet.

What are the possible benefits of taking part?

Beetroot counts towards your 5-a-day, so you will be giving your day a small health boost. Beetroot has also been found to acutely reduce blood pressure,

which has many benefits to health. You will have your height, weight, waist circumference and body fat percentage measured, which are useful to know.

What happens at the end of the research project?

At the end of the project, we will be able to inform you of the results of the study. Results will be presented with the intention to publish in scientific journals. All results will be presented as grouped data and your individual results will not be identifiable.

What if there is a problem?

If you have a concern or complaint about any aspect of the study, Prof Emma Stevenson will deal with this immediately. You can contact her on 0191 208 7865 or write to her at the address detailed below. Medical staff are always on hand in the Clinical Research Facility.

If you prefer to raise your concerns with someone not involved in your care, you can contact the Patient Advice and Liaison Service (PALS). This service is confidential and can be contacted on Freephone: 0800 032 0202

Alternatively, if you wish to make a formal complaint you can contact the Patient Relations Department through any of the details below:

Telephone: 0191 223 1382 or 0191 223 1454

Email: patient.relations@nuth.nhs.uk

Address: Patient Relations Department, The Newcastle upon Tyne Hospitals NHS Foundation Trust, Freeman Hospital, Newcastle upon Tyne, NE7 7DN.

Will my taking part in the project be kept confidential?

Yes, we value your input to this project and all data gathered from you will be treated in the strictest of confidence. Only members of the research team will have access to the data, which will be stored for 5 years, and the results will be anonymous so you cannot be directly identified. Also, although the results may be published, your data will be compiled with others and available as an average, so again you cannot be identified.

What will happen to the results of the research study?

You will not be identified in any report or publication. You will be given a copy of the results once they are published if you wish.

Who has reviewed the study?

This study has been internally reviewed by the research team at Newcastle University and ethical review of the study has been conducted by East of England – Cambridge Central Research Ethics Committee.

Who are the contacts for further information?

Tess Capper
MoveLab, 4th Floor William Leech Building
Newcastle University
NE2 4HH
0191 208 8264

Prof Emma Stevenson
Room 4.078, 4th Floor William Leech Building
Newcastle University
NE2 4HH
0191 208 7865

PART 2

What if relevant new information becomes available?

If new information is published during the course of a study, this can sometimes change how the research should go forward. If this happens, you will be notified immediately and the study will be altered accordingly.

Will anyone else know that I'm doing this?

We will inform your GP that you will be taking part in this study. Only members of the research team will have access to the data. All information that is collected during this research study will be kept strictly confidential and your name and address will be removed so that you cannot be recognised from it. Any information you give or data collected will be retained for a maximum duration of 5 years.

What will happen if I don't want to carry on with the study?

You will be able to withdraw from the study at any time. Measurements already made would still be used if you were to agree to this.

What will happen to samples taken in the study?

We will take samples of blood, urine and saliva during this study. Because we cannot analyse all of the samples straightaway, we will need to place some of the samples in storage. Samples will be taken, processed and then frozen and stored in a controlled-access freezer in the Clinical Research Facility. Each sample will be anonymised using a unique participant code and cannot be traced back to the individual. After samples have been fully analysed they will be destroyed.

What will happen to data from this study?

Personal data will be stored in medical notes created at the Clinical Research Study for use during the study. Only members of the research team will have access to this information and hard files will be stored in a secured filing

cabinet behind controlled-access doors. Personal data will be anonymised by the Chief Investigator and thereafter only codes will be used to identify participant data. Clinical data will not be released to anyone other than the research team. All study data will be analysed at Newcastle University by members of the research team using statistical software. Dr Daniel West (Chief Investigator) will act as custodian for the data generated by the study. After the study has ended, files will be stored under password protection and in locked cabinets and archived appropriately.

Who is organising and funding the research?

This project is funded by Newcastle University as a PhD studentship. The design and organisation of the study is the responsibility of Tess Capper, Prof Emma Stevenson and Dr Mario Siervo, the latter of whom is recognised as an expert in this field.

Design of this information sheet

This document is written in accordance with the requirements of the European Clinical Trials Directive 2001/20/EC, the ICH Good Clinical Practice guidelines and the UK Medicines for Human Use (Clinical Trials) Regulation 2004.

Appendix C: Power calculation, Chapters 2 and 3

The study found overall greater concentrations of plasma nitrate in younger compared to older individuals over a 3-hour sampling period and the estimated effect size f (partial η^2) for a within-between repeated-measure ANOVA was 0.03 (0.175). Using these parameters in the same ANOVA model, we calculated that 12 participants per group ($90/8=11.25$) would be needed to detect a significant difference between doses and age groups with a power of 80% and $P<0.05$. Sample size calculations were performed using G Power 3.1 for Windows.

F tests - ANOVA: Repeated measures, within-between interaction

Analysis: A priori: Compute required sample size

Input:	Effect size f	=	0.1758631
	α err prob	=	0.05
	Power ($1-\beta$ err prob)	=	0.80
	Number of groups	=	8
	Number of measurements	=	8
	Corr among rep measures	=	0.5
	Nonsphericity correction ϵ	=	0.5
	Noncentrality parameter λ	=	22.2680376
Output:	Critical F	=	1.6481176
	Numerator df	=	17.5000000
	Denominator df	=	294
	Total sample size	=	90
	Actual power	=	0.8319245

Appendix D: Nitrate intake questionnaire, chapters 2 and 3

NITRATE INTAKE QUESTIONNAIRE

We are interested in finding out about the amount of nitrate containing food that people consume as part of their everyday lives. The questions will ask you about the types and frequency of food you consume in the **last 7 days**. Think about all the **foods you ate** in the **last 7 days**. Please answer each question or ask the investigators in this study if there are any questions that you are still unsure of.

Please tick on every line.

During the last 7 days how many times did you eat the following foods ?

Food Type	Frequency	Portion Size
a. Green leafy vegetables	<input type="checkbox"/> Everyday	<input type="checkbox"/>
Small (Broccoli, cabbage, spinach, lettuce, etc.)	<input type="checkbox"/> 4 – 6 days	<input type="checkbox"/>
Medium	<input type="checkbox"/> 2 – 3 days	<input type="checkbox"/>
Large	<input type="checkbox"/> 0 – 1 day	
	Frequency	
b. Aubergine, courgette, turnip, pumpkin	<input type="checkbox"/> Everyday	<input type="checkbox"/>
Small	<input type="checkbox"/> 4 – 6 days	<input type="checkbox"/>
Medium	<input type="checkbox"/> 2 – 3 days	<input type="checkbox"/>
Large	<input type="checkbox"/> 0 – 1 day	
	Frequency	
c. Canned Products	<input type="checkbox"/> Everyday	<input type="checkbox"/>
Small (Canned corn, canned peas, canned tomatoes, etc.)	<input type="checkbox"/> 4 – 6 days	<input type="checkbox"/>
Medium	<input type="checkbox"/> 2 – 3 days	<input type="checkbox"/>
Large	<input type="checkbox"/> 0 – 1 day	
	Frequency	
d. Cured Meat and/or bacons	<input type="checkbox"/> Everyday	<input type="checkbox"/>
Small	<input type="checkbox"/> 4 – 6 days	<input type="checkbox"/>
Medium	<input type="checkbox"/> 2 – 3 days	<input type="checkbox"/>
Large	<input type="checkbox"/> 0 – 1 day	
	Frequency	
	Portion Size	

- e. Mature cheese ☐ Everyday ☐
- Small ☐ 4 – 6 days ☐
- Medium ☐ 2 – 3 days ☐
- Large ☐ 0 – 1 day ☐
- Frequency**
- Portion Size**
- f. Beet and beetroot ☐ Everyday ☐
- Small ☐ 4 – 6 days ☐
- Medium ☐ 2 – 3 days ☐
- Large ☐ 0 – 1 day ☐

Please tick every line

During the last 7 days how many times did you eat the following foods?

FOOD TYPE	FREQUENCY			
	Everyday	4 – 6 days	2 – 3 days	0 – 1 day
1. Fresh Cheese				
• Brie	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Ricotta	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Cream Cheese	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Mozzarella	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
2. Cereals				
• Potatoes	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Pasta	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Rice	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Breakfast Cereals	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Bread	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Savoury Biscuits	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3. Meat & Fish				
• Turkey	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Chicken	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Beef	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Pork	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Fish	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Other	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
4. Legumes				
• Bean	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Peas	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Chickpeas	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Lentils	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
5. Vegetables				
• Carrot	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Cucumber	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

• Pepper	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Tomato	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Mushroom	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Cauliflower	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

FOOD TYPE	FREQUENCY			
	Everyday	4 – 6 days	2 – 3 days	0 – 1 day
6. Fruits				
• Apples	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Banana	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Strawberries	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Peach	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Berries	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
7. Sweets				
• Chocolate	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Cakes	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Sweets	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Biscuits	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Ice Cream	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
8. Sauces				
• Ketchup	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Mayonnaise	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
9. Dairy Product				
• Milk	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Yogurt	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
10. Drink				
• Caffeinated	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Non – caffeinated	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Soft Drinks	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• Fruit Juice	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

THANK YOU FOR TAKING YOUR TIME TO COMPLETE THIS QUESTIONNAIRE

Appendix E: Visual analogue scale, chapters 2 and 3

The palatability of the dietary intervention – beetroot

This questionnaire is designed to assess how much you enjoyed the beetroot given to you in this visit. Please make a mark on the lines under each title according to how you felt about each aspect of the intervention.

Visual appeal

0 1 2 3 4 5 6 7 8 9 10
Dislike very much Like very much

Smell

0 1 2 3 4 5 6 7 8 9 10
Dislike very much Like very much

Taste

0 1 2 3 4 5 6 7 8 9 10
Dislike very much Like very much

Texture

0 1 2 3 4 5 6 7 8 9 10
Dislike very much Like very much

Aftertaste

0 1 2 3 4 5 6 7 8 9 10
None Much

Volume of food

0 1 2 3 4 5 6 7 8 9 10
Too much

Appendix F: Section of food diary and restricted food list

[illegible]

Dietary restrictions

The foods below are high in specific polyphenols (antioxidants) and nitrate/nitrite so we would like you to avoid them in order to ensure that any changes that we see in your samples are directly a result of the beetroot or nitrate intervention. If you come across any other foods that you know are high in nitrate or antioxidants, then please avoid these too.

Foods that should be **AVOIDED**:

- Tea, coffee, hot chocolate and alcohol (especially red wine, beer and apple cider)
- Fruit juice (especially Ribena) and soft drinks (coca cola etc.)
- Fruits (especially apples, pears, apricots, prunes, plums, cherries, peaches, blueberries and grapes)
- Vegetables (especially tomatoes, asparagus, cabbage, turnips, leek, garlic, carrots, peppers, onions, broccoli, cauliflower and beetroot)
- Salad leaves and vegetables – spinach, rocket, lettuce, chard, radishes, cucumber, spring onion, celery and Chinese cabbage
- Chocolate and chocolate products
- Wholemeal, granary and brown bread, pasta and rice
- Wholegrain cereals – Weetabix, Bran Flakes, Corn Flakes, oats, muesli
- Beans (baked beans and green beans), lentils and peas
- Curry dishes and spices (paprika, turmeric etc.)
- Herbs – parsley, dill, basil, coriander, chives etc.
- Tomato sauce (ketchup, pasta sauce)
- Cured meats (bacon, sausages, salami, ham, chorizo and hot dogs)

Foods that you may eat:

- White bread
- Butter, vegetable oil (avoid olive oil)
- White pasta, rice and noodles
- Unprocessed meat, fish and seafood
- Eggs
- Peeled white potatoes (mashed, chips, roasted)
- Mushrooms
- Digestive biscuits, rich teas, shortbread and other plain biscuits
- Milk and milk products (plain yoghurt, mild cheese)
- Pastry
- Custard and rice pudding
- Ready salted crisps
- Vanilla ice-cream
- Vanilla milkshake
- Plain scones with butter/cream (no jam)

You can drink water at all times

We are aware that it may be difficult for you to follow such a diet, as it may differ substantially from your normal diet. The reason we ask you to change your normal diet is that the foods listed above contain similar components to what we would like to test during the intervention. Therefore, if you do eat the foods above, it may interfere with the study.

Example diet

Breakfast:

- White toast with butter
- Yoghurt or milk
- Eggs on toast
- Pastries (no jam or chocolate coated)
- Waffles, pancakes
- Rice Crispies with milk

Lunch:

- Sandwich (white bread/bagel/pitta bread/wrap/roll) with any of the following fillings (no salad)
 - Chicken
 - Cheese
 - Tuna
 - Egg
 - Prawns
 - Butter
 - Mayonnaise
- Packet of crisps (plain)
- Burger and chips

Dinner:

- Anything from breakfast or lunch options
- Fish and chips (no ketchup)
- Chicken pie
- Chicken and mushrooms with rice/potato/chips/noodles
- Macaroni cheese
- Tuna/salmon/cod with rice or pasta (no vegetable-based sauce)
- Steak/pork/lamb with potatoes
- Spaghetti with mushrooms and cream sauce

Snacks:

- Digestive/rich tea/shortbread biscuits
- Ready salted crisps
- Natural yoghurt
- Toast and butter

Appendix G: Confirmation of university ethical approval



05/12/17
Tess Capper
Institute of Cellular Medicine

Faculty of Medical Sciences

Newcastle University
The Medical School
Framlington Place
Newcastle upon Tyne
NE2 4HH United Kingdom

FACULTY OF MEDICAL SCIENCES: ETHICS COMMITTEE

Dear Tess,

Title: The effect of dietary nitrate on cognitive function in older adults

Application No: 1432/411/2017

Start date to end date: 22/01/2018 to 23/07/2018

On behalf of the Faculty of Medical Sciences Ethics Committee, I am writing to confirm that the ethical aspects of your proposal have been considered and your study has been given ethical approval.

The approval is limited to this project: **1432/411/2017**. If you wish for a further approval to extend this project, please submit a re-application to the FMS Ethics Committee and this will be considered.

During the course of your research project you may find it necessary to revise your protocol. Substantial changes in methodology, or changes that impact on the interface between the researcher and the participants must be considered by the FMS Ethics Committee, prior to implementation.*

At the close of your research project, please report any adverse events that have occurred and the actions that were taken to the FMS Ethics Committee.*

Best wishes,

Yours sincerely

A handwritten signature in black ink, appearing to read "K. Sutherland".

Kimberley Sutherland

On behalf of Faculty Ethics Committee

cc.

Professor Daniel Nettle, Chair of FMS Ethics Committee

Mrs Kay Howes, Research Manager

*Please refer to the latest guidance available on the internal Newcastle web-site.

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www.ncl.ac.uk

The University of Newcastle upon Tyne trading as Newcastle University

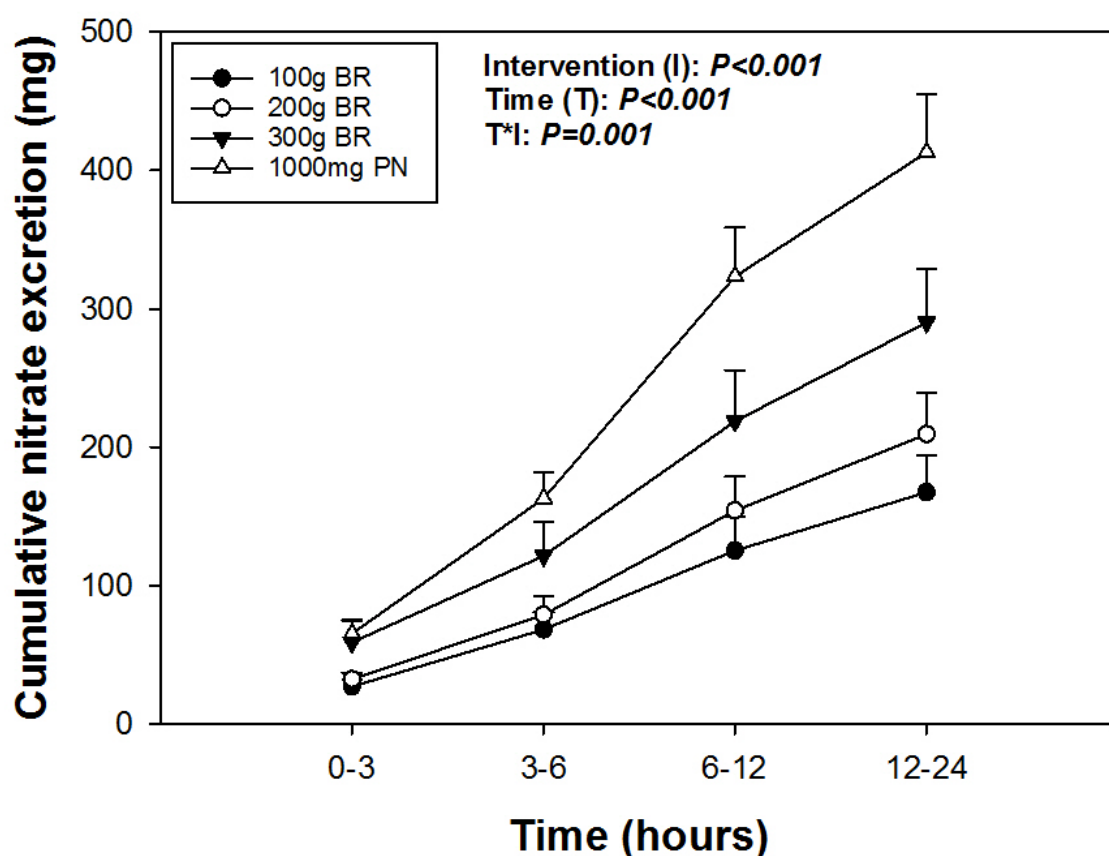


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Appendix H: Chapter 2 supplementary figures

Cumulative Excretion - young

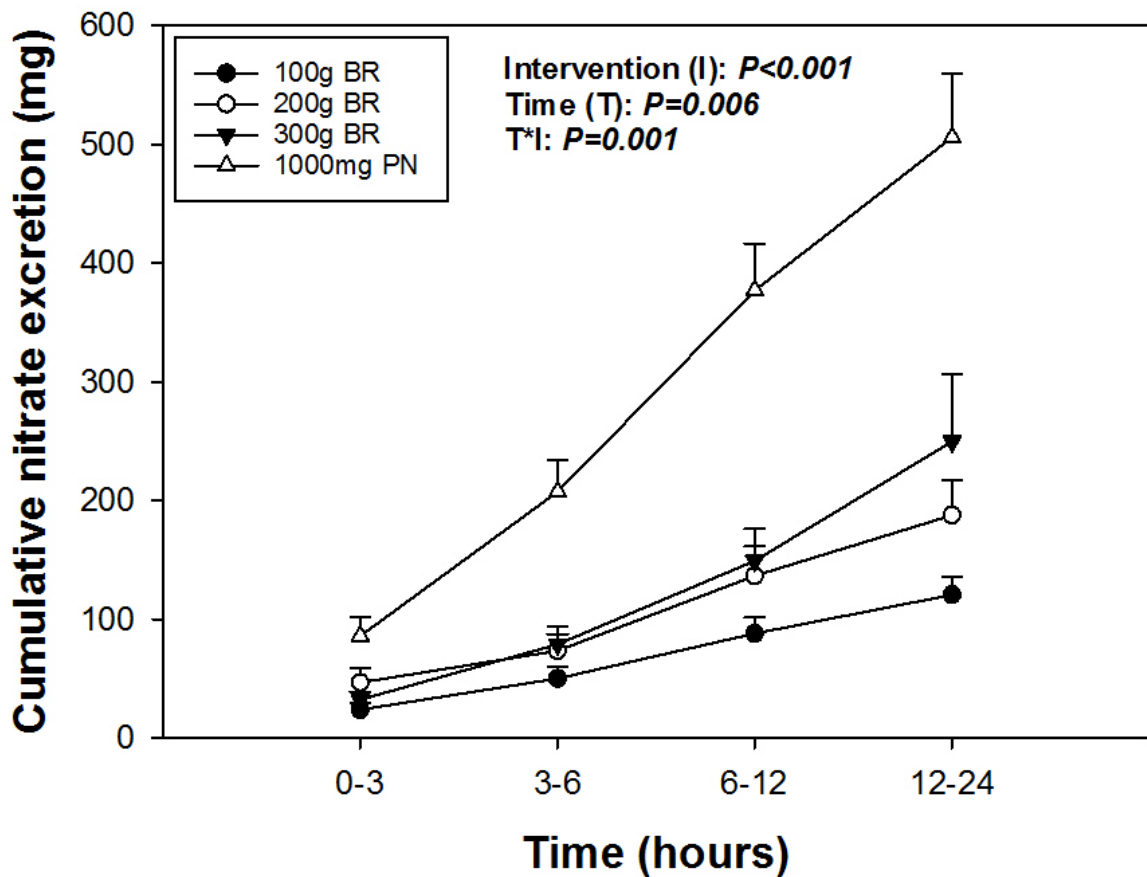
There was a significant effect of time ($P<0.001$) and intervention ($P<0.001$) on urinary nitrate excretion in the young, and an interaction between the two factors ($P=0.001$). Pairwise comparisons show that excretion was significantly higher following PN than 100g beetroot (+144.1mg; $P<0.001$; 95%CI: 80.2, 208.0) and 200g beetroot (+122.5mg; $P=0.004$; 95%CI: 39.5, 205.4). *Post hoc* analyses show that all doses increased excretion over the time period (all $P<0.05$) but cumulative excretion at 12-24hours was significantly higher following PN than 100g beetroot (+245.6mg; $P<0.001$; 95%CI: 126.1, 364.9) and 200g beetroot (+203.6mg; $P=0.01$; 95%CI: 40.5, 366.7).



Cumulative urinary nitrate excretion over 24 hours following incremental doses of nitrate in the young group. Data presented as mean \pm SEM; $n=12$. Statistical analysis using two-factor repeated measures ANOVA (intervention x time). BR = beetroot, PN = potassium nitrate positive control.

Cumulative Excretion – old

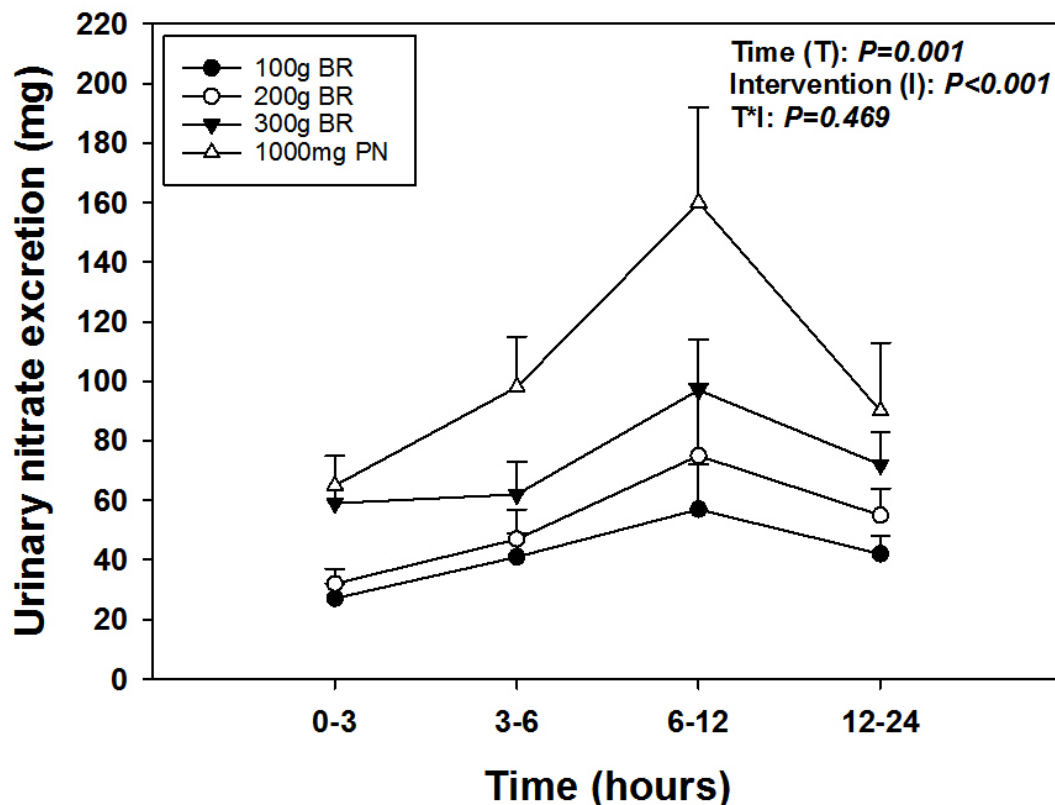
There was a significant effect of time ($P<0.001$) and intervention ($P<0.001$) on nitrate excretion in the old group, and an interaction between the two factors ($P=0.001$). Pairwise comparisons show that excretion was significantly higher following PN than following 100g beetroot (+209.1mg; $P=0.001$; 95%CI: 99.8, 318.4) and following 200g beetroot (+180.9mg; $P=0.003$; 95%CI: 68.3, 293.4), and there was a notable trend following 300g beetroot (+139.0mg; $P=0.08$; 95%CI: -13.9, 292.1). Excretion was significantly higher than baseline at each time point measured (all $P\leq0.001$). *Post hoc* analyses show that cumulative excretion at 12-24hours was significantly greater following PN than 100g beetroot (+359.1mg; $P=0.001$; 95%CI: 161.3, 556.9) and 200g beetroot (309.6mg; $P=0.003$; 95%CI: 116.6, 502.7), demonstrating a dose response.



Cumulative urinary nitrate excretion over 24 hours following incremental doses of nitrate in the old group. Data presented as mean \pm SEM; $n=12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate positive control.

Excretion – young

There was a significant effect of time ($P=0.001$) and intervention ($P<0.001$) on urinary excretion, but no interaction between the two factors ($P=0.47$). Pairwise comparisons show that excretion following PN was significantly higher than following 100g beetroot (+61.4mg; $P<0.001$; 95%CI: 31.5, 91.2) and following 200g beetroot (+50.9mg; $P=0.01$; 95%CI: 10.1, 91.7). Excretion was significantly higher at 6-12hours than at 0-3hours (+51.6mg; $P=0.002$; 95%CI: 19.6, 83.1).

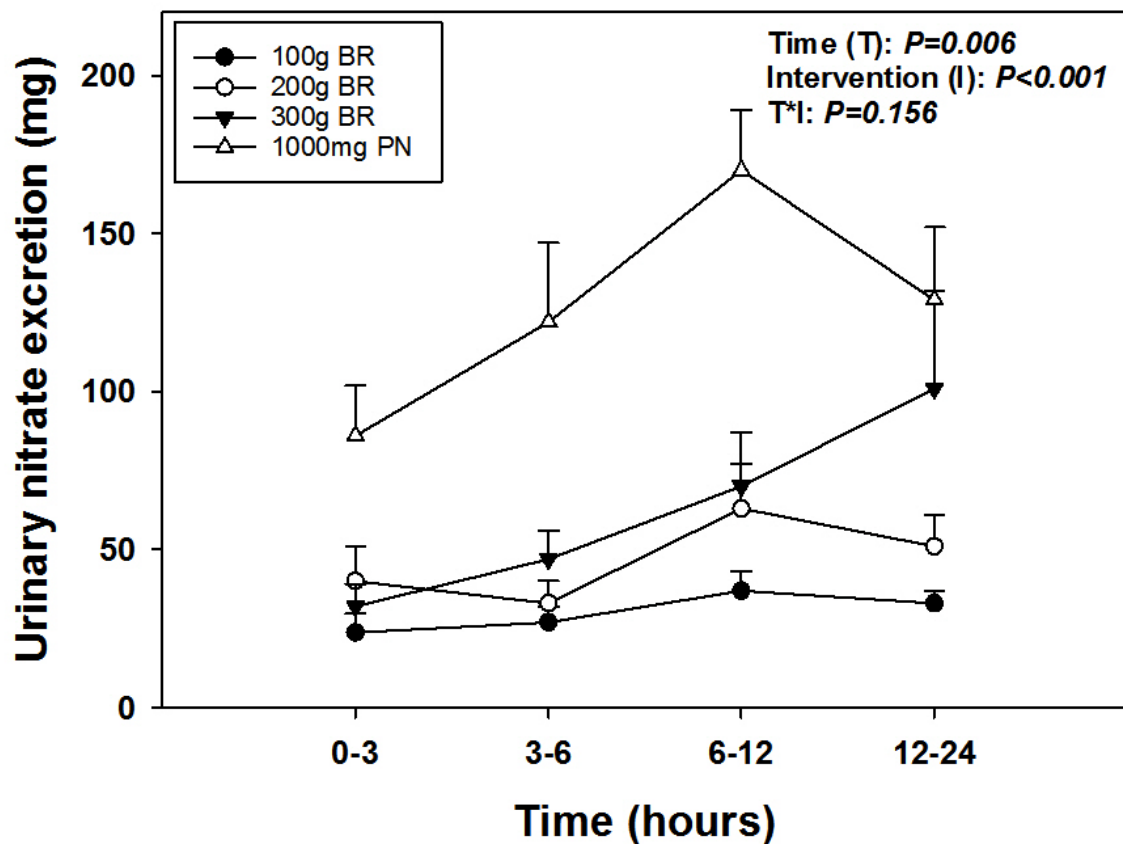


Urinary nitrate excretion over 24 hours in the young group. Data presented as mean \pm SEM; $n=12$. Statistical analysis using two-factor repeated measures ANOVA (intervention \times time). BR = beetroot, PN = potassium nitrate positive control.

Excretion – old

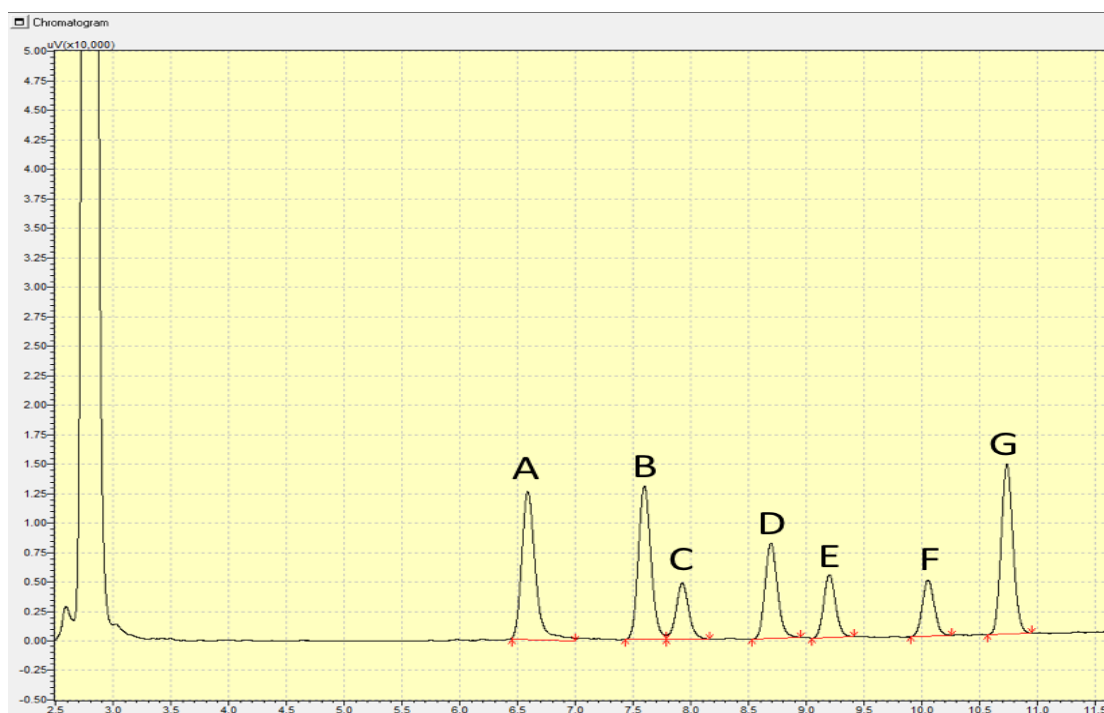
There was a significant effect of time ($P=0.006$) and intervention ($P<0.001$) on urinary excretion in the old, but no interaction between the two factors ($P=0.16$). Pairwise comparisons show that excretion following PN was significantly higher than following 100g beetroot (+89.8mg; $P=0.001$; 95%CI: 40.3, 139.2) and following 200g beetroot (+77.4mg; $P=0.003$; 95%CI: 29.1,

125.7). Excretion was significantly higher at 6-12hours than at 0-3hours (+36.7mg; $P=0.02$; 95%CI: 5.5, 67.8).

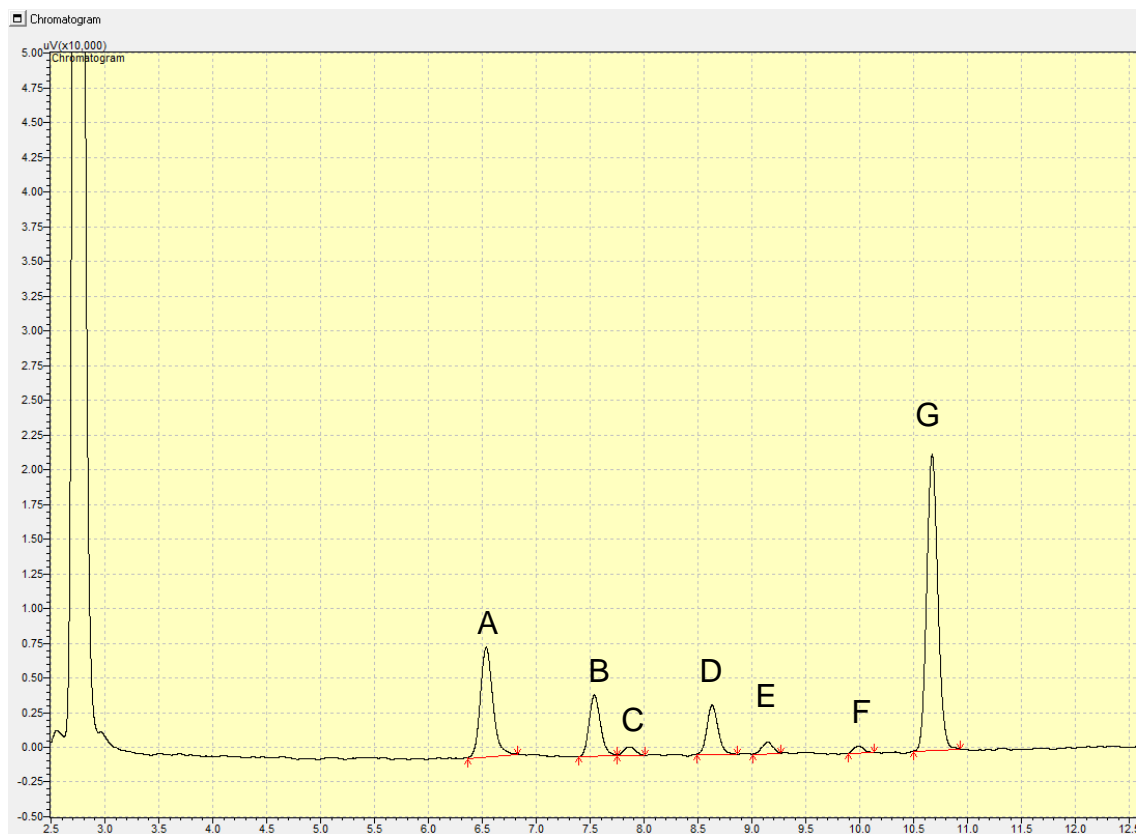


Urinary nitrate excretion over 24 hours in the old group. Data presented as mean \pm SEM; $n=12$. Statistical analysis using two-factor repeated measures ANOVA (intervention x time). BR = beetroot, PN = potassium nitrate positive control.

Appendix I: Short chain fatty acid analysis, Chapter 4



Standard retention times for master mix internal control. Including: acetate (A), propionate (B), isobutyrate (C), butyrate (D), isovalerate (E), valerate (F) and 3MV (G).



Gas chromatography readout for a sample to determine concentration of acetate (A), propionate (B), isobutyrate (C), butyrate (D), isovalerate (E), valerate (F) and 3MV (G).