Dietary Nitrate in Vascular and Brain Health

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Abstract

Nitric oxide (NO) is a highly reactive molecule that is essential for several biological processes, including the regulation of vascular resistance, neurotransmission and muscular energetics. Sufficient NO production is crucial for the maintenance of a healthy vascular system. With ageing, NO synthesis from arginine, catalysed by NO synthase (NOS), is reduced, which may contribute to increased blood pressure (BP), endothelial dysfunction (ED) and impaired brain function. Dietary nitrate (NO_3^-) is an important source of NO production via a non-enzymatic pathway involving the progressive conversion of NO_3^- into nitrite (NO_2^-) by the action of oral bacteria, and then to NO in low pH and hypoxic environments (i.e., stomach and arterial-to-capillary circulation). Whilst several clinical studies have assessed the effect of supplemental dietary NO_3^- intake (often supplied as beetroot juice (BJ)) on vascular and cognitive functions, there is a significant gap in the literature concerning the effects of longer-term intervention, especially in older people. This PhD investigated the effects of BJ supplementation in adult population, including healthy younger and older overweight and obese adult population who is at a higher risk of physiological dysfunctions, including cardiovascular disease and cognitive impairment.

I conducted a systematic review of observational studies to assess NO_3^- intake by adults. The review included 55 articles and found that the median daily NO_3^- intakes were similar in both healthy and patient populations and below the safe upper intake of daily NO_3^- intake (3.7 mg/kg body weight). Then, I performed a meta-analysis of 18 randomised control trials (RCT) to examine the effect of NO_3^- or NO_2^- supplementation on cognitive function and cerebral blood flow (CBF). This meta-analysis revealed no overall effect of NO_3^- or NO_2^- supplementation on cognitive function or CBF. This meta-analysis helped to inform the design of the subsequent feasibility study.

Next, I conducted a small pilot study to examine the validity and reliability of NO_2^- salivary strips (against reference standard laboratory measures) with and without the use of mouthwash. This study showed that these strips have a high level of reproducibility and repeatability in detecting changes in salivary NO_2^-. The study also indicated that the strips can be used to monitor NO_3^- intake in long-term dietary NO_3^- interventions.

The final phase of this project provided evidence of the acceptability and feasibility of an intervention testing the effects of prolonged consumption of incremental doses of NO_3^- in overweight and obese older participants. The findings of this study showed that cognitive

function and CBF were not affected by long-term BJ supplementation. However, there was a non-significant trend towards on systolic BP (SBP) reduction with lower BJ doses.

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Publications and conferences abstracts

PhD research manuscripts published in peer-review journals

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Other Publications

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Conference Abstracts

Poster

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Abbreviations

ADI	Adequate daily intake
AUC	Area under the curve
ADMA	Asymmetric dimethylarginine
AASI	Ambulatory arterial stiffness index
ASVD	Atherosclerotic vascular disease
AMD	Age-related macular degeneration
ASL-MRI	Arterial spin labelling- Magnetic resonance imaging
AD	Alzheimer's disease
AS	Arterial stiffness
AJ	Apple juice
BJ	Beetroot juice
BP	Blood pressure
BC	Breast cancer
BLC	Bladder cancer
BO	Barrett's oesophagus
BCJ	Blackcurrant juice
BH4	Tetrahydrobiopterin
BMI	Body mass index
CVD	Cardiovascular diseases
CV	Cardiovascular
CC	Colorectal Cancer
CI	Confidence interval
COPT	Chronic obstructive pulmonary disease
CHD	Coronary heart disease
CAD	Coronary artery disease
CKD	Chronic kidney disease
CBF	Cerebral blood flow
CW-NIRS	Continuous wave-near infrared spectroscopy
CRT	Choice reaction time
CPT	Continuous performance test
CVRI	Cerebrovascular resistance index

CCID	Carotid characteristic impedance dynes
CBVRD	Carotid bed vascular resistance dynes
CCSA	Carotid cross-sectional area
cGMT	Cyclic guanosine monophosphate
DASH	Dietary approaches to stop hypertension
DBP	Diastolic blood pressure
DH	Diet history
DR	Dietary record
DV	Digit vigilance
DRT	Decision reaction time
DWR	Delayed word recognition
EFSA	European Food Safety Authority
ED	Endothelial dysfunction
EC	Endometrial cancer
EF	Endothelial function
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FAD	Flavin adenine dinucleotide
FMD	Flavin mononucleotide
fMRI	Functional magnetic resonance imaging
FMD	Flow-mediated dilation
FFQ	Food frequency questionnaire
GDP	Gross Domestic Product
GC	Gastric cancer
GNG	Go/No-Go
GF	Grapefruit juice
HFpEF	Heart failure with preserved ejection fraction
ICC	Intraclass coefficient
iNOS	Inducible nitric oxide synthase
IQR	Interquartile range
KC	Kidney cancer

LDI	Laser doppler iontophoresis
L-NMMA	NG-monomethyl-L-arginine
LDL	Low density lipoprotein
MRI	Magnetic resonance imaging
mNOS	Mitochondria nitric oxide synthase
MFT	Mental Fatigue Test
MR	Memory recognition
MZ	Maze
MCAV	Middle cerebral artery blood flow velocity
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NO_3^-	Inorganic nitrate
NO_2^-	Inorganic nitrite
NADPH	Nicotinamide adenine dinucleotide phosphate
NIRS	Near-infrared spectroscopy
NHL	Non-Hodgkin lymphoma
NF-C	Nitrite free capsules
NF-S	Nitrate free solution
NWM	Numeric working memory
NR	Not reported
NUR	Numerical recall
OVC	Ovarian cancer
OJ	Orange juice
PWV	Pulse wave velocity
PPI	Proton pump inhibitors
PC	Pancreatic cancer
PL	Placebo
ROS	Reactive oxygen species
RCT	Randomised clinical trial
RCC	Renal cell carcinoma
RVIP	Rapid Visual Information Processing
sCG	Soluble guanylate cyclase

SBP	Systolic blood pressure
SOD	Superoxide dismutase
SLC17A5	Sialin
SCN^-	Thiocyanate
SMD	Standardized mean differences
SN	Sodium nitrite
SP	Spinach
SS	Serial subtraction
SRT	Simple reaction time
SM	Shape memory
SPM	Spatial memory
SST	Spatial span task
SE	Standard error
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TMT	Trail Making tasks
TC	Thyroid cancer
TVR	Total vascular resistance
UATC	Upper aerodigestive tract cancer
UK	United Kingdom
USA	United States of America
VS-1	Visual interference
VS-2	Verbal interference
WHO	World health organization
WA	Weighted average
24h/48h	24/48-hour recall

Chapter 1. Literature review

1.1. Ageing

Worldwide, there is an increasing median age of the population which is related to declining fertility rates and extended life expectancy (Lutz *et al.*, 2008). Understanding the factors responsible for the continuing and dynamic changes in ageing demographics represents one of the most important global public health challenges to be faced over the coming decades. Specifically, in 2015 the number of people aged 60 and older was about 900 million. This number is projected to increase to more than one billion by 2030 and to more than 2 billion by 2050 (**Figure 1.1**), as reported by United Nations (UN, 2015; ONS, 2018). Also, in 2015, the number of individuals aged 65 and older in the United Kingdom (UK) was 11.5 million, which represents 11.8% of the total population. This number had increased by 21% since 2005 (Shrosbree, 2015) and it is expected that this increasing trend will continue. Over the next 30 years, it has been proposed that the proportion of older people (aged ≥ 65 years) in the population will rise by 25% due to increases in longevity combined with a lower birth rate (Howdon and Rice, 2018).

Age is the main risk factor for chronic health conditions, including cardiovascular diseases (CVD), cancers and neurodegenerative diseases (Niccoli and Partridge, 2012). As the number of older people increases, the prevalence of these chronic diseases will increase, and this will be associated with increasing healthcare demands and greater health expenditure.

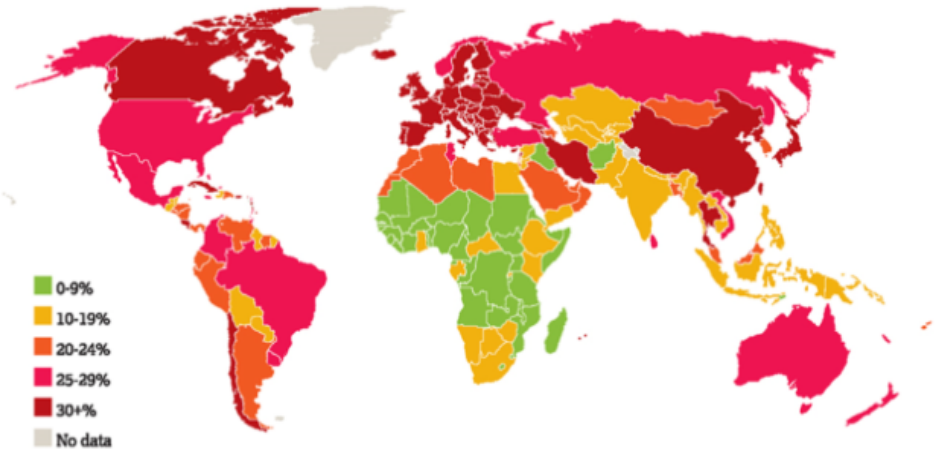

Figure 1.1: Projected proportions of the global population aged 60 years and over in 2050. This figure was taken from (ONS, 2018), ONS; Office for National statistics.

1.1.1. Age-related diseases and physiological changes

Ageing is caused by the accumulation of damage to all cellular macromolecules, including DNA, proteins and lipids (López-Otín *et al.*, 2013), and this damage leads to a progressive deterioration of all cells and tissues, which affects the structure and function of organ systems, including the cardiovascular, nervous and gastrointestinal systems. Consequently, these changes contribute to an incremental risk of chronic diseases. For instance, ageing is associated with several changes in cardiovascular structure and function (Ferrari *et al.*, 2003). The left ventricular muscle becomes thicker and its elasticity is reduced. The heart weight increases slightly, and this is related to myocardial cell hyperplasia (Karavidas *et al.*, 2010). Blood vessels are also affected adversely by ageing. The elasticity of the arterial vessels decreases, and this leads to an increased residual vessel diameter, ultimately resulting in greater wall stiffening and thickening (Fleg and Strait, 2012). These alterations have multiple effects on the function of the cardiovascular system, and increase the risk of CVD, such as hypertension, atherosclerosis, stroke and myocardial infarction (North and Sinclair, 2012).

The oral cavity is also affected by ageing, leading to reduced salivary flow and increased dryness of the mouth, making chewing and swallowing difficult (Lamster *et al.*, 2016). Such oral changes among older people are exacerbated by polypharmacy (Thomson, 2015). Furthermore, there are age-related alterations in the microbiome of both the oral cavity and gut which, in turn, affect digestion and functionality of the immune system, making the body more vulnerable to pathogenic bacteria (Candela *et al.*, 2014; Belibasakis, 2018). Ageing is also associated with structural and functional alterations in the brain which increase the risk of neurovascular dysfunction and neurodegenerative diseases, such as dementia (Aalami *et al.*, 2003), which will be discussed in detail later.

There is increasingly strong evidence that nutrition is a major modifier of the ageing process and that better eating patterns slow ageing (Mathers, 2013), in at least two ways including (i) by reducing pervasive damaging processes such as inflammation, oxidative stress/redox changes and metabolic stress and (ii) by enhancing cellular capacities for damage management and repair (Malcomson and Mathers, 2018). As a consequence, nutritional interventions are an increasingly attractive approach for addressing the increased risk of multiple diseases associated with ageing. For example, the Prevención con Dieta Mediterránea (PREDIMED) Study has shown that CVD in middle-aged people in Spain was reduced significantly in those randomised to a Mediterranean diet (Estruch *et al.*, 2018). The following sections will discuss the vascular and cognitive dysfunctions associated with ageing.

1.1.1.1. Vascular dysfunction

The entire circulatory system is lined with vascular endothelial cells. The endothelium can be considered as a large endocrine organ, which constitutes approximately 1–1.5 kg of body weight and covers a surface area of over 700 m^2 (Böger *et al.*, 2005). Endothelial cells are fundamental in regulating blood vessel tone, blood flow, neutrophil recruitment, thrombosis and haemostasis. These cells release several contracting factors, including endothelin-1, angiotensin-II, thromboxane and reactive oxygen species (ROS). They also release relaxing factors, such as prostacyclin and NO (Cau *et al.*, 2018). A healthy endothelium is characterised by a balance between these vasoconstrictor and vasodilator factors, with a sufficient basal production of NO to mediate normal vascular responses (Deanfield *et al.*, 2007).

Alterations in endothelial regulatory functions, characterised by an imbalanced production of contractile and relaxing agents, result in endothelial dysfunction (ED) (Cau *et al.*, 2018). Ageing is associated with ED which is a precursor of future cardiovascular events (Daiber *et al.*, 2017), and one of its key features is reduced NO availability. Inadequate NO production disturbs vascular haemostasis, and this is involved in the development of atherosclerosis, coronary heart disease (CHD), diabetes and hypertension (El Assar De La Fuente *et al.*, 2012). Older age is associated with lower NO availability due to several mechanisms, which will be discussed in detail in section 1.4. ED reduces blood flow (Sabayan *et al.*, 2014) and has been implicated in the pathogenesis of Alzheimer's disease (AD) (Dede *et al.*, 2007).

1.1.1.2. Cognitive decline and cerebral blood flow

The continuing increase in longevity is leading to increased prevalence of age-related diseases, including CVD and dementia, which are challenges facing society today. One of the most feared aspects of growing old is cognitive decline and dementia. More than 40 million individuals worldwide were estimated to have dementia in 2015, and this number is expected to triple by 2050 (Baumgart *et al.*, 2015). In the UK, cognitive failure accounts for 40% of admissions to care institutions (Deary *et al.*, 2009). Most individuals experience some degree of cognitive decline by age 60, and this decline is exacerbated by age 75 (Williams and Kemper, 2010). These cognitive changes do not involve all cognitive domains. Age-related changes are more likely to occur in cognitive domains related to executive function, memory and processing speed, which reflect the functioning of the prefrontal cortex (Ownby, 2010). The aforementioned cognitive abilities are called "fluid cognition", which is independent of past experiences (Murman, 2015). Changes in fluid cognition are more noticeable and proceed faster in those with dementia (Toepper, 2017). It has been suggested that executive function is the

first to deteriorate with age (West, 1996). Executive function includes problem solving, sequencing and planning responses, decision making and multi-tasking, all of which have been shown to decline with age (Murman, 2015). Other cognitive abilities, called "crystallised abilities", depend on acquired knowledge, such as language and speech, while simple attention tasks typically remain intact with ageing (Murman, 2015).

The brain undergoes substantial structural and functional alterations with age, which seem to be related to changes in cognitive function. The volume of both grey and white matter has been reported to decline with ageing and these changes are correlated with a decreased cognitive performance, particularly in executive tasks (Toepper, 2017). In addition, neurotransmission changes also contribute to reduced cognitive performance. Studies have shown relationships between age-related cognitive dysfunction and reduced concentrations of dopamine (Toepper, 2017) and serotonin (Amin *et al.*, 2005). Furthermore, activity of the enzyme monoamine oxidase, which catalyses oxidation of neurotransmitters in the brain, increases with age and is associated with age-related neurodegenerative disorders (Peters, 2006). It is also worthy to note that early cognitive impairment is associated with reduced CBF (Bangen *et al.*, 2018).

The brain volume comprises 2% of the total body weight, but it receives about 20% of the blood supply and uses approximately 20% of whole-body oxygen and glucose (Rink and Khanna, 2011). The brain obtains most of its energy from aerobic metabolic processes and maintenance of central blood flow ensures adequate supply of nutrients and oxygen to brain cells to maintain normal brain functions (Donnelly *et al.*, 2016). Reduced blood supply to the brain may cause age-related cognitive impairments, and this has been associated with an increased risk of dementia in otherwise healthy older people (Van Beek *et al.*, 2008). The normal brain tissue perfusion is approximately 50–55 ml/100 g/min. During ageing, blood supply to the brain declines gradually leading to greater oxygen extraction from haemoglobin without clinical indications, which can be quantified by arteriovenous difference in oxygen concentration. However, cognitive dysfunction may occur when the blood perfusion falls to 25–30 ml/100 g/min (Bor-Seng-Shu *et al.*, 2012). A recent cross-sectional study conducted on a multi-ethnic cohort (84 Africans, 151 South Asians and 214 Europeans) with a mean age of 71 years found that higher CBF was associated with better cognitive performance (Leeuwis *et al.*, 2018). In a recent large population-based study with 4,759 older participants (median age at baseline, 61.3 years), CBF was measured twice with a 7-year interval between measures. The authors found that reduced CBF at baseline was associated with enhanced cognitive decline and with increased risk of dementia during follow up (Wolters *et al.*, 2017).

Since the microvascular endothelium is involved in many aspects of CBF regulation, it is not surprising that there is a growing body of evidence linking vascular and brain health. Age-associated ED likely contributes to the chronic cerebral hypoperfusion observed in ageing and the consequent cerebral dysfunction, impaired neurovascular coupling and cognitive decline (Toth *et al.*, 2017). These aforementioned dysfunctions have been linked to impaired NO production (Girouard and Iadecola, 2006).

1.2.Obesity and ageing

Globally, obesity is a growing health issue that is associated with rapidly increasing healthcare costs. The World Health Organization (WHO) recently reported that worldwide, 40% of adults are overweight with a body mass index (BMI) $\geq$ 25 kg/m^2, and 13% are obese with a BMI $\geq$ 30 kg/m^2 (WHO, 2016). A recent Australian study showed that overweight and obesity was associated with reduced life expectancy in adults aged 20- 69 years, and this association was stronger with severely obese individuals (Lung *et al.*, 2019). Ageing has been found to be associated with increased fat deposition in skeletal muscle and abdominal white adipose tissue, a condition that accelerates ageing-related disease development (Jura and Kozak, 2016). Ageing and obesity share some features, including increased levels of oxidative stress proinflammatory cytokines and impaired NO availability (Ahima, 2009). Therefore, both are risk factors for numerous functional impairments, including hypertension, type 2 diabetes (T2D), CHD, stroke, cancer and cognitive impairment (Villareal *et al.*, 2005; Hruby and Hu, 2015; Pérez *et al.*, 2016).

1.2.1. Obesity and vascular dysfunction

Arterial functional and structural abnormalities are evident in obese people. Epidemiological evidence has indicated that BMI is a strong predictor of overall mortality and mainly vascular mortality (Prospective Studies Collaboration 2009). In 1996, Steinberg and colleagues were the first to demonstrate that obesity, independent of other risk factors, can result in ED that is indicated by decreased endothelium-dependent vasodilation (Steinberg *et al.*, 1996). This is in line with a more recent study by Heijden et al. (2017) who found that, after adjusting for confounding risk factors, including hypertension, diabetes, smoking and hypercholesteremia, obesity was associated with a reduced ejection fraction. A linear relationship was also found between impaired endothelial function (EF), measured in the forearm, and the severity of obesity (Perticone *et al.*, 2001). In addition, investigation of participants in the Framingham Heart Study revealed an inverse association between flow-mediated dilation (FMD) in the brachial artery and BMI (Benjamin *et al.*, 2004). Increased arterial stiffness is also associated

with obesity (Meyers and Gokce, 2007). In a study of more than 10,000 individuals recruited from 52 centres worldwide, investigators found that BMI is significantly, and positively, associated with both SBP and diastolic BP (DBP) (Dyer and Elliott, 1989). Collectively, these studies link obesity with vascular functional impairments.

1.2.2. Obesity and cognitive decline

The association between obesity and brain health has received increasing attention. A recent systematic review and meta-analysis involving 2.8 million adults (including 57,294 cases of dementia) showed that obesity was positively associated with vascular dementia (Lee *et al.*, 2020). Overweight and obesity are significant risk factors for vascular dementia and AD development in later life (Whitmer *et al.*, 2005; Xu *et al.*, 2011). At mid-life, obese individuals have 74% greater risk of developing dementia later in life (mean 27 years later), while overweight individuals have a 35% greater risk, compared with those of normal body weight (Whitmer *et al.*, 2005). A recent study conducted among 1,545 older adults concluded that older obese adults have a greater incidence of cognitive impairment compared with older adults with normal body weight, and this was independent of other risk factors, such as hypertension and diabetes (Feinkohl *et al.*, 2018). Cross-sectional studies have reported a negative association between BMI and memory-related cognitive domains. Relative to normal weight-matched groups, working memory is significantly impaired in both overweight and obese people (Coppin *et al.*, 2014). However, it is not just memory-related cognitive domains that are impaired with obesity, detriments in other cognitive domains are also evident in obese individual. For instance, impaired executive function has been reported in several studies (Fergenbaum *et al.*, 2009; Fagundo *et al.*, 2012). This observation was independent of age or other comorbidities (Gunstad *et al.*, 2007; Volkow *et al.*, 2009). However, a more recent study did not find an association between BMI and executive measures (Hovens *et al.*, 2019). The inconsistent evidence can be explained by a systematic review that concluded there is insufficient evidence to confirm that cognitive impairments are independent of obesity-related comorbidities or demographic variables (Prickett *et al.*, 2015).

Some studies have suggested that the metabolic activity in the brain might be adversely affected in individuals with higher BMIs due to reduced blood flow to certain brain areas and therefore, reduced cognitive functioning (Gonzales *et al.*, 2010). A significant negative correlation was found between BMI and CBF velocity, and this was associated with reduced cognitive performance (Zhang *et al.*, 2006). This finding was independent of other comorbid factors, such

as T2D and hypertension (Zhang *et al.*, 2006). Altogether, this implies that obesity may pose a risk for impaired brain function.

1.3. Assessment of cognition and CBF

1.3.1. Assessing vascular function

EF can be assessed in either the coronary or peripheral circulation (Flammer *et al.*, 2012). Coronary EF assessments can be performed by measuring epicardial and resistance vessel EF via an acetylcholine infusion (Flammer *et al.*, 2012). However, due to their invasive nature, these methods are limited to research (Flammer *et al.*, 2012). Alternatively, there are non-invasive surrogate methods used to assess EF, including FMD, which is considered the gold standard non-invasive technique for EF assessment. FMD is a marker of NO availability in the endothelium and can predict future cardiovascular events (Matter, 2011). In addition, laser doppler iontophoresis (LDI) is another non-invasive and convenient method to assess EF via the administration of a small amount of drug, usually sodium nitroprusside and acetylcholine, using a small electric current (Alam *et al.*, 2005).

Measuring BP can also help assess cardiovascular events and vascular function indirectly. CVD is the leading cause of mortality, and high BP is one of the major risk factors for CVD and stroke (Wu *et al.*, 2015). Globally, about 47% of CHD and 54% of strokes are due to high BP (Arima *et al.*, 2011). A recent study has shown that high BP is associated with ED among young women without CVD (Adler *et al.*, 2018). The prevalence of hypertension increases with age, with approximately 65% of those over 60 years being affected (Wu *et al.*, 2015). A meta-analysis that included data from 61 observational studies (including 1,000,000 individuals) found that increasing SBP by 20 mmHg is strongly associated with increased mortality from stroke (Lewington and Clarke, 2002), whereas each 10 mmHg decrease in SBP is associated with a significantly lower risk of mortality (Emdin *et al.*, 2015).

1.3.2. Assessing cognitive function

As mentioned earlier, with the increasing number of aged populations, cognitive decline or impairment is becoming a major health issue. Mini-Mental State Examination and the Montreal Cognitive Assessment tools are widely used to measure cognition in clinical practice, mainly as a screening tool.

Today, a plethora of cognitive tasks, either computer- or pencil/paper-based, can be used to assess cognitive function. Some researchers have reported that both computerized and

pencil/paper tests are feasible and equally effective in assessing cognition levels in older people, but computer-based tests are more acceptable to researchers and participants (Collerton *et al.*, 2007). The utility of a number of computer-based test batteries to assess cognition in older people has been reviewed (Wild *et al.*, 2008). Computerised cognitive assessments have several advantages. Computerised batteries can be highly standardised, sensitive and they can record precisely the speed and accuracy of responses and allow an assessment of multiple cognitive functions. Furthermore, they represent potential cost and time savings for researchers (Wild *et al.*, 2008). The use of computerised tests helps to quantify the effects of several types of intervention, including medications, nutrients and physical or cognitive training, on cognitive function in older adults (Oliveira *et al.*, 2014).

Traditional pencil/paper assessments, such as Trail Making tasks (TMT), which are based on the connection of numbered circles with a line or the connection of numbered and lettered circles in order, have been widely used in research and have shown their sensitivity with older adults (LaRoche *et al.*, 2014; Justice *et al.*, 2015; Zhou *et al.*, 2017). A combination of both methods (TMT and computerised-based tests) was used in the current project to determine functioning in certain cognitive domains. Recently, with advances in technology, an increasing number of studies have assessed cognition in older adults using smartphones and digital devices. These were found to be effective and more convenient for older participants in terms of time, place barriers and frequency (Chinner *et al.*, 2018; Koo and Vizer, 2019).

The variation in the results of cognitive tests is related with the repetition of cognitive tests, suggesting a practice or learning effect (Duff *et al.*, 2001; Beglinger *et al.*, 2005). A learning effect can be defined as an improvement in a cognitive performance without having any intervention that could justify it. This could be due to an increased familiarity with cognitive tasks or reduced anxiety. A previous study observed an improvement in cognitive performance in healthy adults, even in those who had not undergone the drug intervention throughout the six repetitions, showing high levels of learning (Beglinger *et al.*, 2004). A significant learning effect was also found in older individuals when the computerised cognitive test was applied twice with a two-week interval. This is due to an increased confidence in the use of the computer (Raymond *et al.*, 2006). To overcome this issue, using a pre-baseline cognitive test is an appropriate solution (Goldberg *et al.*, 2015).

1.3.3. Assessing cerebral blood flow

Cerebral hypoperfusion has been associated with an increased risk of dementia and AD in healthy older adults. Several studies have reported that patients with dementia or cognitive

impairments have lower CBF compared with healthy controls (Binnewijzend *et al.*, 2013; Binnewijzend *et al.*, 2016). Therefore, reduced CBF can be a crucial contributor to cognitive decline.

The number of millilitres of blood per 100 g of brain tissue per minute is the standard unit of CBF measurement. As stated earlier, in human grey matter, a frequently observed value is about 55 ml/100 g/min, which represents about 1 ml of blood for every 100 g of brain tissue per second. Assuming an average brain tissue density of 1g/ml, this means that per second, approximately 1% of the total tissue volume is provided with freshly delivered blood (Joris *et al.*, 2018). Radioactive tracers were used in earlier studies to measure the absolute CBF by single-photon emission computed tomography or positron emission tomography. However, these techniques have several limitations that restrict their use in clinical research, including costs, the requirement for a specialised imaging unit and additional invasive and technically demanding steps (Catafau, 2001). Moreover, these techniques need time intervals between several measurements to avoid overexposure to radiation (Joris *et al.*, 2018). Therefore, recent developments of non-invasive techniques, such as magnetic resonance imaging (MRI), have received increasing attention in measuring cerebral perfusion in humans (Herold *et al.*, 2018). However, some drawbacks for the fMRI include the relatively high costs, the fact that it is a noisy measurement, and the fact that it is sensitive to head movements (Scarapicchia *et al.*, 2017).

CBF can also be assessed indirectly by several techniques, including transcranial doppler ultrasound imaging, phase contrast MRI and near-infrared spectroscopy (NIRS). The NIRS technique was first described in 1977 by Frans Jobsis (Jobsis, 1977). NIRS indirectly assesses CBF by measuring the changes in oxyhaemoglobin and deoxyhaemoglobin via the transmission of light through the intact scalp. Both deoxyhaemoglobin and oxyhaemoglobin are light absorbing chromophores, and they have slightly different wavelengths (600–750 nm and 800–940 nm, respectively) (Herold *et al.*, 2018). NIRS can quantify their concentrations by measuring the amount of light absorbed. The changes in oxyhaemoglobin and deoxyhaemoglobin detected by NIRS indirectly represent neuronal activity (Herold *et al.*, 2018). In activated brain regions, increased oxyhaemoglobin and decreased deoxyhaemoglobin concentrations are observed (Herold *et al.*, 2018).

Different NIRS systems are available. The most commonly used system is continuous wave NIRS (CW-NIRS) to assess brain haemodynamics. However, CW-NIRS is unable to determine the absolute concentrations of oxyhaemoglobin and deoxyhaemoglobin and generates concentration changes only. Thus, it is not appropriate for measuring changes in CBF between

different recording sessions (Wightman *et al.*, 2015a). This issue has been overcome using the frequency-domain system that produces absolute values for changes in oxyhaemoglobin and deoxyhaemoglobin concentrations (Jackson and Kennedy, 2013), and this system was used in the present project.

1.3.3.1. Application of NIRS in nutrition

With the increased interest in the involvement of nutrition in cognitive health, NIRS applications in nutritional neuroscience have also been gaining attention. Nonetheless, to date, NIRS has been utilised in only a handful of studies to evaluate the cerebral haemodynamic effects of dietary components. There are several double-blind placebo-controlled studies conducted in healthy adults to assess the effects of nutritional interventions on CBF by NIRS, mainly in the prefrontal cortex. For example, NIRS has shown a vasoconstricting effect of caffeine in healthy adults (Kennedy and Haskell, 2011). NIRS has also indicated a dose-related increase in CBF during cognitive task performances after acute administration of resveratrol (Kennedy *et al.*, 2010), and a decrease in CBF was observed after the acute administration of epigallocatechin, which is present in green tea (Wightman *et al.*, 2012). NIRS has also been used following the acute administration of a combination of several nutrients such as resveratrol with piperine (Wightman *et al.*, 2014).

NIRS has been used not only in acute nutritional studies but also in longer duration nutritional interventions. The effect of 12 weeks of daily dietary supplementation with polyunsaturated fatty acids, including docosahexaenoic acid and eicosapentaenoic acid, on cerebral haemodynamics has been assessed by NIRS (Jackson *et al.*, 2012). In this case, only docosahexaenoic acid was associated with increased CBF during task performance (Jackson *et al.*, 2012). NIRS did not detect any changes in CBF after chronic supplementation (28 days) of resveratrol or multivitamins over 8 weeks (Wightman *et al.*, 2015a; Kennedy *et al.*, 2016). A more recent study has shown a modulation in CBF, as assessed by NIRS, after 56 days administration of *Zanthoxylum armatum*, which is a traditional Asian culinary spice and medicinal compound (Kennedy *et al.*, 2019).

All aforementioned nutritional trials were conducted with healthy young adults. Few nutritional studies have used NIRS in older age groups. To our knowledge, there is only one recent study that has focused on older people to assess the acute and chronic effects of Greek mountain tea on CBF (Wightman *et al.*, 2018). In that study, NIRS detected modulation in CBF following acute supplementation only, with no additional effects after chronic supplementation (28 days).

1.4. Nitric oxide, ageing and obesity

1.4.1. Nitric oxide history

In the early 1770s, the chemist Joseph Priestley discovered NO and for more than two centuries this colourless and odourless gas was widely known as a toxic gas (Habib and Ali, 2011). In environmental research, NO content in the air is measured routinely to monitor air quality and pollution levels (Kreuzer and Patel, 1971). NO is a component of cigarette smoke and is produced by motor vehicles during the combustion of hydrocarbon fuel. It has also been shown to destroy the ozone layer (Seinfeld and Pandis, 2016). Later, after several discoveries, research showed that NO is an essential molecule within human physiology.

In 1980, the relaxation of vascular smooth muscles was considered to be effected by the action of endothelial cells that release a substance, called at that time, endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980). Seven years later, several studies by Moncada and Ignarro suggested that EDRF is NO (Ignarro *et al.*, 1987; Palmer *et al.*, 1987), which is produced by the oxidation of the amino acid L-arginine (Palmer *et al.*, 1988). Today, the molecule, once known only as an environmental pollutant, is recognised as one of the most important gasotransmitters in living organisms (Zoccali *et al.*, 2009). It is also a key molecule in several biological processes, including the regulation of vascular resistance, neurotransmission and muscular energetics (Moncada *et al.*, 1991; Kasparek *et al.*, 2008). The biological importance of NO was officially recognized with the award of the Nobel Prize to Furchgott, Ignarro and Murad in 1998 for the discovery of the biological actions of NO (Furchgott *et al.*, 1998).

1.4.2. Nitric oxide production (L-arginine/NO synthase pathway)

The endogenous generation of NO depends on an enzyme NOS acting on the substrate amino acid, L-arginine. This pathway was first discovered by Moncada and co-workers in 1989 (Moncada *et al.*, 1989). NOS catalyses the oxidation of a guanidino nitrogen from L-arginine yielding L-citrulline and NO in the presence of oxygen, using NADPH as a source of electrons. In this reaction, NOS is activated by the Ca^{++}/calmodulin complex (Ca^{++} is released from intracellular stores and binds to calmodulin). There are three isomers of NOS: endothelial NOS (eNOS or NOS3); neuronal NOS (nNOS or NOS1); and inducible NOS (iNOS or NOS2) (McNally *et al.*, 2016). In addition, evidence indicates that mitochondria might have another form (mNOS) (Dynnik *et al.*, 2020). This reaction involves several cofactors that have specific binding sites on the enzyme, including: i) the N-terminal oxygenase has a binding site for the

tetrahydrobiopterin cofactor (BH4) and haem and substrates L-arginine and oxygen; and ii) the C-terminal reductase contains binding sites for one molecule each of flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH) (Andrew and Mayer, 1999; Jobgen *et al.*, 2006). Calmodulin binding domains separate the two domains in NOS (oxygenase and reductase). A deficiency in any of these cofactors will limit NO production, and in many cases NOS will produce superoxide instead (a situation called uncoupling NOS) (Bruckdorfer, 2005). Critical factors that can affect the NO synthetic pathway include: a low availability or increased degradation of L-arginine, a low availability of essential cofactors (e.g. BH4), or the presence of natural inhibitors of NOS, such as asymmetric dimethylarginine (ADMA) (Bruckdorfer, 2005). An overview of the endogenous NO production pathway is provided in **Figure 1.2.**

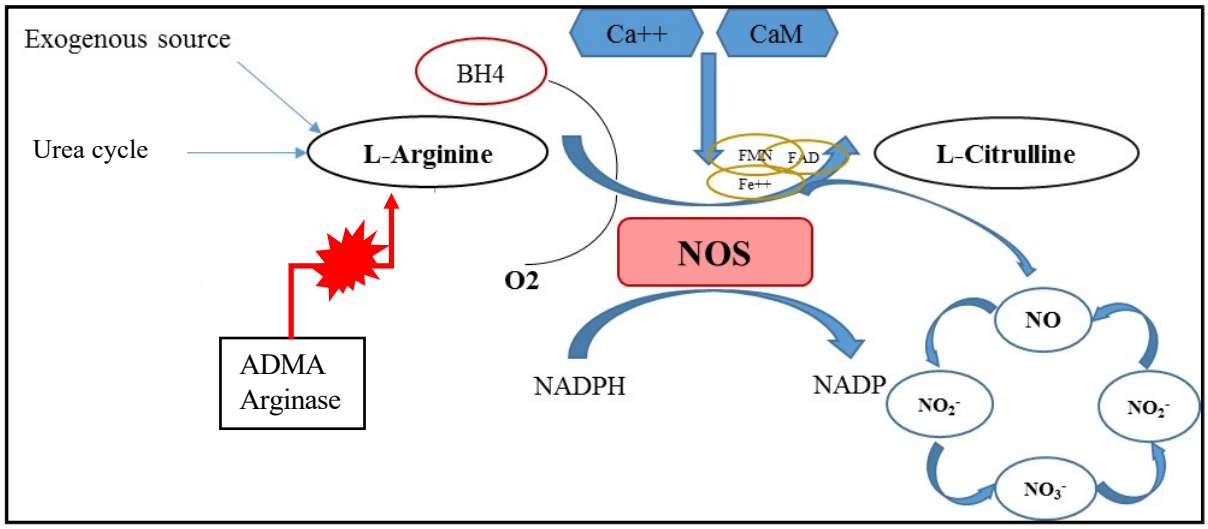

Figure 1.2: Synthesis of nitric oxide (NO).
Arginine is the precursor for NO synthesis and can be produced in the body via turnover of protein, the diet or *de novo* synthesis. The concentration of L-arginine can be affected by the activity of arginase enzyme and asymmetric dimethyl L-arginine (ADMA), which is a nitric oxide synthase (NOS) inhibitor. L-arginine is converted to L-citrulline and NO and NOS catalysis this reaction in the presence of several cofactors. NO can be oxidized to nitrite (NO_2) and nitrate (NO_3) in the circulation.

1.4.3. Physiology and pathophysiology of nitric oxide

NO is primarily known for the regulation of blood flow and vascular tone and these occur by the activation of the soluble guanylate cyclase enzyme (sGC) in smooth muscle tissue. The sGC is a heterodimeric haem protein that acts as the primary NO receptor (Montfort *et al.*, 2017). NO has a very short half-life, ranging from milliseconds to a few seconds, depending on the surrounding chemicals (Thomas *et al.*, 2001). Once NO is synthesized, it diffuses rapidly into

the vessel and binds to a haem group of sGC, which results in an increase in cyclic guanosine monophosphate (cGMP) concentration that decreases intracellular Ca and stimulates vascular relaxation (Luiking *et al.*, 2010). The activation of cGMP by NO lowers BP via dilating the resistance type of blood vessels (Lehners *et al.*, 2018) and genetic impairment of this activity result in elevated BP in mouse mutants (Friebe *et al.*, 2007), suggesting that NO-cGMP regulates BP. In platelets, increased cGMP, by the action of NO, inhibits platelet aggregation, activation and adhesion to the vascular wall (Radomski *et al.*, 1987).

NO also inhibits smooth muscle proliferation via preventing platelet-derived growth factors. NO also has anti-inflammatory properties by inhibiting leucocyte adhesion in the vessel wall. Due to these effects, NO plays a primary cardioprotective role and possesses an anti-atherosclerotic action (Omer *et al.*, 2012). These important cardiovascular regulatory actions also explain the close association between decreased NO availability and the pathogenesis of CVD, such as hypertension, stroke and CHD (Naseem, 2005).

The function and effects of NO go beyond its ability to regulate vascular tone: it also plays an important role in the nervous system. NO plays a role as a neurotransmitter in a variety of neuronal functions, including memory and learning processes (Garthwaite, 1993). The neurotransmitter action of NO is achieved by stimulating sGC and the formation of cGMP (Susswein *et al.*, 2004). The importance of the NO-cGMP pathway in memory consolidation has been experimentally tested in various animal models (Kendrick *et al.*, 1997; Harooni *et al.*, 2009). The inhibition of NO synthesis was found to block long-term potentiation, leading to an impairment of memory and learning processes (Böhme *et al.*, 1991).

NO also has several effects that are independent of cGMP. For example, NO can competitively inhibit cytochrome c oxidase, which is the terminal enzyme in the mitochondrial electron transport system (Cleeter *et al.*, 1994); thus it is involved in the regulation of oxygen consumption. In addition, NO has cytotoxic effects against pathogens, including viruses, bacteria and fungi, that occur via a cGMP-independent mechanism (Lundberg *et al.*, 2004). However, these physiological effects of NO might be affected adversely when the body is unable to produce sufficient NO under certain circumstances. In the next section, the mechanisms related to the insufficiency of NO formation in high-risk populations will be discussed.

1.4.4. Insufficiency of nitric oxide with ageing and obesity

The endogenous pathway works efficiently in healthy individuals and can generate sufficient amounts of NO to maintain health (approximately 0.65–1 μmol/kg/h) (Siervo, 2010).

Nonetheless, some conditions such as ageing, and obesity are associated with reduced NO production.

Ageing and obesity result in lower NO availability due to a gradual decrease in the efficiency of the enzymatic synthetic pathway. The ageing effect on NO production is sizable and it has been estimated that endothelium-derived NO is 75% lower in older (70–80 years) individuals compared with healthy young people (20 years) (Egashira *et al.*, 1993). The ageing- and obesity-related NO insufficiency is multifactorial, but oxidative stress has been suggested as the primary factor (Seals *et al.*, 2011; Torregrossa *et al.*, 2011). Oxidative stress causes an imbalance between antioxidant mechanisms and ROS (Friederich *et al.*, 2009). It is well known that ROS play an important role in the ageing process (Liochev, 2013). Ageing-associated mitochondrial dysfunction accounts for about 90% of the total ROS production (Friederich *et al.*, 2009). NO can be easily scavenged by ROS, mainly superoxide, and thus form a potent oxidant called peroxynitrite (Herrera *et al.*, 2010). As a result, the concentration of NO in cells is reduced (Van Der Loo *et al.*, 2000). Furthermore, excessive production of superoxide leads to uncoupling of the NOS system by oxidizing BH_4, which is a key factor for NO production. Consequently, this leads to further superoxide production rather than NO (Luo *et al.*, 2014). The antioxidant defence mechanisms include superoxide dismutase (SOD), catalase and glutathione peroxidase and, under physiological conditions, these help to preserve the balance between NO production and superoxide (Herrera *et al.*, 2010). SOD catalyses the dismutation of superoxide to oxygen and hydrogen peroxide (Younus, 2018). However, these free radical scavenging enzymes are reduced during ageing (Tatone *et al.*, 2006) and in obesity (Torkanlou *et al.*, 2016; Čolak *et al.*, 2019).

In addition to oxidative stress, there are other mechanisms associated with ageing and obesity that could compromise NO availability. For example, NOS expression decreases and arginase (the enzyme that breaks down arginine substrate for NOS) activity increases with ageing (Pie *et al.*, 2002; Berkowitz *et al.*, 2003). Animal and human studies have reported that arginase activity increases with obesity leading to reduced arginine and NO availability (Johnson *et al.*, 2015; El Assar *et al.*, 2016). In addition, ageing and obesity have been associated with higher ADMA concentration, which is an endogenous NOS inhibitor (Holguin, 2013; Rezaei *et al.*, 2019) associated with increased mortality older group (Rezaei *et al.*, 2019).

In older people, reduced NO availability is associated with increased CVD risk, including hypertension, atherosclerosis, heart failure and thrombosis, and these risks may be accelerated by obesity (Jura and Kozak, 2016). Some evidence has pointed to an association between impaired NO production and increased risk of AD (Cifuentes *et al.*, 2017). Therefore, given

that NO is a fundamental molecule that helps maintain health and prevent disease, restoring NO homeostasis in high-risk populations may prevent or delay the onset of some diseases. Nutritional supplementation may be an effective therapeutic strategy for preserving physiological functions by increasing circulating NO in older adults.

NO biosynthesis is highly dependent on arginine, which is the main substrate for the enzymatic pathway. The beneficial effect of dietary supplementation with arginine has been studied widely in both animals (Gianotti *et al.*, 1993; Shi *et al.*, 2000; Kohli *et al.*, 2004) and humans (Wolf *et al.*, 1997; Siani *et al.*, 2000). These studies have shown that the benefits of arginine are largely mediated by the arginine-NO pathway (Wu *et al.*, 2009). However, harmful effects have been detected with long-term L-arginine supplementation (Chen *et al.*, 2003; Wilson *et al.*, 2007), suggesting that its effect may depend on the duration of the supplementation (Xiong *et al.*, 2014).

Studies that have investigated the effect of arginine supplementation in older populations are limited and contradictory results have been reported (Chauhan *et al.*, 1996; Blum *et al.*, 2000; Bode-Böger *et al.*, 2003; Wilson *et al.*, 2007). This suggests that this strategy may not be effective with populations at greater risk. A recent systematic review and meta-analysis concluded that oral arginine supplementation did not elicit improvements in blood flow or production of NO metabolites (Rodrigues-Krause *et al.*, 2019).

Researchers have found there is an alternative pathway to produce NO that depends on dietary inorganic NO_3^- intake (a non-enzymatic pathway). A growing body of evidence has shown that inorganic NO_3^- boosts NO availability, and several investigators have found that consumption of food rich in NO_3^- was associated with improvements in several physiological functions. The following sections will discuss the nature of NO_3^-, its dietary sources and how dietary NO_3^- could help boost NO production. In addition, these sections will discuss how dietary NO_3^- was considered a contaminant from the historical perspective.

1.5. Inorganic nitrate and nitrite

Inorganic NO_3^- has a relative molecular mass of 62.005. It is a water-soluble inorganic compound that can be found naturally in water and soil and is a fundamental component of the nitrogen cycle (l'Hirondel, 2001). Fixation of atmospheric nitrogen by plants and animals is important in forming amino acids. Atmospheric nitrogen is converted to ammonia and subsequently to NO_3^- in a series of reactions (Bernhard, 2010). Inorganic NO_3^- is different from organic NO_3^-, which is a commonly used medicine for CVD (e.g. glyceryl trinitrate) (Lundberg *et al.*, 2011). Both NO_3^- and nitrite (NO_2^-) can be produced endogenously in humans via

oxidation of NO. NO_3^- can be formed directly from the reaction between NO and oxyhaemoglobin (Gow *et al.*, 1999), while NO_2^- can be produced through auto-oxidation of NO, which is catalysed by plasma protein ceruloplasmin (Shiva *et al.*, 2006). In 1995, Zweier and associates conducted animal experiments and reported that NO can be formed under acidotic conditions by the reduction of the large pool of systemic NO_2^-, and this formation was not blocked even after NOS inhibition (Zweier *et al.*, 1995). These findings have also been observed in humans after the infusion of 75 mg of sodium NO_2^- into the forearms of healthy individuals, which resulted in increasing blood flow by 175%. Interestingly, they also reported that the generation of NO was not blocked after NOS inhibition by the infusion of NG-monomethyl-L-arginine (L-NMMA; NOS inhibitor). These authors concluded that systemic NO_2^- represents a storage pool for NO generation (Cosby *et al.*, 2003). One year later, Lundberg and Govoni reported that NO_3^- can be used as a substrate for systemic NO_2^- formation after observing a significant increase in plasma NO_3^- and NO_2^- following a NO_3^- load (Lundberg and Govoni, 2004).

All aforementioned studies have suggested that NO_3^- and NO_2^- can be recycled physiologically in tissues to synthesise NO independently of the enzymatic NOS pathway and heavily dependent on entero-salivary circulation of the NO_3^- pathway (Lundberg *et al.*, 2008). This pathway offers a backup system to promote NO production when endogenous NO generation via the NOS pathway is impaired (Carlström *et al.*, 2010), and it is a key focus of current research.

1.5.1. Entero-salivary circulation of nitrate

This pathway is simpler than the enzymatic pathway as it is only based on the reduction of NO_3^- to NO_2^- without multiple co-factors (Lundberg *et al.*, 2008). As mentioned earlier, NO_3^- can be synthesized in our body via the oxidation of endogenously generated NO. NO_3^- is also ingested as part of food consumption. Consequently, circulating NO_3^- levels depend on the activity of NOS and the type of food consumed (Weitzberg and Lundberg, 2013). The dietary sources of NO_3^- will be discussed in more detail in section 1.5.4.

After the ingestion of high-NO_3^- food, the NO_3^- is absorbed rapidly and efficiently from the upper gastrointestinal tract (~100% bioavailability) into the blood (Van Velzen *et al.*, 2008), where it mixes with the endogenous NO_3^- that comes from the oxidation of NO, primarily by oxyhaemoglobin. The concentration of NO_3^- in blood then rises significantly within 15 minutes and reaches a peak after around 30 minutes (Weitzberg and Lundberg, 2013). Blood NO_3^- concentrations remain high for about 5–6 hours, and then start to decline gradually. About 60-

70% of the plasma NO_3^- is excreted via the kidneys in urine. The remaining NO_3^- (about 25%) is actively taken up by the salivary glands and concentrated in saliva (Omar *et al.*, 2015). The exact mechanisms are not known; however, it has been proposed that the sialin protein (SLC17A5) plays a role as a NO_3^- transporter from the blood to the saliva (Qin *et al.*, 2012).

Salivary NO_3^- concentrations are 10–20 times greater than plasma NO_3^- concentrations (Lundberg and Weitzberg, 2009). Salivary NO_3^- is secreted into the oral cavity where ~20% of the NO_3^- (~5% of ingested NO_3^-) is reduced to NO_2^- by facultatively anaerobic bacteria located on the dorsal surface of the tongue (Duncan *et al.*, 1995). Examples of these bacteria are *Actinomyces*, *Staphylococcus*, *Veillonella*, *Propionibacterium* and *Rothia* (Doel *et al.*, 2005), which express NO_3^- reductase enzymes. These bacteria use NO_3^- as an alternative final electron acceptor to obtain ATP in the absence of oxygen. The fundamental role of these bacteria in NO_3^- metabolism has been confirmed in several studies by using antiseptic mouthwash (Govoni *et al.*, 2008; Kapil *et al.*, 2013; Woessner *et al.*, 2016); this will be discussed later in the chapter. As a result of oral NO_3^--reducing bacteria, salivary NO_2^- levels become very high (> 1,000 fold) compared with plasma NO_2^- levels (Lundberg and Govoni, 2004).

Once salivary NO_2^- is swallowed, some NO_2^- is protonated and converted to nitroso acid, which decomposes into NO and other nitrogen intermediates (e.g. nitrosothiols, ethyl nitrite, nitrated fatty acids and nitrosamine) because of the acidity of the stomach environment (Benjamin, 1994). The stomach acidity is fundamental for NO_2^- reduction; thus, prevention of acid secretion by antacid medications adversely affects NO production in the stomach (Lundberg *et al.*, 1994), as will be shown later in this section 1.8.2. There are other factors, such as vitamin C (Carlsson *et al.*, 2001) and polyphenols (reducing compounds) (Peri *et al.*, 2005; Gago *et al.*, 2007), in addition to pH, that may promote and enhance the reduction of NO_2^- to NO and other nitrogen oxides. A description of the entero-salivary pathway is provided in **Figure 1.3.**

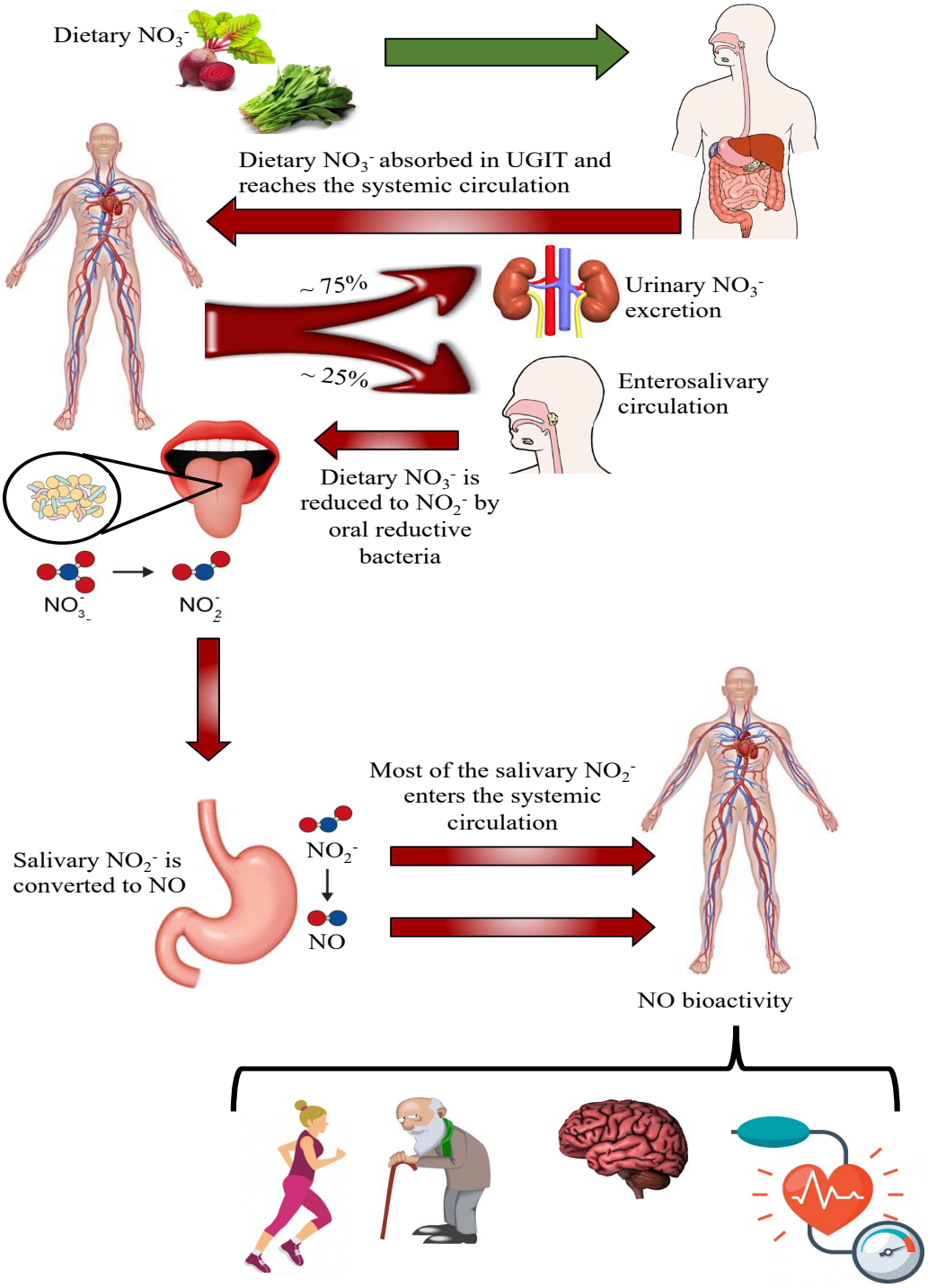

Figure 1.3: Entero-salivary pathway for nitrate (NO_3^-) NO_3^- ingested is rapidly absorbed in upper gastrointestinal tract (UGIT) into the circulation.
Most NO_3^- is excreted by the kidney (70%), while a significant portion is taken up by the salivary glands (25%). Facultative anaerobic bacteria in the mouth reduce NO_3^- to nitrite (NO_2^-). Swallowed NO_2^- is reduced to NO by the acidity in the stomach and elicits a variety of physiological effects in the body. This figure has been modified from (McDonagh *et al.*, 2019).

1.5.2. Generation of nitric oxide from systemic nitrite

After an intake of an oral dose of either NO_3^- or NO_2^-, systemic NO_2^- levels increase rapidly (Larsen *et al.*, 2007). When NO_2^- is swallowed and enters the acidic gastric environment, a small proportion is reduced to NO, while the majority of the salivary NO_2^- is absorbed into the circulation (Lundberg and Govoni, 2004) where it can be further reduced to NO by specialized enzymes with reductase activity. These enzymes include xanthine oxidoreductase, deoxyhaemoglobin, deoxymyoglobin, mitochondrial proteins, carbonic anhydrase, cytochrome P450, eNOS and aldehyde oxidase (Weitzberg and Lundberg, 2013). These reactions are greatly enhanced during conditions like hypoxia and low pH levels (Weitzberg and Lundberg, 2013). The half-life of NO_2^- is about 20 minutes (Weitzberg *et al.*, 2010), and it is considered a stable reserve for NO availability (Lundberg and Weitzberg, 2005). During fasting conditions or after a low NO_3^- diet, the enzymatic pathway accounts for the majority of systematic NO_2^- (Rhodes *et al.*, 1995).

It is important to mention that NO_3^- represents the main dietary source of NO_2^- (Lundberg and Govoni, 2004; Bryan and Ivy, 2015). Furthermore, it has been reported that plasma NO_2^- concentrations increase 4–5 fold after the ingestion of an oral dose of NO_3^-, which is then available as a substrate for systemic NO formation. Greater systemic levels of NO_2^- after NO_3^- intake would be associated with an increased NO generation and associated physiological signalling.

1.5.3. Historical health concerns of nitrate consumption

Inorganic NO_3^- had a notoriously bad reputation as it was considered a toxic component due to its supposed carcinogenic effects and its association with the development of methemoglobinemia in infants (Hmelak Gorenjak and Cencič, 2013). Therefore, the WHO set the adequate daily intake (ADI) of NO_3^- at 3.7 mg/kg of body (WHO, 2003).

It is believed that the carcinogenic effect of NO_3^- is mediated by its reduction to NO_2^-. This observation originated long ago when researchers found the formation of nitrosamine (carcinogenic substance) in some rodents after the ingestion of NO_2^- (Mirvish, 1975). In addition, a small number of cases of lymphoma were observed in rats after chronic NO_2^- ingestion (Newberne, 1979); however, the doses administered were supra-physiological. In 2010, the WHO stated “there is inadequate evidence in humans for carcinogenicity of NO_3^- in food” (WHO, 2010), which means that the available evidence on the association between dietary NO_3^- and the cancer risk is inconclusive and still in doubt. Recent epidemiological studies have not provided evidence that high NO_3^- intake is associated with an increased risk of

cancer. A recent meta-analysis found that the consumption of NO_3^--rich food is associated with a decreased risk of gastric cancers (Song *et al.*, 2015). In addition, another recent meta-analysis found no significant association between NO_3^- intake and several types of cancers (Xie *et al.*, 2016). In 2012, Bryan et al. published a critical review of epidemiological studies and animal toxicology research and they concluded, "Newly published prospective epidemiological cohort studies indicate that there is no association between estimated intake of NO_2^- and NO_3^- in the diet and stomach cancer. This new and growing body of evidence calls for a reconsideration of NO_2^- and NO_3^- safety".

Regarding the associated risk of NO_3^- with methemoglobinemia or cyanosis, this concern was raised based on studies conducted in the 1940s. Methaemoglobin is synthesised when ferrous iron (Fe^{2+}) in haemoglobin is oxidised to ferric iron (Fe^{3+}) by NO_2^-, and this condition leads to anoxia (Fewtrell, 2004). Comly was the first to investigate well water that had contacted NO_3^- and its purported relationship to developing methemoglobinemia in infants aged less than 6 months (Comly, 1945). As a result of this concern, the WHO raised guidelines implementing a safe NO_3^- content in drinking water (≤ 50 mg/L) (Sayre, 1988). This purported relationship was questioned for several years until Avery reported that cases of methemoglobinemia were more likely related to bacterial contamination (Avery, 1999). In the UK and other developed countries, the levels of NO_3^- in drinking water have been strictly controlled to be not more than 50 mg/L, while 44 mg/L in the United States (WHO, 2004a). These guidelines for NO_3^- in drinking water have been recommended to primarily protect infants from developing blue baby syndrome or methemoglobinemia (Bryan and van Grinsven, 2013). Thus, to avoid contaminating the water supply, the agriculture industry has been requested to minimise the use of NO_3^- fertilisers (Directive, 1991).

Concerns about NO_3^- significantly changed when it was discovered that it can be converted to NO inside the body. Today, NO_3^- that is widely contained in our daily food is considered a robust exogenous source for NO availability.

1.5.4. Dietary sources of nitrate

Vegetables contain relatively high concentrations of NO_3^-; thus, they can be considered a main source of NO_3^- intake in humans. Vegetables contribute approximately 60–80% of total NO_3^- intake (Weitzberg and Lundberg, 2013), which is estimated to be about 81–106 mg/day (Coles and Clifton, 2012). The NO_3^- content in vegetables is influenced by two main factors, which are the vegetable species and the NO_3^- content in the soil (Ma *et al.*, 2018). Vegetables can be classified into different categories according to the amount of NO_3^-. For example, spinach,

rocket, beetroot and lettuce are vegetables considered to have a high NO_3^- content (> 1,000 mg/kg), while cabbage, green beans, turnips, cucumbers and carrots have a medium NO_3^- content (100–1,000 mg/kg), and onions and tomatoes are low NO_3^- content vegetables (Lidder and Webb, 2013). **Figure 1.4** shows the amount of NO_3^- in commonly consumed vegetables. The amount of NO_3^- in different vegetables is extremely variable and varies between regions and countries (Ranasinghe and Marapana, 2018). In addition, NO_3^- contents can vary in different samples coming from the same vegetable. These variations are attributed to various environmental factors, such as fertilizer use, seasonal differences, light intensity, temperature, growing conditions, and cooking and storage conditions (Hord *et al.*, 2009; Weitzberg and Lundberg, 2013).

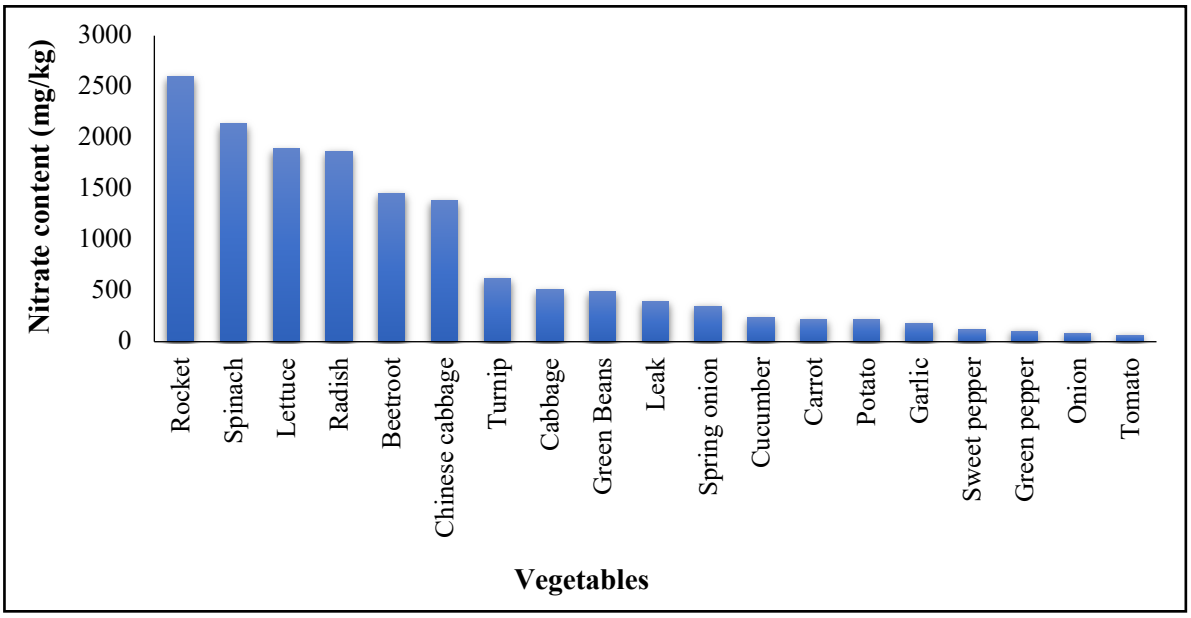

Figure 1.4: Nitrate content of commonly consumed vegetables.
Data for this figure have been obtained from (Lidder and Webb, 2013).

Due to the association between fruit and vegetable intake and reductions in CVD risk, including stroke, hypertension and CHD, the WHO recommends consuming 400 g of fruit and vegetables daily (WHO, 2004b). At the same time, the ADI of NO_3^- recommended by the WHO is 3.7 mg/kg/day (WHO, 2003). This amounts to 240 mg for a 65-kg person. Vegetables contain as much as 1,000-fold more NO_3^- than fruits (Weitzberg and Lundberg, 2013). Therefore, adherence to the recommended daily intake of 400 g of fruit and vegetables, which is mainly green vegetables, would exceed the recommended ADI for NO_3^-. Consuming only 100 g of raw green leafy vegetables per day would contain 300–400 mg of NO_3^-, and this clearly exceeds the ADI for NO_3^- (Weitzberg and Lundberg, 2013; Ranasinghe and Marapana, 2018). Conforming this, high amounts of vegetable intake are a characteristic of several dietary patterns, such as

the the Dietary Approaches to Stop Hypertension (DASH) and a traditional Japanese diet, all of which have been shown to be protective against CVD (Hobbs *et al.*, 2013; Weitzberg and Lundberg, 2013). Adherence to the DASH diet would exceed the ADI for NO_3^- by 700% (Weitzberg and Lundberg, 2013). NO_3^- intake was assessed in 25 healthy individuals after following the traditional Japanese diet over a 10-day period, and researchers found that the dietary NO_3^- provided by this diet exceeded the ADI for NO_3^- by five times (Sobko *et al.*, 2010).

In addition to vegetables, water and food preservatives that are usually added to animal-based products to improve appearance and taste are also sources for NO_3^- intake, and they contribute 15–20% and 10–15% of the total NO_3^- intake, respectively (Weitzberg and Lundberg, 2013). The NO_3^- concentration levels in drinking water differ depending on regional rules regarding safe levels of NO_3^- in water and geographical location (WHO, 2011). NO_3^- levels in bottled mineral waters differ from brand to brand with some containing < 0.1 mg/L, as with Buxton water, while some contain up to 8 mg/L, as with Perrier water. Therefore, usually, Buxton water is recommended for use in interventional studies when there is a need to control NO_3^- intake (Siervo M, Personal Communication).

1.6. Potential physiological effects of dietary NO_3^- in the form of beetroot juice

1.6.1. Blood pressure

The regulation and maintenance of BP is vital, and body NO concentrations are an important factor, as outlined before. The beneficial role of NO as a vasodilator has been explored in several studies. Dietary NO_3^- was shown to play an essential role in vascular control for the first time in 2006, when Larsen et al. showed a mean reduction in DBP of 3.2 mmHg in healthy young individuals following the ingestion of 6.2 mg/kg of sodium NO_3^- for 3 days. The NO_3^- dose used in their study was equivalent to ~150–250 g of NO_3^--rich vegetables. This was the first evidence to demonstrate the physiological effects of dietary NO_3^- on cardiovascular health (Larsen *et al.*, 2006), and, since then, numerous studies have investigated the potential effects of dietary NO_3^- on BP reductions.

In 2008, Webb et al. (2008) confirmed the positive effects of NO_3^- on BP when supplementing healthy individuals with BJ as a rich dietary source of NO_3^- (500 ml providing 1,042 mg NO_3^-), and the SBP and DBP decreased by 10 and 8 mmHg, respectively. A meta-analysis from our group summarised results from clinical trials conducted between 2006 and 2012 and found that dietary NO_3^- was associated with a significant reduction in SBP (-4.4 mmHg) and DBP (-2.2 mmHg) (Siervo *et al.*, 2013). This review also suggested that higher doses of NO_3^- were positively associated with reduction in SBP and the range of NO_3^- doses was from 156–2,790

mg (Siervo *et al.*, 2013). Hobbs et al. (2013), Gee and Ahluwalia (2016), Ashworth et al. (2017) and Jackson et al. (2018) extensively reviewed the studies from 2006 to 2016 and indicated beneficial effects of different forms of NO_3^- supplementation with various doses on BP reduction. These reviews demonstrated that BJ was the dominant source of NO_3^- used and suggested that dietary NO_3^- could be an effective dietary intervention for improving cardiovascular health. They also indicated that administering dietary NO_3^- at doses ranging from 130–1,490 mg is capable of inducing reductions in BP.

The majority of these studies have evaluated the BP-lowering effects of dietary NO_3^- in healthy young adults, but some studies have also reported a BP-lowering effect of dietary NO_3^- in healthy older individuals (Kelly *et al.*, 2013; Raubenheimer *et al.*, 2017; Vanhatalo *et al.*, 2018; Coggan *et al.*, 2019). These effects have also been reported in older adults with various health conditions, such as hypertension (Ghosh *et al.*, 2013; Ashworth *et al.*, 2015; Kapil *et al.*, 2015; Shaltout *et al.*, 2017; Kerley *et al.*, 2018), peripheral artery disease (Kenjale *et al.*, 2011), chronic kidney disease (Kemmner *et al.*, 2017), heart failure (Shaltout *et al.*, 2017) and chronic pulmonary disease (Berry *et al.*, 2015; Curtis *et al.*, 2015; Kerley *et al.*, 2015). Table 1.1 summarizes interventional studies published between 2017 and 2020 that used BJ as a source of dietary NO_3^-.

There are also some studies that have failed to identify beneficial effects of dietary NO_3^- on BP in older individuals (Gilchrist *et al.*, 2013; Bondonno *et al.*, 2015; Shepherd *et al.*, 2015; Blekkenhorst *et al.*, 2018; Oggioni *et al.*, 2018; Kerley *et al.*, 2019; Sundqvist *et al.*, 2020). The discrepancies in these findings may be attributed to several factors. For example, some studies included participants who consumed hypoglycaemic or anti-hypertensive medications, which might interfere with the dietary NO_3^- effects. However, a more recent study suggests that the beneficial vascular effect of dietary NO_3^- was independent of antihypertensive medications, but depends on the degree of BP elevation (Broxterman *et al.*, 2019). From that study, it also appears that BP baseline levels can impact an individual's responsiveness to dietary NO_3^- (Broxterman *et al.*, 2019). According to Gee and Ahluwalia (2016), the lack of dietary NO_3^- efficacy on lowering BP could be associated with a well-controlled BP at baseline. This is also supported by the recent systematic review by Lara and colleagues that showed the hypotensive effects of dietary NO_3^- appear to be enhanced with higher baseline BPs (Lara *et al.*, 2016). In line with this, a recent study conducted by Kerley et al. (2017) showed that BJ selectively reduced BP in uncontrolled but not in controlled hypertensives. According to Ashworth and Bescos "The conclusion that can be drawn from studies reporting non-significant changes to BP is that the overall understanding of the efficacy of NO_3^- supplementation is still unclear,

especially in patients with pre-existing clinical conditions such as obesity" (Ashworth and Bescos, 2017).

There are also other potential factors related to the dose of NO_3^- used or the duration of the intervention. For example, a recent study by Bondonno and colleagues indicated that NO_3^- supplemented at a high dose (800 mg/day for 7 days) in older hypertensive subjects did not produce any beneficial effects on BP. Whereas, in the same year, Kapil et al. (2015) conducted a study in comparable patients and reported a sustained and significant reduction in BP following a daily consumption of 390 mg NO_3^- for 4 weeks that was administered via BJ. Although Kapil et al. used almost half of the dose used by Bondonno et al., it is possible that the three times longer intervention period intensified the NO_3^--induced BP-lowering effect. However, more recent studies have shown that a longer intervention duration is not always effective when lower NO_3^- doses are used. For example, Blekkenhorst et al. (2018) did not observe any BP-lowering effects following 4 weeks of ingestion of NO_3^--rich vegetables (200 g/day containing ~ 150 mg of NO_3^-) in comparison with the ingestion of low NO_3^- vegetables, in hypertensive older adults. Two years later, a larger study conducted by Sundqvist et al. (2020), in comparable patients, indicated that daily consumption of green leafy vegetables or NO_3^- salts containing 300 mg NO_3^- for 5 weeks did not reproduce BP lowering effects.

Collectively, the evidence to date indicates that dietary NO_3^- in the form of BJ has a potential BP-lowering effect under acute conditions in both young and old adults. From 2006 to 2020, only four studies supplemented BJ in older adults for longer than 2 weeks. Therefore, the focus on a prolonged intervention period in older adults, particularly those at greater risk, is warranted. It is also important to further investigate various doses of NO_3^- to indicate a range in which a NO_3^- dose is effective after a long period of supplementation.

Table 1.1: Summary of studies investigating the effect of dietary nitrate in the form of BJ on blood pressure and other relevant variables

Study*	Subjects characteristics	Study design	Beetroot juice dosage[1]	Duration	Blood pressure (mmHg)	Other findings
Studies conducted on older adults						
Raubenheimer *et al.*, (2017)	12 healthy older adults (age range 57-71)	RCT, double-blind, crossover trial	1-BJ (140 ml, ~12.6 mmol NO_3^-) 2- PL	Single bolus	SBP, DBP, and MAP reduced ($P < 0.05$)	Plasma NO_3^- and NO_2^- increased.
Kemmner *et al.*, (2017)	17 CKD old patients aged 72 ± 6, BMI: 32.6 ± 3.6 kg/m^2	RCT, open label, crossover trial	1-BJ (200 ml, 300 mg NO_3^-) 2-Water	Single bolus	SBP reduced by 8.2 ($P < 0.05$). DBP reduced by 8.5 ($P < 0.05$).	Renal resistive index reduced ($P < 0.05$).
Shaltout *et al.*, (2017)[3]	20 old patients with HFpEF aged 69 ± 7, BMI 33.5±5.8 kg/m^2	Pilot, RCT trial	1-BJ (70 ml, ~ 6.1 mmol NO_3^-) + exercise 2- PL+exercise	3 days/ week for 4 weeks	Resting SBP was reduced following both interventions ($P < 0.05$).	Plasma NO_3^- and NO_2^- increased following BJ. Dietary NO_3^- in form of BJ did not represent and additive beneficial effect when combined with exercise.
Shaltout *et al.*, (2017)[3]	26 controlled hypertensive old adults aged 65±5, BMI 32.3 ±4.6 kg/m^2	RCT, double-blind trial	1-BJ (70 ml, ~ 8 mmol NO_3^-) + exercise 2- PL+exercise	3 days/ week for 6 weeks	Resting SBP reduced following BJ ($P < 0.05$), but the between group comparison was not significant.	Plasma NO_3^- and NO_2^- increased following BJ. Dietary NO_3^- in form of BJ did not represent and additive beneficial effect when combined with exercise.
Kerley *et al.*, (2017)	11 controlled hypertensive aged 60.9 ± 9.1, BMI 30.8 ± 2.4 and	Uncontrolled, pilot study,	BJ (140 ml, ~ 800 mg NO_3^-)	14 days	Ambulatory BP reduced only in uncontrolled hypertensive ($P < 0.05$).	Serum NO_3^- and NO_2^- increased following BJ. AASI and LDL cholesterol reduced in uncontrolled hypertensives only.

Study*	Subjects characteristics	Study design	Beetroot juice dosage[1]	Duration	Blood pressure (mmHg)	Other findings
	8 uncontrolled hypertensive aged 49.3 ± 14.5, BMI 29.5± 6					
Kerley *et al.*, (2018)	20 uncontrolled hypertensive aged 62.5 ± 13.1, BMI: 30.7 ± 5.8, Baseline BP: 137/80	RCT, double-blind, crossover trial	1-BJ (140ml, 800 mg NO_3^-) 2- PL	7 days	SBP reduced by 8 ($P < 0.05$). DBP reduced by 4 ($P < 0.05$).	Plasma NO_2^- levels increased following BJ.
Vanhatalo *et al.*, (2018)	9 healthy old adults aged 75 ± 3. And 9 young adults aged 20 ± 1	RCT, double-blind, crossover trial	1-BJ (140 ml, ~800 mg NO_3^-) 2- PL	10 days	SBP and DBP reduced in old but not young participants ($P < 0.05$).	Plasma NO_3^- and NO_2^- increased following BJ in old and young adults. Salivary microbiome was altered in young and old adults following BJ.
Ramick *et al.*, (2018)[2]	15 CKD older adults aged 61±4	RCT, double-blind, crossover trial	1-BJ (~12.6 mmol NO_3^-) 2- PL	Single bolus	MAP reduced ($P < 0.05$).	Plasma NO_3^- and NO_2^- increased following BJ. Microvascular function was improved. PWV or EF was not affected.
Caldwell *et al.*, (2019)	10 hypertensive post-menopausal women aged	RCT, double-blind, crossover trial	1-BJ (140 ml, 800 mg NO_3^-) 2- PL	Single bolus	No effect	Plasma NO_2^- increased following BJ. Improvement functional sympatholysis by ~50% .

Study*	Subjects characteristics	Study design	Beetroot juice dosage[1]	Duration	Blood pressure (mmHg)	Other findings
	56±1, BMI 31 ± 5 kg/m^2					
Coggan *et al.*, (2019)[2]	6 healthy older aged 69±3	RCT, double-blind, crossover trial	1-High dose BJ (~1750 mg NO_3^-), 2-Low dose BJ (~850 mg NO_3^-) or 3- PL	Single bolus	MAP in: High BJ reduced by 8 ($P < 0.05$) Low BJ educed by 4 ($P > 0.05$).	Enhance muscle contractility with lower dose.
Kerley *et al.*, (2019)	8 CKD old patients aged 62.9 ± 7.1 , BMI: 25.6 ± 5.1 kg/m^2	RCT, double-blind, crossover trial	1-BJ (800 mg NO_3^-) 2- PL	14 days	No effect	Plasma NO_3^- and NO_2^- increased in BJ. Pulmonary function was not affected.
Kim *et al.*, (2019)	13 healthy postmenopausal women aged 63 ± 1 And a reference control cohort of 10 young premenopausal women aged 22 ± 1	RCT, double-blind, crossover trial	1-BJ (140 ml, ~9.7 mmol NO_3^-) 2- PL	Single bolus	In post-menopausal women, brachial and aortic SBP reduced after BJ ($P < 0.05$).	Plasma NO_3^- and NO_2^- increased following BJ. The pulse pressure amplification increased in post-menopausal women. PWV was not affected.

Study*	**Subjects characteristics**	**Study design**	**Beetroot juice dosage[1]**	**Duration**	**Blood pressure (mmHg)**	**Other findings**
La Salle *et al.*, (2019)[2]	14 controlled hypertensive aged 53±11 and 14 uncontrolled hypertensive aged 49±13	RCT, double-blind, crossover trial	BJ and PL (dosage NR)	3 days	SBP, DBP and MAP reduced significantly in uncontrolled hypertensive ($P < 0.05$), but not on controlled hypertensive.	Leg blood flow increased during exercise in uncontrolled hypertensive following BJ. *Biological fluid results were NR.
Walker *et al.*, (2019)	15 healthy older males aged 69 ± 4	RCT, double-blind, crossover trial	1-BJ (140 ml, ~800 mg NO_3^-) 2- PL	Single bolus	No effect	Plasma NO_3^- and NO_2^- increased following BJ. PWV was not affected. EF was improved.
Broxterman *et al.*, (2019)	13 controlled hypertensive aged 53 ± 12 14 uncontrolled hypertensive aged 49 ± 13	RCT, double-blind, counterbalanced measures trial	1-BJ (70 ml, ~6.2 mmol NO_3^-) 2- PL	3 days	No effect	Plasma NO_3^- and NO_2^- increased following BJ. EF was not affected.
Stanaway *et al.*, (2019)	11 healthy older adults aged 56 ± 6 13 healthy young adults aged 25 ± 3	RCT, double-blind, crossover trial	1-BJ (150 ml, ~10.5 mmol NO_3^-) 2- PL	Single bolus	SBP reduced in old and young adults ($P < 0.05$), but no interaction effect was found. DBP reduced in old adults ($P < 0.05$).	Plasma NO_3^- and NO_2^- increased following BJ. Reaction time was improved in the Stroop test following BJ for both groups.

Study*	Subjects characteristics	Study design	Beetroot juice dosage[1]	Duration	Blood pressure (mmHg)	Other findings
Jones *et al.*, (2019b)	20 healthy older adults aged 63 ± 6	RCT, double-blind, crossover pilot trial	1-BJ (70 ml, 400 mg NO_3^-) 2- PL	2 weeks	SBP reduced by 6 DBP reduced by 4 Both *(*$P < 0.05$).	Plasma NO_3^- increased. Findings confirmed the presence of NO_3^- reducing bacteria in the oral cavity. No effect was detected on microvascular function.
Siervo *et al.,* (2020)	47 middle-aged and older participants aged 61 ± 6, BMI 28 ± 5 kg/m^2)	RCT, double-blind, paralleled feasibility trial	1-BJ (70 ml, 400 mg NO_3^-)+folic acid (∼5 mg) 2-BJ+ PL 3- NO_3^-depleted BJ+ PL	2 months	24-h SBP reduced by −10.8 ($P < 0.001$), −6.1 ($P < 0.05$).), and −0.3 ($P > 0.05$).) in the BJ + PL, BJ + folic acid, and NO_3^-depleted BJ+Placebo groups, respectively. There was a significant decrease in 24-h DBP in the BJ + PL group (−5.4, $P < 0.05$).	Compliance to the interventions was high (>90%) in all groups. A significant increase in NO_3^- and folic acid concentrations in plasma and saliva samples.
Studies conducted on younger adults						
Bailey *et al.*, (2017)	9 healthy adults aged 20±1	RCT, crossover trial	1-BJ (140 ml, 800 mg NO_3^-)+Potassium gluconate (198 mg), 2-BJ (140 ml, 800 mg NO_3^-)+Potassium Iodide (450 µg) or	6 days	SBP reduced in BJ and BJ with iodide ccompared to control ($P < 0.05$). DBP reduced only in BJ compared to PL ($P < 0.05$).	Salivary and plasma NO_3^- and NO_2^- increased in BJ and BJ with iodide compared to control. Iodide supplementation did not compromise the hypotensive effects of nitrate supplementation.

Study*	Subjects characteristics	Study design	Beetroot juice dosage[1]	Duration	Blood pressure (mmHg)	Other findings
			3-Control			
Shannon *et al.*, (2017)	8 trained young male aged 28.3 ± 5.8	RCT, double-blind trial	1-BJ (140 ml, ~12.5 mmol NO_3^-) 2- PL	Single bolus	No effect	Plasma NO_3^- and NO_2^- increased following BJ. BJ improved performance only during shorter compared to longer-distance treadmill running time-trials.
Eglin *et al.*, (2017)	14 healthy young participants aged 25.8±4.2	RCT, double-blind, crossover trial	1-BJ (140 ml, ~11.9 mmol NO_3^-) 2- PL	Single bolus	No effect	Plasma NO_3^- and NO_2^- increased following BJ. Extremely rewarming, EF were not affected.
Triantafyllou *et al.*, (2017)[2]	13 untreated hypertensive aged 42.3 ± 12.3	RCT, double-blind, crossover trial	1-BJ (8.1 mmol NO_3^-) 2- PL	Single bolus	SBP and DBP reduced *(*$P < 0.05$). *was measured during exercise.	-
Ormesher *et al.*, (2018)	40 pregnant women	RCT, double-blind, feasibility trial	1-BJ (70ml, 400 mg NO_3^-) 2- PL	8 days	No effect	Salivary and plasma NO_3^- and NO_2^- increased in BJ. A highly significant correlation between changes in plasma NO_2^- concentrations and changes in DBP after BJ.
Kent *et al.*, (2018)	12 trained cyclists aged 27 ± 6	RCT, double-blind repeated-measures, counter-	1-BJ (6.5 mmol NO_3^- for 2 days and 13 mmol NO_3^- 2 h prior to exercise)	2 days	No effect	Salivary NO_3^- and NO_2^- increased following BJ. No differences in skin blood flow or muscle oxygenation.

Study*	Subjects characteristics	Study design	Beetroot juice dosage[1]	Duration	Blood pressure (mmHg)	Other findings
		balanced fashion	2- PL			
Amano *et al.*, (2018)	8 heathy young adults aged 24 ± 4	RCT, double-blind, crossover trial	1-BJ (140 ml, ~8 mmol NO_3^-) 2- PL	3 days	MAP was not affected at rest but reduced during exercise following BJ ($P < 0.05$).	Plasma NO_3^- and NO_2^- increased following BJ. Local sweating and cutaneous vascular responses were not affected.
Wong *et al.*, (2018)	7 healthy young adults (age range 20-26)	Uncontrolled pilot trial	BJ (70 ml, ~5 mmol)	3 days	No effect was observed on SBP and MAP. DBP reduced ($P < 0.05$).	Cutaneous reactive hyperaemia was not affected *Biological fluid results were NR
Craig *et al.*, (2018)	9 healthy young adults aged 25 ± 2	RCT, double-blind, crossover trial	1-BJ (140 ml, ~13 mmol NO_3^-) 2- PL	Single bolus	SBP reduced by 7 DBP reduced by 4 MAP reduced by 6% (all $P < 0.05$).	Plasma NO_2^- increased following BJ. exercise tolerance was not affected. Muscle blood flow was not affected during exercise.
Kukadia *et al.*, (2019)	15 healthy adults aged 29.2 ±8.3,	RCT, double-blind, crossover trial	1- BJ (70 ml, ~ 400 mg NO_3^-). 2- PL	Single bolus	SBP reduced by 5.2 (CI 1.9-8.5). No effect on DBP.	-
de Lima Bezerra *et al.*, (2019)	14 obese adults aged 25.3 ± 4.7, BMI 35.8 ± 3.3 kg/m^2	RCT, crossover trial	1-BJ (200ml, ~ 800 mg NO_3^-)+exercise. 2-Fruit soda (200 ml) +exercise. 3-Water (200 ml)	Single bolus	Ambulatory SBP reduced by 5.3 ($P < 0.05$). No effect on DBP.	The plasma NO_3^- and NO_2^- following BJ.

Study*	Subjects characteristics	Study design	Beetroot juice dosage[1]	Duration	Blood pressure (mmHg)	Other findings
de Vries and DeLorey, (2019)	12 young male aged 23 ± 5	RCT, double-blind, crossover trial	1-BJ (~12.6 mmol NO_3^-) 2- PL	Single bolus	No effect	Plasma NO_3^- and NO_2^- increased following BJ. Sympathetic vasoconstrictor responsiveness was not affected.
Zafeiridis *et al.*, (2019)	18 drug-naïve hypertensives aged 44 ± 2.6, BMI 28.5 ± 1.2	RCT, double-blind, crossover trial	1-BJ (500 mg NO_3^-) 2- PL	Single bolus	SBP and DBP reduced at rest and during exercise following BJ ($P < 0.05$).	Plasma NO_2^- increased following BJ. PWV was not affected. Muscle microvascular reactivity was improved.
Burleigh *et al.*, (2019)[2]	11 healthy males aged 30 ± 7	RCT, double-blind, crossover trial	1-BJ (70 ml, ~6.2 mmol NO_3^-) 2- PL	7 days	BP decreased following BJ ($P < 0.05$).	Plasma and salivary NO_3^- and NO_2^- increased following BJ. EF was improved. BJ supplementation results in meaningful alterations to the oral microbiome.
Fowler *et al.*, (2020)	11 healthy males aged 25 ± 5	RCT, double-blind, crossover trial	1-BJ (9.2 mmol NO_3^-) 2- PL	5 days	MAP reduced following BJ *(*$P < 0.05$).	Plasma NO_3^- and NO_2^- increased. No difference was detected on exercise tolerance test in heat.
O'Gallagher *et al.*, (2020)	11 healthy males aged 23 ± 4	RCT, single-blind, crossover trial	1-BJ (70 ml, 400 mg NO_3^-)+GF(250 ml) 2-BJ+Buxton water 3-PL+GF	Single bolus	SBP reduced in BJ+GF compared to BJ+Buxton water and PL+GF *(*$P < 0.05$).. DBP increased in BJ+GF compared to BJ+Buxton	Plasma and salivary NO_2^- decresed in BJ+GF compared to BJ+Buxton water.

Study*	Subjects characteristics	Study design	Beetroot juice dosage[1]	Duration	Blood pressure (mmHg)	Other findings
					water (P < 0.05), but DBP decreased in PL+GF compared to BJ+GF (P < 0.05).	
Nogueira Soares *et al.*, (2020)	13 HIV-infected aged 36±10 old) and 18 healthy adults aged 27±8	RCT, double-blind, crossover trial	BJ and PL (dosage NR)	Single bolus	No effect	BJ improved FMD in HIV and control groups. No difference was detected on PWV.
van der Avoort *et al.*, (2020)	30 healthy adults aged 24±6	Randomised, crossover trial	1-BJ (~400mg) 2- NO_3 rich vegetables (~400 mg)	1 week	SBP and DBP decreased throughout both intervention (P < 0.05), with no differences between BJ and NO_3^- -rich vegetables	Plasma NO_3^- and NO_2^- increased with both interventions.

AASI; ambulatory arterial stiffness index, AS; Arterial stiffness, BJ; Beetroot juice, BMI; Body mass index, CKD; Chronic kidney disease, DBP; Diastolic blood pressure EF; Endothelial function, FMD; Flow mediated dilation, GF; Grapefruit juice, HFpEF; Heart failure with preserved ejection fraction, NO_3^- ; nitrate, NO_2^-; nitrite, NR; not reported LDL; Low density lipoprotein, PL; Placebo, PWV; Pulse wave velocity, RCT; Randomised clinical trial; SBP; Systolic blood pressure,T2D; Type 2 diabetes.

* Table shows published studies between 2017 and 2020.

[1]The dosage of BJ reported as in the published papers

[2] This is an abstract

1.6.2. Cognitive function and CBF

Lower CBF is associated with CVD and with vascular risk factors, and is a potentially important contributor to cognitive decline and dementia (Leeuwis *et al.*, 2018). Reduced NO availability is associated with reduced cerebral perfusion, which could lead to disruption of neurovascular function (Poels *et al.*, 2008). In addition, decreased NO synthesis could impair cerebral energy metabolism (e.g. by reducing glucose delivery to the brain), which increases the risk of neurodegeneration and cognitive deficits (Clifford *et al.*, 2015). These findings have stimulated further research into the effects of dietary NO_3^- interventions aimed at targeting the NO pathway on CBF as a strategy to protect cognitive function especially in older people.

Pre-clinical studies have indicated that NO_2^- infusion is associated with an increased CBF (Rifkind *et al.*, 2007). In 2011, Presley and co-authors were the first to examine the effect of dietary NO_3^- on CBF in healthy older adults. They found that high NO_3^- consumption for 3 days increased the perfusion in the frontal cortex, the region of the brain that is responsible for control of executive function, but cognitive function was not assessed in that study (Presley *et al.*, 2011). Several other studies then assessed the effect of supplemental dietary NO_3^-, mainly via BJ, on CBF in both young and older adults, but equivocal findings were reported.

In 2013, Kelly and co-authors conducted a double-blind, randomized, crossover study in healthy older adults and found that BJ supplementation for 3 days did not alter cognitive function (Kelly *et al.*, 2013). A longer-duration study with a similar design was conducted in older diabetic patients and found that the daily ingestion of BJ for 14 days improved reaction time. However, other measures of cognitive performance, including rapid processing, memory and decision time, were not affected (Gilchrist *et al.*, 2015), which may suggest NO_3^- supplementation in older adults improves certain specific components of cognitive function only. Stanaway et al. (2017) suggested that the discrepancies in findings between Gilchrist et al. and Kelly et al. can be related to the duration of the intervention, as Gilchrist et al. used a much longer intervention period compared to Kelly et al. Furthermore, the older participants with clinical conditions, such as diabetes, may be more responsive to supplemental NO_3^- than healthy older adults . Justice et al. (2015) showed that 10 weeks of NO_2^- supplementation improved cognitive performance (reduced the time to complete TMT tasks) in both middle-aged and older adults.

The effect of dietary NO_3^- on cognitive function has also been investigated in younger adults, and the majority of these studies have seen the effect of dietary NO_3^- in combination with exercise. All studies were reviewed systematically as part of this PhD project, and a meta-

analysis was conducted to identify the effect of dietary NO_3^- on cognition and CBF. The findings are reported in Chapter 3.

1.7. Beetroot juice as a nitrate supplement in long-term interventional trials

There are a variety of dietary NO_3^- sources, but the most widely used source in research is BJ. In the literature, two types of BJ with different volumes have been used, either unconcentrated BJ with 250–500 ml (Webb *et al.*, 2008) or 70 ml concentrated shots (Jajja *et al.*, 2014). Due to different processing techniques and various seasons of harvest, the NO_3^- content in BJ also varies. A recent study measured NO_3^- content in 16 commercial juices and large variations were found, ranging from 0.01 to 2.4 g/L (Wruss *et al.*, 2015). This discrepancy in NO_3^- levels was also reported in concentrated BJ shots that were provided from the same company. The analysis by Jajja et al. (2014) showed that the bottles contained 165 ± 2 mg of NO_3^-, while 400 mg was reported on the bottles. The issue of these variations in NO_3^- content can be of concern, especially if different batches were used, which sometimes is unavoidable, particularly in long-term studies. This should generally be an acknowledged limitation of research using such products.

There are several advantages of using BJ in research. For instance, it is easily transportable, practical and more convenient for consuming effective concentrations of NO_3^- in relatively small amounts of drink. Dietary NO_3^- administration in the form of vegetable beetroot needs to be offered in large amounts to reach similar effective NO_3^- concentrations. As a result, it might be difficult for participants to comply, particularly in longer-term interventions. Recently, a NO_3^- depleted BJ, which is indistinguishable from the normal BJ, was developed specifically for research purposes, and not for public use. This is considered a major benefit of using BJ in research, allowing randomized placebo-controlled double-blind interventional trials, which might be a major challenge when using a raw vegetable in research.

In recent years, other forms of beetroot have been developed and used in research, such as beetroot-enriched bread (Hobbs *et al.*, 2012), beetroot gel (Silva *et al.*, 2016) and beetroot powder and chips (Vasconcellos *et al.*, 2016). These were all effective in increasing the NO metabolites in biological fluids, thus NO availability, and likely to have favourable physiological effects, like BP reduction. However, the creation of new beetroot products needs to be tested in further studies. All aforementioned studies have tested these products in acute application only, and it is unclear whether they will have longer-term acceptance. In addition, to establish a real comparison, the NO_3^- content should be matched in these sources. Vasconcellos et al. (2016) compared the NO_3^- contents of four different forms of beetroot (BJ,

chips, beetroot powder and cooked beetroot) and found the NO_3^- content was significantly higher in BJ than in the other sources.

1.8. Aspects to consider when conducting dietary nitrate interventional studies

Dietary NO_3^- consumption can increase the level of NO bioavailability, and thus, may have beneficial effects on cardiovascular health. Nevertheless, some factors generated from daily habitual practices and some lifestyle choices are known to disrupt the enterosalivary circulation of NO_3^- and can exert a major influence on the effectiveness of the NO_3^- physiological response. These factors need to be considered when designing a protocol for NO_3^- intervention studies.

1.8.1. Antiseptic mouthwash & antibiotics

As mentioned earlier, dietary NO_3^- can raise the level of NO precursors, such as NO_2^-, in biological fluids, and this action is usually associated with some physiological effects, such as reductions in BP. Circulating NO_2^- is a storage pool for NO. The presence of NO_2^- is dependent on the action of oral bacteria that use NO_3^- as a final electron acceptor in their respiration, as mentioned earlier. Therefore, the elimination of or reduction in commensal oral bacteria will affect the reduction of dietary NO_3^- to NO_2^- and consequently to NO.

Govoni and co-authors showed that the acute use of antibacterial mouthwash (chlorhexidine), which could be a daily routine on the part of some individuals, significantly attenuates the rise in plasma and salivary NO_2^- concentrations after an acute consumption of 10 mg of sodium NO_3^- (Govoni *et al.*, 2008). Similar results have been found by Petersson's group when they used chlorhexidine mouthwash spray in rats twice daily for 5 days while they were giving 140 mg of NO_3^- dissolved in drinking water (Petersson *et al.*, 2009). The use of mouthwash was also found to eradicate the antihypertensive effects of dietary NO_3^- (Petersson *et al.*, 2009).

Studies continued in this area in 2013 when Kapil and co-authors found that rinsing the mouth with chlorhexidine mouthwash twice daily for 7 days decreased plasma and salivary NO_2^- concentrations by 25% and 90%, respectively (Kapil *et al.*, 2013). This reduction in NO_2^- concentration was accompanied by an increase in BP. These findings were confirmed in older treated hypertensives by Bondonno's group, who reported that using antibacterial mouthwash over 3 days decreased oral NO_3^- reduction and resulted in higher BP in adults (Bondonno *et al.*, 2014b). However, Sandqvist et al. (2016) did not observe an alteration in BP level after 3 days of antibacterial mouthwash in healthy females. Studies then focused on testing the effect of different types of mouthwashes, based on their strength, on dietary NO_3^- reduction activity and its beneficial vascular effect. They found that different strengths of mouthwash may have

different effects on salivary and plasma NO_2^- concentrations, and the stronger mouthwashes, like chlorhexidine and Cepacol, increase BP compared to control and weaker antiseptic mouthwashes (McDonagh *et al.*, 2015; Woessner *et al.*, 2016). This finding was confirmed in a recent systematic review that included eight studies and investigated relationships between mouthwash use and the pharmacokinetics of NO_3^- and NO_2^- on BP. The authors concluded that mouthwashes of varying strengths had differential effects on outcome measures. In addition, they showed that salivary and plasma NO_3^- and NO_2^- concentrations were altered negatively with mouthwash use, with an associated rise in BP (Senkus and Crowe-White, 2019).

The chronic use of mouthwash has also been evaluated. Recent studies have found that the disruption of entire salivary NO_3^- reduction by chronic mouthwash use over 3 years is associated with an increased risk of diabetes and hypertension (Joshipura *et al.*, 2017; Joshipura *et al.*, 2019). In addition, it has been found that using mouthwash was associated with reduced insulin sensitivity in obese individuals (Beals *et al.*, 2017).

In addition to the effect of antibacterial mouthwash, the use of the broad spectrum of antibiotic has been found to disturb the enterosalivary circulation of NO_3^- and attenuate the production of salivary NO_2^-. Dougal and co-authors found that administration of amoxicillin in 10 healthy adults reduced salivary NO_2^-, following a 200 mg of potassium NO_3^- (Dougall *et al.*, 1995). Subsequent enterosalivary pathway-derived adverse events following antibiotic exposure remain unexplored.

From these findings, the observations provide strong evidence of the fundamental role of dietary NO_3^- reductase activity by oral microflora. The elimination of these bacteria by an antibacterial mouthwash or antibiotics may impact NO bioavailability and, thus, its associated beneficial effects.

1.8.2. Stomach acidity

Even if oral bacteria work efficiently and reduce ingested NO_3^- to NO_2^-, there is another factor that should also be considered if one wishes to obtain optimal effects of dietary NO_3^-. This factor is stomach acidity. As described earlier, most of the gastric juice NO_2^- is derived mainly from dietary NO_3^-, and this swallowed salivary NO_2^- becomes protonated in the acidic stomach to form NO. Thus, any disturbance of gastric acidity may impair the NO_3^- to NO_2^- to NO pathway. Previous ex vivo studies found that at a stomach pH of 1.5 or 2.5 (with the presence of ascorbic acid), the majority of salivary NO_2^- is converted to NO within a few seconds (~10 s), whereas the formation of NO becomes slower at a pH of 3.5 and is substantially reduced at a pH of 4.5 (Iijima *et al.*, 2003). In addition, as gastrointestinal dysfunction is a consequence of

critical illness, a study by Boivin et al. found that a failure in gastric acidification in critically ill patients was responsible for markedly decreased NO concentrations in the stomach, compared with the healthy control group (Boivin *et al.*, 2006).

Proton pump inhibitors (PPIs), which are a type of antacid, have been shown to shut down NO production by inhibiting stomach acid production, and thus, increasing gastric pH. The same study by Biovin et al. found that, after treating a healthy control group with Omeprazole, gastric NO production was significantly reduced (Boivin *et al.*, 2006). Interestingly, there is evidence that using PPIs, such as Omeprazole, almost completely abolishes the BP-lowering effects of orally administered NO_2^- (Pinheiro *et al.*, 2012). Pinherio and co-authors suggested that PPI impairs the cardiovascular responses to dietary NO_3^- and reduces NO activity (Pinheiro *et al.*, 2014). In agreement with this suggestion, a recent study by Lundberg's group found that the robust reduction in SBP following the ingestion of sodium NO_2^- was abolished after the administration of esomeprazole in healthy adults (Montenegro *et al.*, 2017).

The use of PPIs has increased significantly among older adults owing to the increased incidence of gastric ulcers (Fezzi *et al.*, 2009). However, this marked increase in using these medications is not entirely due to gastrointestinal disorders, which suggests that many older people are using these medications inappropriately (Hamzat *et al.*, 2012). It is possible that the beneficial cardiovascular effects following the ingestion of NO_3^--rich vegetables, such as beetroot, may be lost in those older groups who use PPIs due to an impairment of the NO_3^- to NO_2^- to NO pathway (Montenegro *et al.*, 2017). Effects of PPIs may extend beyond the NO exogenous pathway from dietary NO_3^-. Some evidence has indicated that even endogenous NO formation is affected by PPIs by increasing ADMA levels in human endothelial cells (Ghebremariam *et al.*, 2013; Montenegro *et al.*, 2017). Therefore, using such antacids can be a confounding factor for effective dietary NO_3^- intervention, and this needs to be considered, especially when targeting older individuals for a dietary NO_3^- intervention study.

1.8.3. Smoking

As described earlier, the remaining ingested NO_3^- (~25%) is actively taken up by the salivary glands from the systemic circulation and then concentrated in the oral cavity, but the exact mechanism is still not fully understood. There is some evidence that circulating thiocyanate (SCN^-), whose levels increase with cigarette smoking, competitively inhibits salivary NO_3^- uptake (Mervish *et al.*, 2015). Accordingly, increased exposure to SCN^- could interfere with dietary NO_3^- metabolism and may consequently decrease the effectiveness of the NO_3^- physiological response.

Previous studies have indicated that salivary NO_3^- concentration is lower in cigarette smokers than in non-smokers after NO_3^- consumption (Ladd *et al.*, 1984; Knight *et al.*, 1987a). A recent study evaluated the effect of NO_3^- supplementation on salivary and plasma NO_2^-, NO_3^- and SCN^- levels, as well as BP levels, in smokers and non-smoking controls. They demonstrated that an increase in plasma NO_2^- concentration and a decrease in BP were seen only in non-smokers (Bailey *et al.*, 2016). The attenuation of the increase in salivary and plasma NO_3^- in smokers was associated with higher levels of salivary and plasma SCN^- (Bailey *et al.*, 2016). Therefore, to investigate the beneficial effect of dietary NO_3^-, tobacco smoking should be controlled and the exclusion of smokers might be needed in designing a dietary NO_3^- intervention study.

Serum SCN^- also increases after ingesting vegetables of *Brassica*, such as cabbage, broccoli and cauliflower. As a result, some researchers have hypothesised that the consumption of SCN^--rich vegetables with NO_3^--rich vegetables would be associated with an impaired NO_3^- metabolism and its protective effects (Dewhurst-Trigg *et al.*, 2018). Interestingly, it was found that the consumption of these vegetables together prevents an increase in salivary NO_2^-, but not NO_3^-, and prevents the hypotensive effects observed with NO_3^--rich vegetables without SCN^--rich vegetables (Dewhurst-Trigg *et al.*, 2018). Although the consumption of these vegetables might be considered a confounder in testing the effects of dietary NO_3^-, it would be difficult to control the dietary habits of participants, particularly in longer-term interventional studies.

1.9. Hypotheses, aims and objectives

The overall hypothesis for this PhD project was that prolonged supplementation with dietary NO_3^- will reduce BP and will enhance brain health in older individuals at greater risk of cognitive dysfunction. In addition, I hypothesised that such effects would occur in a dose-dependent manner.

The aim of this project was to test the above hypotheses by investigating the effect of prolonged BJ supplementation (as rich source of dietary NO_3^-) in a range of doses over a 13-week period on BP, cognitive function and CBF in older overweight and obese people.

The specific objectives of this project included:

1) To carry out a systematic review to assess dietary daily NO_3^- intake in humans.
2) To carry out a systematic review and meta-analysis to examine the effects of dietary NO_3^- and NO_2^- on cognitive function and CBF.
3) To undertake a small validation study on the accuracy and reliability of salivary NO_2^- strips that can be used as a monitoring tool for NO_3^- intake during prolonged dietary NO_3^- intervention studies. This study also investigated the effect of antibacterial mouthwash use on utility of these strips.
4) To evaluate the feasibility and acceptability of a prolonged intervention randomised trial with BJ in a range of doses in older overweight and obese individuals.
5) Within the study outlined in 4) above, to undertake preliminary investigation of whether prolonged BJ supplementation improves BP, cognitive function and CBF.

Chapter 2. Assessment of dietary nitrate intake in humans: a systematic review

This chapter has been adapted with some modification from our published paper in *American Journal of Clinical Nutrition:*

Babateen, A.M., Fornelli, G., Donini, L.M., Mathers, J.C. and Siervo, M. (2018) 'Assessment of dietary nitrate intake in humans: a systematic review', *The American Journal of Clinical Nutrition*, 108(4), pp. 878-888 (**Appendix 2.1**).

2.1. Introduction

Inorganic NO_3^- is a water-soluble inorganic compound that can be naturally found in water and soil as part of the nitrogen cycle (l'Hirondel, 2001). NO_3^- and NO_2^- are commonly used as food additives in processed meats to increase shelf life and to avoid bacterial growth, which, in addition, causes the pink coloration of processed meats (Nummer and Andress, 2002). High consumption of processed meats has been linked with greater risk of cancer of the upper gastrointestinal tract, which may be due to the formation of the highly reactive and mutagenic N-nitrosamines (Spiegelhalder *et al.*, 1976; Tannenbaum *et al.*, 1976; Lin, 1990). As a result of these detrimental effects, the WHO established the ADI of NO_3^- as 3.7 mg/kg body weight (WHO, 2003), and this estimate was confirmed recently by the European Food Safety Authority (EFSA, 2017). However, diets with a high NO_3^- content, such as vegetarian or DASH dietary patterns, are associated with beneficial effects on cancer risk and other health outcomes (Key *et al.*, 2009; Katz and Meller, 2014), and current reviews have confirmed a lack of association between dietary NO_3^- consumption and cancer risk (Lidder and Webb, 2013; McNally *et al.*, 2016). In addition, several clinical trials have reported beneficial effects of dietary NO_3^- supplementation, as beetroot or NO_3^-salts, on oxidative stress, BP and EF (Lara *et al.*, 2016; Ashworth and Bescos, 2017; Lundberg *et al.*, 2018). However, most of these studies included relatively small numbers of participants and were of relatively short duration. Moreover, longitudinal studies have reported protective effects of a higher intake of NO_3^--rich vegetables against risk for cardiovascular mortality and incidence of CHD and stroke (Lidder and Webb, 2013).

The putative health benefits of higher dietary NO_3^-consumption appear to be related to an increased NO generation via the NO_3^- - NO_2^- -NO pathway (McNally *et al.*, 2016). NO is a free

radical gas and highly reactive molecule, which is involved in various physiological functions including vascular regulation, nerve transmission and cellular energetics (Weitzberg and Lundberg, 2013). Two distinct pathways regulate the synthesis of NO. The first pathway, called the enzymatic pathway, utilizes L-arginine as a precursor which is converted into NO and citrulline by NO synthases (Bruckdorfer, 2005). The second pathway involves the progressive reduction of NO_3^- into NO_2^- and NO in a series of reactions involving oral bacterial reductases, gastric acidic environment and molybdenum-containing enzymes such as xanthine-oxido reductases or aldehyde dehydrogenase (Weitzberg and Lundberg, 2013). These two pathways are not independent and a cross-talk appears to exist between them to maintain a normal NO production (Carlström *et al.*, 2015).

Green vegetables contain relatively high concentrations of NO_3^- and they can be considered as a main source of NO_3^-intake in humans (60-80% of total NO_3^-intake) (Weitzberg and Lundberg, 2013). Examples of NO_3^--rich vegetables are rocket, spinach, lettuce and beetroot (Lidder and Webb, 2013). However, the NO_3^-content in vegetable products is influenced by environmental and agronomic conditions. In addition, NO_3^-occurs in widely differing concentrations in drinking water, which can contribute (≤15–20% of total NO_3^- intake) (Weitzberg and Lundberg, 2013). These factors combined with the lack of reliable NO_3^- data in most food-,beverage-, and water-composition databases all contribute to the difficult task of estimating daily NO_3^- intake in humans (Weitzberg and Lundberg, 2013).

After dietary NO_3^-consumption, urinary NO_3^- concentration peaks after 4–6 hours and returns to baseline within 24 hours (Wagner *et al.*, 1983a; Pannala *et al.*, 2003). Urinary NO_3^- concentration is affected strongly by NO_3^- consumption, but only 60–70% of consumed $NO_3^-$is excreted in urine, so that 24-h urinary NO_3^- output may not reflect quantitatively long-term dietary NO_3^- intake (Wagner *et al.*, 1983b; Schultz *et al.*, 1985).

Currently, there is no established consensus on a reference dietary method to assess dietary NO_3^- intake, which is a critical factor in explaining the large heterogeneity observed in estimates of intake between studies. Therefore, we conducted a systematic review of observational studies to try to define these sources of heterogeneity in the assessment of dietary NO_3^- intake in humans, as well as to quantify daily NO_3^- intake in adult populations. Specifically, we evaluated the application and compilation of different food databases and dietary assessment methods in food products that were used to quantify the amount of daily NO_3^- intake. Secondary aims included investigation of factors influencing NO_3^- intake, such as health and disease status and stage of economic development of the countries included in the systematic review.

2.2. Methods

The protocol for this systematic review has been registered in Prospero (CRD 42017060354). This systematic review was conducted according to Cochrane and the Centre for Reviews and Dissemination guidelines (Higgins and Green, 2011) and is reported according to PRISMA guidelines (Liberati *et al.*, 2009).

2.2.1. Literature search

Studies to be included were identified by searching 3 databases Pubmed (https://www.ncbi.nlm.nih.gov/pubmed/), Web of Science (http://wok.mimas.ac.uk/), and Embase (https://www.elsevier.com/en-gb/solutions/embase-biomedical-research) from inception to February 2018. The search was restricted to human studies. To maximize the inclusiveness of the search and to ensure the inclusion of articles in press, these databases were searched for titles and abstracts and without any restriction for type of studies. Hand-searching was performed for relevant articles and reviews to retrieve potential, eligible studies not found in the primary search.

The following keywords were entered in the databases to conduct the searches: nitrate, inorganic, diet, dietary, food content, food frequency, dietary recall, 24-h recall, dietary record, food composition, ingestion, intake, consumption, exposure, effect, cross sectional, case control, cohort, longitudinal, prospective, ecological, and epidemiology. We used Boolean terms (i.e., AND) to build different algorithms to increase the sensitivity of the search strategy. A summary of the specific search algorithms is reported in **Table 2.1**.

The results for each individual search were exported to a reference manager software (ENDNOTE X7.7.1, Clarivate Analytics, Philadelphia, United States). A master file was created to contain all of the results of the search. Duplicates were subsequently removed.

Table 2.1: Description of keywords included in the search strategy.

Component 1 (Nutritional)	Boolean terms	Component 2 (Compound)	Boolean terms	Component 3 (Epidemiology)
Inorganic	AND	Nitrate	AND	Ingestion
Diet	AND		AND	Intake
Dietary	AND		AND	Consumption
Food content	AND		AND	Exposure
Food frequency	AND		AND	Effect
Dietary recall	AND		AND	Cross sectional
24h recall	AND		AND	Case control
Dietary record	AND		AND	Cohort
Food composition	AND		AND	Longitudinal
			AND	Prospective
			AND	Ecological
			AND	Epidemiology
			AND	Retrospective
			AND	Survey
			AND	Observational
			AND	Association

2.2.2. Study selection

Articles were selected for inclusion in the systematic review according to the following criteria: *1*) observational study (cohort, case-control, cross-sectional), *2*) populations with a mean age ≥18 y, *3*) quantification of dietary nitrate consumption (in milligrams per day or millimoles per day), and *4*) nitrate consumption was reported for the whole diet. Studies were excluded if they were intervention studies, conducted in non-adult populations, and if nitrate intake was not reported or was assessed only for selected foods or beverages (e.g., vegetables, water, or meat products). Only articles published in English were included. Two investigators screened the titles and abstracts of the articles independently to assess eligibility for inclusion. If agreement was reached, articles were either excluded or moved to the next stage (full-text). If agreement was not reached, the article was moved to the full-text stage. The full texts of the selected articles were critically evaluated by 3 reviewers (AMB, GF and MS) to determine final eligibility for inclusion in the systematic review. Disagreements were resolved by discussion between the reviewers until consensus was reached.

2.2.3. Data Extraction

Once the eligible articles were selected, the following information were extracted: authors' name, journal and year of publication, country where the analysis was conducted, sample size of population, age, gender, disease status, dietary assessment method, daily NO_3^- intake, method used to assess NO_3^- content in food declared by authors and measurement of NO_3^- in biological fluids, if reported. In articles that included population of different age group, only data of targeted age population (mean age 18 years old) were extracted and included in the systematic review.

2.2.4. Quality assessment

The quality assessment of the studies was assessed according to specific criteria that were adapted from previous quality-assessment checklists (Crichton *et al.*, 2011; Cericato *et al.*, 2015), including the study design, description of the study objective, results, and study limitations. Additional criteria were also developed assessing dietary methods, approaches used to quantify NO_3^- content in foods and beverages, and measurement of NO_3^- in biological samples. A description of the study quality checklist and quality assessment of studies is shown in **Appendix 2.2.** The appraisal of the quality of the studies was carried out by two investigators. Discrepancies in the assessment of study quality were resolved by consensus. Low-quality studies were not excluded from the systematic review to inform discussion of the strengths and limitations of the current evidence, to enhance the representativeness of the results, and as a basis for developing suggestions for the conduct of future investigations of NO_3^- intake.

2.2.5. Calculation of nitrate intake

The descriptive statistics used by the selected studies to report NO_3^- intake were primarily mean or median for the study cohort. However, several articles reported NO_3^- intake after stratification of the population into separate groups (e.g. quintiles of NO_3^- intake) and did not report NO_3^- intake for the total population. In these studies, the weighted average (WA) of total NO_3^- intake by each group was calculated as this approach takes into account the number of individuals in each group. A clear description of how NO_3^- intake was calculated can be found in **Appendix 2.4.** In case of limited data, authors were contacted directly to obtain the missing information. When it was not possible to use the available data to adjust NO_3^- intake, the article was excluded. A sensitivity analysis was conducted to evaluate the contribution of water consumption to total NO_3^- intake and we investigated possible differences in NO_3^- intake according to study design (cross-sectional, case-control, cohort)**.**

2.2.6. Nitrate intake and disease outcomes

Phenotypic characteristics of the populations including disease status at baseline as well as incident disease cases were extracted. This information offered the opportunity to describe the frequency and distribution of articles that reported associations of NO_3^- intake with disease risk. In addition, we calculated the median NO_3^- intake for patient populations (i.e., disease cases reported in cohort and case-control studies) and compared it with the median NO_3^- intake derived from healthy populations.

2.2.7. Gross Domestic Product, globalization index, and nitrate intake

An ecological analysis was conducted to assess the relations between NO_3^- intake and Gross Domestic Product (GDP) per capita of respective countries, as a measure of the economic development of each included country. Relevant GDP data were obtained from www.data.worldbank.org/indicator/NY.GDP.PCAP.CD (in US dollars). Because globalization also affects food supplies and food intake patterns, the relation between NO_3^- intake and the KOF Globalization Index was assessed in a further ecological analysis. The KOF Globalization Index measures the economic, social, and political dimensions of globalization. Relevant KOF data were obtained from www.kof.ethz.ch/en/forecasts-and-indicators/indicators/kof-globalisation-index.html. Year-specific GDP and KOF index information was extracted according to the article's year of publication (the most recent data were used for the articles published in the last 2 or 3 y). NO_3^- intakes from healthy populations only were included in this analysis (53 articles). The relevant GDP estimates and KOF index values are provided in **Appendix 2.5.** An important assumption of these analyses was that the NO_3^- intake calculated in each study was representative of the total population of the country.

2.2.8. Statistical analysis

NO_3^- intake is reported as medians (IQRs). NO_3^- intake and GDP were not normally distributed and were log-transformed before analysis. Independent *t* tests were used to determine differences in NO_3^- intake between healthy and disease groups. Pearson's correlation and linear regression were performed to explore the association of NO_3^- intake with GDP and the KOF Globalization Index. $P< 0.05$ was considered to be significant. The Statistical Package (IBM SPSS, Version 23, NY, USA) was used to perform the analysis.

2.3. Results

2.3.1. Search results

A summary of screening process and selection of studies is shown in **Figure 2.1**. The initial literature review identified 9550 articles, 1376 duplicate articles were removed, 8174 articles were screened and 8053 were excluded. Sixty-six of the remaining 121 articles were excluded as they did not meet our inclusion criteria. One article was found through hand-search (Ellen *et al.*, 1990). Fifty-five articles met the inclusion criteria for entry in the systematic review. The main reason for excluding studies was that they did not provide sufficient information to determine their eligibility.

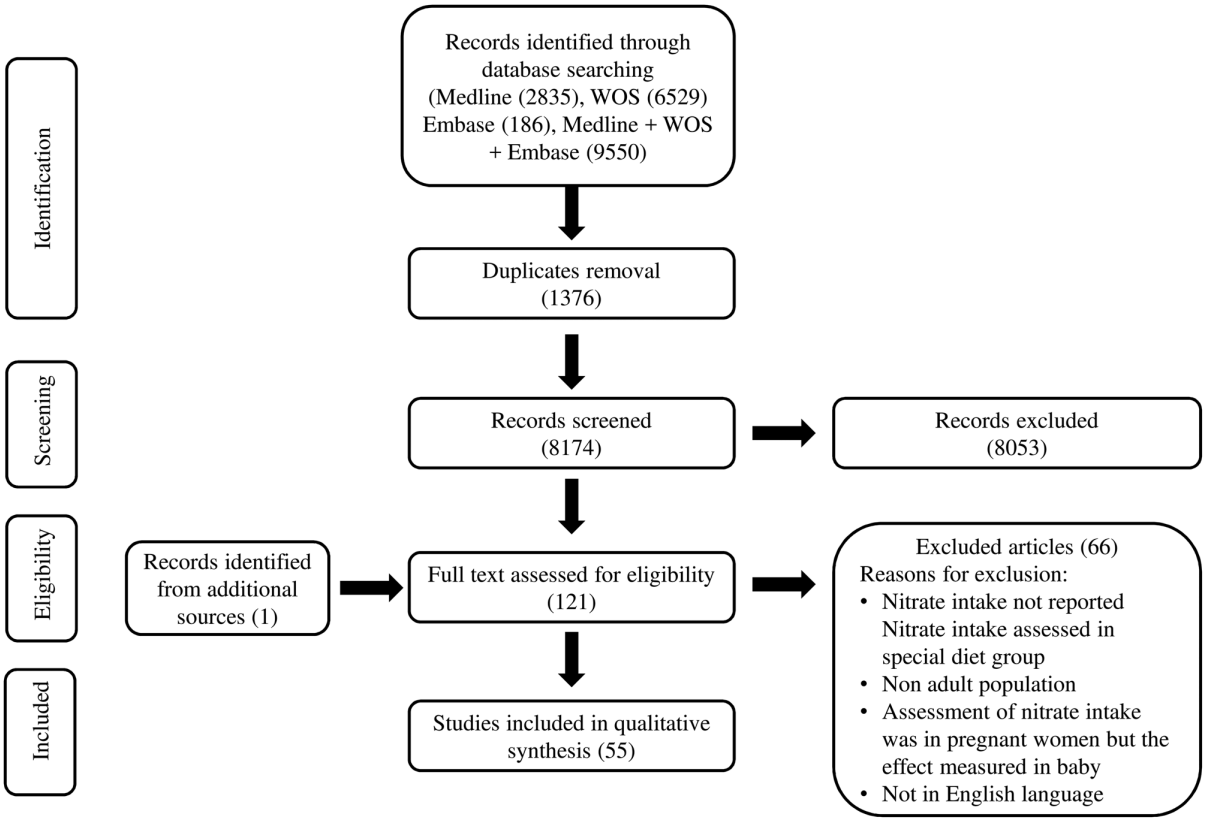

Figure 2.1: Flow diagram of the selection process of the observational studies included in the systematic review.

2.3.2. Studies characteristics

The general characteristics of the studies are summarized in **Table 2.3**.

2.3.2.1. Study design and country

The 55 observational studies included in the systematic review recruited a total of 3,430,148 participants. There were 19 case control (Barbone *et al.*, 1993; Gonzalez *et al.*, 1994; Hansson *et al.*, 1994; La Vecchia *et al.*, 1994; Virtanen *et al.*, 1994; Pobel *et al.*, 1995; Rogers *et al.*, 1995; Palli *et al.*, 2001; Chen *et al.*, 2002; Coss *et al.*, 2004; Ward *et al.*, 2006; Kim *et al.*, 2007; Hernandez-Ramirez *et al.*, 2009; Kilfoy *et al.*, 2010; Yang *et al.*, 2010; Kilfoy A *et al.*, 2012; Kilfoy A *et al.*, 2013; Espejo-Herrera *et al.*, 2016a; Espejo-Herrera *et al.*, 2016b) (**Table 2.4**), 22 cohort (van Loon *et al.*, 1998; Zeegers *et al.*, 2006; Michaud *et al.*, 2009; Dubrow *et al.*, 2010; Ward *et al.*, 2010; Kilfoy *et al.*, 2011a; Kilfoy *et al.*, 2011b; Inoue-Choi *et al.*, 2012; Kilfoy *et al.*, 2012; DellaValle *et al.*, 2013; Keszei *et al.*, 2013; Kilfoy *et al.*, 2013; Dellavalle *et al.*, 2014; Keszei *et al.*, 2014; Inoue-Choi *et al.*, 2015; Bahadoran *et al.*, 2016a; Jones *et al.*, 2016; Kang *et al.*, 2016; Bahadoran *et al.*, 2017; Blekkenhorst *et al.*, 2017a; Jones *et al.*, 2017; Quist *et al.*, 2018) (**Table 2.5**) and 14 cross sectional (Stephany and Schuller, 1980; Knight *et al.*, 1987b; Vandenbrandt *et al.*, 1989; Ellen *et al.*, 1990; Knight *et al.*, 1990; Laitinen *et al.*, 1993; Dich *et al.*, 1996; Vaessen and Schothorst, 1999; Mitacek *et al.*, 2008; Griesenbeck *et al.*, 2010; Temme *et al.*, 2011; Anyzewska and Wawrzyniak, 2014; Inoue-Choi *et al.*, 2016; Jonvik *et al.*, 2017) (**Table 2.6**) studies. One study had an unclear study design (Anyzewska and Wawrzyniak, 2014). Twenty-five studies were conducted in North America (1 from Mexico 24 and from United States) (Barbone *et al.*, 1993; Rogers *et al.*, 1995; Chen *et al.*, 2002; Coss *et al.*, 2004; Ward *et al.*, 2006; Hernandez-Ramirez *et al.*, 2009; Michaud *et al.*, 2009; Dubrow *et al.*, 2010; Griesenbeck *et al.*, 2010; Kilfoy *et al.*, 2010; Ward *et al.*, 2010; Kilfoy *et al.*, 2011a; Kilfoy *et al.*, 2011b; Inoue-Choi *et al.*, 2012; Kilfoy A *et al.*, 2012; Kilfoy *et al.*, 2012; DellaValle *et al.*, 2013; Kilfoy A *et al.*, 2013; Inoue-Choi *et al.*, 2015; Inoue-Choi *et al.*, 2016; Kang *et al.*, 2016; Jones *et al.*, 2017; Quist *et al.*, 2018), 23 studies were conducted in Europe (8 studies from The Netherlands (Stephany and Schuller, 1980; Ellen *et al.*, 1990; van Loon *et al.*, 1998; Vaessen and Schothorst, 1999; Zeegers *et al.*, 2006; Keszei *et al.*, 2013; Keszei *et al.*, 2014; Jonvik *et al.*, 2017), 3 studies from Italy (Knight *et al.*, 1990; La Vecchia *et al.*, 1994; Palli *et al.*, 2001), 2 studies from Spain (Gonzalez *et al.*, 1994; Espejo-Herrera *et al.*, 2016b), 1 study conducted between Spain and Italy (Espejo-Herrera *et al.*, 2016a), 3 from Finland (Laitinen *et al.*, 1993; Virtanen *et al.*, 1994; Dich *et al.*, 1996) and United Kingdom (Knight *et al.*, 1987b; Vandenbrandt *et al.*, 1989), 1 study from Belgium (Temme *et al.*, 2011), Sweden (Hansson *et al.*, 1994) France (Pobel *et al.*, 1995), and Poland (Anyzewska and Wawrzyniak, 2014)), 5 studies in Asia (2 studies from each of China (Kilfoy *et al.*, 2013; Dellavalle *et al.*, 2014) and Korea (Kim *et al.*, 2007; Yang *et al.*, 2010) and 1 study from

Thailand (Mitacek *et al.*, 2008)), 2 studies were conducted in the Middle East (both studies in Iran (Bahadoran *et al.*, 2016a; Bahadoran *et al.*, 2017)) and 1 study from Australia (Blekkenhorst *et al.*, 2017a). The study specific quality assessment scores are shown in **Appendix 2.3**.

Table 2.2: General characteristic of the studies

Characteristic of studies		Number of studies	Percentage (%)
Study Design	Cohort	22	41
	Case-control	19	34
	Cross sectional	13	24
	Unclear	1	2
Dietary Assessment Methods	FFQ	43	80
	Measurement of nitrate in biological samples	6	11
	DH	3	6
	DR	3	6
	FFQ and 24-hr recall	2	4
	48-hr recall	1	2
	24-hr recall	1	2
	Not reported	1	2
Methods Used to Estimate Nitrate Content in Food	Published literature	37	69
	Food composition tables and databases	11	20
	Direct analysis of duplicate 24-hr diet samples	3	6
	Measured during study and also used published literature	2	4
	Not reported	1	2
Countries	North America	25	45
	Europe	23	41
	Asia	5	9
	Middle East	2	3
	Australia	1	2

DH; Diet history, DR; Dietary record, FFQ; Food frequency questionnaire, 24-hr/48-hr; 24/48-hour recall.

Table 2.3: Characteristics of case-control studies

Study	Country	Cases	Sample size (Case/Control)	Age (Years)	Gender (M/F)	Duration (Years)	Dietary Assessment
Barbone *et al.*, (1993)	USA	EC	103/236	63-64	All F	4	116-item FFQ
Chen *et al*, (2002)	USA	Glioma	236/ 449	≥21	390 /295	3	Modified 48-item FFQ
Coss *et al.*, (2004)	USA	PC	189/1244	40-85	877 / 556	27	55-item FFQ
Espejo-Herrera *et al.*, (2016b)	Spain	BC	1245/1520	58 (mean)	All F	7	Validated 140-item FFQ
Espejo-Herrera *et al.*, (2016a)	Spain /Italy	CC	1869/3530	20-85	4190 /3051	6	Validated 140-item FFQ
Gonzalez *et al.*, (1994)	Spain	GC	354/354	65 (mean)	Available only for cases: 235/119	2	Diet history
Hansson *et al.*, (1994)	Sweden	GC	338/479	40-79	NR	20 prior the interview	45-item FFQ
Hernandez-Ramirez *et al.*, (2009)	Mexico	GC	257/478	49-70	NR	2	Validated 127-item FFQ
Kilfoy A *et al.*, (2012)	USA	NHL	586/NA	21-84	All F	4	120-item FFQ
Kilfoy *et al.*, (2010)	USA	NHL	594/710	67 (mean)	All F	4	120-item FFQ
Kilfoy A *et al.*, (2013)	USA	NHL	348/470	20-75	NR	3	FFQ
Kim *et al.*, (2007)	Korea	GC	136/136	57 (mean)	190 / 88	1	Validated 109-item FFQ
La Vecchia *et al.*, (1994)	Italy	GC	103/236	63-64	All F	7	29-item FFQ
Palli *et al.*, (2001)	Italy	GC	382/561	50-64	567 / 376	2	181-item FFQ
Pobel *et al.*, (1995)	France	GC	92/128	66 (mean)	133/87	3	Diet history
Rogers *et al.*, (1995)	USA	UATC	645/458	NR	NR	3.5	125-item FFQ
Virtanen *et al.*, (1994)	Finland	T1D	1168/1168	NR	1096/1240	3	FFQ
Ward *et al.*, (2006)	USA	NHL	361/276	63 (mean)	329/309	2	Modified 117-item FFQ

Study	Country	Cases	Sample size (Case/Control)	Age (Years)	Gender (M/F)	Duration (Years)	Dietary Assessment
Yang *et al.*, (2010)	Korea	BC	362/362	30-65	All F	1	121-item FFQ

BC; Breast cancer, CC; Colorectal Cancer, EC; Endometrial cancer, FFQ; Food frequency questionnaire, GC; Gastric cancer, NHL; Non-Hodgkin lymphoma, NR; Not reported, PC; Pancreatic cancer, T1D; Type I diabetes, UATC; Upper aerodigestive tract cancer, USA; United States of America

Table 2.4: Characteristics of cohort studies

Study	Country	Cases	Sample size	Age (Years)	Gender (M/F)	Duration (Years)	Dietary Assessment
Bahadoran *et al.*, (2017)[1]	Iran	T2D	Total (N=2139) Cases (n=143)	20-70	971/1168	6	Validated 168-item FFQ
Bahadoran *et al.*, (2016a)[1]	Iran	CKD and HT	Total (N=2799) Cases of CKD (n=1780) and HT (n=1878)	20-70	806/1072	6	Validated 168-item FFQ
Blekkenhorst *et al.*, (2017a)	Australia	ASVD	Total (N=1226) Dead (n=238)	70-85	All F	15	74-items FFQ
DellaValle *et al.*, (2013)	USA	RCC	Total (N=491841) Cases (n=1816)	50-71	292125/198069	9	Validated 124-item FFQ
Dellavalle *et al.*, (2014)	China	CC	Total (N=73118) Cases (n=619)	40-70	All F	5	Validated 77-item FFQ
Dubrow *et al.*, (2010)	USA	Glioma	Total (N=545770) Cases (n=585)	50-71	322347/ 223423	1	124-item FFQ
Inoue-Choi *et al.*, (2015)[2]	USA	OVC	Total (N=28555) Cases(n=315)	55-69	All F	1	Validated 126-item FFQ
Inoue-Choi *et al.*, (2012)[2]	USA	BC	Total (N=41836) Cases (n=2875)	55-69	All F	1	127-item FFQ
Jones *et al.*, (2016)	USA	BLC	Total (N=33964) Cases (n=258)	55-70	All F	21	Validated 127-item FFQ
Jones *et al.*, (2017)	USA	KC	Total (N=33964) Cases (n=256)	55-69	All F	21	Validated FFQ
Kang *et al.*, (2016)	USA	Glaucoma	Total (N=104987) Cases (n=1483)	>40	63893/41094	2	Validated 116-item FFQ
Keszei *et al.*, (2013)	Netherlands	EC and GC	Total (N= 4959) Cases (n= 927)	55-69	1947/2085	16.3	Validated 150-item FFQ
Keszei *et al.*, (2014)	Netherlands	BO	Total (N=3717), Cases (n=43)	55-69	2074/2076	16.3	150-items FFQ
Kilfoy *et al.*, (2012)	USA	OVC	Total (N=151316) Cases (n=709)	50-71	All F	10	Validated 124-item FFQ

Study	Country	Cases	Sample size	Age (Years)	Gender (M/F)	Duration (Years)	Dietary Assessment
Kilfoy *et al.*, (2013)	China	TC	Total (N=73317) Cases (n=164)	40-70	All F	11	Validated 77-item FFQ
Kilfoy *et al*., (2011)[3]	USA	PC	Total (N=492226) Cases (n=1728)	50-71	293491/198735	10	Validated 124-item FFQ
Kilfoy *et al.*, (2011b) [3]	USA	TC	Total (N=490194) Cases (n=370)	50-71	292125/198069	7	Validated 124-item FFQ
Michaud *et al.*, (2009)	USA	Glaucoma	From three studies: 49935 (HPFS), 92468 (NHS I), 95391 (NHS II) Cases (n= 335)	25-75	49935/187856	>24	131-item FFQ
Quist *et al*., (2018)	USA	PC	Total (N=32,242) Cases (n=313	55-69	All F	25	127-items FFQ
van Loon *et al.*, (1998)	Netherlands	GC	Total (N=3405) Cases (n= 282)	55-96	1525/1598	6.3	Validated 150-item FFQ
Ward *et al.*, (2010)	USA	TC	Total (N=20651) Cases (n=40)	61 (mean)	All F	19	126-item FFQ
Zeegers *et al.*, (2006)	Netherlands	BLC	Total (N=5230) Cases (n= 871)	62 (mean)	2391/330	9.3	Validated 150-item FFQ

[1] These two studies have used the same cohort, but studied different outcomes.

[2] These two studies have used the same cohort, but studied different outcomes.

[3] These two studies have used the same cohort, but studied different outcomes.

ASVD; Atherosclerotic vascular disease, BC; Breast cancer, BLC; Bladder cancer, BO; Barrett's oesophagus, CC; Colorectal Cancer, CKD; Chronic kidney disease, EC; Esophageal cancer, FFQ; Food frequency questionnaire, GC; Gastric cancer, HT; Hypertension, KC; Kidney cancer, OVC; Ovarian cancer, PC; Pancreatic cancer, RCC; Renal cell carcinoma, TC; Thyroid cancer, T2D; Type 2 Diabetes, USA; United States of America.

Table 2.5: Characteristics of cross-sectional studies

Study	Country	Health status	Sample size	Age (Years)	Gender (M/F)	Dietary Assessment
Anyzewska and Wawrzyniak, (2014) [1]	Poland	Healthy	NR	NR	NR	NR
Dich *et al.*, (1996)	Finland	Healthy	10054	>15	5,304/ 4,750	Diet history
Ellen *et al.*, (1990)	Netherland	Healthy	110	42 (mean)	57/53	DR
Griesenbeck *et al.*, (2010)	USA	Healthy	5958	NR	All F	58-item FFQ
Inoue-Choi *et al.*, (2016b)	USA	Healthy	490194	50-71	292125 /198069	124-item FFQ and 24-hr recall
Jonvik *et al.*, (2017)	Netherlands	Healthy (Athletes)	553	NR	226/327	24-hour recall
Knight *et al.*, (1987b)	UK	Healthy	747	15-74	284/463	16-item FFQ
Knight *et al.*, (1990)	Italy	Healthy	313	16-60	159/172	22-item FFQ
Laitinen *et al.*, (1993)	Finland	Healthy	747	15-24	582/632	48-hr recall
Mitacek *et al.*, (2008)	Thailand	Healthy	467	19-<60	212/255	97-item FFQ and analyzing real sample
Stephany and Schuller, (1980)	Netherlands	Healthy	100	NR	70/30	DR
Temme *et al.*, (2011)	Belgium	Healthy	3083	< 15	1,546/1,537	FFQ and two-24-hr recall
Vaessen and Schothorst, (1999)	Netherlands	Healthy	123	18-74	60/63	DR
Vandenbrandt *et al.*, (1989)	UK	Healthy	59	29.7 (mean)	35/24	120-item FFQ

DR; Dietary record, FFQ; Food frequency questionnaire, 24-hr/48-hr; 24/48-hour recall, NR; Not reported, UK; United Kingdom, USA; United States of America.
[1]Unclear study design.

2.3.2.2. Participants and health status

Thirteen studies assessed NO_3^- intake in healthy individuals and 42 studies investigated the association between NO_3^- intake and disease risk. The majority of these studies (88%) examined the association between NO_3^- intake and various types of cancer. Single studies were found for other six conditions including T1D and T2D (Virtanen *et al.*, 1994; Bahadoran *et al.*, 2017), glaucoma (Kang *et al.*, 2016), kidney failure (Bahadoran *et al.*, 2016a) hypertension (Bahadoran *et al.*, 2016a), and atherosclerotic vascular disease (Blekkenhorst *et al.*, 2017a).

2.3.2.3. Dietary Assessment Methods

Studies were characterized by substantial variation in the type of dietary assessment methods used to estimate NO_3^- intake. The majority of the studies (43 studies) used food frequency questionnaires (FFQ) (Knight *et al.*, 1987b; Vandenbrandt *et al.*, 1989; Knight *et al.*, 1990; Barbone *et al.*, 1993; Hansson *et al.*, 1994; La Vecchia *et al.*, 1994; Virtanen *et al.*, 1994; Rogers *et al.*, 1995; van Loon *et al.*, 1998; Palli *et al.*, 2001; Chen *et al.*, 2002; Coss *et al.*, 2004; Ward *et al.*, 2006; Zeegers *et al.*, 2006; Kim *et al.*, 2007; Hernandez-Ramirez *et al.*, 2009; Michaud *et al.*, 2009; Dubrow *et al.*, 2010; Griesenbeck *et al.*, 2010; Kilfoy *et al.*, 2010; Ward *et al.*, 2010; Yang *et al.*, 2010; Kilfoy *et al.*, 2011a; Kilfoy *et al.*, 2011b; Inoue-Choi *et al.*, 2012; Kilfoy A *et al.*, 2012; Kilfoy *et al.*, 2012; DellaValle *et al.*, 2013; Keszei *et al.*, 2013; Kilfoy A *et al.*, 2013; Kilfoy *et al.*, 2013; Dellavalle *et al.*, 2014; Keszei *et al.*, 2014; Inoue-Choi *et al.*, 2015; Bahadoran *et al.*, 2016a; Espejo-Herrera *et al.*, 2016a; Espejo-Herrera *et al.*, 2016b; Jones *et al.*, 2016; Kang *et al.*, 2016; Bahadoran *et al.*, 2017; Blekkenhorst *et al.*, 2017a; Jones *et al.*, 2017; Quist *et al.*, 2018). Diet history was used in three studies (Gonzalez *et al.*, 1994; Pobel *et al.*, 1995; Dich *et al.*, 1996). Two studies combined both FFQ and 24-hour recall methods (Temme *et al.*, 2011; Inoue-Choi *et al.*, 2016), three studies used dietary records (Stephany and Schuller, 1980; Ellen *et al.*, 1990; Vaessen and Schothorst, 1999), one study used 48-hour recall (Laitinen *et al.*, 1993) and another 24-hour recall (Jonvik *et al.*, 2017). The method of dietary assessment was not reported in one study (Anyzewska and Wawrzyniak, 2014). Six studies reported the measurement of NO_3^- concentrations in biological samples (plasma, urine or saliva) as an objective measure of exposure to dietary NO_3^- (Stephany and Schuller, 1980; Knight *et al.*, 1987b; Vandenbrandt *et al.*, 1989; Knight *et al.*, 1990; Bahadoran *et al.*, 2016a; Bahadoran *et al.*, 2017).

2.3.3. Daily nitrate intake

The daily NO_3^- intake in each study can be found in **Appendix 2.6**. The estimated median (IQR) NO_3^- intake in 52 studies including healthy participants was 108 mg/day (87-145 mg/day). (**Figure 2.1A**). The estimated median NO_3^- intake in 41 studies including patients was 110 mg/day (89-153 mg/day) (**Figure 2.1B**). There was no significant difference in daily nitrate intake between healthy participants and patients (P=0.61). Total NO_3^- intake was significantly higher in studies that did not account for the contribution of NO_3^- from water intake compared with those which included NO_3^- from water (P=0.005). **(Figure 2.2).** Dietary NO_3^- intake was similar in studies using different study designs (P=0.30) **(Figure 2.3).**

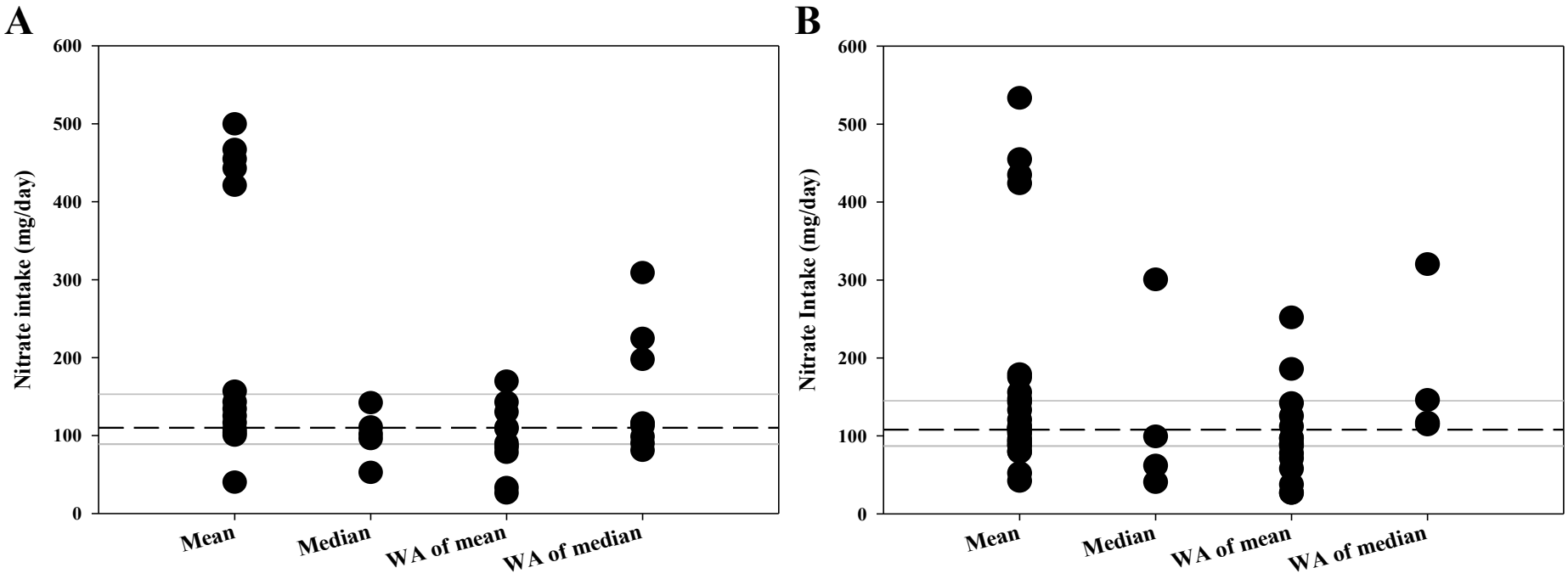

Figure 2.2: NO_3^- intake assessment in unhealthy and healthy individuals.

Daily NO_3^- intake reported by each of the included articles in patients (A) (disease cases from case-control studies or individuals who developed diseases during follow-up; n = 42; median (IQR) = 110 (89–153) mg/day) and in healthy individuals (B) (n = 52; median (IQR) = 108 (87–145) mg/day).WA, weighted average.

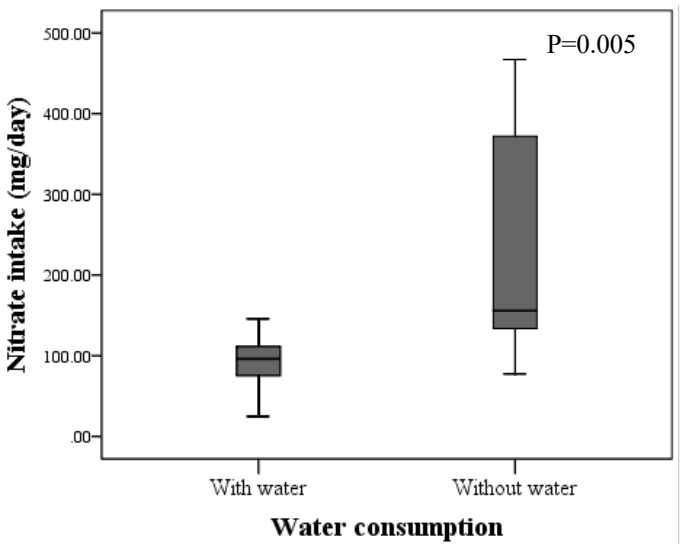

Figure 2.3: Daily NO_3^- intake in studies.

Daily NO_3^- intake in studies including water (n=25) and excluding water (n=11). Data are represented as median and error bars are interquartile range (25th and 75th centiles). Data were log-transformed prior to the analysis by independent t test to test differences in NO_3^- intake between countries. (In this analysis we excluded studies that they did not clearly declared their inclusion or exclusion criteria).

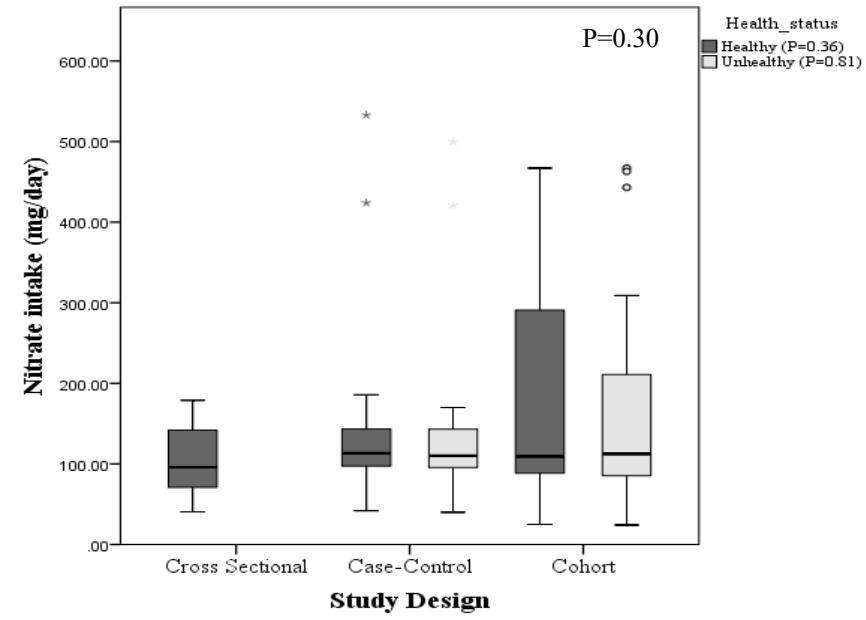

Figure 2.4: Daily NO_3^- intake in studies.

Daily NO_3^- intake in cross sectional (n=14), case-control (n=22) and cohort (n=19) studies. Data are represented as median and error bars are interquartile range (25th and 75th percentiles). Data were log-transformed prior to the analysis by ANOVA differences in NO_3^- intake between studies with different design. (Data were re-analysed after excluding one study (Anyzewska et al., 2014), which had unclear study design; however, the exclusion of the study did not affect the results).

2.3.4. Dietary nitrate intake, gross domestic product and globalization index

We found a significant inverse association between daily NO_3^- intake and GDP ($n = 53$; β coefficient $\pm$ SE $= -0.44 \pm 0.12$ mg/day, $r = -0.46$, $P < 0.001$) (**Figure 2.4A**). There was a similar inverse association between NO_3^- intake and the KOF Globalization Index ($n = 53$; β coefficient $\pm$ SE $= 1.73 \pm 0.73$ mg/day, $r = -0.31$, $P = 0.02$) (**Figure 2.4B**). Daily NO_3^- intake was higher in low- or middle-income countries such as Mexico, China, or Thailand than in countries with a higher per capita income such as the United States or the Netherlands.

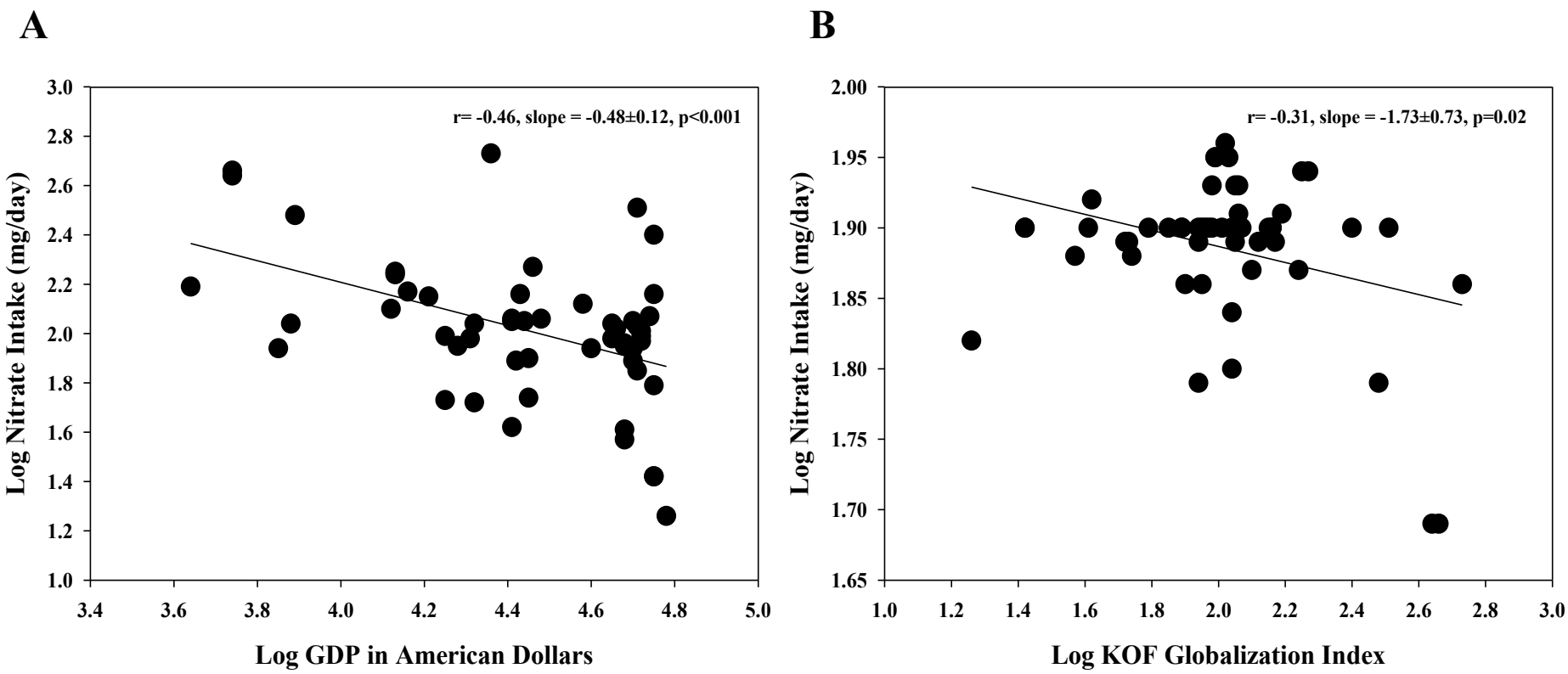

Figure 2.5: Association between daily NO_3^- intake and GDP and KOF Globalization Index.

NO_3^- intake was inversely associated with GDP (A) (n = 53; slope = −0.44 ± 0.12 mg/d; r = −0.46, P < 0.001) and with KOF Globalization Index (B) (n = 53; slope = 1.73 ± 0.73 mg/d; r = −0.316, P = 0.02). Data were log transformed before analysis (n = 53 studies). Pearson's correlation was used to test the association between variables. GDP, Gross Domestic Product.

2.4. Discussion

2.4.1. Main findings

The estimated NO_3^- intakes for healthy individuals and patients were very similar i.e. 108 and 110 mg/day, respectively. There was much heterogeneity in types of dietary assessment methods and in data sources used for estimation of the NO_3^- composition of foods and beverages. The results also indicated that the majority of epidemiological studies which considered health outcomes have focused on the association between NO_3^- intake and cancer risk and very few articles have assessed associations between NO_3^- intake and risk for cardiovascular and metabolic diseases.

2.4.2. Assessment method used for estimating dietary nitrate intake

The accurate assessment of daily NO_3^- intake is important for studies of relationships between NO_3^- exposure and human health. This review shows that several methods have been used to estimate NO_3^- intake with FFQ as the most commonly used tool (Pérez Rodrigo *et al.*, 2015). The main advantages of FFQ are self-completion, cost-effectiveness and easy application in large populations as well as rapid computerised calculations of energy and nutrient intake. Additionally, FFQ provides a representative estimate of dietary intake over a relatively long period of time (e.g. 12 months) (Thompson and Byers, 1994). However, there are several specific issues when using FFQ for assessing NO_3^- intake. One of these issues is the grouping together within the FFQ of multiple foods rich in NO_3^- e.g. green vegetables. Since the NO_3^- content of green vegetables can vary greatly and range from 1 mg/kg to 4800 mg/kg (i.e., Brussel sprouts and rocket, respectively) (Hmelak Gorenjak and Cencič, 2013), failure to quantify intakes of individual green vegetables could lead to significant under- or over-estimation of NO_3^- intake and this could be exacerbated by the decisions made by investigators in specifying the NO_3^- content of green vegetables used in their calculations. Moreover, because of optimistic bias and the structure of questions used, FFQs can overestimate intakes of fruits and vegetables (Shu *et al.*, 2004; Zhang *et al.*, 2015; Steinemann *et al.*, 2017), which may lead to over-reporting of NO_3^- intake.

Currently, there is no standard database containing detailed and accurate information on NO_3^- content. The majority of the epidemiological studies included in this review relied on estimates of food NO_3^- content obtained from various sources such as peer-reviewed articles, official reports from government bodies or position statements from scientific working groups. Some studies used databases from other countries because of the lack of information on NO_3^- content

in their local foods, which could affect the reliability of the results. Factors such as seasonality, temperature, light exposure, and use of fertilizers contribute to the variable NO_3^- content of foods and complicate the comparison of daily NO_3^- intake between countries (Weitzberg and Lundberg, 2013). In addition, the majority of the databases contain data on NO_3^- contents of raw foods and do not take into account effects of preservation and cooking processes on NO_3^- in foods as eaten. Methods used for processing of food, especially vegetables, may affect NO_3^- content. Boiling has been reported to reduce NO_3^- content in leafy vegetables by 47-65%, (presumably because of leaching into the accompanying water) while frying in soybean oil increased the NO_3^- content as much as 159-307% (presumably though concentration because of evaporation of water) (Prasad and Chetty, 2008). A recent Iranian study found that boiling potatos may markedly decrease the content of NO_3^- by 59.7%, while frying increased it by 52% (Ebrahimi *et al.*, 2020). In addition, since NO_3^- is highly water soluble, canned foods have lower NO_3^- content than the corresponding fresh food, as much of the water is discarded when the can is opened (Bednar *et al.*, 1991).

Recently, a group from USA developed a reference database for NO_3^- and NO_2^- in foods and used it to estimate NO_3^- and NO_2^- intakes among participants in the National Institutes of Health-AARP Diet and Health Study from FFQ and 24-hour recalls (Inoue-Choi *et al.*, 2016). More recently, another group of researchers from Australia have developed a reference database for assessing dietary NO_3^- from a large variety of vegetables (Blekkenhorst *et al.*, 2017b). The later work has considered some of the processing factors such as cooking and preservation methods that could influence the NO_3^- concentration in vegetables. There is a need to apply similar approaches to establish the concentrations of NO_3^- in other food types and in other countries. Since our systematic review was published, nine further studies have been identified that also assessed NO_3^- intake in different population groups (Gopinath *et al.*, 2018; Jackson *et al.*, 2018a; Bahadoran *et al.*, 2019; Jackson *et al.*, 2019; Jones *et al.*, 2019a; Liu *et al.*, 2019; Sim *et al.*, 2019; Zheng *et al.*, 2019; Barry *et al.*, 2020). The estimated NO_3^- intake in these 9 studies can be seen in **Appendix 2.7**. The measurement of NO_3^- concentrations in biological fluids such as plasma, saliva or urine could be useful to assess the validity of estimated NO_3^- intake. However, only six studies have reported the associations between dietary intake and NO_3^- concentrations in saliva (Stephany and Schuller, 1980; Knight *et al.*, 1987b; Knight *et al.*, 1990) and urine (Vandenbrandt *et al.*, 1989; Bahadoran *et al.*, 2016a; Bahadoran *et al.*, 2017). Two Iranian studies reported analyses conducted on the same cohort and collected a single urine sample but they did not provide a description of the collection protocols and timing of urine collection (Bahadoran *et al.*, 2016a; Bahadoran *et al.*, 2017). The studies reported significant correlations between dietary intake of NO_3^- and NO_2^- with their corresponding urinary

concentrations (r = 0.59, p<0.001, and r = 0.29, p<0.001). The other study collected total overnight urine specimens on two occasions and separated by two weeks, (Vandenbrandt *et al.*, 1989). Such overnight urinary NO_3^- outputs are likely to provide an indication of short-term NO_3^- intake only. Given the day-to-day heterogeneity in food intake and the high variability in NO_3^- content of foods, it is probable that urine sampling on multiple days would be needed to provide an objective and accurate assessment of long-term typical exposure to dietary NO_3^-. Hence, there is a clear research gap in developing and validating objective biomarkers of NO_3^- intake in humans.

Three studies have estimated NO_3^- intake using the duplicate-portion sampling method which combines dietary assessment with the determination of NO_3^- content from similar food samples collected by the participants. However, this approach is expensive and time-consuming and, therefore, unsuitable for use when estimating NO_3^- intakes by large numbers of people.

2.4.3. Estimated nitrate intake

Using data on NO_3^- content of foods from the UK, the total daily NO_3^- intake for the UK population was estimated to be 95 mg/day (Knight *et al.*, 1987b). The *IARC Monograph on the Evaluation of Carcinogenic Risks to Humans* report (IARC, 2010) estimated that total dietary NO_3^- intake globally ranged from 58 to 218 mg/day. In the present study, the estimated intake of dietary NO_3^- from the included studies was within this range with median intakes of 108 and 110 mg/d for healthy individuals and for those with disease, respectively. Regardless of the dietary assessment method or food composition database used, estimated NO_3^- intakes were below the safe upper intake of 3.7 mg/kg body weight of NO_3^- ion (~250 mg nitrate/day) established by WHO and confirmed recently by EFSA (EFSA, 2017). The more recent estimates of NO_3^- intakes from studies published after we published this systematic review, were also within that range (58-218 mg/day) (**Appendix 2.7**). Using information on cultural meal patterns, a recent paper has estimated NO_3^- intake from four different countries (USA, China, Japan and India) and found similar values to those reported here (Keller *et al.*, 2017). We found that studies that did not include water in their estimates of ingested NO_3^- reported significantly higher NO_3^-intake than studies that included water in their calculations. These results need careful interpretation, as water is an important, if variable, source of ingested NO_3. Therefore, our comparison is likely to be confounded by differences in the characteristics of studies in each group (with and without inclusion of water) such as geography or contamination of water sources with $NO_3^-$from fertilizer run-off or leaching from organic sources. Five studies that did not include $NO_3^-$from water (three from Iran (two articles) (Bahadoran *et al.*, 2016a;

Bahadoran *et al.*, 2017) and two from China (Kilfoy *et al.*, 2013; Dellavalle *et al.*, 2014)) reported very high daily NO_3^- intakes (greater than 300 mg/day). Removal of these studies from the analyses significantly modified the results and showed that total daily NO_3^- intake was comparable between studies which did (104 mg/day) and did not (115 mg/day) include NO_3^- intake from ingested water.

2.4.4. Associations between gross domestic product, globalization index and nitrate intake

Ecological analysis showed that NO_3^- intake was inversely associated with GDP and KOF Globalization Index of the countries in which the studies were conducted. These analyses are speculative since they are based on the assumption that the dietary NO_3^- intake of each study is representative of the entire county. A large proportion of the studies included in the analysis (20 out of 53 are from the USA), and this might have confounded the association by inflating the numbers of countries with a high GDP and high KOF Globalization Index. However, the removal of the studies from United States from the analysis did not modify the findings and NO_3^- intake remained inversely associated with GDP (n=33; slope= -0.57±0.15 mg/day, r= -0.57, p<0.001) and with the KOF Globalization Index ($n = 33$; β coefficient ± SE = -2.29 ± 0.85 mg/d, $r = -0.57$, $P < 0.001$). The significant inverse association that we observed suggests that NO_3^- intake may fall as countries become more developed economically. This may be because economic development is often followed by westernization of dietary habits and, therefore, that reduced NO_3^- intake may be a consequence of progress though the nutrition transition (Popkin, 1994). The main dietary changes likely to lead to lower NO_3^- intake are higher intake of animal products and reduced intake of NO_3^- -rich fruits and vegetables (Popkin *et al.*, 2012). In addition, ethnicity, religion and cultural food patterns are key determinants of the diversity of food choices (Dindyal and Dindyal, 2003), and thus may affect NO_3^- intake.

2.4.5. Strengths and limitations

A major strength of this review is the inclusion of data from over 3 million participants from 15 different nations. Additional strengths include the sensitivity analyses performed to evaluate possible confounding effects of study design or water intake on estimates of NO_3^- intake. Limitations of the study include the exclusion of non-English articles which could result in selection bias. In addition, we did not stratify NO_3^- intake by dietary assessment methods as the majority of studies used FFQ to estimate nitrate intake and, therefore, the results were described narratively. Moreover, as mentioned earlier, after the current review was published, 9 more

studies were identified and a summary table for these new studies can be found in **Appendix 2.7.** However, including the estimates of NO_3^- intake from these studies makes little change in the overall estimates of NO_3^- intake reported here. For example, the estimated median NO_3^- intake for healthy individuals becomes 109.4 (88-185) mg/day, and the estimated median NO_3^- intake for those with health conditions becomes 110 (87-156) mg/day.

2.5. Conclusions

This review provides an evidence-based evaluation of the methods used to assess NO_3^- intake in humans and estimates the median daily NO_3^- consumption in both healthy and patient populations. An important finding of the review is the high heterogeneity in dietary assessment methods and in use of food composition tables. In addition, few studies have attempted to validate their dietary NO_3^- intake data using biomarkers such as NO_3^- excretion in urine. As a consequence, the accuracy of published estimates of NO_3^- intake remains uncertain. In summary, the outcomes of this systematic review highlight the need for a consensus initiative to delineate guidelines to improve and standardize the assessment of NO_3^- intake in humans.

Chapter 3. Effect of inorganic nitrate and nitrite consumption on cognitive function and cerebral blood flow: a systematic review and meta-analysis of randomised clinical trials.

This chapter has been adapted with some modification from our published paper in *Critical Reviews in Food Science and Nutrition:*

Clifford, T., **Babateen, A.,** Shannon, O.M., Capper, T., Ashor, A., Stephan, B., Robinson, L., O'Hara, J.P., Mathers, J.C.,Stevenson, E. and 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*, pp. 01-31 (**Appendix 3.1**).

3.1. Introduction

Cognitive impairment and major neurocognitive disorders like dementia are global health problems (Hugo and Ganguli, 2014). They have remarkable consequences for the health care system and the economy due to the costs associated with management and treatments (Hugo and Ganguli, 2014). The number of people with dementia is increasing enormously. According to the WHO, there are around 10 million new cases per year (WHO, 2019). It has been estimated that the number of demented people will reach more than 100 million by 2050 (Wortmann, 2012). Therefore, over the past decade research has focused to investigate effective interventions to prevent cognitive decline and dementia onset. Today, there is major interest in dietary approaches to maintain better cognitive health.

Cognitive functions are regulated by several factors whose mechanisms are still not fully understood. Impairment NO production was thought to be related to cognitive decline and development of AD (De la Torre and Stefano, 2000; Toda *et al.*, 2009). NO is one of the most important gaso-transmitters in living organisms and known for its pleiotropic biological roles (Kasparek *et al.*, 2008). Amongst several physiological effects, NO possesses inflammatory mediator, vasodilator and neuromodulator effects, which all lead to a protective effect on brain function (Balez and Ooi, 2016). NO can be produced in the brain and many other organs by the activity of NOS (eNOS, iNOS, and nNOS) (Weitzberg and Lundberg, 2013; Reis *et al.*, 2017). Some evidence has shown that blood flow decrease after inhibition of eNOS synthesis, and therefore it may contribute to the development of cognitive dysfunction (de la Torre and Aliev,

2005). On the other hand, administration of NO-donors to prevent NO deficiency can provide a protective effect in cognitive decline (Manukhina *et al.*, 2008).

In addition to the NOS system, dietary NO_3^- and NO_2^- are important sources to generate NO as mentioned earlier in chapter 1. Dietary NO_3^- and NO_2^- are present in a wide range of concentrations in a variety of foods with the higher content found in green leafy vegetables, beetroot, or meat products that have had NO_2^- salts added as preservatives (Lidder and Webb, 2013). An accumulating body of evidence has shown that ingestion of NO_3^- and NO_2^- could improve vascular and metabolic outcomes by increased production of NO (Weitzberg and Lundberg, 2013; McDonagh *et al.*, 2019). Recent evidence also has demonstrated their beneficial effects on cognition, brain metabolic and vascular health (McDonagh *et al.*, 2019).

Presley and co-workers provided a possible mechanism for this cognitive improvement by measuring cerebral perfusion using Arterial spin labelling- Magnetic resonance imaging (ASL-MRI) after the administration of high NO_3^- diet versus low NO_3^- diet. They showed that cerebral perfusion in the prefrontal cortex, the brain region associated with executive function and working memory, of older adults was stimulated after high NO_3^- diet (Presley *et al.*, 2011). However, mixed findings were found in subsequent studies that measured CBF or cognitive function (Kelly *et al.*, 2013; Clifford *et al.*, 2015). Therefore, it remains unclear whether consumption of dietary NO_3^- or NO_2^- salts are an effective strategy for increasing CBF and/or improving cognitive deficits.

As a result, a systematic review and meta-analysis was conducted of RCTs to examine the effect of dietary NO_3^- and NO_2^- supplementation on cognitive function and CBF in adult healthy participants or those with health conditions. We set out to determine whether the ingestion of NO_3^- -rich foods or NO_2^- -salts enhances CBF and improves cognitive function and to estimate effect sizes. In addition, to examine whether BMI, age, supplement dose, intervention duration, test conditions (e.g., exercise vs. rest), and studies' quality modified the effects of dietary NO_3^- or NO_2^- on CBF and cognitive function. These findings will help to inform whether NO_3^- or NO_2^- supplementation holds promise as a relatively inexpensive strategy for augmenting CBF and reducing cognitive decline.

3.2. Methods

This systematic review was conducted according to Cochrane and the Centre for Reviews and Dissemination guidelines (Higgins and Green, 2011) and is reported according to PRISMA guidelines (Liberati *et al.*, 2009). I was involved in conducting the data extraction, quality assessment and meta-analysis.

3.2.1. Literature search

The literature search was conducted by another researchers. Two databases; PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) and Embase (https://www.elsevier.com/en-gb/solutions/embase-biomedical-research) were searched for articles from inception until May 2017. In addition, included reviews and eligible full text articles were searched manually to identify other suitable articles to be included in the systematic review. The following terms and keywords were entered, and Boolean terms were used to increase the sensitivity of the search strategy: nitrate, nitrite, beetroot, rocket, cabbage, lettuce, spinach, green leafy vegetables, cognition, brain, dementia, cerebral, memory, executive, attention, motor skills, blood flow, vascular flow, perfusion.

3.2.2. Study selection

The study selection was performed by another researchers. Titles and abstracts were screened using pre-defined eligibility criteria in accordance with the PICOS (population, intervention, comparator, outcome, study design) framework before retrieval of the full-text articles. The following inclusion criteria were used to assess the eligibility of articles for inclusion in this systematic review: 1) RCT (no exclusion criteria were used for study design, or blinding); 2) trials recruiting adult participants (≥18 years) and no exclusion criteria were applied in relation to participants' health status; 3) trials based on NO_3^- or NO_2^- supplementation were included if they provide information on the type of NO_3^- salt (potassium or sodium), dose, formulation, frequency and route of administration. Trials based on BJ supplementation or ingestion of NO_3^- -rich foods were included in the analyses if they provided information on the frequency and amount of NO_3^- -containing food provided; 4) trials reporting effects of NO_3^- or NO_2^- on global and domain-specific cognitive function and CBF measured by different techniques including MRI, ultrasound or NIRS; 5) English-language restriction but not time restriction was applied in searching the databases; 6) Full text papers and abstracts were included (if they contained sufficient information to complete qualitative and quantitative analysis). Two investigators independently evaluated the titles and abstracts to check eligibility for inclusion. If the reviewers agreed, each article was either excluded or moved to the next stage (full-text). If agreement was not achieved, the article was moved for evaluation after retrieval of the full-text. The selected full-texts were then reviewed to confirm their inclusion in the systematic review. Disagreements were discussed with a third reviewer and resolved by consensus.

3.2.3. Data extraction

Relevant information was extracted and tabulated separately for cognitive function and CBF. If information was not available from the full text, authors were contacted to obtain the relevant data.

Cognitive function

The following information was extracted independently by two investigators from eligible articles: 1) authors and year of publication; 2) study characteristics (design, sample size); 3) participant characteristics (age, male/female ratio, health status and baseline values for BMI; 4) route, dose and duration of dietary NO_3^- and NO_2^- supplementation; and 5) cognitive tests and exercise condition. Any disagreements in data extraction were resolved through discussion until consensus was reached.

Cerebral blood flow

Two independent reviewers extracted relevant information from the eligible articles: 1) authors and year of publication; 2) study characteristics (design, sample size); 3) participant characteristics (age, male/female ratio, health status and baseline values for BMI, 4) route, dose and duration of dietary NO_3^- / NO_2^- supplementation, and 5) method to assess CBF and testing conditions (i.e., exercise, mental stimulation). Any disagreements in data extraction were resolved through discussion until consensus was reached.

3.2.4. Quality assessment

The modified Jadad score was applied to evaluate the risk of bias of the trials. Specific questions linked to randomisation procedure, blinding and description of dropout or attrition rates were used for rank the quality of the trials (Jadad *et al.*, 1996). Scores ranged from 0 to 5; a score less than 3 indicates a low-quality trial where a score greater or equal to 3 indicates the high-quality trial.

3.2.5. Statistical analysis

The primary outcomes of the meta-analysis were changes in cognitive function and CBF after dietary NO_3^- or NO_2^- supplementation. Random effect models were applied to address the heterogeneity related to differences in study design and application of different and concomitant methods for the evaluation of cognitive function and CBF. In addition, some trials used several cognitive tests to assess domain-specific changes in cognitive function and CBF, as shown in

Table 3.1 and **3.2**. This may lead to reduced independence of measurements and to consequential over-estimation of the effect size derived from the meta-analysis. These methodological aspects were taken into account in the analysis by averaging the standardised effect sizes for each trial with the aim of providing a more conservative estimate of the effect size. Forest plots were created to summarise and illustrate the individual and overall effects of dietary NO_3^- and NO_2^- supplementation on cognitive function and CBF. The meta-analysis was conducted using Comprehensive Meta-Analysis software (Biostat, Engelwood, New Jersey). Results are described as standardized mean differences (SMDs) and 95% confidence intervals (95%CI). If data were not available in the main text or in tables, figures were used to extract the information.

Sensitivity analyses were performed to investigate whether the effects of dietary NO_3^- and NO_2^- supplementation on cognitive function and CBF were influenced by testing conditions (i.e., exercise or mental stimulation). A random-effect meta-regression model was applied to examine the associations between effect sizes for cognitive function and for CBF and age, BMI, dose of NO_3^- or NO_2^- supplementation, duration of the trial and Jadad score. Funnel plots and Egger's regression tests were performed to evaluate the publication bias (Egger *et al.*, 1997). Heterogeneity was assessed by using Cochrane Q statistic; $P > 0.1$ indicates significant heterogeneity. The I^2 test was utilised to assess heterogeneity across trials where a value < 25% indicates low risk, 25-75% indicates moderate risk, and > 75% indicates a high risk (Higgins *et al.*, 2003).

3.3. Results

3.3.1. Search results

The screening process and the number of the studies included in the systematic review are described in **Figure 3.1**. The initial search of the two electronic databases produced 12865 articles which was reduced to 5387 after the deletion of duplicates. No relevant studies were found by manual search of relevant reviews and studies. After the first title and abstract selection phase, 23 full-text articles were identified for further assessment and, from these, 18 trials were included in the systematic review. Thirteen trials and nine trials were included in the meta-analysis to investigate effects of NO_3^- and NO_2^- supplementation on cognitive function and on CBF, respectively.

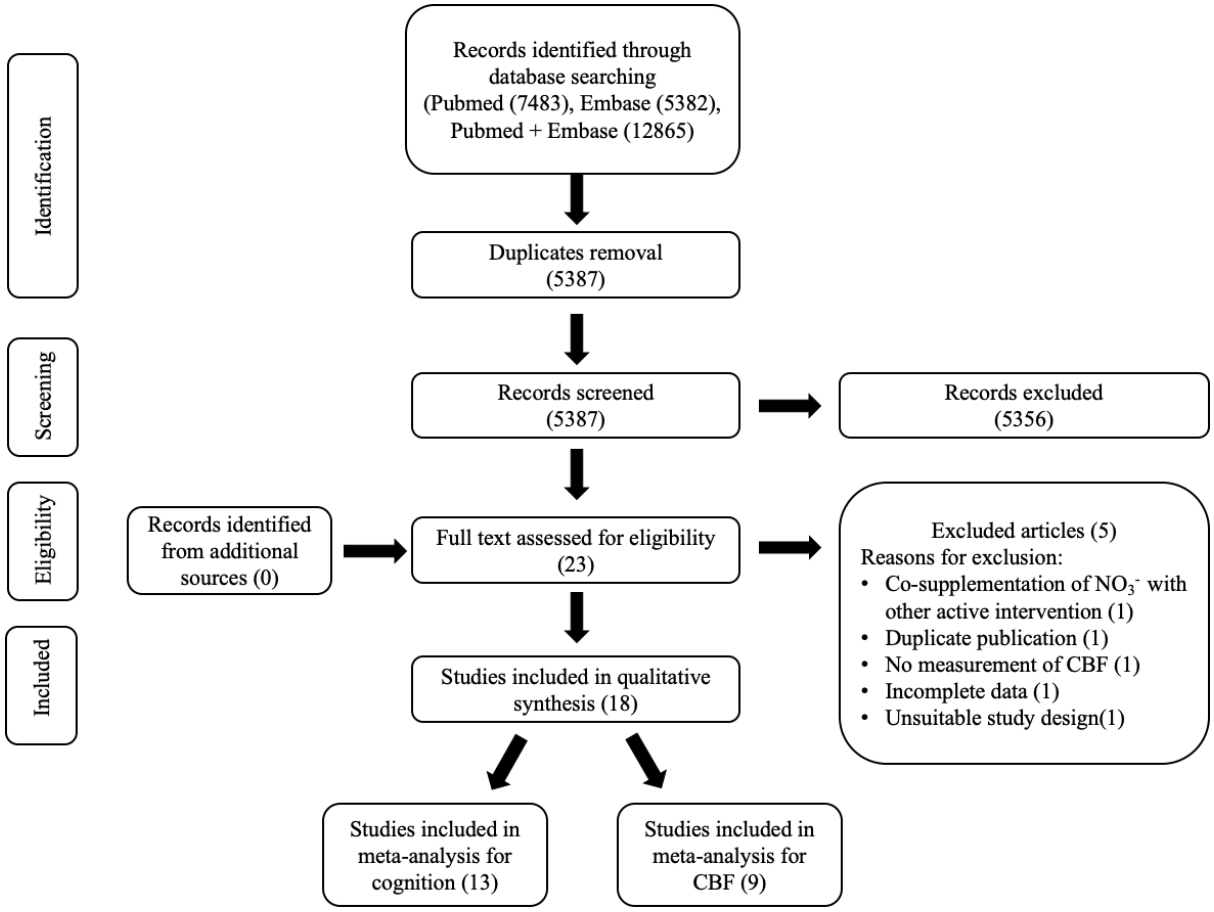

Figure 3.1: Flow diagram of the process used in selection of the randomised controlled trials included in this systematic review and meta-analysis.

3.3.2. Study characteristics (cognitive function)

3.3.2.1. Study design and supplementation

The trials included in the systematic review reported on a total of 297 participants with a median of 23 (range 10-48) participants per trial. The median age of the participants was 36 (range 21 –73) years. The systematic review includes 2 parallel and 11 crossover trials and 12 of them were double-blind. Six of these studies included an exercise component as part of the protocol to evaluate the effects of dietary NO_3^- and NO_2^- on cognitive function at rest and during exercise. The large majority (12 of 13 studies) supplemented with NO_3^- or vegetable- NO_3 rich foods; eleven trials used beetroot and one trial used spinach as sources of dietary NO_3^-, and one study supplemented with sodium NO_2^- (**Table 1**). As placebo, eight trials used NO_3^- -depleted BJ (Kelly *et al.*, 2013; Gilchrist *et al.*, 2015; Rattray *et al.*, 2015; Thompson *et al.*, 2015; Lefferts *et al.*, 2016; Thompson *et al.*, 2016; Vanhatalo *et al.*, 2016; Shannon *et al.*, 2017b), one study

employed NO_2^- -free capsules (Justice *et al.*, 2015), two trials combined apple and blackcurrant juice (Thompson *et al.*, 2014; Wightman *et al.*, 2015b) and one study did not report information on the control group (Bondonno *et al.*, 2014a).

3.3.2.2. Participant health status and intervention duration

Two trials included patients with T2D (Gilchrist *et al*., 2014; Shepherd *et al.*, 2015), four trials included middle-aged and older healthy participants (Kelly *et al.*, 2013; Bondonno *et al.*, 2014; Justice *et al.*, 2015; Vanhatalo *et al.*, 2016) and the remaining seven trials recruited young healthy participants (**Table 3.1**). The median BMI of the adults included in the trials was 24.6 kg/m^2 (range: 24.0 –30.8 kg/m2). The duration of interventions ranged from 90 minutes to 10 weeks but ten trials (out of 13) had a duration of less than 7 days. For NO_3^- supplementation studies, the median dose of dietary NO_3^- provided was 7.2 mmol/day (range: 2.9 –12.8 mmol/day); the trial using NO_2^- involved supplementation with 2.4 mmol/day of sodium NO_2^- (Justice *et al.*, 2015).

The greatest source of heterogeneity in the cognitive function trials was the type of cognitive assessment with 23 different tests being reported. Three trials used a single cognitive function test (Rattray *et al.*, 2015; Thompson *et al.*, 2015; Vanhatalo *et al*., 2016) whereas one trial employed eight different cognitive function tests (Lefferts *et al*., 2015). A summary of the distribution of cognitive tests per trial is provided in **Table 3.1.**

3.3.2.3. Meta-analysis

Overall, dietary NO_3^- or NO_2^- supplementation did not improve cognitive function (SMD +0.06, 95% CI: -0.06, 0.18, P = 0.32) and we observed no significant heterogeneity between studies (I^2 = 0%; P = 0.68) (**Figure 3.2**). There was one study which involved supplementation of dietary NO_2^- in healthy older individuals for 10 weeks, which reported a significant improvement in cognitive function (Justice *et al*., 2015). When stratified by inclusion of exercise testing in the protocols, there was no significant effect of dietary NO_3^- supplementation in either the exercise (N=6, SMD +0.13, 95% CI: -0.05, 0.32, P = 0.16) or non-exercise (N=7, SMD +0.02, 95% CI: -0.15, 0.21, P = 0.76) trials. Meta-regression analysis did not reveal any significant association between cognitive function effect size and age (β: -0.002, SE: 0.003, P = 0.33), BMI, (β: -0.02, SE: 0.02, P = 0.23), dose (β: 0.002,SE: 0.004, P = 0.63), study duration (β: 0.0005, SE: 0.0002, P = 0.07) or Jadad score (β: 0.09, SE: 0.18, P = 0.09) (**Table 3.1**).

3.3.2.4. Study Quality and Publication bias

The quality of the trials ranged from 2 to 5 (median: 3) on the Jadad score and only one study had a score < 3 (Bondonno *et al*., 2014), indicating the overall high quality of the trials **(Table 3.1).** Visual inspection of the Funnel Plot revealed a study with a large positive effect size and the presence of publication bias was also confirmed by the Egger's Regression test (P = 0.01; **Figure 3.3**). Exclusion of the study (Justice *et al*., 2015) with the largest positive effect size removed the publication bias (N=12, Egger's test, P=0.13).

Table 3.1: Characteristics of the studies included in the systematic review and meta-analysis of the effects of dietary nitrate or nitrite on cognitive function

Reference	Study Design	Sample Size	Health Status	Age	Males	Nitrate Dose (mmol/day)	Type of Intervention	Placebo/ control	Duration	Baseline BMI (Kg/m^2)	Cognitive Tests	Exercise Testing?	Jadad Score
Bondonno *et al.*, (2014)	RCT, Crossover, non-blind	30	Healthy Middle-Aged	47.3	6/24	2.9	SP	LNC	150 min	23.6	SRT, DV, CRT, SM, NWM, DWR	NO	2
Gilchrist *et al.*, (2015)	RCT, crossover, double-blind	27	T2D	67.2	18/9	7.5	BJ	ND-BJ	14 days	30.8	SRT, SM, RVIP, DRT, SPM	NO	3
Justice *et al.*, (2015)	RCT, parallel, double blind	30	Healthy Older	62	16/14	1.2/2.4	SNi	NF-C	10 weeks	24.9	TMT-A TMT-B	NO	4
Kelly *et al.*, (2013)	RCT, crossover, double-blind	12	Healthy Older	64	6/6	9.6	BJ	ND-BJ	3 days	24.1	RVIP, SS, NUR	NO	3
Lefferts *et al.*, (2015)	RCT, crossover, double-blind	20	Healthy, Young	23	All M	6.5-7.0	BJ	ND-BJ	120 min	24.6	MR, ER, DV, AST, CRT, MZ, CPT, GNG	YES	3
Rattray *et al.*, (2015)[1]	RCT, crossover, double-blind	12	Healthy, Young	NR	NR	12	BJ	ND-BJ	120 min	NR	Stroop	YES	-
Shannon *et al.*, (2017)	RCT, crossover, double-blind	10	Healthy, Young	23	All M	12.5	BJ	ND-BJ	175 min	23.9	SST, AST, RVIP	YES	3

Reference	Study Design	Sample Size	Health Status	Age (years)	Gender (M/F)	Nitrate Dose (mmol/day)	Type of Intervention	Placebo/ control	Duration	Baseline BMI (Kg/m^2)	Cognitive Tests	Exercise Testing?	Jadad Score
Shepherd *et al.*, (2014)[1]	RCT, crossover, double-blind	48	T2D	63.3	NR	6.4	BJ	ND-BJ	4 days	30.1	SRT, SPM, Stroop	NO	
Thompson *et al.*, (2015)	RCT, crossover, double-blind	16	Healthy, Young	24	All M	12.8	BJ	ND-BJ	7 days	24.6	Stroop, DRT	YES	3
Thompson *et al.*, (2014)	RCT, crossover, double-blind	16	Healthy, Young	24	All M	5	BJ	BCJ+AJ	90 min	24.1	RVIP, Stroop	YES	3
Thompson *et al.*, (2016)	RCT, crossover, double-blind	36	Healthy, Young	24	All M	6.4	BJ	ND-BJ	5 days	24.6	Stroop	YES	3
Vanhatalo *et al.*, (2016)[1]	RCT, crossover, double-blind	30	Healthy Older	73	NR	12	BJ	ND-BJ	10 days	25	RVIP	NO	-
Wightman *et al.*, (2015)	RCT, parallel, double-blind	40	Healthy, Young	21	12/28	5.5	BJ	ND-BJ	90 min	24	SS, RVIP, MFT	NO	3

AST; Attention Switching Task, BCJ+AJ; Blackcurrant cordial juice and apple juice, BJ; Beetroot juice, BMI; Body mass index, CRT; Choice Reaction Time, CPT; Continuous performance test, DRT; Decision reaction time, DWR; Delayed word recognition, DV; Digit vigilance, ER; Emotion Recognition, GNG; Go/No-Go, NF-C; Nitrite free capsules, RCT; Randomised clinical trial, RVIP; Rapid Visual Information Processing, SNi; Sodium nitrite, SP; Spinach, SS; Serial subtractions, SRT; Simple reaction time, SM; Shape memory, SPM; Spatial memory, SST; Spatial span task, T2D; Type 2 diabetes, TMT-A; Trail Making Tests A, TMT-B; Trail Making Test-B, MFT; Mental fatigue test, MR; Memory recognition, MZ; Maze, NWM; Numeric working memory, NUR; Number recall, VS-1; Visual interference, VB-1; Verbal interference. [1] These are abstracts and the quality assessment was not performed.

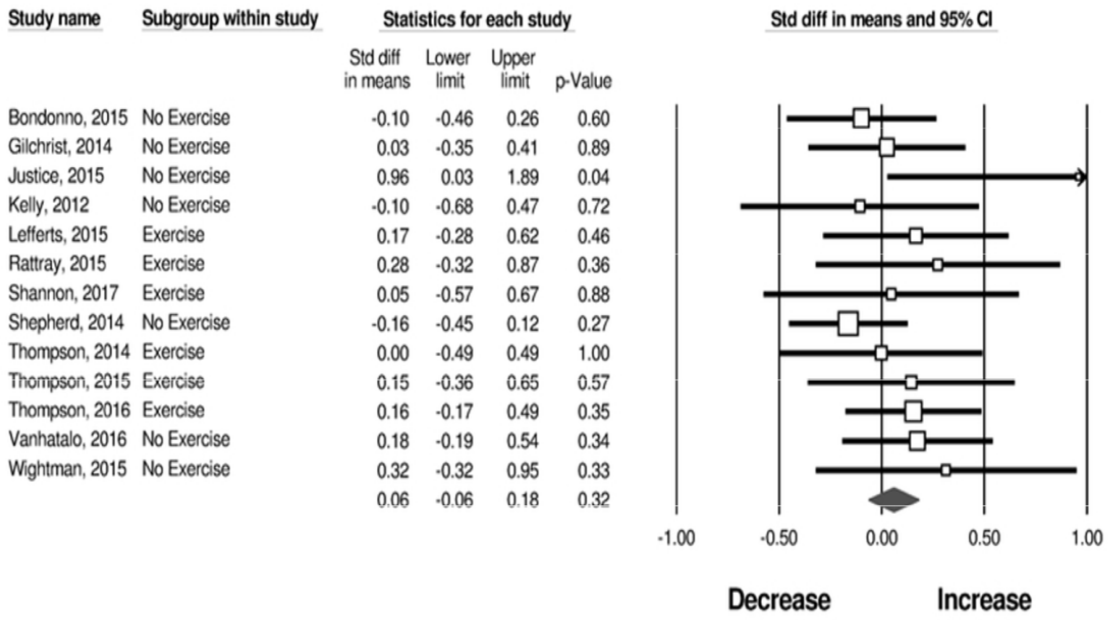

Figure 3.2: Forest plots showing the effect of dietary NO_3^- and NO_2^- supplementation on cognitive function.

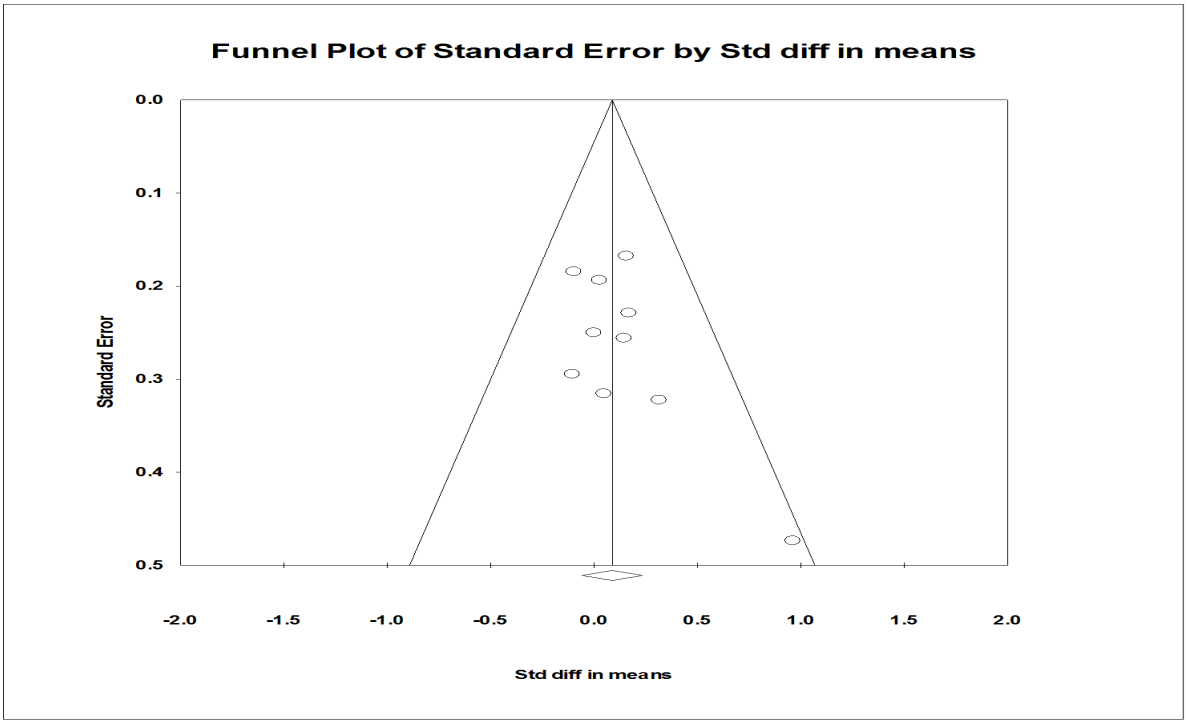

Figure 3.3: Funnel plot to evaluate publication bias of trials testing the effects of NO_3^- and NO_2^- on cognitive function.

Egger's test, (n=13), P=0.01. Exclusion of the study with the largest positive effect size removed the publication bias (n=12, Egger's test, P=0.13).

3.3.3. Study characteristics (Resting and stimulated cerebral blood flow)

3.3.3.1. Study design and supplementation

Nine trials assessed changes in CBF in resting conditions and included a total of 163 participants (sample size range: 10 –40); the overall median age of the participants was 22 years (range 20 –70). Five of these studies also assessed CBF under stimulated conditions (i.e., exercise (Bond *et al.*, 2013; Curry *et al.*, 2016; Lefferts *et al*., 2016; Thompson *et al.,* 2014), or mental stimulation (Wightman *et al*., 2015).

One study employed a parallel study design (Wightman *et al*., 2015) whereas all remaining eight trials used a cross-over design (Presley *et al.*, 2011; Aamand *et al.*, 2013; Bond *et al.*, 2013; Thompson *et al.*, 2014; Rattray *et al.*, 2015; Curry *et al.*, 2016; Lefferts *et al.*, 2016; Chirinos *et al.*, 2017) (**Table 3.2**). Most studies (seven) used BJ as a source of dietary NO_3^- but high NO_3^- foods or sodium nitrite were also used in some studies (**Table 3.2**).

3.3.3.2. Cerebral blood flow tests

Four studies reported the effect of dietary NO_3^- supplementation on middle cerebral artery blood flow velocity (MCAV) (Aamand *et al.,* 2013; Curry *et al*., 2016; Lefferts *et al*., 2016; Rattray *et al.,* 2015) and two reported the effect of dietary NO_3^- on CBF measured by arterial spin labelling (Presley *et al.,* 2011; Aamand *et al.,* 2013). Additional measurements used to assess CBF included NIRS (Thompson *et al.,* 2014; Wightman *et al.,* 2015), cerebrovascular resistance index by Transcranial Doppler Ultrasonography (Bond *et al*., 2013) and evaluation of changes in Carotid Characteristic Impedance, Carotid Cross-Sectional Area and Carotid Bed Vascular Resistance (Chirinos *et al*., 2017).

3.3.3.3. Participant health status and intervention duration

Eight trials recruited healthy individuals and one trial recruited patients with heart failure (Chirinos *et al*., 2017) (**Table 3.2**). The duration of the dietary NO_3^- supplementation ranged from 3 hours to 3 days. The dose of dietary NO_3^- ranged from 5.5 to 24 mmol/day (median dose: 9.8 mmol/day).

3.3.3.4. Meta-analysis

Overall, dietary NO_3^- did not improve CBF under either resting (SMD +0.14, 95% CI: -0.13, 0.41, P = 0.31), or under stimulated conditions (SMD + 0.23, 95% CI: -0.11, 0.56, P= 0.19). We observed moderate heterogeneity between studies testing the effect of dietary NO_3^- on CBF

at rest and stimulated conditions (I^2= 56.7%; P = 0.01; I2 = 44.1%; P = 0.12, respectively) (**Figure 3.4**). Meta-regression analysis produced no evidence for significant associations of resting CBF effect size with age (β: 0.001, SE: 0.006, P = 0.98), BMI, (β: 0.016, SE: 0.019, P = 0.41), dose (β: -0.01, SE: 0.019, P = 0.58),or Jadad score (β: 0.03, SE: 0.13, P = 0.79). However, there was a significant negative association between CBF effect size and study duration (β: -0.001, SE: 0.0006, P = 0.02) (**Table 3.3**).

3.3.3.5. Study quality and publication bias

The quality of the trials ranged from 2 to 4 (median: 2) according to the Jadad score. On this scoring system, 4 studies showed a score ≥3 (Chirinos *et al.,* 2017; Lefferts *et al*., 2015; Thompson et al, 2014; Wightman *et al.,* 2015) (**Table 3.2**). We could not assess the quality of one study (Rattray *et al.,* 2015), as it was an abstract. Visual inspection of the Funnel Plot revealed no evidence of publication bias and this was confirmed by the Egger's Regression test for both resting (P = 0.43) and stimulated (P = 0.58) CBF (**Figure 3.5**).

Table 3.2: Characteristics of the studies included in the systematic review and meta-analysis of the effects of dietary nitrate- or nitrite on cerebral blood flow

Reference	Study Design	Sample Size	Health Status	Age	Gender (M/F)	Nitrate Dose (mmol/day)	Type of Intervention	Placebo / control	Duration	Baseline BMI (Kg/m^2)	CBF Assessment	Exercise Testing?	Effect at resting	Effect in stimulated conditions	Jadad Score
Aamand *et al.*, (2013)	RCT, crossover, double-blind	20	Healthy, Young	25	All M	7.7	SNA	NaCl	3 days	-	ASL	NO	No change	-	2
Bond *et al.*, (2013)	RCT, crossover	12	Healthy, Young	20	All F	5-6	BJ	OJ	120 min	24.4	CVRI, MCAV	YES	Positive	Positive	1
Chirinos *et al.*, (2017)	RCT, crossover, double-blind	16	HFpEF	65	14/2	12.9	BJ	ND-BJ	150 min	34.4	CCID, CCSA, CBVRD	NO	Positive	-	4
Curry *et al.*, (2016)	RCT, crossover	10	Healthy, Young	20	All F	24.2	BJ	OJ	120 min	23.5	CAIx	YES	No change	Positive	1
Lefferts *et al.*, (2015)	RCT, crossover, double-blind	20	Healthy, Young	23	All M	6.5-7.0	BJ	ND-BJ	120 min	24.6	MCAV	YES	No change	No change	3
Presley *et al.*, (2011)	RCT, crossover	16	Healthy, Old	≥ 70	NR	12.4	HN diet	LN diet	2 days	-	ASL	NO	Positive (reginal cerebral perfusion)	-	2
Rattray *et al.*, (2015)[1]	RCT, crossover, double-blind	12	Healthy, Young	NR	NR	13	BJ	ND-BJ	120 min	-	MCAV	YES	Positive	-	-
Thompson *et al.*, (2014)	RCT, crossover, double-blind	16	Healthy, Young	24	All M	5	BJ	BCJ+AJ	90 min	24.1	NIRS	YES	Positive	Positive	3
Wightman *et al.*, (2015)	RCT, parallel, double-blind	40	Healthy, Young	21	12/28	5.5	BJ	ND-BJ	90 min	24	NIRS	NO	Positive	Negative	3

ASL; Arterial spin labelling, BCJ+AJ; blackcurrant cordial Juice and apple juice, BJ; Beetroot juice, BMI; Body mass index, CVRI; Cerebrovascular resistance index, CCID; Carotid characteristic impedance dynes, CBVRD; Carotid bed vascular resistance dynes, CCSA; Carotid cross-sectional area, HN; High nitrate, HFpEF; Heart failure preserved left ventricular ejection fractio, LN; Low nitrate, MCAV; Middle cerebral artery blood velocity, NIRS; Near-infrared spectroscopy, NaCl; Sodium chloride, ND-BJ; Nitrate depleted beetroot juice, NF-C; nitrite free capsules, OJ; Orange juice, RCT' Randomised clinical trial, SNA; Sodium Nitrate, TVR; Total vascular resistance. [a] This is an abstract and the quality assessment was not performed.

Table 3.3: Meta-regression analysis to evaluate whether age, BMI, dose of nitrate and duration of the intervention modified the effects of nitrate or nitrite supplementation on cognitive and cerebral blood flow

	Slope (β)	SE	Q (df)	*P*
Cognitive function (n= 13)				
Age (years)	-0.002	0.003	0.92 (1)	0.33
BMI (kg/m^2)	-0.02	0.02	1.38 (1)	0.23
Dose (mg/day)	0.002	0.004	0.22 (1)	0.63
Duration (hours)	0.0005	0.0002	3.2 (1)	0.07
Resting CBF (n= 8)				
Age (years)	0.001	0.006	0.09 (1)	0.75
BMI (kg/m^2)	0.02	0.032	0.58 (1)	0.45
Dose (mg/day)	-0.02	0.023	0.43(1)	0.51
Duration (hours)	-0.007	0.003	4.67 (1)	0.03

BMI, Body mass index; SE, Standard error; CBF, Cerebral blood flow.

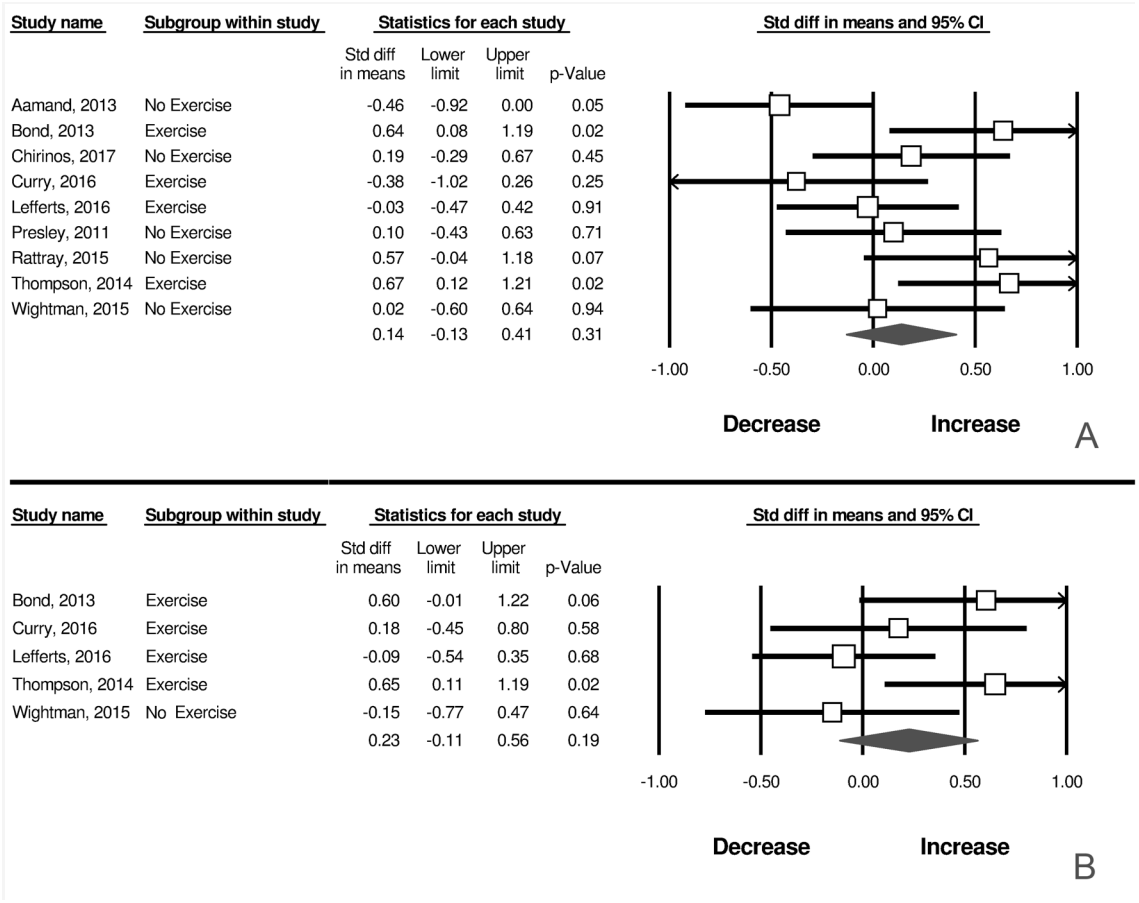

Figure 3.4: Forest plots showing the effect of dietary NO_3^- and NO_2^- supplementation on cognitive function, cerebral blood flow at rest (A) and in stimulated conditions (B).

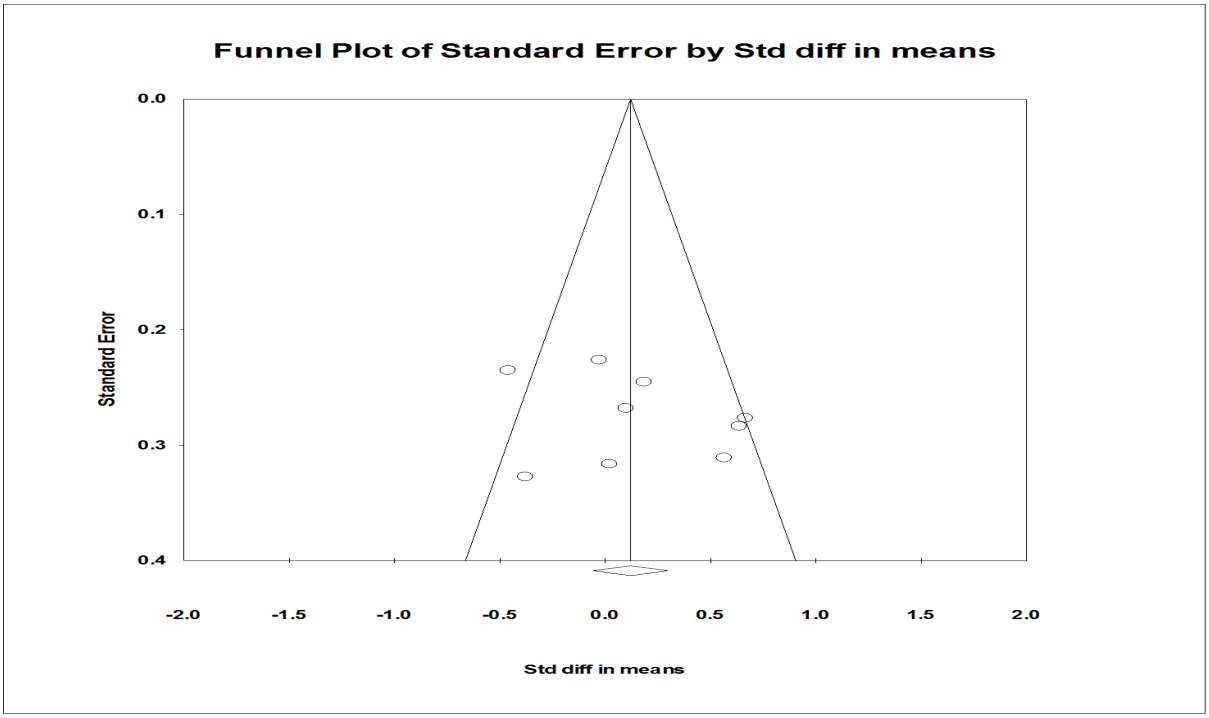

Figure 3.5: Funnel plot to evaluate publication bias of trials testing the effects of NO_3^- on resting cerebral blood flow.
Egger's test, (n=9), P=0.43.

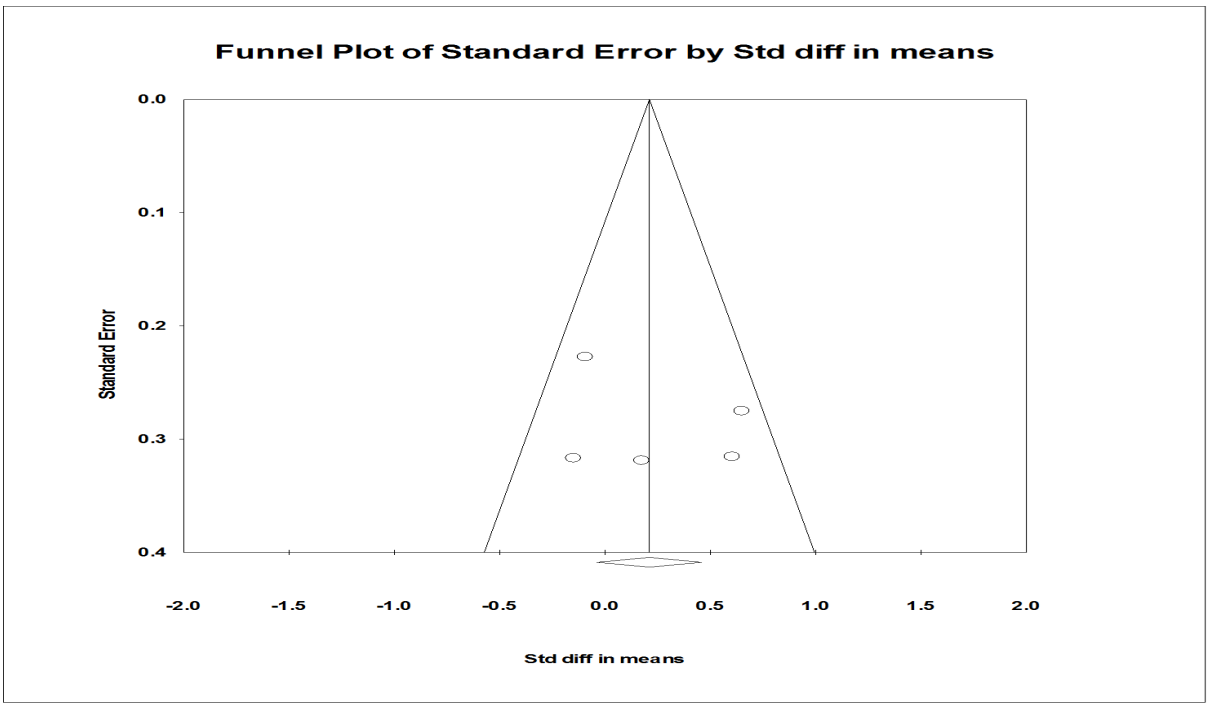

Figure 3.6: Funnel plot to evaluate publication bias of trials testing the effects of NO_3^- on stimulated cerebral blood flow.
Egger's test, (n=9), P = 0.43.

3.4. Discussion

The current systematic review and meta-analysis demonstrated that cognitive function and CBF were not improved following dietary NO_3^- and NO_2^- supplementation. The combined SMD (placebo vs. intervention) was +0.06 for cognitive function and +0.14 and +0.23 for CBF at rest and in simulated conditions, respectively. I investigated the potential influence of several factors including: participants' age and BMI, the dose of dietary NO_3^- or NO_2^-, and whether the tests were conducted at rest or during exercise but none of these factors modified findings for cognitive function or CBF. Overall, included studies were characterised by small sample size and short duration. Thus, it is difficult to draw definitive conclusions regarding the efficacy of dietary NO_3^- or NO_2^- in modulating cognitive function and CBF.

The quality of the studies evaluating cognitive function was generally higher than that of those evaluating CBF. Although all of them used a randomised design, double blinding was employed in only 6 out of 9 studies that assessed CBF. In contrast, all but one study that assessed cognitive function used a double-blind design (the exception was Bondonno *et al.,* 2014). Generally, included studies are characterised by small sample size. Prior power calculations suggest that approximately 30 participants were sufficient to detect subtle effects of treatments, as suggested by Bondonno et al., (2014). However, studies on cognitive function had a median sample size of 23 suggesting that many of these studies were not adequately powered to detect potential effects of the NO_3^- or NO_2^- treatment on cognitive function. Type 2 error is highly associated with such lower sample sizes. Moreover, most of the included studies (19 out of the 21 studies) did not report that they had undertaken *a priori* power calculations which indicates that their findings might not be sufficient to draw robust conclusions. These limitations of previous studies can be overcome by recruiting larger sample sizes in future studies that are sufficiently powered to detect potential effects of NO_3^- or NO_2^- on CBF and cognitive function.

The participants in most studies were healthy males with normal BMI and who were not suffering from a cognition-related disease. Only two studies investigated the effect of dietary NO_3^- on cognitive function in obese diabetic patients and these studies reported conflicting results (Gilchrist *et al.,* 2014, Shepherd *et al.,* 2015). The remaining 11 studies that assessed the effect of NO_3^- or NO_2^- on cognitive function were conducted on participants with a BMI $\leq$ 25 kg/m^2. Obesity is associated with decreased NO bioavailability (Chen *et al.*, 2018), thus individuals with a BMI greater than 30 kgm^{-2} might be more responsive to NO_3^- or NO_2^- induced vascular or metabolic effects (Ashor *et al.*, 2016). Also, future studies should focus on comparing the effects of NO_3^- or NO_2^- supplementation on cognitive function between normal weight and obese individuals. To date, only one study has assessed the effects of dietary NO_3^-

supplementation on CBF in non-healthy, obese participants. This study by Chirinos et al. (2017) investigated effects of NO_3-rich BJ among heart failure patients and observed no significant change in carotid artery hemodynamics. Since heart diseases are associated with increased age, it is important to conduct studies on older people, particularly those with cognitive disorders. It may be easier to detect positive effects of dietary NO_3^- and NO_2^- supplementation on cognitive function and CBF among older people with some cognitive dysfunction than in healthy young adults where the scope for improvement would be much less. Few studies have included female participants. Although there is no strong a priori rationale to anticipate that the impact of such supplementation would differ by sex, women live longer than men and the prevalence of dementia increases with age (Zarulli *et al.*, 2018). Thus, it is necessary to conduct more studies to establish if there are any significant differences between males and females on effects of dietary NO_3^- or NO_2^- supplementation on cognitive function and CBF, particularly in later life. The importance of potential sex differences with dietary NO_3^- supplementation has been highlighted in a recent review with other physiological functions such as BP and exercise performance (Wickham and Spriet, 2019). This review indicates that women are under-represented in research related to dietary NO_3^- supplementation.

The current meta-regression analysis revealed that the duration of NO_3^- or NO_2^- supplementation had a moderate influence on CBF. Longer duration of supplementation led to a small improvement in CBF. However, this finding needs to be confirmed by further studies to reveal the exact influence of supplement duration since the majority of trials were of short duration. Two of nine studies on the effects of supplement on CBF observed positive impacts. The first study by Presley et al. (2011) employed a randomized crossover trial where healthy older adults received either a high NO_3^- (12.6 mmol/day) or low NO_3^- (0.9 mmol/day) diet for 2 days prior to measurements of cerebral perfusion using MRI. They found that participants on the high NO_3^- diet had significantly higher frontal cortex perfusion compared with those on the low NO_3^- diet. In another study by Aamand et al. (2013), the CBF measured by MRI did not change following 3 days of supplementation with sodium NO_3^- compared to participants given NO_3^- -free saline. However, the authors found that there was a decrease in the haemodynamic lag of the blood oxygenation level dependent (BOLD) response in the visual cortex of healthy, young males following sodium NO_3^- supplementation. These results suggest that more studies are needed to investigate the effect of duration on efficacy of NO_3^- or NO_2^- on CBF.

Most studies included in this review provided NO_3^- in the form of BJ or NO_3^- -rich foods such as green leafy vegetables while few provided NO_3^- salts *per se*. This poses the question as to whether mode of NO_3^- delivery affects its efficacy on cognitive function and CBF. Recent

studies have shown greater effects of NO_3^--rich vegetable products on BP (Jonvik *et al.*, 2016), the oxygen cost of exercise (Flueck *et al.*, 2015) and post-exercise recovery (Clifford *et al.*, 2017) in comparison with NO_3^- salt. These results suggest possible synergistic effects of NO_3^- and of other compounds present in vegetables. Indeed, polyphenol compounds such as catechins, anthocyanins, and other flavonoids, and carotenoids (Gómez-Pinilla, 2008; Macready *et al.*, 2009) are reported to have beneficial effects on CBF and cognitive function through NO-dependent mechanisms. These plant compounds have vasodilative effects that are beneficial for cognitive function and CBF (Sokolov *et al.*, 2013). Other previous studies have also indicated positive effects of several plant-derived compounds, other than NO_3^-, on cognitive function and CBF (Macready *et al.*, 2009; Desideri *et al.*, 2012; Ide *et al.*, 2014).

To date, there is no evidence to suggest that beetroot, the main vehicle used in the included RCTs, contains high quantities of the polyphenolic compounds showing potential for cognitive modulation. Other than NO_3^-, betanin is the most abundant bioactive compound in beetroot, whose potential effects on cognitive function are unknown. Other NO_3^--rich plants may contain additional bioactive compounds that influence cognitive function which introduces potential complexity when interpreting the effects of these plants with those of NO_3^- / NO_2^- salts. The independent effects of these bioactive compounds and of the NO_3^- / NO_2^- in these foods is an important question for future research.

The current study has a number of other limitations. First, a wide range of assessments and methods were employed by different researchers to evaluate effects of NO_3^- on cognitive function, several of which were domain specific, thus pooling the average effects size for all tests overlooks potential changes for isolated tests. We used an average effect size as the main outcome measure in order to provide a more conservative estimate. A second limitation is the heterogeneity between studies that investigated effects on CBF; this is attributed to wide variability in participant age and health status, CBF measures used, and the dose and duration of the NO_3^- or NO_2^- interventions used in each study. As noted in a recent study, heterogeneity between studies can obscure the benefits seen in single studies whose conditions are well controlled (Barnard *et al.*, 2017). Setting specific conditions in single experiments allows the observation of accurate effects of treatments. The possibility of heterogeneity must be considered when interpreting our findings. During the period since this study was published, 12 more RCT have been identified and a summary tables for these new studies can be found in **Appendix 3.2**.

3.5. Conclusion

There is no strong evidence that dietary NO_3^- or NO_2^- supplementation influences CBF or cognitive function. Most studies were conducted on young adults or in older (<75 years old) healthy participants. Hence, these findings might not be generalizable to much older people, those with higher BMI or those with reduced cognitive ability. In addition, all available trials were characterized by small sample sizes and short intervention durations thus, most of the studies may not have been powered adequately to show potential benefits of NO_3^- or NO_2^- , if they existed. Therefore, it can be concluded that there is not sufficient evidence to show that supplemental NO_3^- or NO_2^- could improve cognitive function or CBF. Given the growing interest in use of dietary approaches for maintenance and improvement of cognitive function during ageing, there is a need for further studies with well-controlled and sufficiently powered trials, especially in more at-risk populations such as older people with evidence of cognitive dysfunction. In addition, there is a need for studies of longer duration (to determine whether any benefits are sustainable) with different doses of NO_3^- or NO_2^- supplementation to determine the optimal dose.

Chapter 4. Validity and reliability of test strips for the measurement of salivary nitrite concentration with and without the use of mouthwash in healthy adults

This chapter has been adapted with some modification from our published paper in *Nitric Oxide:*

Babateen, A.M., Shannon, O.M., Mathers, J.C. and Siervo, M. (2019) 'Validity and reliability of test strips for the measurement of salivary nitrite concentration with and without the use of mouthwash in healthy adults', *Nitric Oxide*, 91, pp. 15-22 (**Appendix 4.1**).

4.1. Introduction

NO is a reactive gas which is involved in numerous physiological processes, including blood flow regulation, immune defence and neurotransmission (Bruckdorfer, 2005). NO can be synthesised endogenously from l-arginine in a reaction catalysed by the NOS enzymes. Additionally, NO can be generated via an alternative pathway that depends on the entero-salivary circulation of NO_3^- – an inorganic anion which is present in a range of commonly consumed foods (Weitzberg and Lundberg, 2013). Ingested NO_3^- is absorbed rapidly from the upper gastrointestinal tract into the blood. Approximately 25% of circulating NO_3^- is taken up by the salivary glands and concentrated, prior to being excreted into the mouth in saliva (Omar *et al.*, 2015). The protein sialin (SLC17A5) was identified as the principal NO_3^- transporter in the salivary glands and knockdown of sialin expression reduced NO_3^- transport (Qin *et al.*, 2012). Once in the mouth, a portion of the NO_3^- is reduced to NO_2^- by commensal facultative anaerobic bacteria which reside predominantly on the dorsal surface of the tongue (Doel *et al.*, 2005). The resulting NO_2^- is then swallowed in saliva and may be further reduced to NO via enzymatic and non-enzymatic pathways to help support or maintain NO-signaling, especially in acidic (Omar *et al.*, 2015) and ischemic (Weitzberg and Lundberg, 2013) conditions.

Dietary supplementation with NO_3^-, which increases NO production via the NO_3^--NO_2^--NO pathway, elicits multiple beneficial effects on physiological outcomes. Effects include a significant reduction in BP (Kapil *et al.*, 2010), improved exercise performance (Bailey *et al.*, 2009; Berry *et al.*, 2015), and enhanced cognitive function (Gilchrist *et al.*, 2015), although this latter finding was not confirmed in the meta-analysis reported in Chapter 3. The NO_3^- reducing bacteria which reside in the oral cavity play a fundamental role in facilitating these beneficial effects following dietary NO_3^- ingestion (Doel *et al.*, 2005). Indeed, several studies have shown

that using antibacterial mouthwash diminishes considerably the colony size of the bacteria (Bondonno *et al.*, 2014b; Woessner *et al.*, 2016). This reduction affects the production of NO_2^- in the oral cavity, concomitantly lowering the concentration of NO_2^- in saliva and plasma, and diminishes the physiological effects that may otherwise manifest following NO_3^- supplementation (Govoni *et al.*, 2008; Kapil *et al.*, 2013; Bondonno *et al.*, 2014b; Woessner *et al.*, 2016). These studies, in addition to other human and animal studies, were included in a recent systematic review which concluded that using mouthwash reduces the plasma and salivary NO_3^- and NO_2^- concentrations (Senkus and Crowe-White, 2019). This systematic review showed that short term use (3-7 days) of mouthwash abolishes the BP lowering effect of dietary NO_3^- supplementation (Senkus and Crowe-White, 2019). The long-term effect of mouthwash use on cardiovascular and metabolic health was shown in a recent longitudinal study conducted on > 1000 individuals over 3 years follow-up. This longitudinal cohort study found that frequent use of mouthwash is associated with increased risk of hypertension (Joshipura *et al.*, 2019) and diabetes (Joshipura *et al.*, 2017). Thus, whilst antibacterial mouthwash could help to maintain oral health, its regular use may interfere adversely with the beneficial effects of NO_3^- on cardiovascular health.

Evidence that dietary NO_3^- may improve NO bioavailability and thus enhance a range of physiological functions has attracted researchers to develop a range of simple techniques to monitor systemic NO-bioavailability, including, amongst others, NO salivary test strips. These strips allow the estimation of salivary NO_2^- concentration, which could provide a non-invasive indication of NO availability and provide information on compliance with dietary NO_3^- interventions. The strips have been validated in non-supplemented (Modi *et al.*, 2017) and supplemented subjects (McDonagh *et al.*, 2017) and correlate significantly with salivary NO_2^- measured via gold-standard techniques (i.e. ozone-based chemiluminescence). However, to our knowledge, no study has investigated whether antiseptic mouthwash could affect the sensitivity of the salivary strips by inhibiting the conversion of NO_3^- into NO_2^- . In addition, previous studies validated these strips using simple correlation analyses (McDonagh *et al.*, 2017), and have not applied the Bland-Altman method to evaluate the magnitude, variability and direction of the measurement bias (Bland and Altman, 1986).

Therefore, the purpose of this study was to test the validity of these strips against reference standard laboratory measures (i.e. ozone-based chemiluminescence) of salivary NO_2^- and NO_3^- concentrations with and without the use of mouthwash, using the Bland-Altman method. In addition, as these strips are based on a modified Griess reagent reaction, we also measured salivary NO_2^- using the Griess method for further comparison. Finally, we also took the

opportunity to investigate the effect of both NO_3^- supplementation and mouthwash on salivary sialin concentrations, which functions as a NO_3^- transporter in the plasma membrane of salivary glands.

4.2. Methods

4.2.1. Participants

Ten healthy, non-smoking, normal weight or overweight (BMI range: 20-29.9 kg/m^2) participants aged ≥ 20 years were recruited via email from Newcastle University staff and students to take part in this study. Exclusion criteria included: smoking, history of clinical conditions and medical treatments likely to interfere with the study outcome, pregnancy and breastfeeding. All participants were fasting for at least 12 h prior to participating in the experiment. All participants were provided with a detailed information sheet and written informed consent was received before entry to the study. The study was approved by the Faculty of Medical Sciences, Newcastle University (1459/3414/2018) (**Appendix 4.2**).

4.2.2. Experimental protocol

This study was a cross-over, randomised, validation study consisting of two experimental trials (with or without mouthwash) conducted on two separate visits and with a washout period of 24 hours. At present, there is limited *in vivo* evidence on the minimum time taken for the oral NO_3^--reducing microbiome to recover following administration of antibacterial mouthwash (Rundegren *et al.*, 1992; Shen *et al.*, 2016). For practical reasons, a washout period of 24 hours was selected and this also allowed us to elucidate whether the oral microbiome remained compromised a day after mouthwash use. Eligible participants were invited for their first experimental visit early in the morning (~8.30-9.00 am) after an ~12-h overnight fast and having avoided consumption of high NO_3^- foods for the previous 24hours. Body weight was measured, and participants were asked to collect a baseline saliva sample followed by the application of two NO Test Strips (Berkeley Test®, CA, USA), as per the manufacturer's instructions. Baseline resting BP was then measured, and participants were randomised to either rinse their mouth with 20 ml of low NO_3^- water (Buxton water) or 20 ml of antiseptic mouthwash (Corsodyl, Chlorhexidine Digluconate 0.2%, UK) for 2 minutes. After 15 minutes, participants consumed one 70 ml 'shot' of concentrated BJ (Beet-it, James White Company). This juice contains approximately 400 mg (~6.5 mmol) of NO_3^-, which is roughly equivalent to eating a large portion 200-300 g of lettuce or rocket. Participants were asked to collect saliva samples, apply the salivary strips, and measure their resting BP at 1, 2, 3, 4 and 5 hours post-

consumption. During this period, participants were asked not to eat any food, except for consumption of a low NO_3^- chocolate bar after collection of the third saliva sample. The consumption of low NO_3^- water was allowed *ad libitum*, but was prohibited in the 15 minutes prior to the collection of each saliva sample. An overview of the protocol is shown in **Figure 4.1**. Dietary instructions and necessary materials for the collection of saliva samples were provided. Participants were asked to refrain from using mouthwash in the 24h period before each trial, and throughout each experimental trial.

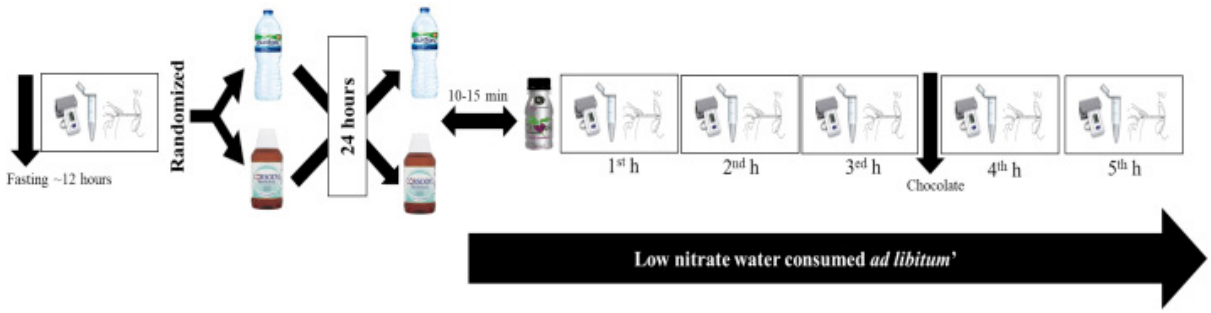

Figure 4.1: Overview of the study protocol.

4.2.3. Blood pressure measurements

Baseline resting BP was measured in duplicate using an automated BP monitor (Omron M3, Omron Healthcare Ltd., Kyoto, Japan). The mean of the two records was taken as the baseline BP. At 1, 2, 3, 4 and 5 h post administration of BJ, participants measured their own BP in duplicate.

4.2.4. Saliva samples collection

For the measurement of NO_3^-, NO_2^- and sialin concentrations, saliva samples were collected by chewing a cotton ball for 1–2 min. The cotton ball was then placed in a 20 ml syringe, which was used to squeeze the saliva into a 1.5 ml Eppendorf tube prepped with 4µl of NaOH to avoid degradation of salivary NO_3^- and NO_2^-. Samples were stored at −20 °C within 30 minutes of collection for further analyses.

4.2.5. Salivary nitrite assessment using strips

Salivary NO_2^- strips (Berkeley Test strips, CA, USA) were used as per the manufacturer's guidelines. Specifically, the test strip with the 'saliva here' side was placed on the tongue and swabbed over a 10 s period covering different areas including the dorsal surface of the tongue. The two ends of the strip were folded and pressed gently for 10 s. The colour of the NO test pad

was then allowed to develop over a 45 s period. The intensity of the colour was compared with a colour chart using a mobile phone-based application developed by the manufacturers The application has a long colour chart and each colour is associated with a quantitative value for NO_2^- concentration, with darker colours corresponding to higher NO_2^- concentrations. To evaluate the repeatability of this method, participants estimated their salivary NO_2^- concentration using two, separate test strips, with a 1-min interval between them. In addition, five observers read each of the strips independently to quantify inter-observer reproducibility.

4.2.6. Salivary nitrate and nitrite analysis

Salivary NO_3^- and NO_2^- concentrations were quantified using gas-phase chemiluminescence and a colorimetric Griess assay as described below:

4.2.6.1. Chemiluminescence

Salivary NO_3^- and NO_2^- concentrations were analysed using a Sievers gas-phase chemiluminescence NO analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). Sodium iodide in acetic acid was used as a reductant for NO_2^- to NO, while vanadium chloride in hydrochloric acid at 95 °C was used to determine NO_3^- concentrations by the reduction of NO metabolites to NO and subsequent subtraction of NO_2^- concentration. The concentrations of NO_3^- and NO_2^- were determined by plotting signal area (mV) against a calibration plot of known concentration NO_3^- and NO_2^- standards and data were analysed using the Sievers® NO Analysis™ Software Version 3.2. All saliva samples were diluted 1:100 with deionised water in preparation for the analysis. The standard curves used for the quantification of NO_3^- and NO_2^- concentrations can be found in **Appendix 4.3.** Details of chemiluminescence procedure can be found in **Appendix 4.4.**

4.2.6.2. Griess method

A commercial kit (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical, Ann Arbor, MI, US) was used to measure NO_3^- and NO_2^- concentrations using the Griess method. Using this kit, NO_2^- was measured directly but NO_3^- was reduced enzymatically to NO_2^- prior to the diazotization reaction. Griess reagents were then added to convert NO_2^- into a purple-coloured azo compound and the concentrations of the azo compound were then monitored spectroscopically at 540 nm. Saliva samples were diluted in assay buffer 1:100 for NO_2^- analysis and 1:10 for NO_3^- analysis. Details of the procedure can be found in **Appendix 4.5.**

4.2.7. Sialin (SLC17A5) analysis

Sialin (SLC17A5) concentrations in saliva were quantified using a commercial BioAssay™ ELISA Kit (Human) from Stratech Scientific Ltd, in a 96-well format. This Elisa kit is based on the sandwich enzyme-linked immunoassay technique. To determine the optimum dilution factor, different saliva dilutions were tested (1:100, 1:50, 1:10 and 1:2), and a dilution of 1:2 was chosen as the optimum saliva concentration for the assay. Details of the procedure can be found in **Appendix 4.6.**

4.2.7. Statistical analysis

A two factor ANOVA for conditions (water and mouthwash) with repeated measures for sampling time was applied to determine the effects of the BJ intervention on BP, salivary NO_3^- and NO_2^-, and salivary sialin. Bland-Altman analysis was applied (Bland and Altman, 1986) to provide a visual representation of the agreement between methods used to analyse salivary NO_3^- and NO_2^- concentrations. Normal distribution was checked via the Shapiro-Wilk test, and data were log transformed when necessary. Spearman's correlation analysis was performed to evaluate whether changes in NO_3^- and NO_2^- concentrations were associated with changes in BP and sialin concentrations. In addition, we evaluated whether changes in salivary NO_2^- concentrations measured by salivary strips and Griess and chemiluminescence methods were significantly associated. To evaluate the effects of mouthwash/water use on recovery time of the NO_3^- reducing capacity of oral bacteria the area under the curve (AUC) of NO_2^- concentrations for participants receiving the water on the first day ($n = 5$) was compared with the AUC derived from participants receiving the water on the second day ($n = 5$) (independent sample *t*-test). All data are presented as mean ± SEM unless otherwise indicated. Statistical significance was accepted when $P < 0.05$. The Statistical Package for Social Sciences (IBM SPSS, version 23, NY, USA) was used to perform the analysis.

4.4. Results

4.4.1. Participants' baseline characteristics

Ten healthy participants were recruited (6 females and 4 males) with an age range of 20–45 years and a BMI range of 21.1–29.8 kg/m^2 (**Table 4.1**). Baseline SBP and DBP were not different between the water and mouthwash experiments ($P = 0.91$ and $P = 0.60$ for SBP and DBP, respectively).

Table 4.1: Baseline characteristics of the participants

Characteristic	Mean	SD
Age (years)	31.2	8.7
Height (cm)	163.0	12.9
Weight (kg)	65.5	13.8
Body mass index (kg/m^2)	24.3	2.7
SBP (mmHg):		
Water experiment	115.4	8.3
Mouthwash experiment	115.0	7.9
DBP (mmHg):		
Water experiment	71.5	9.7
Mouthwash experiment	73.6	9.3

SBP, Systolic blood pressure; DBP, Diastolic blood pressure (n=10).

4.4.2. Salivary nitrate concentration

There was no significant difference in salivary NO_3^- concentration at baseline between the mouthwash and water conditions, as determined by both chemiluminescence ($P = 0.34$) and Griess methods ($P = 0.43$). Following BJ ingestion, salivary NO_3^- concentration rose rapidly to peak within 1–3 h after which concentrations declined. Time to peak appeared to be delayed after use of the mouthwash. This pattern of response in salivary NO_3^- concentrations was similar when measurements were made by the chemiluminescence and Griess methods but, overall, concentrations determined by the Griess method were ~12% lower (**Figure 4.2**). Salivary NO_3^- concentration was higher than baseline at all-time points after BJ ingestion ($P < 0.001$).

4.4.3. Salivary nitrite concentration

There was a significant main effect for time on salivary NO_2^- concentration (chemiluminescence: $P < 0.001$; Griess: $P = 0.004$). In addition, a significant effect for condition (both $P < 0.001$) and interaction between time*condition (both $P < 0.001$) was observed. Following BJ ingestion, salivary NO_2^- concentration rose significantly to peak within 2–3 h in participants drinking water, and it remained elevated until the end of the observation period. However, this increase in salivary NO_2^- concentration vanished after using anti-bacterial mouthwash (**Figure 4.3**). This pattern of response in salivary NO_2^- concentration was similar when measurements were made by the chemiluminescence and Griess methods but, overall, measurement derived from the Griess method were ~38% and ~27% lower for the water and mouthwash conditions, respectively. There was no significant difference ($P = 0.32$) in the AUC

for NO_2^- concentration measured in participants receiving the water on the first day compared with participants receiving it on the second day (**Appendix 4.7**).

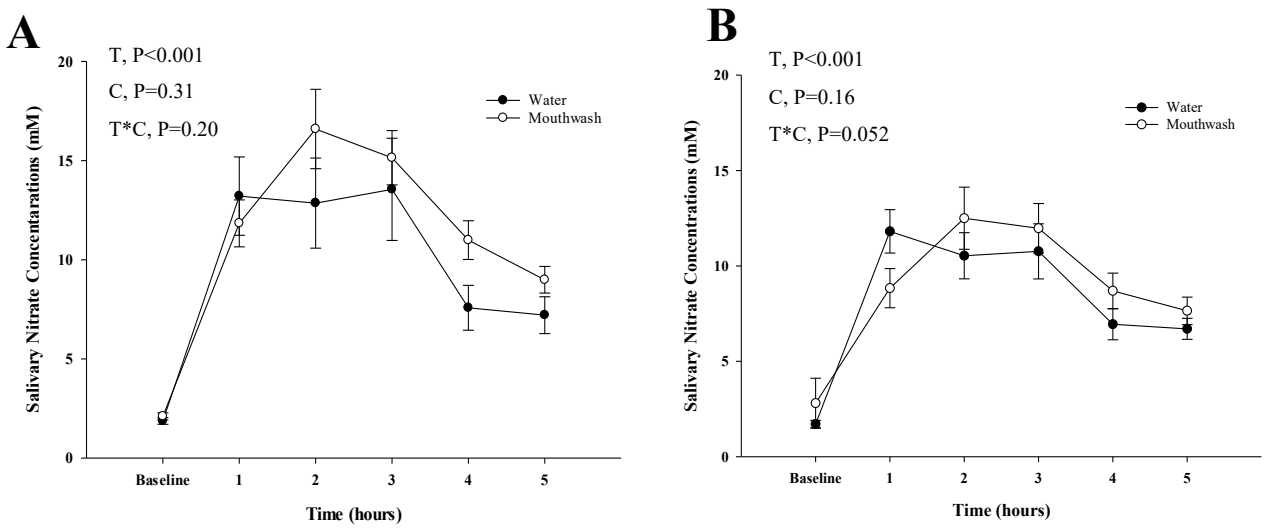

Figure 4.2: Mean salivary NO_3^- concentrations measured by chemiluminescence (A) and Griess (B) methods after acute ingestion of BJ (70 ml).

Filled circles represent times when individuals rinsed their mouth with water 15 min before the BJ ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time*condition) ANOVA. Data are expressed as mean ± SEM, (n = 10).

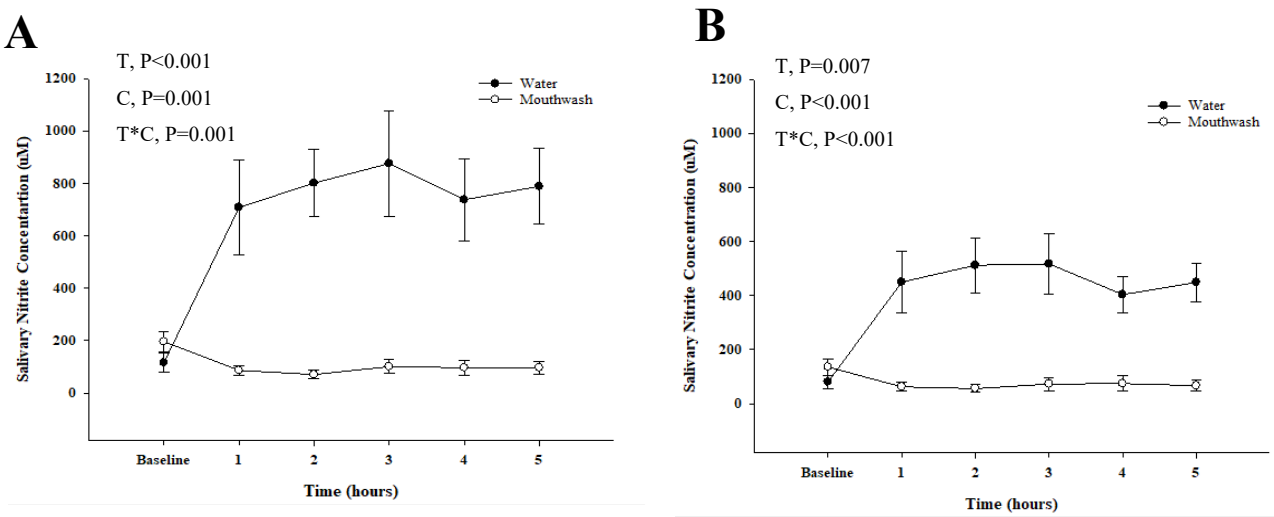

Figure 4.3: Mean salivary NO_2^- concentrations measured by chemiluminescence (A) and Griess (B) methods after acute ingestion of BJ (70 ml).

Filled circles represent times when individuals rinsed their mouth with water 15 min before BJ ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time*condition) ANOVA. Data are expressed as mean ± SEM, (n = 10).

4.3.4. Salivary nitrite strips

The effects of BJ on salivary NO_2^-, as determined by the salivary strips, are presented in **Figure 4.4**. Overall, the salivary NO_2^- strips detected changes in salivary NO_2^- concentration following BJ supplementation similar to those measured by the chemiluminescence and Griess methods. The response was virtually abolished when participants rinsed their mouth with antibacterial mouthwash before consuming the BJ. There were significant main effects for time, conditions and for their interaction (time*condition) ($P < 0.01$). Overall, in the water experiment, the strips underestimated NO_2^- concentration by more than 50% and by ~27% compared with chemiluminescence and Griess methods, respectively.

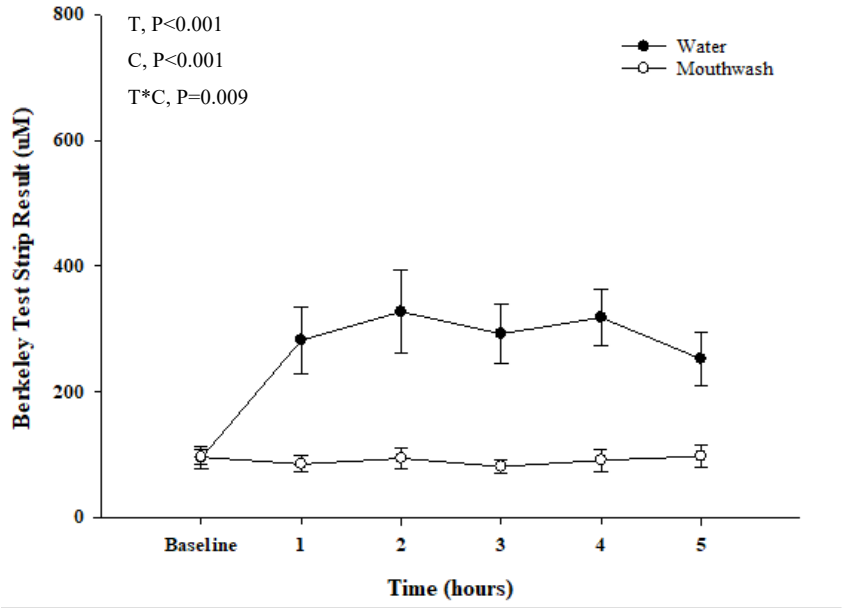

Figure 4.4: Mean salivary NO_2^- concentrations measured by salivary NO_2^- strips after acute ingestion of BJ (70 ml).
Filled circles represent times when individuals rinsed their mouth with water 15 min before BJ ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time*condition) ANOVA. Data are expressed as mean ± SEM, (n = 10).

4.3.5. Agreement analysis (Bland & Altman method)

Concentrations of salivary NO_2^- estimated using the salivary strips were significantly and strongly correlated with measurements obtained using the chemiluminescence (rho = 0.77, $P < 0.001$) and Griess (rho = 0.83, $P < 0.001$) methods. Similarly, changes in salivary NO_2^- measured by the strips were significantly correlated with changes in concentration measured by the chemiluminescence (rho = 0.79, $P < 0.001$) and Griess (rho = 0.80, $P < 0.001$) methods. As

expected, there was a significant, strong correlation between salivary NO_3^- concentration measured by the chemiluminescence and Griess methods ($P < 0.001$, $r = 0.86$). However, despite these statistically significant correlations, the limits of agreement between methods illustrated in the Bland Altman analysis (**Figure 4.5**) were wide, indicating a lack of accuracy of the Griess method. In addition, the Griess and salivary strips methods showed a significant differential bias as magnitude of the differences became larger with increasing concentrations. For salivary NO_2^- measured by Griess and chemiluminescence, the estimated bias was −150 μM (95% CI −193 to −107, $P = 0.0001$) and the 95% limits of agreement were fairly wide (−618, 318 μM). For salivary NO_2^- measured by the salivary strips and chemiluminescence the estimated bias was −201 μM (95% CI −266 to −136, $p = 0.0001$) and the 95% limits of agreement were also wide (−909, 506 μM). For salivary NO_2^- measured by the salivary strips and Griess the estimated bias was −64 μM (95% CI -97 to −32, $p = 0.0001$) and the 95% limits of agreement ranged from −418 to 289 μM. The differences between measurements increased with higher NO_2^- concentrations. For salivary NO_3^- measured by Griess and chemiluminescence, the estimated bias was −2 mM (95% CI− 2 to −1, $p = 0.0001$) and 95% limits of agreement were between −8 and 5 mM).

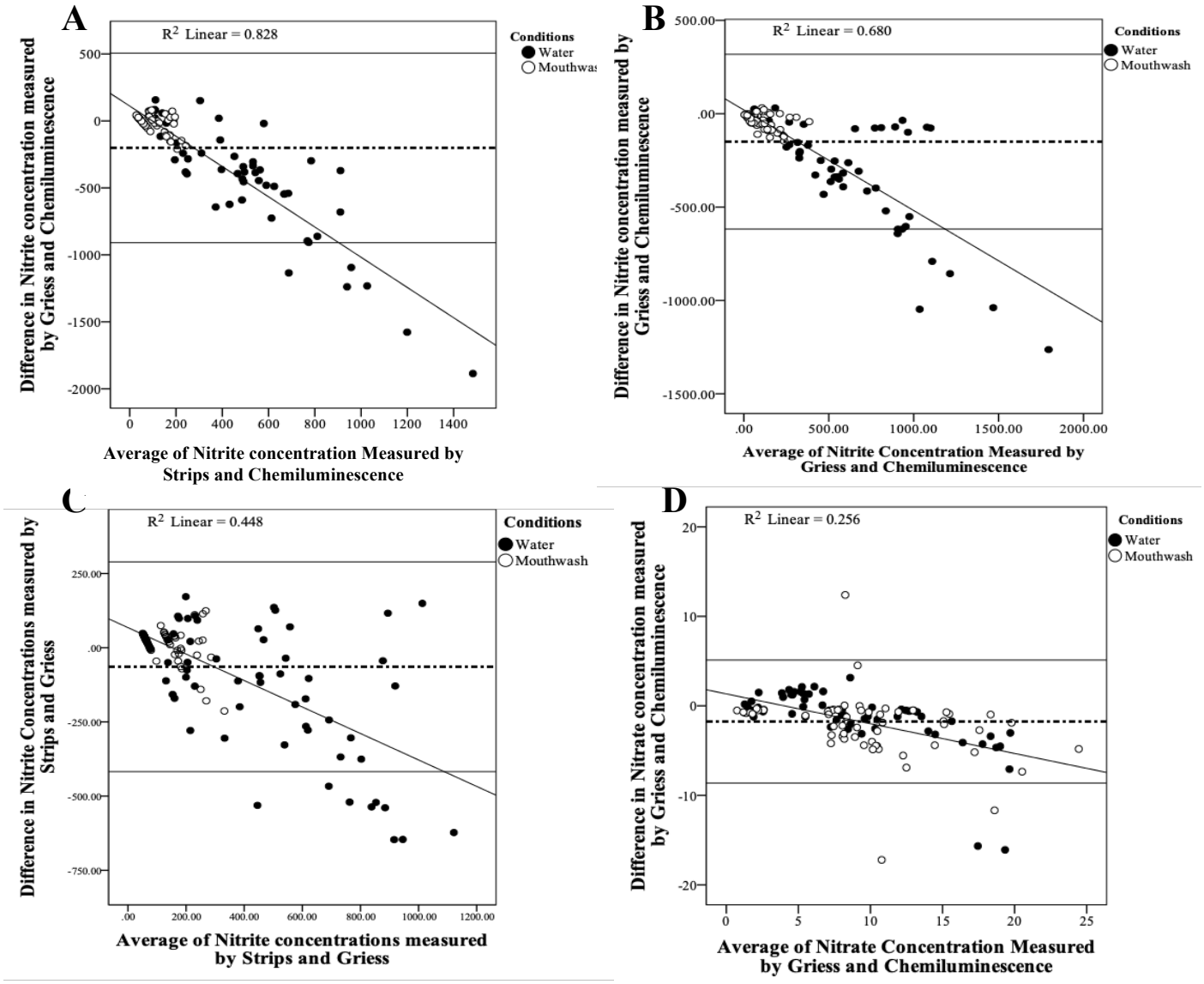

Figure 4.5: Comparison of mean salivary NO_2^- (A, B and C) and NO_3^- (D) concentrations measured by two different methods.

Black dash horizontal line shows the mean difference and the ± 2 SD. range (fine, black line). A regression line was fitted to the points to evaluate differential bias.

4.3.6. Reliability of salivary nitrite strips

4.3.6.1. Reproducibility

The mean concentrations of salivary NO_2^- estimated using the salivary strips for all 10 participants at each time-point obtained from five different observers is shown in **Table 4.2**. The intra-class correlation coefficient (ICC) showed a high reproducibility between the five observers.

Table 4.2: Inter-observer reproducibility of strips

Observer 1	Observer 2	Observer 3	Observer 4	Observer 5	ICC	P-value
176±147	200±140	193±117	157±72	75±129	0.91	<0.001

ICC, intraclass correlation coefficient (absolute agreement). Salivary nitrite values are presented as mean ± SD. The average of two strips' readings of salivary nitrite strips was reported. P-value indicate that the strips reading are significantly correlated between observers.

4.3.6.2. Repeatability

The results of NO_2^- measurements by salivary strips performed by five observers on two different occasions are shown in **Table 4.3**. The high ICCs indicated a high repeatability of the salivary NO_2^- strips.

Table 4.3: Intra-observer repeatability of the two strips used at each time point

Observers	Reading 1	Reading 2	ICC	P-value
Observer 1	173±145	179±153	0.938	<0.001
Observer 2	200±147	200±146	0.813	<0.001
Observer 3	185±127	174±103	0.833	<0.001
Observer 4	158±76	156±72	0.918	<0.001
Observer 5	76±141	73±137	0.720	<0.001

ICC, intraclass correlation coefficient (absolute agreement). Salivary nitrite values are presented as mean ± SD. P-values indicate that the readings of the two strips used at each time point are significantly correlated between observers.

4.7. Salivary sialin

There was no significant difference between salivary sialin concentrations measured at baseline between the water and mouthwash conditions ($P = 18$). There were no significant effects of condition (water v. mouthwash; $P = 0.54$) or time ($P = 0.49$), or time*condition ($P = 0.41$) interaction. We found a trend for an increase in sialin in the mouthwash experiment compared to water experiment, but this increment was not significant at any of the time points ($P > 0.05$) (**Figure 4.6**). A weak but significant correlation was found between the change in salivary sialin and the change in salivary NO_2^- concentration ($r = -0.20$, $P = 0.04$). Conversely, there was a

weak positive correlation between the change in salivary sialin and the change in salivary NO_3^- concentration (r = 0.18, P = 0.06) (**Appendix 4.7**).

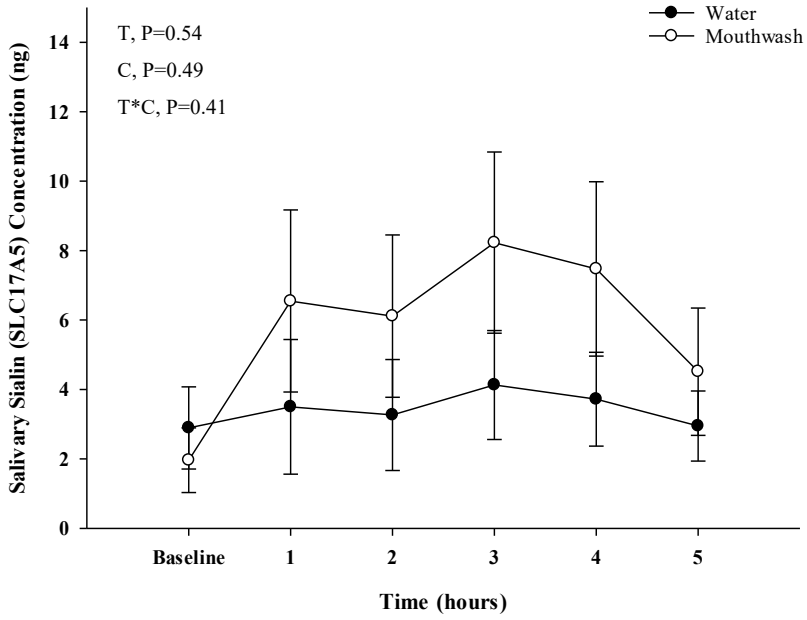

Figure 4.6: Mean salivary sialin concentrations.
Filled circles represent times when individuals rinsed their mouth with water 15 min before BJ ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time*condition) ANOVA. Data are expressed as mean ± SEM, (n = 10).

4.8. Blood pressure

There was no significant difference in SBP and DBP at baseline between the mouthwash and water conditions (P = 0.91 and P = 0.60, respectively). Over the 5 h following ingestion of BJ, there were no significant changes in SBP and DBP (P > 0.05) with, or without, use of the mouthwash (**Figure 4.7**).

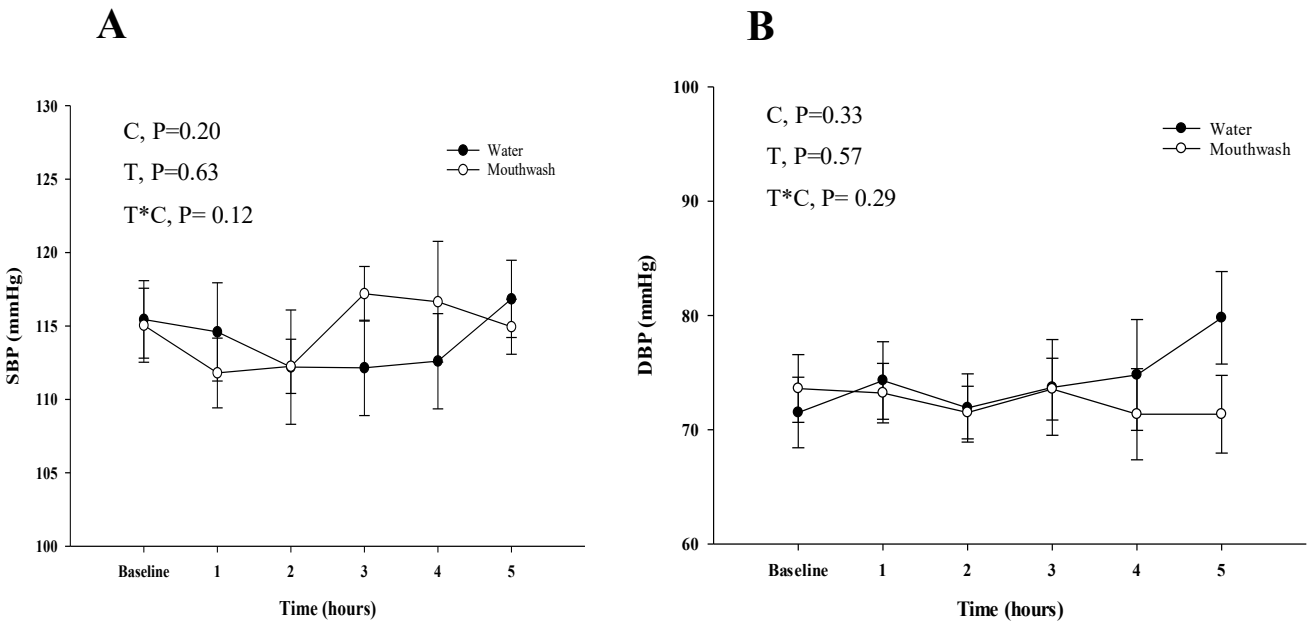

Figure 4.7: Mean systolic blood pressure (SBP) and diastolic blood pressure (DBP).
Filled circles represent times when individuals rinsed their mouth with water 15 min before BJ ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time*condition) ANOVA. Data are expressed as mean ± SEM, (n = 10).

4.4. Discussion

This study investigated for the first time the validity of salivary NO_2^- strips against the gold standard chemiluminescence technique and Griess methods after acute consumption of NO_3^- rich BJ with and without the use of mouthwash. Furthermore, this study evaluated, for the first time, whether acute NO_3^- supplementation, with and without mouthwash, altered sialin concentrations in saliva. Overall, salivary strips provide a simple and user-friendly method to detect changes in salivary NO_2^- concentrations after the consumption of high-NO_3^- foods, which could be useful for the monitoring of compliance in longer-term high-NO_3^- nutritional interventions. The strips are also characterised by a high repeatability and reproducibility, but they underestimated NO_2^- concentration when compared with the other laboratory methods (Griess and chemiluminescence) especially at higher salivary NO_2^- concentrations. In addition, when study participants used the mouthwash, salivary sialin concentration tended to increase following the ingestion of BJ and salivary sialin concentrations correlated with salivary NO_2^- concentration.

Clodfelter and colleagues tested different brands of NO saliva test strips and found that they reacted with a solution containing sodium NO_2^- ($Na\ NO_2^-$) but not with sodium NO_3^-, indicating

that these strips can be utilised for the selective detection of NO_2^- in biological fluids (Clodfelter *et al.*, 2015). The study found that the colour intensity of the strips increased with greater concentrations of $NaNO_2^-$ and that the lowest limit of detection was 10 μM. However, the Clodfelter et al., study was performed *ex-vivo*. When the strips were applied on the tongue, we observed that colour intensity increased after the consumption of high NO_3^- BJ. However, there was no increase in colour on the strips after the use of mouthwash which confirmed the lack of change in salivary NO_2^- concentration when measured by standard laboratory methods (chemiluminesence and Griess). This finding clearly indicates the sensitivity of the strips in detecting the effect of the antibacterial mouthwash, which is known to block the activity of the oral bacterial NO_3^- reductase and thus inhibit the conversion of NO_3^- into NO_2^- (Bondonno *et al.*, 2014b). This study also demonstrated a high level of repeatability and reproducibility of the strips. In addition, our study revealed the capacity of the strips to detect changes in salivary NO_2^- concentrations following an acute oral dose of NO_3^- rich BJ (400 mg). This is in agreement with McDonagh and colleagues who found that the strips measured changes in salivary NO_2^- concentrations following the ingestion of a range of doses of NO_3^- (~5.76 and ~1.40 mmol of NO_3^-) (McDonagh *et al.*, 2017).

The current study revealed significant strong correlations between the strips and other laboratory methods for the measurement of absolute salivary NO_2^- concentrations and also for the measurement of changes in salivary NO_2^- concentrations. Overall, the strength of the correlations found in this study (rho = 0.80 and 0.79 for the Griess and chemiluminescence methods, respectively) was greater than the correlation (r = 0.57) reported by McDonagh et al., (2017). The different strength of the associations reported in the two studies may be explained by the use in our study of a mobile phone-based application that provides a more detailed colour chart providing an assigned, quantitative value of NO_2^- concentration to each colour. McDonagh et al. used a simple colour chart which classified NO_2^- concentrations as depleted, low, threshold, target and high. McDonagh et al. (2017) concluded that salivary strips are a practical method to estimate salivary NO_2^- concentrations after the consumption of dietary NO_3^-. However, the poor level of agreement and the significant bias between the methods may limit the application of the strips for absolute measurement of salivary NO_2^- concentrations (Ludbrook, 2002). We used the Bland Altman method to assess the agreement between the strips and Griess and chemiluminescence methods and found that the limits of agreement between salivary strips and other, more precise, laboratory methods are wide, suggesting that salivary strips may not provide accurate estimates of salivary NO_2^-concentrations. However, the strips detected changes in response to acute ingestion of high doses of NO_3^- and therefore they may be useful for monitoring the compliance in nutritional interventions testing the effects

of inorganic NO_3^-. Indeed, these strips were shown to be effective in monitoring NO_3^- intake in longer intervention studies (14 days) (Hohensinn *et al.*, 2016). A more recent 13-week observational study also used a similar tool to monitor salivary NO_2^- changes over study period (Tripp *et al.*, 2019). Thus, use of these strips could be a simple cost-effective method of monitoring NO_3^-intake as the results can be seen almost instantaneously and this method can also provide an indication of NO availability, without requiring access to expensive laboratory equipment. Further, this method could represent a convenient and effective solution for research studies conducted in situations where the collection and storage of saliva samples for later analysis may be problematic (e.g. studies conducted in rural areas and/or developing countries).

In pigs, Qin and co-authors identified sialin as the primary NO_3^- transporter in salivary glands and observed inhibition of NO_3^- transport after sialin expression was knocked down (Qin *et al.*, 2012). To our knowledge, the present study is the first study to examine the association between salivary sialin concentrations and changes in salivary NO_2^- and NO_3^- concentrations in humans. The acute dose of NO_3^- rich BJ did not affect sialin concentrations in saliva as concentrations remained similar to baseline levels during the experimental period. Sialin concentrations tended to increase, however, after blocking the NO_3^- conversion into NO_2^- via mouthwash and a weak but significant correlation between salivary sialin and salivary NO_2^- concentrations was observed. Tentatively, these results may suggest the existence of a feedback mechanism linking NO_3^- transport and conversion to sialin expression, but mechanistic studies with larger sample size are needed to confirm this suggestion.

Previous studies have shown that SBP can be reduced after 3 h of BJ consumption (Webb *et al.*, 2008; Kapil *et al.*, 2010). In addition, in a recent study, Woessner and co-workers found a significant difference in SBP changes between water and mouthwash over a 4-h period post BJ consumption (Woessner *et al.*, 2016). In our study, we found no effect of BJ on SBP or on DBP and no effect of use of mouthwash. A possible explanation for this difference is the higher SBP baseline values in the study by Woessner and colleagues compared with our study (124 vs 115 mmgH), which may make individuals more responsive to the BP lowering effect of NO_3^- (Ashworth and Bescos, 2017).

In this study we administered mouthwash on one occasion only, which contrasts with several previous investigations where mouthwash has been administered two or more times daily over several days (Kapil *et al.*, 2013; McDonagh *et al.*, 2015; Sundqvist *et al.*, 2016a; Ashworth *et al.*, 2019). We found that acute mouthwash use abolished the increase in salivary NO_2^- concentration consequent to BJ ingestion, suggesting that one-time use of mouthwash is sufficient to blunt, at least temporarily, the NO_3^--reducing capacity of the oral microbiome.

However, in contrast with some (e.g. (Kapil *et al.*, 2013)) but not all (e.g. (Sundqvist *et al.*, 2016a; Ashworth *et al.*, 2019)) prolonged mouthwash studies, we did not observe a mouthwash-induced increase in BP. Future studies are warranted to determine potential differential effects of acute versus chronic mouthwash use on markers of NO availability and physiological responses.

A limitation of the current study is the small sample size, which reduced the power to detect significant changes in BP and sialin concentrations after BJ ingestion. However, the primary purpose of the study was to test the validity of the salivary strips for which the study size was deemed adequate based on the sample sizes of previous studies with a similar study design (Govoni *et al.*, 2008). The short washout period between the two experiments (mouthwash and water) could be considered a potential limitation if the oral microbiota had not recovered from the acute use of mouthwash. However, there was no significant difference in the AUC for salivary NO_2^- ($P = 0.34$) between the participants who used water on the first experimental day and those who used water on the second day (received the mouthwash 24-hour prior to water). This appears to indicate that the NO_3^--reducing capacity of the oral bacteria may recover within 24 h following the use of mouthwash. It is challenging to reconcile this finding with the currently limited evidence on the effects of antiseptic mouthwash on oral bacteria. *Ex vivo* studies have shown that, after exposure to chlorhexidine digluconate (0.2%) for 3 min, the proportion of viable bacteria is reduced by approximately 30% within a few hours and that full recovery of the bacteria requires up to 5 weeks (Shen *et al.*, 2016). The vitality of plaque bacteria after treatment with chlorhexidine digluconate (0.2%) was investigated in 6 volunteers studied over four days (Rundegren *et al.*, 1992). At 24 h after the last exposure to the mouthwash, plaque bacteria vitality was 60%. Our observation of no increase in NO_2^- concentrations over the 5 h following use of mouthwash indicates immediate, and total, suppression of the NO_3^--reducing capacity of the bacteria. However, 24 h after mouthwash use, the capacity of oral NO_3^--reducing bacteria has been re-established as we observed no difference in the AUCs of salivary NO_2^-concentrations measured during the two water experiments (**Appendix 4.8**). These findings suggest differential kinetics after mouthwash use for total plaque bacteria viability and for the specific bacteria responsible for NO_3^--reduction. The effects of frequent mouthwash treatment on NO_3^--reducing oral bacteria and its impact on the recovery the bacterial flora after stopping the treatment are very relevant research questions that should be explored in future studies.

4.5. Conclusion

The commercially available salivary NO_2^- strips applied in this study showed a high level of reproducibility and repeatability in detection of changes in salivary NO_2^-concentrations following acute ingestion of inorganic NO_3^-. However, Bland Altman plots indicated that there is a poor agreement between salivary strips, chemiluminescence and Griess methods, which means that these strips are not sufficiently accurate for the measurement of absolute concentrations of NO_2^- in saliva. Salivary strips may be a cost effective and simple method for monitoring changes in salivary NO_2^- concentrations and for monitoring compliance in intervention studies focussed on increasing dietary NO_3^- intake. Our preliminary findings suggesting an association between salivary NO_2^- concentrations and the salivary NO_3^- transporter (sialin) are intriguing and should be explored in future mechanistic studies.

Chapter 5: Acceptability and feasibility of a 13-week pilot randomised controlled trial testing the effects of incremental doses of beetroot juice in overweight and obese older adult (Part I)

The protocol and the results of this study has been published in *Contemporary Clinical Trials Communications* and *Nutrients,* respectively*:*

1-Babateen, A.M., Rubele, S., Shannon, O., Okello, E., Smith, E., Mcmahon, N., O'Brien, G., Wightman, E., Kennedy, D., Mathers, J.C. and Siervo M (2020) 'Protocol and recruitment results from a 13-week randomized controlled trial comparing the effects of different doses of nitrate-rich beetroot juice on cognition, cerebral blood flow and peripheral vascular function in overweight and obese older people', *Contemporary Clinical Trials Communications*, p. 100571 (**Appendix 5.1**).

2-Babateen, A. M.; Shannon, O.M.; O'Brien, G.M.; Okello, E.; Khan, A. A; Rubele, S.; Wightman, E.; Smith, E.; McMahon, N.; Olgacer, D.; Koehl, C.; Fostier, W.; Mendes, I.; Kennedy, D.; Mathers, J.C.; Siervo, M. (2021). "Acceptability and Feasibility of a 13-Week Pilot Randomised Controlled Trial Testing the Effects of Incremental Doses of Beetroot Juice in Overweight and Obese Older Adults" *Nutrients* 13, no. 3: 769. https://doi.org/10.3390/nu13030769.

5.1. Introduction

Increased life expectancy is associated with a concomitant rise in the occurrence of age-related medical conditions, including cardiovascular, pulmonary and neurological diseases (Niccoli and Partridge, 2012). In the UK, the number of individuals aged 60 years and over is estimated to increase by 8.6 million over the next five decades (Storey, 2018). Therefore, it is of paramount importance to promote and protect the quality of life of older people. Since diet has a major influence on ageing and on the risk of age-related diseases (Malcomson and Mathers, 2018), the identification of nutritional and lifestyle factors associated with healthy ageing is greatly needed. However, conducting nutritional intervention studies in older populations is often challenging.

Recruitment of older participants in clinical research studies can be challenging (Ridda et al., 2010). Older participants are more likely to be excluded from studies due to pre-existing

medical conditions, such as limited mobility, polypharmacy or the presence of chronic diseases (Florence et al., 2018). These factors may make recruitment a more difficult and time-consuming process despite the fact that older participants are often more willing to participate in clinical trials compared with younger participants (Cherubini and Gasperini, 2017). In addition, given the medical complexities associated with ageing, attrition rates from studies may be increased, which is another concern from the researcher's perspective (Knechel, 2013). Compliance with dietary interventions or study protocols may be challenging for older people. Factors with potentially adverse effects on compliance include high frequency of intake, rigid condition for ingestion times, and onerous preparation of meals in study protocols (Cusack and O'toole, 2013). Although some strategies to address reasons for non-participation in clinical research and to improve compliance with study protocols have been proposed (Crichton *et al.*, 2012; Knechel, 2013; Akmatov *et al.*, 2017), there is considerable need for improved design and performance of intervention studies in older people. This gap can be addressed through sharing of experiences, challenges and successes in recruiting and retaining older adults in nutritional intervention studies and related clinical research.

The purpose of this chapter is to report of the evaluation of the feasibility and acceptability of a pilot RCT designed to test the effects of incremental doses of BJ in overweight and obese older individuals. Whilst a number of studies have investigated the effects of BJ in older adults (Jajja et al., 2014; Vanhatalo et al., 2016; Raubenheimer et al., 2017), these studies have been characterised by short duration and, which may pose fewer issues with compliance than longer-term studies. To our knowledge, the present 13-week study is one of the longest RCT that has tested the effect of different BJ doses in older overweight and obese participants. This chapter describes the study protocol and reports the challenges associated with recruitment and retention of older adults in this study and assesses the compliance with the study protocol including the BJ interventions.

5.2. Methods

5.2.1. Ethical approval

The study was approved by the Faculty of Medical Sciences, Newcastle University (1503/4477/2018). The intervention study was registered with the clinical trial ISRCTN registry (ISRCTN14746723) (**Appendix 5.2**).

5.2.2. Study design and randomization

This was a randomised, single-blind, placebo-controlled, four-arm parallel feasibility trial. The intervention had a duration of 13 weeks. The study was conducted at the NU-Food research facility at Newcastle University and in the Brain Performance Nutrition Research Centre at Northumbria University. After a screening assessment for the evaluation of inclusion and exclusion criteria, eligible participants were randomised to one of the four intervention groups:

1- High dose of NO_3^- (HN); two 70 ml shots of concentrated BJ per day (400 mg of NO_3^- per shot), one every morning (~8am) and one every evening (~9pm).
2- Medium dose of NO_3^- (MN); one shot of concentrated BJ every evening (~9pm).
3- Low dose of NO_3^- (LN); one shot of concentrated BJ every other evening (~9pm).
4- The control group receiving the placebo (PL); one shot of NO_3^- depleted BJ (0.001 mg of NO_3^-) every other evening (~9pm).

The BJ was provided by the same manufacturer (Beet-it, James White Company) during the study but different batches were used, so a slight variation in the NO_3^- content is likely, as reported previously by Jajja et al. (2014). Sixty randomisation codes were generated in advance using the RAND function in Excel (Excel Microsoft software, Microsoft corp, Redmond, WA, USA). After confirming eligibility during the screening visit, each participant was then allocated to the next available intervention in the code list. All participants were instructed to avoid using mouthwash and to maintain their normal daily dietary and physical activity habits for the duration of the study.

5.2.3. Blinding

This was a single blind study; participants were not informed about whether they were allocated the NO_3^- -rich BJ or NO_3^- -depleted BJ (PL). Blinding of the researchers to the intervention was not possible due to the nature of the dietary interventions and study design as the frequency and volume of the BJ given to each participant revealed the nature of the interventions to the researchers.

5.2.4. Recruitment strategies

Participant recruitment occurred between July 2018 and April 2019 (10 months). Potential participants were identified through several recruitment strategies:

- Approximately 1500 flyers were distributed in mail-boxes in different areas of Newcastle. Additionally, flyers were displayed in several local facilities such as libraries, coffee shops, gyms, supermarkets and community centres (an example of the flyer can be seen in **Appendix 5.3**).
- Advertisements in local newspapers and newsletters (*Evening Chronicle*, *Journal* and *Sunday Sun*) were placed in September 2018 and January and February 2019 (11 advertisements in total). The total cost of these advertisements was £1729.80 (The newspaper advertisement can be seen in **Appendix 5.4**).
- Emails were circulated to Newcastle University members of staff.
- Potentially eligible participants were identified in recruitment electronic databases containing contact details and basic health-related information on individuals who have consented previously to be contacted to take part in future research studies.
- The study was also advertised through Voice (https://www.voice-global.org/) to reach older participants who might be interested in participating. The advertisement was placed for one year from July 2018.
- There was an attempt to advertise the study in the weekly bulletin of St. Mary's Cathedral, Newcastle, during March 2019. Flyers were also given to individuals attending the church.
- The study was advertised on Facebook. The title of the page created was "Nutritional research at Newcastle University" and the Facebook advertisement was created using the standard Facebook procedure (https://www.facebook.com/advertising). To narrow down the target population, Facebook has a feature that allows it to target people based on gender, age and location. Although we wished to recruit adults aged 60 to 75 years old, we placed the advertisement targeting males and females within the age range 18 to >65 years old, with the intention that young adult Facebook users could inform their parents or grandparents about the study. The advertisement was broadcast within a radius of 30 miles (48 km) around Newcastle upon Tyne. Those interested in participating in the study were asked to register on this google drive link (https://docs.google.com/forms/d/16huyt9XmrkB1oYuMWS9WSwF-1fWbOj_PnwmcnxIEzhQ/edit). This online form requested their name, age, gender, email, telephone number and whether or not they were taking any kind of medication. Only those who registered via that link were contacted. The advertisement ran for 10 days (20/02 – 2/03, 2019) at a cost of £100. The advertisement was reviewed and approved by the Facebook team before it was run.

- Recruited participants were asked to invite their friends and relatives who may fit the inclusion criteria and who may have had an interest in participating in the study.

All advertisements included a brief description of the study, the aim of the study, the main inclusion criteria, the duration of the study and also the name and email/telephone number of some members of the research team. In addition, they indicated that reasonable travel expenses would be covered and that participants would receive a £60 voucher at the end of the study.

5.2.5. Retention strategies

We adopted several strategies to maximise compliance with the study protocol and to minimise the drop out of participants during the study. All participants were provided with contact details of researchers who were available to answer any queries during the duration of the intervention. Furthermore, reminder emails were regularly sent to participants to remind them to complete specific tasks at home such as the scheduled dietary assessments using Intake24 every two weeks and collection of biological samples including urine and saliva samples every 4 weeks (the details ca. Regular contact was established with participants via emails and/or text messages to monitor adherence and health status during the intervention. All appointments were scheduled at the participant's convenience. All participants were compensated for their time with a £60 shopping voucher which was given on completion of the study. Retention in the study was quantified as the percentage of participants who completed the 13-week assessments.

5.2.6. Participants

5.2.6.1. Inclusion and exclusion criteria

Participants were included if they were aged 60 to 75 years, male or female, overweight or obese (BMI range: 25-40 kg/m^2) and if they were non-smoker and non-vegetarian. Participants on prescribed medications such as hormonal therapies, anti-hypertensive or anti-lipidemic medication were included if the dose had not been started/been changed in the previous 3 months. Potential participants were excluded if they were on medications such as antacids and diuretics (which affect NO_3^- metabolism), anti-anxiety or anti-depressant medications, if they reported excessive alcohol intake weekly (> 14 units for female, > 21units for male), were using antibiotic for the last month, were diagnosed with chronic or acute metabolic and inflammatory conditions interfering with the study outcome, were participating in another clinical intervention study, had insulin-dependent diabetes, had allergy or intolerance with the intervention or had uncontrolled hypertension (SBP > 160 mmHg or DBP >100 mmHg).

Participants were also excluded if they could not comply with the intervention e.g. because of being on holiday or out of the country for ≥4 weeks.

5.2.6.2. Screening

Individuals who contacted the research team and expressed an interest to participate received detailed information about the study either by email or by post (**Appendix 5.5**). A telephone screening was then arranged to assess eligibility based on date of birth, medical history and medication use and commitment and availability to participate in the study over a period of three months. If potential participants met the eligibility criteria at the telephone screening, they were invited to an onsite screening visit for further assessments to confirm their eligibility.
During the on-site screening visit, a member of the research team explained the study protocol and measurement procedures and answered any queries from participants. Potential participants were asked to read and sign the consent form (**Appendix 5.6**). Next, body weight and height were measured to calculate BMI and resting clinic BP was measured in triplicate after resting for about 10 minutes. Participants were included in the study if BMI and BP were within the range specified in the study protocol. At the end of the screening visit, eligible participants started a familiarisation session for the computerized cognition tasks, which included the completion of three consecutive cognitive tasks (i.e. 3 repetitions). This aimed to minimise the risk of a learning effect between baseline and end of study assessments.

Before they left the research centre, the participants were provided with a list of high-NO_3^- foods (see **Appendix 5.7**) and instructed to avoid foods on the list for 24 hours before the baseline and end-of-study visits. In addition, a urine container (Mid-stream urine collection set, UN252, Shermond, UK) was given to them to be used on the morning of the first study visit and they were asked to bring the sample with them to the research facility.

5.2.7. Outcome measures

5.2.7.1. Primary

The primary outcomes of the trial were measures of feasibility, acceptability, and compliance with the interventions and with the study protocol. This was achieved through documentation of adherence to the intervention and measurement protocol, recording of any adverse events, as well as measurements of changes in health outcomes that may occur during the intervention period.

5.2.7.2. Secondary

The study protocol included the investigation of changes in:

- Cognitive function.
- CBF.
- Resting clinic BP.
- NO biomarkers (urinary, plasma and salivary NO_2^- and NO_3^-).
- Validation of salivary NO strips for assessment of long-term compliance with NO_3^- -based nutritional interventions.

There were also other objectives included in the study protocol and data were collected by the researcher. However, these data are being investigated by other team members and, therefore, are not included in my PhD. These objectives are:

- Endothelial-dependent and independent microvascular blood flow measured by Laser Doppler Iontophoresis (LDI).
- Pulmonary function measured by portable spirometer.
- Whole-body NO production using stable isotopic methods.

5.2.8. Data collection procedures

All data were collected over one year between July 2018 and July 2019. Baseline measurements were taken on two separate days, as the measurements were conducted in two different locations. On the first day, participants arrived at the research facility (NU-Food, Newcastle University) in the morning (between 9:30-10:00) in the fasting condition (~ 12 h after the previous meal). Participants were reminded to follow a 24-hour run-in period to standardise their NO_3^- intake which included avoidance of green leafy vegetables, and to avoid alcohol and caffeine consumption 24h before the visit. To assess their NO_3^- intake (excluding the intervention supplement) during the study, participants were asked to record their food intake every two weeks using the online tool Intake24 (https://intake24.co.uk/, six dietary records in total).

Body composition, waist circumference, biological samples (saliva, salivary NO strips and blood samples), BP, physiological functions including pulmonary, endothelial and cognitive functions were all collected in this visit. Before they left the research facility, participants were provided with a small snack (orange juice and muffin) and were asked to continue following the low NO_3^- diet for the rest of the day. Participants were also provided with instructions and the necessary materials for the collection of saliva and urine samples and the salivary strip to be collected the next morning. Participants were asked to complete the international physical

activity questionnaire (IPAQ) at home (**Appendix 5.8**) to estimate their physical activity over the week prior to the visit (Craig *et al.*, 2003).

On the following day (the second day of the baseline measurements), participants were asked to collect saliva and urine samples and apply the salivary NO_2^- strips in the morning at home after an overnight fast (~12 hours). All participants were requested to follow the same instructions except from fasting (following low NO_3^- diet and limit alcohol and caffeine consumption) before they arrived at the Brain Performance Research Facility at Northumbria University, in the afternoon, to perform the CBF measurement. They were asked to have their lunch around 12 Pm (low in NO_3^- meal). Due to the limited availability of the qNIRS device at Northumbria University, all measurements of CBF were made between 15:00 and 16:00. Before leaving the research facility, the participants were provided with their BJ allowance for the next 6 weeks.

An interim visit was performed at approximately 6 weeks at NU-Food to check their compliance to the study, to gather information on safety and adverse events that may have occurred during the first part of the study and to provide the participants with the final batch of BJ bottles to complete the study. BP and body composition were measured. To remind participants of the cognitive tasks, they performed the same computerised cognitive tasks, but the time of each task was reduced to limit the burden on participants.

The last two end study visits were performed after 13-weeks following the same order of the baseline measurements. These two visits were scheduled to occur ~12 hours after consumption of the last BJ dose for HN and MN groups, and ~36 hours after consumption of the last BJ dose for LN and PL groups. A summary of the study protocol is provided in **Figure 5.1**.

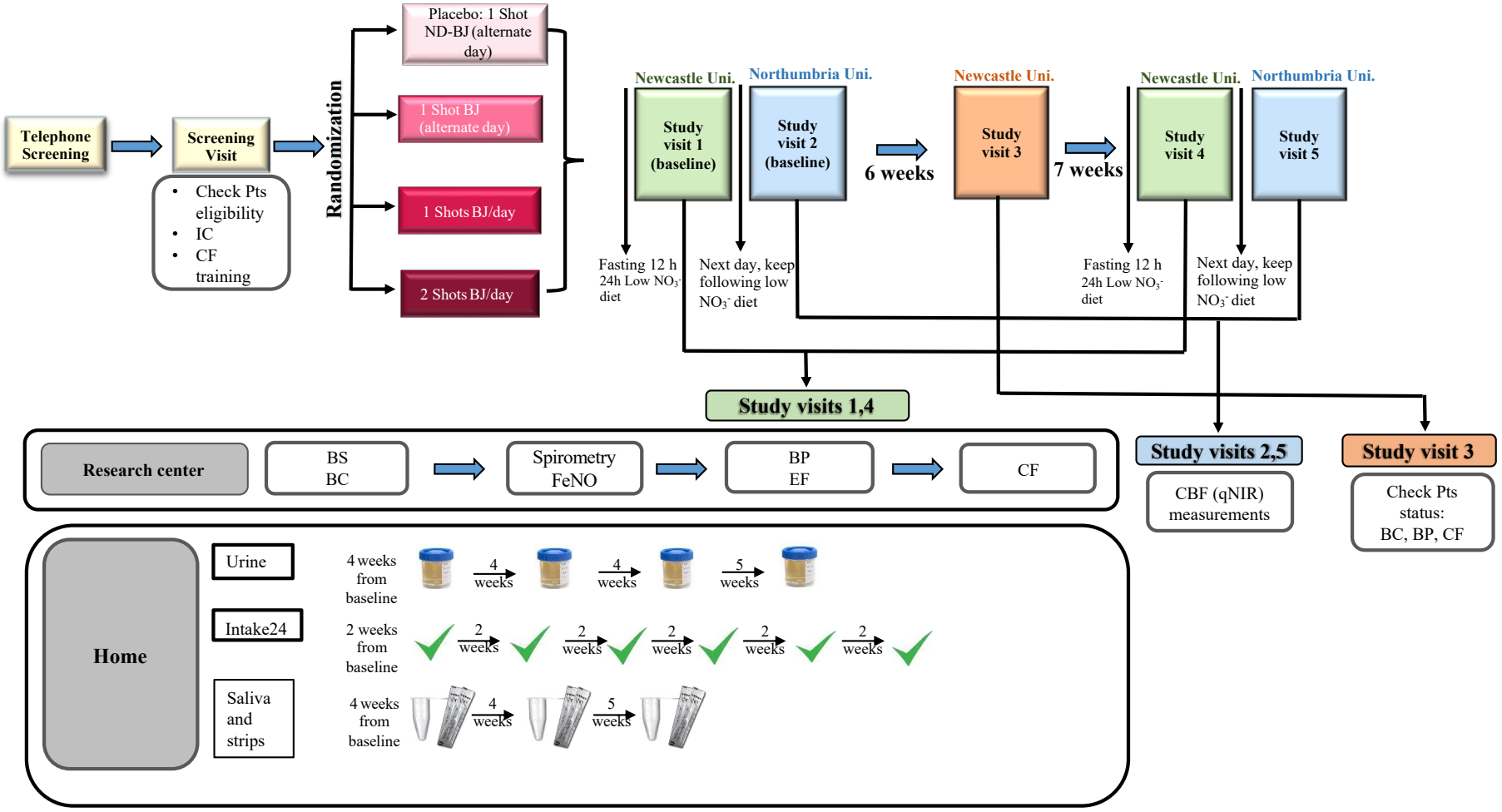

Figure 5.1: Overview of study protocol.

Pts: participants, BJ: Beetroot juice, ND-BJ: Nitrate depleted beetroot juice, IC: Informed consent signing, BC: Body composition, BS: Blood sample, CF: Cognitive function test, CBF: Cerebral blood flow test, BP: Blood pressure, EF: Endothelial function test.

5.2.9. Compliance measures

All participants were provided with verbal and written instructions on the tasks to be completed at home during the trial. Participant compliance was checked primarily by a daily compliance log which was used to record the time at which they consumed the BJ (**Appendix 5.9**). In addition, they were asked to return any missed, unused bottles to the study team. Compliance with the intervention was also assessed objectively by measuring dietary NO_3^- and NO_2^- concentrations in saliva and urine samples collected every 4 weeks during the study and in plasma samples collected at baseline and 13 weeks. Also salivary NO_2^- strips that have been described in Chapter 4, were collected concomitantly with saliva samples as part of a validation sub-study aiming at further validating the utility of non-invasive salivary NO_2^- strips for the assessment of compliance with the supplement in prolonged NO_3^--based nutritional interventions (more details on sample collection and analysis are reported in the next section). NO_3^- and NO_2^- concentrations were determined as a measure of adherence to the intervention. We expected to find greater concentrations in samples collected from the high NO_3^- dose group compared with lower NO_3^- dose groups and the PL group.

Attendance of visit appointments, collection of biological samples at home, delivery of samples every 4 weeks and completion of food recalls using the online software Intake 24 every 2 weeks were also used as indicators of compliance with the study protocol. All participants were contacted either by phone (text) messages or by email two days prior to a scheduled visit to remind them of the upcoming visits and to ask them to follow any instructions in preparation for the study visit. All participants were instructed not to change their dietary habits or physical activity patterns and to avoid using mouthwash during the study.

5.2.10. Intake24 data collection

To assess NO_3^- intake for the participants during the trial, they were asked to record their food and drinks once every two weeks using an online dietary recall Intake 24 (6 entries in total for each participant). Intake24 is an online tool developed by a group of nutritionists and computer scientists at Newcastle University as a web-based tool that is to be quick and simple to use. This online tool has been developed from an earlier system called SCRAN24 (Foster *et al.*, 2014).

Login details for Intake24: At the baseline visit, each participant was provided with written details including an unique username and password and the URL for the platform (https://intake24.co.uk/surveys/NCS) . In addition, login information was sent by a reminder email/text message to each participant whenever they were asked to report their dietary intake.

Participants were asked to watch a tutorial video (https://www.youtube.com/watch?v=5vB4NgI4ATc) in the system to help them to use Intake 24. This video shows several features such as how to add any food items at different mealtimes, how to delete a meal, how to change the times of the meal, and also how to add any missing food items. To estimate the portion size of consumed food and drinks, Intake 24 includes a series of >3000 food photographs to help participants to remember how much they ate or drank. If any participant did not find a specific food item, they were asked to select the closest match.

5.2.11. Calculation of nitrate intake

During this study, we collaborated with a group at Queensland University, Australia (led by Dr Michael Leveritt) who have created a comprehensive database that includes the NO_3^-, NO_2^- and nitrosamine concentrations in 3498, 2134 and 954 individual food and beverages, respectively, estimated using data from sixty different countries. I used this database to calculate the NO_3^- content of food and beverages consumed by my participants using the data collected from Intake24. The unit of NO_3^- contents within this database is (mg/100g), thus, the total was divided by 100 and then multiplied by the portion size. Tips followed in the calculation of NO_3^- intake can be found in (**Appendix 5.10**).

5.2.12. Collection of biological samples

The timings of collection of biological samples during the study is shown in **Figure 5.2.**

5.2.12.1. Blood

Blood samples were collected after an overnight fast (~12 hours) at baseline and 13 weeks. Blood samples were collected by venepuncture by trained phlebotomists in 3 tubes (4ml each), containing (LH) lithium heparin, EDTA (Ethylenediaminetetraacetic acid) and sodium fluoride and potassium oxalate, respectively.

The samples were processed within 10 minutes of collection. Samples were spun at 4000rpm for 10 minutes at room temperature and plasma aliquots were stored at -80ºC until further analysis. Samples were used to measure NO_3^- and NO_2^- concentrations.

5.2.12.2. Urine and saliva samples and salivary nitrite strips

Salivary NO_2^- strips, urine and saliva samples were collected at baseline, 4 weeks (3 consecutive days), 8 weeks (3 consecutive days), and 13 weeks.

Urine

A mid-stream urine sample was collected at the baseline and end of study visits using a sterile, collection kit (Mid-stream urine collection set, UN252, Shermond, UK). After 4 and 8 weeks, urine samples were collected at home by each participant for three consecutive days using a urine collection kit (Biological substance Category B, Shuttlepac, UK). A pre-paid box with sealed envelopes for the storage of biological samples was provided and each participant was asked to mail the samples to the research team using a fast delivery service (Royal mail). During each three-day collection period, participants were asked to keep urine samples in the fridge. A urine collection was fully assembled/address labelled and loaded with 6 urine vacutainer tubes (7.5 ml) (in case any vacutainer was to fail to work), 3 straws and a collapsible urine collection cup. All participants were instructed verbally on how to use this kit. Each kit also contained detailed illustrated instruction with pictures explaining how to use it (**Appendix 5.11**). Participants were asked to collect the samples in the morning at around the same time after overnight fasting. During public holidays (i.e. Christmas, Easter or bank holidays), participants were asked to keep the samples in a freezer until they could post them. After arrival at the laboratory, all urine samples were stored at -20ºC until further analyses. Urine samples were used for the measurement of NO_3^- concentrations.

Saliva

The procedure of saliva collection was explained in Chapter 4, section 4.2.4. Participants were asked to repeat this saliva collection procedure at home as required by the study protocol. Samples collected after 4 and 8 weeks were posted (together with urine samples (see above)), and participants were provided with all necessary materials needed and instructed to keep the saliva samples in the freezer until they were put in the post. After arrival at the laboratory, saliva samples were stored at -20ºC until required for analysis of NO_3^- and NO_2^- concentrations.

Salivary nitrite strips

Berkeley strips (Berkeley Test®, CA, USA) were used to measure salivary NO_2^- concentrations. Participants were instructed on how to use the strips as per the manufacturer's guidelines to monitor participant's compliance (see details in Chapter 4, section 4.2.5). The strips collected at 4 and 8 weeks were posted (with the urine and saliva samples) to the research team. Participants were asked to keep the strips in a dry place until they were mailed to the research team. The colour of the strips was recorded as soon as they were received.

5.2.13. Processing and analysis of biological samples

5.2.13.1. Plasma, urine and saliva samples

Before analysis, plasma samples were deproteinised using cold ethanol that was first chilled to 0 °C. 250 µl of each plasma sample was placed in a 1.5 ml microcentrifuge tube and 500 µl of cold ethanol was added and the tubes were then vortexed for 10 seconds. The tubes were then centrifuged at 14,000 RPM for 5 minutes. The supernatant was removed to a pre-labelled tube. Urine and saliva samples were diluted to 1:100 in deionized water prior to the analysis. Deproteinised plasma and diluted saliva samples were used for the determination of NO_3^- and NO_2^- , while diluted urine samples were used for the determination of NO_3^- by ozone based chemiluminescence. To minimise possible confounding due to batch effects during analyses, each batch of samples for analysis contained samples from all intervention groups.

The general procedure of the ozone-based chemiluminescence (Sievers NOA 280i, Analytix Ltd, Durham, UK) is described in **Appendix 4.5**. The standard curve for both NO_3^- and NO_2^- was created to calculate the concentrations of NO_3^- and NO_2^-. All glassware and injection syringes were thoroughly cleaned before use. The diluted samples were injected into the purge vessel with an injection volume ranging from 10 µl to 100 µl. The quality of the peaks was checked visually. Samples were re-diluted with a lower dilution factor (1:20) if no peaks were detected. The area under the curve of each peak was used to calculate the concentration of NO_3^- and NO_2^-. Each analysis run was started with the injection of a standard with known concentration, and this was used to calculate a correction factor to ensure the accuracy of the results.

5.2.13.2. Analysis of salivary nitrite strips

A similar strip tool used in Chapter 4 (Berkeley Test Application, CA, USA), was used in this study. However, in this chapter, the analysis of salivary strips was based on the visual assessment of changes in the colour displayed on the strip pad and compared against the colour concentration chart provided by the manufacturer (1=Depleted, 2=Low, 3=Threshold, 4=Target and 5=High). The colour chart is shown in (**Appendix 5.11**). This method has been previously applied in other studies (Hohensinn *et al.*, 2016; McDonagh *et al.*, 2017).

Although the application that was used to analyse the strips in Chapter 4 was more accurate than the current method, the update of the application becomes based on setting up an image capture system to photograph the colour change on the test strip pads. In fact, we did not find the new system very accurate as it was affected by the ambient light and background.

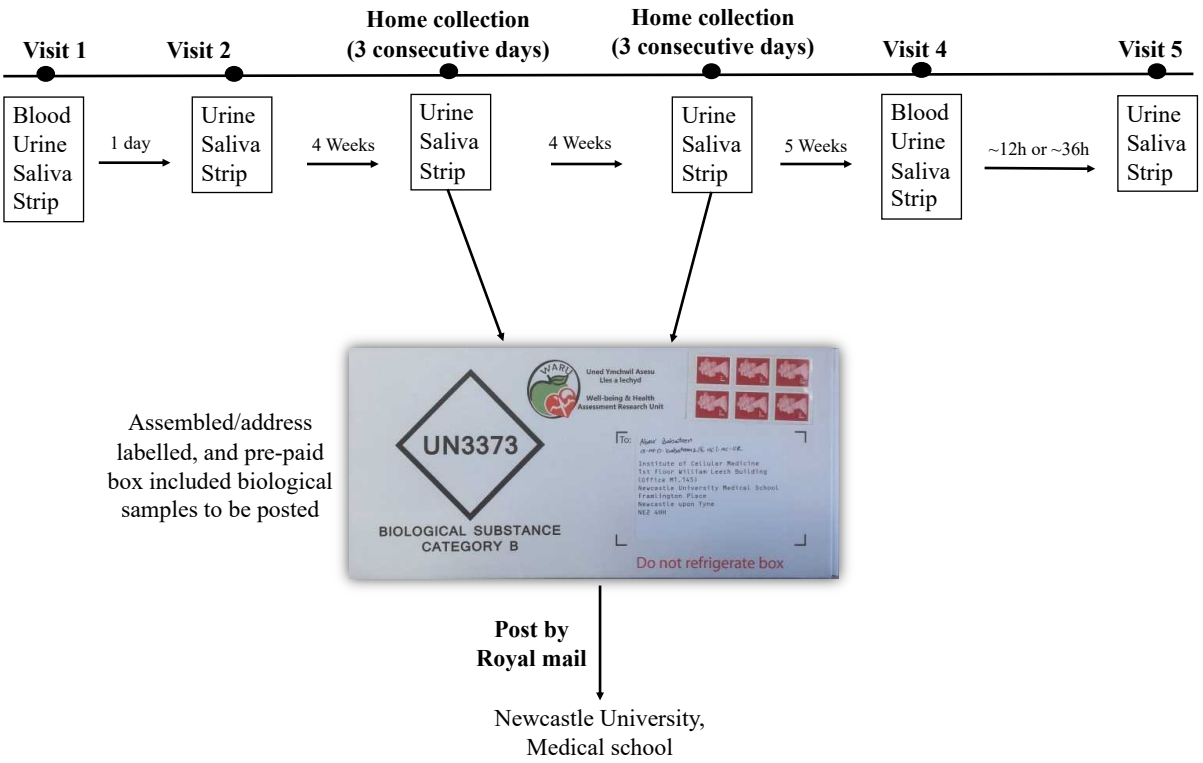

Figure 5.2: Summary of the collection of biological samples during the study.
Blood samples were collected in the research centre at baseline and after 13-weeks (visit 1 and visit 4). Urine, saliva and salivary nitrite (NO_2^-) strips were collected at baseline, after 4 weeks (for 3 consecutive days), 8 weeks (for 3 consecutive days), and at 13-weeks.

5.2.14. Participant feedback

After completing the study intervention, participants completed an anonymous 25-item questionnaire to obtain detailed feedback on the intervention and study protocol. The questionnaire took approximately 10 minutes to complete. Questions were designed to obtain feedback on the following specific topics: 1) reasons for joining the study and expectation from the study, 2) the duration of the study, 3) nutritional supplementation and 4) measurement protocols. The questionnaire included a range of closed and open questions. Based on the participants' responses to the open questions, the answers were categorised into specific topics and the frequency of each response topic was calculated. Responses were analysed for all participants whereas responses specifically related to the nutritional supplementation were analysed separately for each intervention.

5.2.15. Sample size calculation

This was a pilot study designed to assess the feasibility and acceptability of the proposed intervention. A sample size of 15 per group was based on i) the predicted effect size of the intervention on cognitive changes (Trail making test-B) (Justice *et al.*, 2015) and ii) the guidelines indicated by (Whitehead *et al.*, 2016) who provides guidance on sample size calculation for pilot studies with the aim of maximising use of resources and avoiding type II errors. Specifically, a sample size of 15 individuals per group would provide a 90% power to detect a medium effect size between 0.3 and 0.7.

5.2.16. Statistical analysis

Continuous data are summarised as mean ± SD and categorical data are presented as percentage (%). One-way analysis of variances (ANOVA) was used to compare baseline characteristics between intervention groups, except for gender and medication use which were analysed by Chi-square test.

Feasibility and compliance: Feasibility of the intervention was evaluated by collating quantitative information on eligibility, and recruitment and retention of participants in the trial. Retention was estimated by calculating the proportion of enrolled participants who dropped out and were lost to follow up. Intervention compliance rate was estimated as the proportion of BJ shots consumed relative to the total dispensed shots i.e. (number of BJ bottles consumed/ total number of BJ required to be consumed during the 13-weeks) X 100. Participants were considered compliant if they reported consuming 80% or more of their BJ during the 13 weeks of the study. Compliance to the intervention was also assessed by measuring changes in biomarkers of dietary NO_3^- intake including plasma, urine and salivary concentrations of NO_3^- and NO_2^-. Compliance with other aspects of the study protocol, especially those that were conducted at home, including collection of biological samples and posting the samples to the laboratory, and recording dietary intakes using Intake24, was evaluated.

Biomarkers analysis: Summary data are presented as mean ± SEM in figures, and 95% confidence intervals (CI) in the text. Only data from participants who completed the study were included in the analysis. Normality distribution of variables was checked by visual inspections of histograms and by Shapiro-Wilks test. Paired t-test was used to assess the between-day repeatability of biomarkers for the two visits conducted at baseline and also at the end of the study. To evaluate changes in biomarkers concentrations post-supplementation, the mean value for both test days was used in the analyses. Changes over time were analysed using two-factor repeated-measure ANOVA with time as a within-subjects factor and intervention as a between-

subjects factor. Models were checked for sphericity assumptions and multivariate models were applied if assumptions were violated. If the model was significant, Dunnett's test was used to compare the effects of different doses of NO_3^-. The change from baseline was calculated for each individual and then treatments were compared using one-way ANOVA. One-way ANOVA was also used to determine whether delays in delivery of the samples collected at home affected salivary and urinary NO_3^- and NO_3^- concentrations (all interventions were combined together in this analysis). To investigate the dose response relationship between supplemental doses of NO_3^- and concentrations of NO_3^- and NO_2^- in biofluids, polynomial regression analysis was used. Associations between salivary NO_2^- concentrations measured by chemiluminescence and the values obtained from salivary strips were investigated using Pearson's correlation analysis. Statistical significance was set at $P<0.05$.

Nitrate intake and its association with baseline biomarkers: The NO_3^- intake data of participants was excluded from this analysis if they missed making 2 or more estimates of dietary intake (out of the required 6 estimates) using Intake24. The mean NO_3^- intake from the 6 estimates was calculated for each individual and compared with the mean for baseline NO_3^- and NO_3^- concentrations in biological samples using Pearson's correlation analysis. All statistical analyses were completed using (IBM SPSS, version 23, NY, USA).

5.3. Results

5.3.1. Baseline characteristics of participants

The summary of baseline characteristics of the participants (**Table 5.1)** shows that the participants in each intervention group were well-matched for anthropometric variables, age and BP. The age of participants ranged from 60 to 73 years (mean±SD, 66±4) and 62% were men (n=38). Fifty-six percent of participants were overweight and the remaining 44% were obese. BMI ranged from 25 to 39 kg/m^2 (30.4±4 kg/m^2). SBP ranged from 110 to 167 mmHg (135±15 mmHg) and DBP ranged from 60 to 100 mmHg (77±10 mmHg). Six participants were hypertensive and on antihypertensive medication and 27 participants were on other medications as illustrated in **Table 5.1**.

Table 5.1: Baseline characteristics of the study participants including use of medication

	All	HN	MN	LN	PL	P-value
Characteristics						
Number	62	16	17	14	15	-
Gender, M/F	24/38	10/6	12/5	4/10	5/10	0.16
Age (years)	66.3±3.7	64.7±3.5	66.7±4.2	67.3±2.7	65.7±3.9	0.16
Education (years)	15.3±3.0	16.0± 3	15.7± 2.6	15.0±3.1	14.6±3.1	0.73
Body weight (kg)	84.9±12.6	90.9±13.4	84.6±10.5	80.1±12.5	83.9±12.6	0.15
BMI (kg/m^2)	30.3±3.7	30.5±3.6	30.5±3.2	29.9±3.4	30.3±4.8	0.99
WC (cm)	102.4±9.2	104.5±10.3	100.6±9.6	102.1±8.9	102.6±8.2	0.59
FM (kg)	32.4±8.7	32.1±8.7	34.5±9.0	31.3±7.2	31.5± 10.0	0.90
FM (%)	37.8±7.8	35.2±8.2	39.2±7.8	39.3±6.6	37.1±8.1	0.41
TBW (kg)	38.6±6.3	41.3±7.4	38.6±4.5	35.6±6.3	38.8±5.9	0.18
SBP (mm Hg)	135.1±14.7	130.8±12.0	136.1±10.4	139.5±13.2	134.1±12.9	0.46
DBP (mm Hg)	76.9±9.4	75.8±9.7	77.3±9.1	77.8±8.1	76.9±11.2	0.96
PA (METs/wk)	3667±5604	2741±1522	3257±1845	2262±1933	6280±10512	0.20
Medication use						
Antihypertensive	6 (9.8%)	1 (6%)	1 (6 %)	1 (7%)	3 (20%)	-
Hormonal therapy						
Thyroxin	9 (14.5%)	3 (19%)	1 (6%)	4 (29%)	1 (7%)	-
Testosterone	1 (1.6%)	1	0	0	0	-
Antihistamine	1 (1.6%)	0	0	1 (7%)	0	-
Lipid lowering agents	10 (16%)	5 (31%)	2 (12%)	1 (7%)	2 (13%)	-
Vitamin D	3 (3%)	1 (6%)	2 (12%)	0	0	-
Aspirin	1 (1.6%)	0	0	0	1 (7%)	-
Corticosteroid inhalers	2 (3%)	0	1 (6%)	0	1 (7%)	-
No therapy	35 (56%)	10 (63%)	9 (53%)	7 (50%)	9 (60%)	-

M/F, male/female; BMI, body mass index; WC, waist circumference; FM, Fat mass; FFM, Fat free mass; SBP, systolic blood pressure; DBP, diastolic blood pressure; PA, physical activity; PA, physical activity; Data are expressed as mean ± SD. Medications are presented as n (%). P values are based on one-way ANOVA, except for gender which were based on Chi Square test.

5.3.2. Recruitment

In total, 249 responses were received from our recruitment strategies. As shown in **Figure 5.3**, the Facebook advertisement generated the highest number of responses with 77 responses (31% of the total) within just 10 days, followed by 68 responses (28% of total) received from emails that were sent to previous participants enrolled in existing databases. In addition, 61 responses (24%) were obtained from local newspapers, 13 responses (5%) from the advertisement in Voice, 12 responses (5%) from an advertisement in St Mary's Cathedral newsletter and 11 responses (4%) from participants who heard about the study from other participants. The least effective strategy was the distribution of flyers (7 responses, 3% of total).

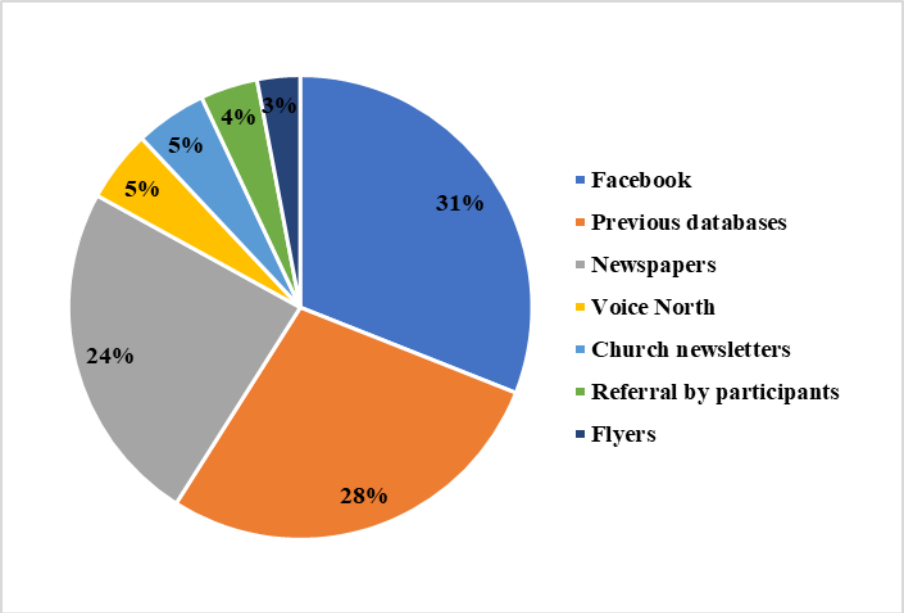

Figure 5.3: Response rate from recruitment strategies.

Thirty-five (45%) of the 77 respondents received from the Facebook advertisement were excluded as they were taking medications (e.g. antacids, antidepressants or diuretics) that were exclusion criteria. Seventeen respondents (22%) could not be reached by phone or email after several attempts.

Overall, 105 respondents were either unreachable or declined to participate. **Figure 5.4** summarises the recruitment and retention of respondents in the study. The initial telephone screening was conducted with 144 individuals. At this stage, 68 potential participants were excluded (or lost) for the following reasons: taking medications such as antacids, antidepressants or diuretics (n=37, 54%), co-existing health conditions including cancers, cardiovascular disease, kidney disease, T1D or epilepsy (n=13, 19%). In addition, some individuals declined to take part after reading the information sheet (n=8, 12%), some were unreachable (n=4, 6%), some were smokers (n=3, 4%) and some were over 75 years old (n=3, 4%).

A total of 76 participants attended the on-site screening visit to confirm their eligibility. Seventy participants were eligible and 62 participants gave their consent and were enrolled. Five participants (7%) changed their mind and 9 participants (11%) were excluded at the screening visit for reasons that included high BP (55%), normal BMI < 24.9 kg/m^2 (33%) and morbid

obesity (BMI > 40 mg/m^2) (11%). Additional reasons not to participate included: 1) no longer interested to take part (4%), 2) personal bereavement (1%) and 3) being unwell (1%). The recruitment target was 60 participants, but two participants who dropped out of the study immediately after the start of the intervention were replaced and, therefore, 62 participants were randomized and had a baseline visit assessment.

The sixty-two participants were randomised to one of the four intervention groups as follows: 16 participants were allocated to Group 1 (2 shots of BJ/day, one every morning and the other in the evening), 17 participants to Group 2 (1 shot of BJ/day, in the evening), 14 participants to Group 3 (1 shot of BJ every other evening) and 15 participants were allocated to Group 4 (PL, 1 shot of NO_3^- depleted BJ every other evening).

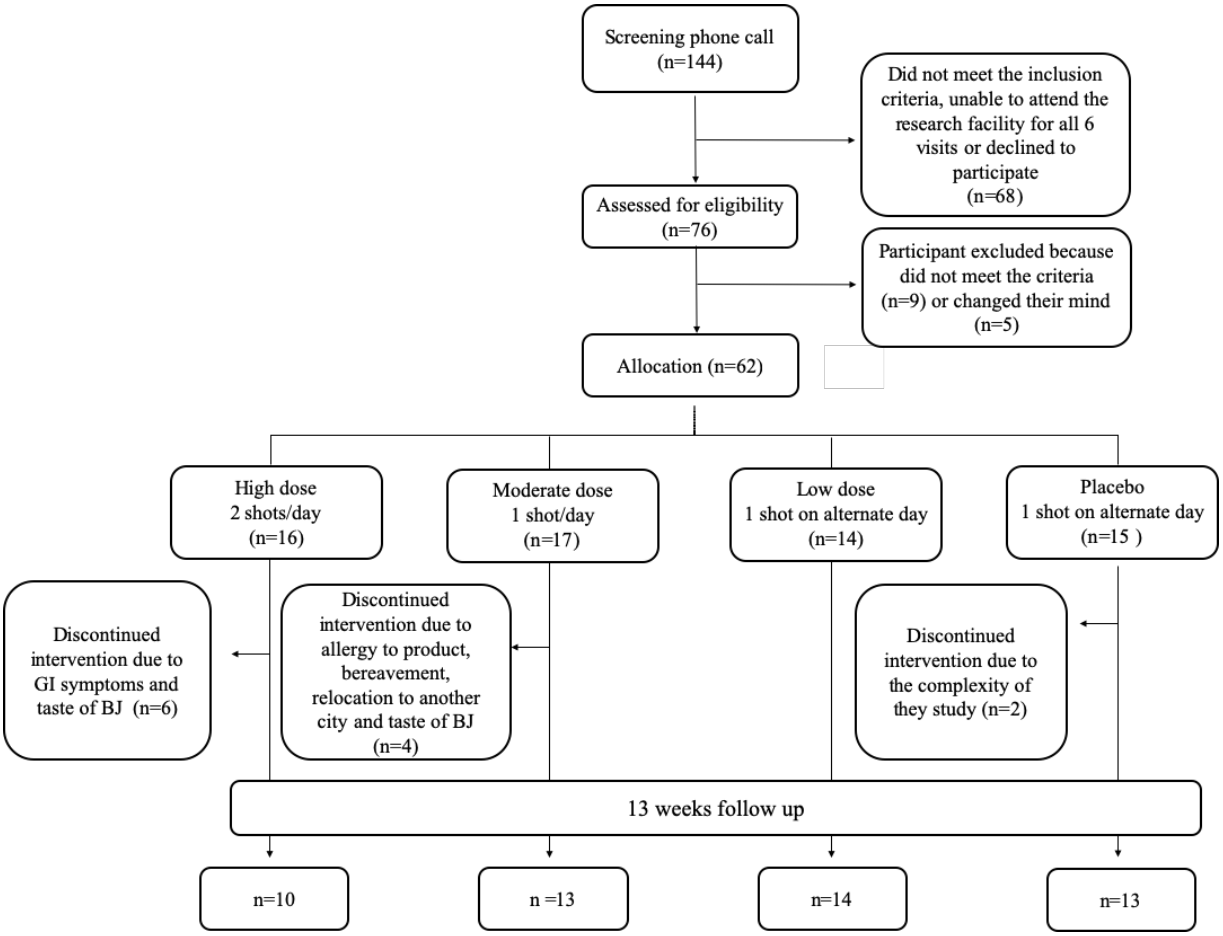

Figure 5.4: Flowchart describing the recruitment of participants into the trial.

5.3.3. Retention and attrition

Of the 16 participants who were randomly assigned to Group 1 (HN), 12 participants completed their 6 weeks' assessment (interim visit), and 10 participants completed their 13 weeks assessment. A total of six participants dropped out from this group. Five participants withdrew before the interim assessment due to: 1) uncomfortable bowel movement (n=2), nausea due to smell and taste of BJ (n=2) or; 3) prescription of antiseptic mouthwash due to dental problems (n=1). One participant in this group dropped out after the interim assessment due to dental problems and intolerance to the sugar content of the BJ.

Seventeen participants were randomly assigned to Group 2 (MN) and 13 participants completed the study. One participant withdrew immediately after the first baseline visit due to allergy (self-reported) to the lemon juice extract contained in the BJ. Two participants dropped out before the 6-week assessment due to bereavement and relocation to another city. One

participant withdrew after the interim assessment due to aversion to the smell and taste of the BJ.

Fourteen participants were assigned to Group 3 (LN)، and all of them completed the study. Fifteen participants were assigned to Group 4 (PL) and 13 participants completed the study. The two participants who dropped out did so because they found the study too complicated.

The overall attrition rate for the study was 19% with a total of 12 participants withdrawing from the study. Reasons offered by participants for withdrawing from the study are summarised in **Table 5.2**. During the recruitment and data collection there was an attempt to replace some of the participants who dropped out at initial stages, and, as discussed above, 2 participants were replaced.

Table 5.2: Primary reasons for withdrawal from study

Reasons	N (%)	Specific reasons provided by participants
Uncomfortable bowel movement	3 (25)	"*The number of times I spent to the toilet increased, I go to the toilet up to eight times a day, it is obvious that something in the supplement has a certain effect on me*" "*I drank the BJ, within fifteen minutes I had to rush to the lavatory with diarrhoea*". "*I need to give up, BJ seems to be giving me extremely loose bowels and I have had to stop taking it*"
Taste and smell of BJ	3 (25)	"*The smell, taste and texture are all extremely unpleasant, leaving me with a feeling of nausea for a long time after taking the BJ (Sometimes hours)*". "*I am so sorry I have to pull out, because of the horrible taste of BJ, I was cheating can't drink all of the bottle, don't want to spoil your study*" "*There is a fundamental problem in that I can't tolerate the juice. I hate the juice; the consistency makes me feel sick and it is way too sweet. Much as I love beetroot, I cannot drink this*".
Moving out of the area	1 (8)	"*I regret to say due to an enormous amount of travelling I have had to endue recently, I will have to drop out from your research scheme*"
Complicated study	2 (16)	"*Sorry but I find it all a chore,* *with travelling in and out of town. Sorry I would like to exit from tria l*"
Teeth problems	1 (8)	"*Unfortunately, I do think I will need to stop the study.* *too much sugar in the juices which are spoiling my teeth, I often have headaches after I drink them*"
Bereavement family	1 (8)	-
Mouthwash use	1 (8)	-

5.3.4. Compliance

Intervention Intake

The daily log sheets used to record BJ consumption indicated excellent compliance with the intervention for the large majority of participants. Across all intervention groups, the overall compliance was more than 97% (**Figure 5.5**).

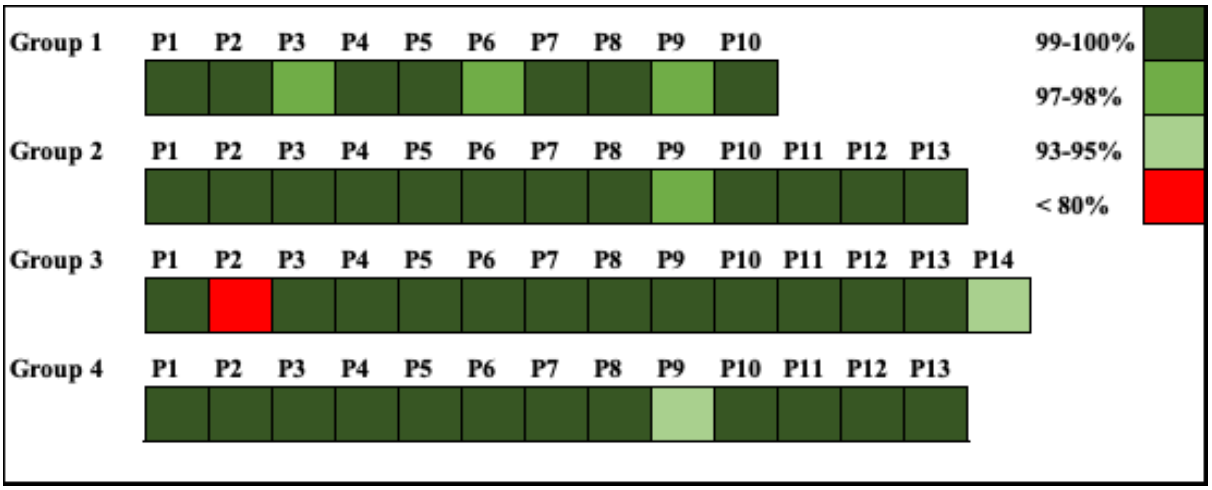

Figure 5.5: Compliance with the intervention.
Compliance with the intervention was assessed as the proportion (%) of BJ shots consumed by each participant in each intervention group. P; participant.

Sample collection at home and delivery by postg to the research centre

All participants who completed the study adhered to the protocol for home collection of the biological samples during the study. Participants did not report any difficulties with posting the samples using the pre-paid delivery boxes. We expected to receive the samples within 2 days from posting and we asked participants specifically to avoid posting the samples during weekends or on public holidays. However, we experienced delays in receiving some of the samples. In total, 16 samples were delivered at least 3 days after being posted. **Figure 5.6** shows the number of days between posting and receipt of the samples at the research centre. During the first month of the intervention, 22 boxes were received the next day after posting, 8 were received after 2 days, and 8 boxes were received at least 3 days after posting. During the second month of the study, 12 boxes were received the next day after posting, 13 boxes were received after 2 days, and 8 boxes were delivered after at least 3 days. Some samples were also collected from the participants' homes (n=5), and in some cases participants took the samples directly to the research centre (n=28).

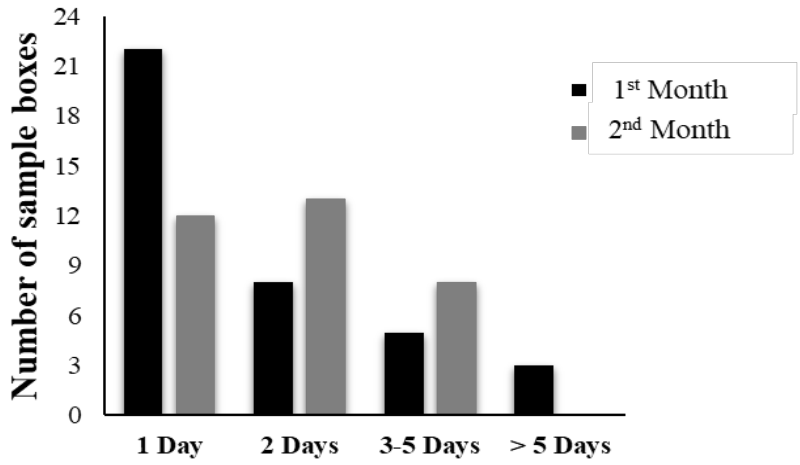

Figure 5.6: Distribution of number of days between samples being posted by the participants and receipt by the researcher.

Recording dietary intake using Intake24

Thirty-eight (62%) participants completed all 6 dietary intake records. Of those, 3 participants reported their intakes on paper due to difficulty with accessing the software, and dietary data were then uploaded to Intake24 by a member of the research team. Sixteen (26%) participants had incomplete dietary intake records due to dropping out or non-adherence. Among that group, 9 participants had less than three dietary records. Six participants did not collect any dietary data at all, as they dropped out within the first two weeks (**Figure 5.7**).

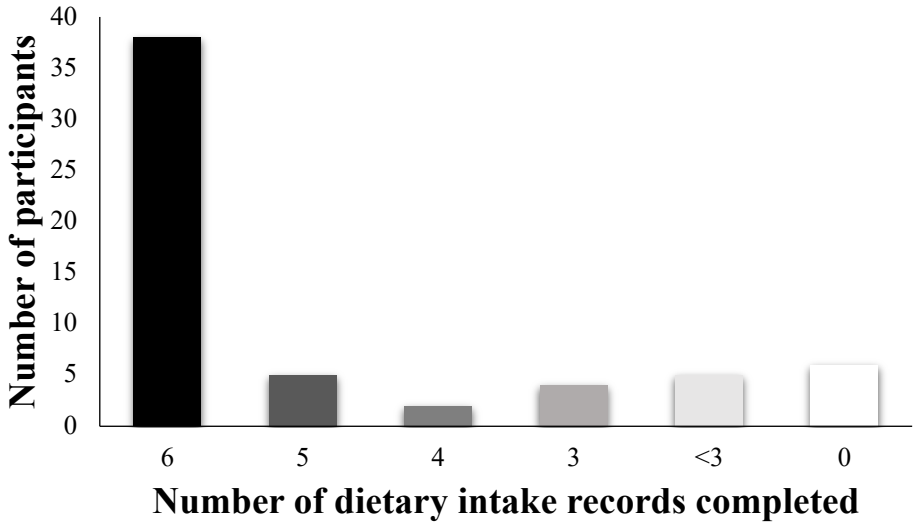

Figure 5.7: Number of dietary intake records completed by participants. Participants used Intake24 software to record their dietary intakes every two weeks during the trial.

5.3.5. Plasma nitrate and nitrite concentrations

Due to difficulty to collect the sample from one participant, data from 49 participants were included in the analysis. One-way ANOVA reveals that there was a statistically significant difference between intervention groups in the change from baseline in plasma NO_3^- concentration at 13-weeks ($P<0.001$). Compared with PL, the change in plasma NO_3^- concentrations increased significantly after HN (Δ 236 µM/L, 95% CI: 126, 346 µM/L, $P<0.001$) and MN (Δ 111 µM/L, 95% CI: 10, 212 µM/L, $P=0.02$) doses. No significant differences were found between the LN and PL doses (Δ 18 µM/L, 95% CI: -82, 119 µM/L, $P=0.94$) (**Figure 5.8**). However, there was only a trend towards significant difference between intervention groups in the change from baseline in plasma NO_2^- ($P=0.054$). Relative to PL, the change from baseline in plasma NO_2^- was significantly increased only after the MN dose (Δ 130 µM/L, 95% CI: 12, 248µM/L, $P=0.02$). There were no significant differences in the change from baseline for plasma NO_2^- for the HN or LN doses compared with PL ($P>0.05$) (**Figure 5.9**). The mean of percentage of change (mean±SD) in plasma NO_3^- in HN, MN, LN and PL at 13-weeks relative to baseline are 1038±447%, 615±300%, 95±90% and -4±45%, respectively. Whereas, the percentages of the change in plasma NO_2^- in in HN, MN, LN and PL are 83±119%,73±80%, 45±90% and -2±36%, respectively.

Using polynomial regression analysis, there was a significant positive linear relationship between NO_3^- dose and the change in plasma NO_3^- concentration ($R^2 = 0.71$, $P < 0.001$) (**Figure**

5.10 A). However, there was a non-linear relationship (cubic model) between NO_3^- doses and the change from baseline in plasma NO_2^- concentration ($R^2 = 0.24$, P = 0.006) (**Figure 5.10 B**).

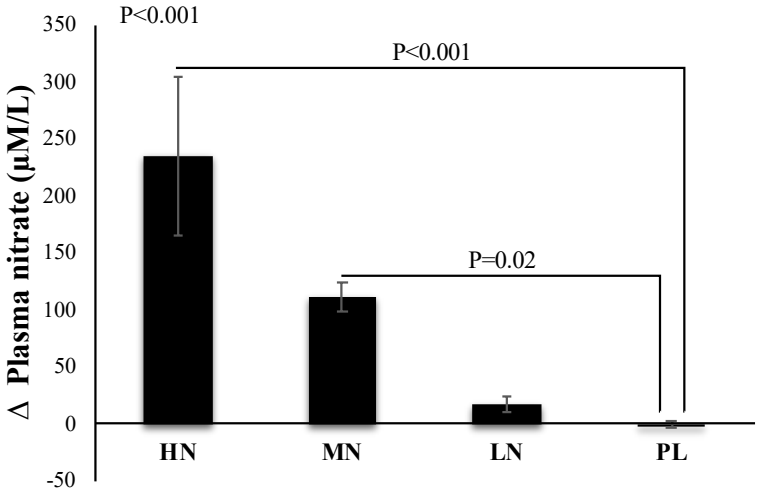

Figure 5.8: Mean changes in plasma NO_3^- concentrations of incremental doses of dietary NO_3^- in form of BJ in older overweight and obese adults.
HN (High NO_3^- ; two 70ml shots of BJ/day, morning and evening), MN (Medium NO_3^- ; 70 ml of BJ/day), LN (Low NO_3^-; 70 ml of BJ every alternate days) and PL (placebo; 70 ml of NO_3^- depleted BJ). Each shot of BJ contains 400 mg of NO_3^-. The change at 13-weeks from baseline data were analysed with one-way ANOVA. Data are expressed as mean ± SEM, (n = 49).

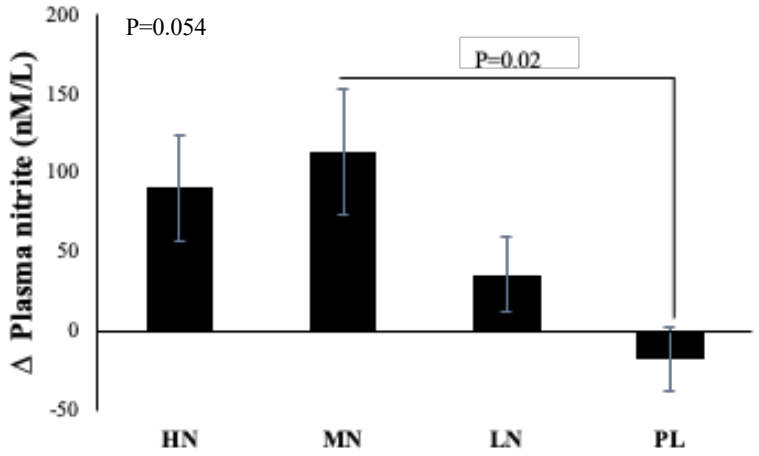

Figure 5.9: Mean changes in plasma NO_2^- concentrations of incremental doses of dietary NO_3^- in form of BJ in older overweight and obese adults.
HN (High NO_3^- ; two 70ml shots of BJ/day, morning and evening), MN (Medium NO_3^- ; 70 ml of BJ/day), LN (Low NO_3^-; 70 ml of BJ every alternate days) and PL (placebo; 70 ml of NO_3^- depleted BJ). Each shot of BJ contains 400 mg of NO_3^-. The change at 13-weeks from baseline data were analysed with one-way ANOVA. Data are expressed as mean ± SEM, (n = 49).

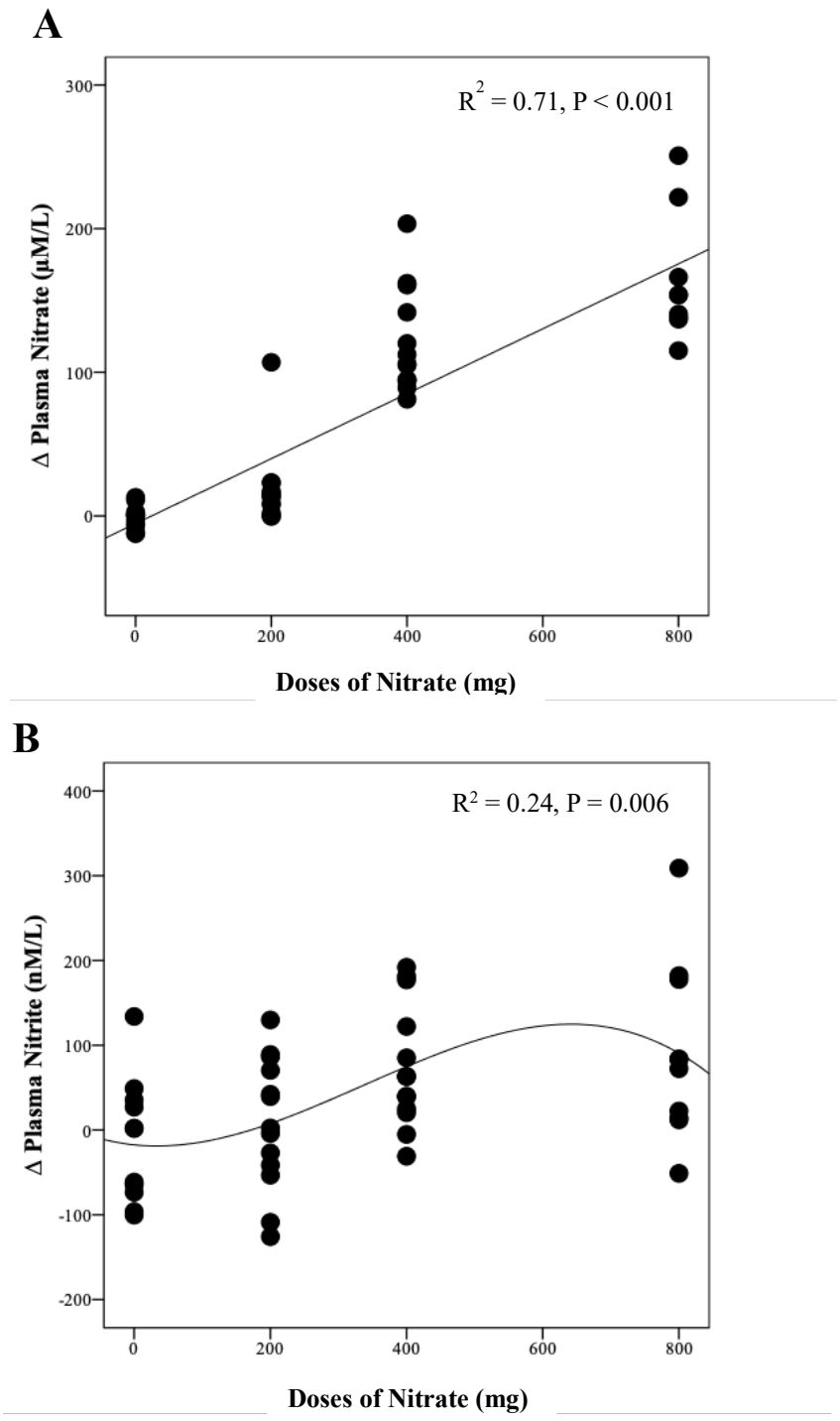

Figure 5.10: Relationship between changes in plasma NO_3^-, NO_2^- and doses of NO_3^-
This figure shows the relationship between changes in plasma NO_3^- (A) and NO_2^- (B) and doses of NO_3^-. Regression lines are fitted to each distribution, (A) is linear and (B) is cubic, (n=49).

5.3.6. Salivary nitrate and nitrite concentrations

There was no significant difference between and within the intervention groups in salivary NO_3^- and NO_2^- concentrations measured at the two baseline visits (P>0.05), confirming that participants complied with the instruction of low NO_3^- diet for both baseline visits (Visit 1 at Newcastle University, and Visit 2 at University of Northumbria) (**Figure 5.11 A**). For salivary NO_3^-, repeated measures analysis showed a significant effect of time (P<0.001), intervention (P<0.001), and a significant time*intervention interaction (P<0.01) (**Figure 5.11 A**). There was no significant difference in salivary NO_3^- concentrations between the two end of-study measurements (Visit 4 at Newcastle Uni and Visit 5 at Northumbria Uni) within the intervention groups (P>0.05) (**Figure 5.11 A**). One-way ANOVA revealed a statistically significant difference between intervention groups in the change from baseline in salivary NO_3^- at 13-weeks (P<0.01). Compared with PL, the change from baseline in salivary NO_3^- concentrations at 13-weeks significantly increased after HN and MN (Δ 2.7 μM/L, 95% CI: 0.4, 4.9, P=0.02), (Δ 2.9 μM/L, 95% CI: 0.8, 5.0, P=0.004), respectively, but there was no significant difference between LN dose and PL (P>0.05) (**Figure 5.11 B**). The mean percent changes (mean±SD) in salivary NO_3^- in HN, MN, LN and PL at 13- weeks relative to baseline were 1182±780%, 1092±608%, 318±440% and 20±54%, respectively.

For salivary NO_2^-, repeated measures analysis showed a significant effect for time (P<0.001) intervention (P<0.001), and a significant interaction term (P=0.045). There was no significant difference in salivary NO_2^- concentrations between the two end of-study measurements within the intervention groups (P>0.05) (**Figure 5.12A**). One-way ANOVA reveals that there was a statistically significant difference between intervention groups on the change from baseline in salivary NO_2^- at 13-weeks (P=0.001). Compared to PL, the change from baseline at 13-weeks in salivary NO_2^- concentrations increased significantly after HN and MN (Δ 319 μM/L, 95% CI: 119, 520 μM/L, P=0.001) , (Δ 217 μM/L, 95% CI: 34, 401 μM/L, P=0.02), respectively (**Figure 5.12 B**). No significant difference was found in the change of salivary NO_2^- between LN dose and PL (P>0.05). The mean percent changes in salivary NO_2^- in HN, MN, LN and PL at 13- weeks relative to baseline were 521±397%, 431±500%, 145±257% and 64±129%, respectively.

The distinct pattern of changes in concentrations of NO_3^- and NO_2^- for the LN group becomes even clearer when data from week 4 and week 8 are combined (**Figure 5.13 A and B**). A significant difference between time points for both NO_3^- and NO_2^- (P=0.003 and 0.002, respectively) is observed. Compared to baseline, salivary NO_3^- concentrations increased significantly on the 1st and 3rd day (based on combined data obtained at week 4 and week 8) ((Δ

2.3 mM/L, 95% CI: 0.5, 4.2 mM/L, P=0.009) and (Δ 2.8 mM/L, 95% CI: 0.9, 4.6 mM/L, P=0.002), respectively (Figure 5.13 A). Similarly, salivary NO_2^- concentrations increased significantly on the 1st and 3rd days ((Δ 454.8 µM/L, 95% CI: 65.2, 844.5 µM/L, P=0.02) and (Δ 587.7 µM/L, 95% CI: 198.0, 977.4 µM/L, P=0.001), respectively (**Figure 5.14 B**). No significant difference was found between data collected on the 2nd day, at baseline and end of the study for both salivary NO_3^- and NO_2^- concentrations (P>0.05).

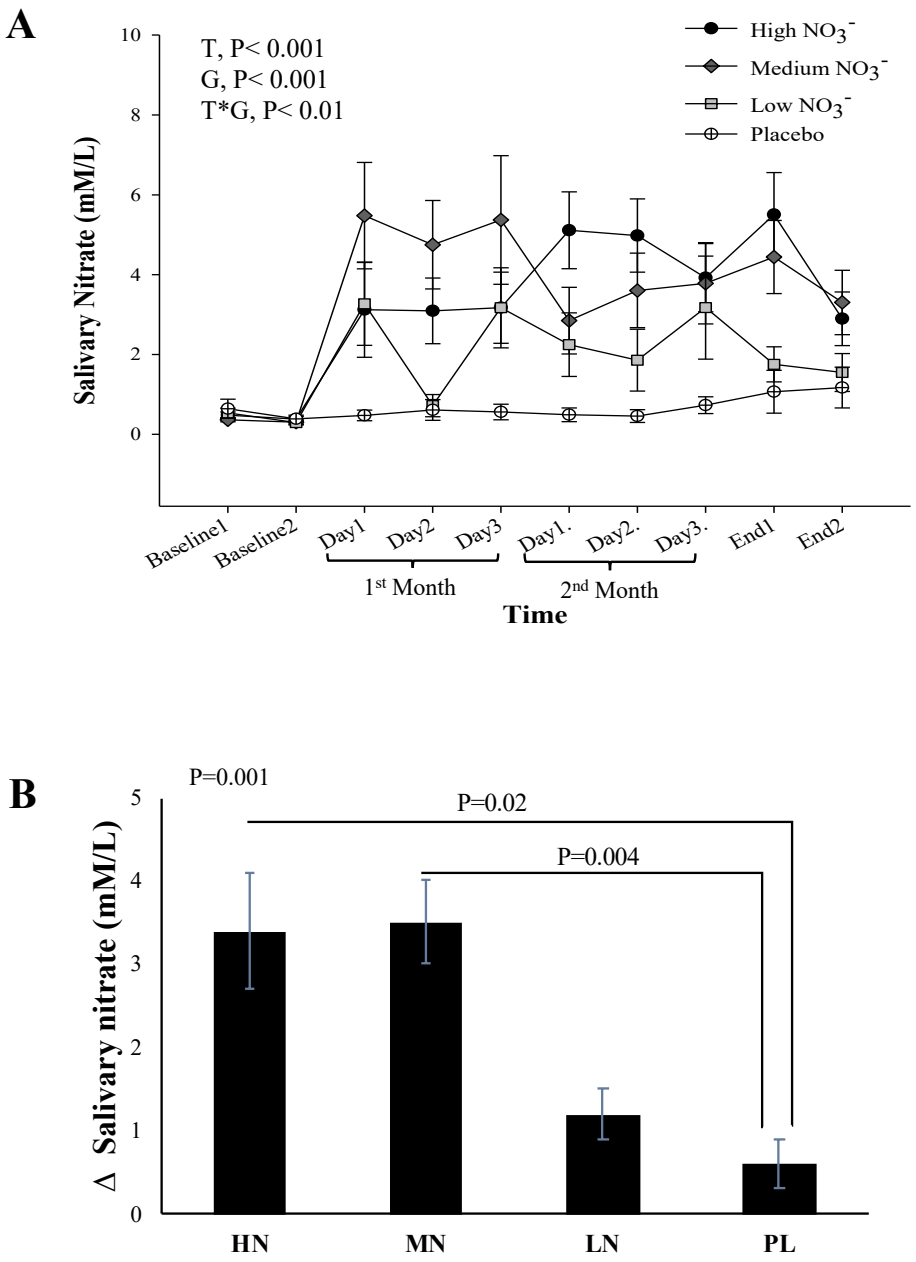

Figure 5.11: Mean salivary NO_3^- concentrations for each of the intervention groups.
(A) Mean salivary NO_3^- concentrations measured at baseline, 4 weeks, 8 weeks, and after 13-weeks for each of the intervention groups. HN (High NO_3^-; two 70ml shots of BJ/day, morning and evening), MN (Medium NO_3^-; 70 ml of BJ/day), LN (Low NO_3^-; 70 ml of BJ every alternate days) and PL (placebo; 70 ml of NO_3^- depleted BJ). Each shot of BJ contains 400 mg of NO_3^-. Data were analysed using a 2-factor repeated measures ANOVA (time*group of intervention). (B) The Mean change in salivary NO_3^- concentrations between baseline and week 13 for each of the intervention groups. These changes of from baseline were analysed with one-way ANOVA. Data are expressed as mean ± SEM, (n = 50).

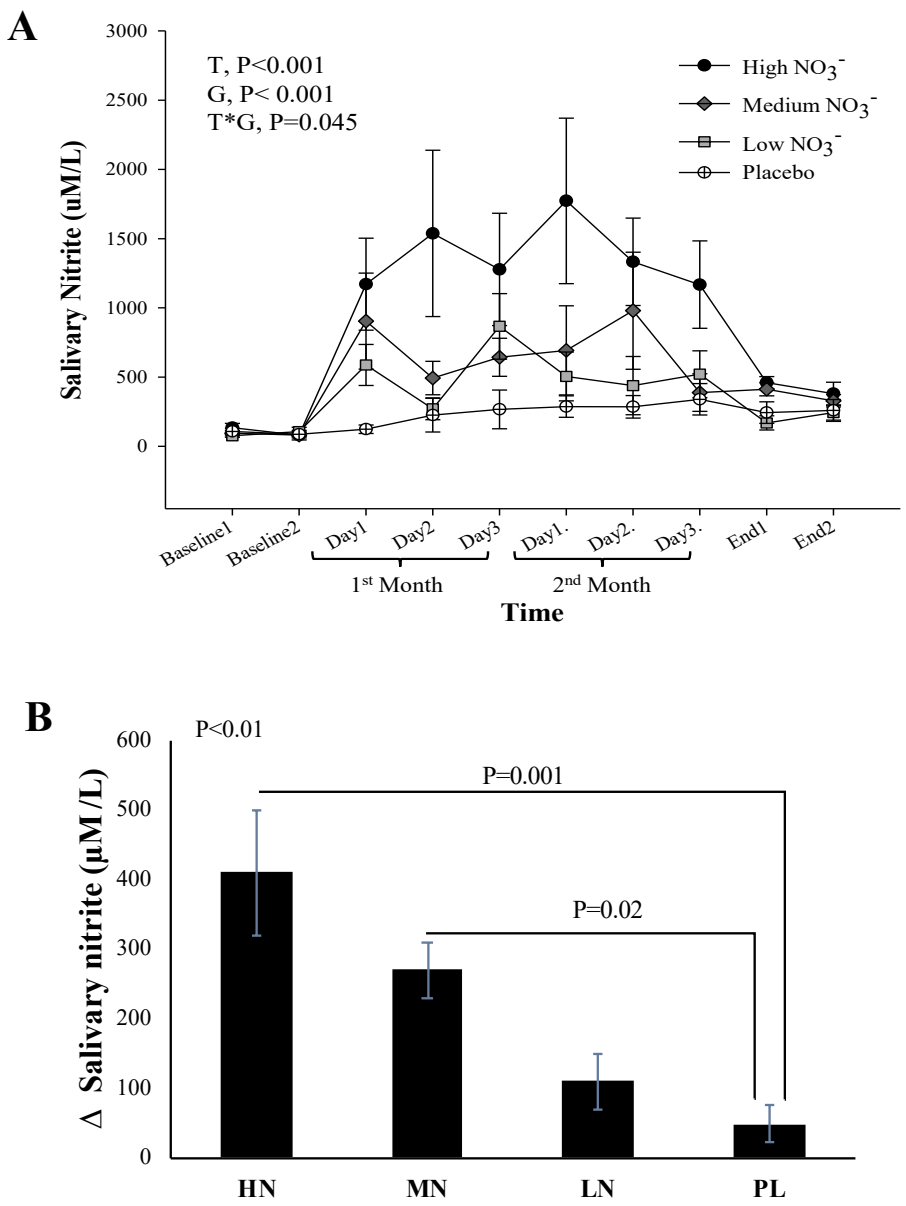

Figure 5.12: Mean salivary NO_2^- concentrations for each of the intervention groups.
(A) Mean salivary NO_2^-concentrations measured at baseline, 4 weeks, 8 weeks, and after 13-weeks for each of the intervention groups. HN (High NO_3^- ; two 70ml shots of BJ/day, morning and evening), MN (Medium NO_3^- ; 70 ml of BJ/day), LN (Low NO_3^-; 70 ml of BJ every alternate days) and PL (placebo; 70 ml of NO_3^- depleted BJ). Each shot of BJ contains 400 mg of NO_3 Data were analysed using a 2-factor repeated measures ANOVA (time*group of intervention). (B) The Mean change in salivary NO_2^- concentrations between baseline and week 13 for each if the intervention groups. These changes from baseline were analysed with one-way ANOVA. Data are expressed as mean ± SEM, (n = 50).

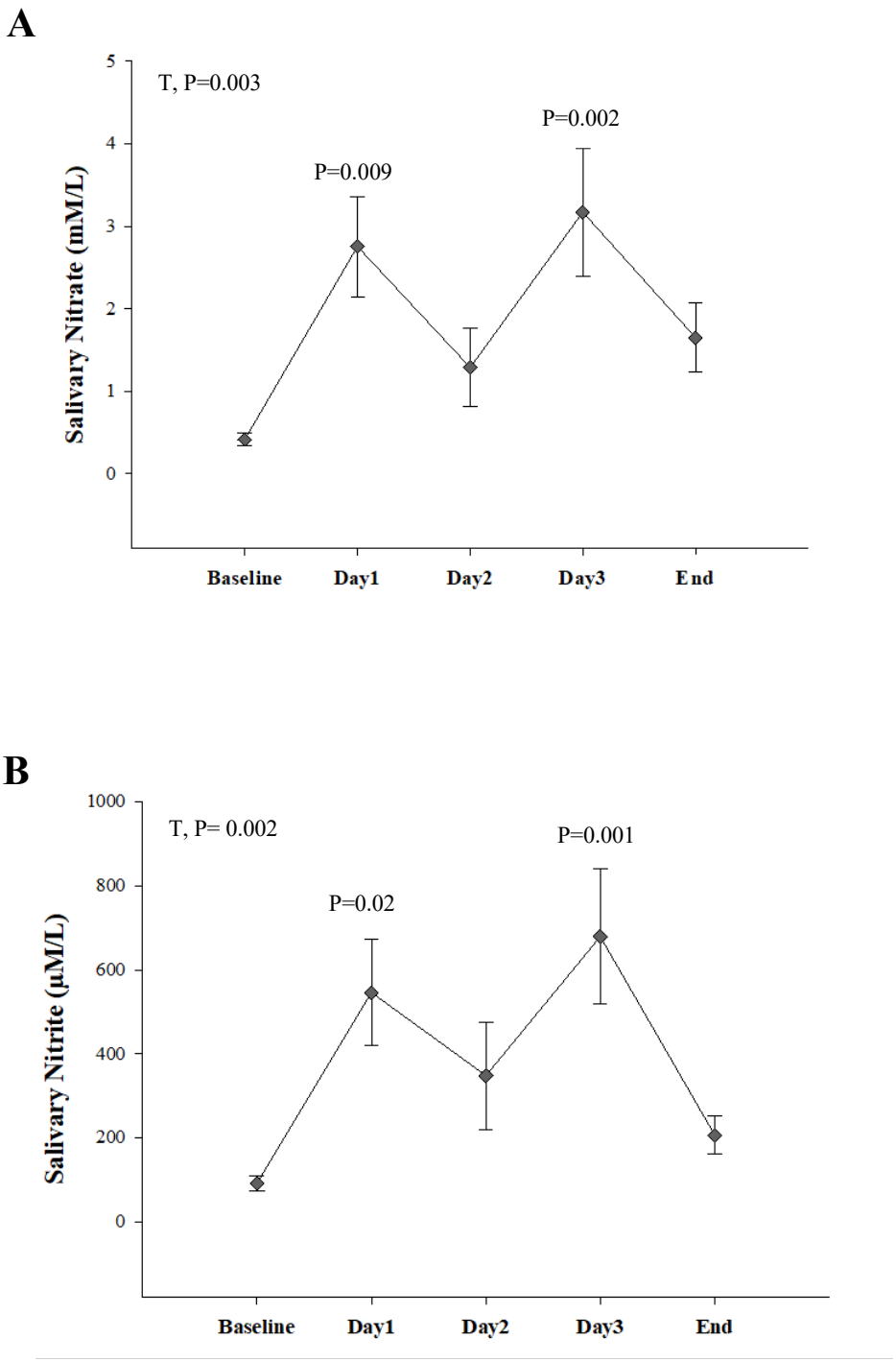

Figure 5.13: Mean of salivary NO_3^- and NO_2^- concentrations.
Mean of salivary NO_3^- (A) and NO_2^- (B) concentrations in the group consumed a LN (low NO_3^- dose; 70 ml of BJ every alternate days). Statistical analysis using one factor (time) repeated measure ANOVA. Saliva samples were collected on three consecutive days at week 4 and week 8. Data collected on the week 4 and week 8 were combined. Data are expressed as mean ± SEM, (n = 50).

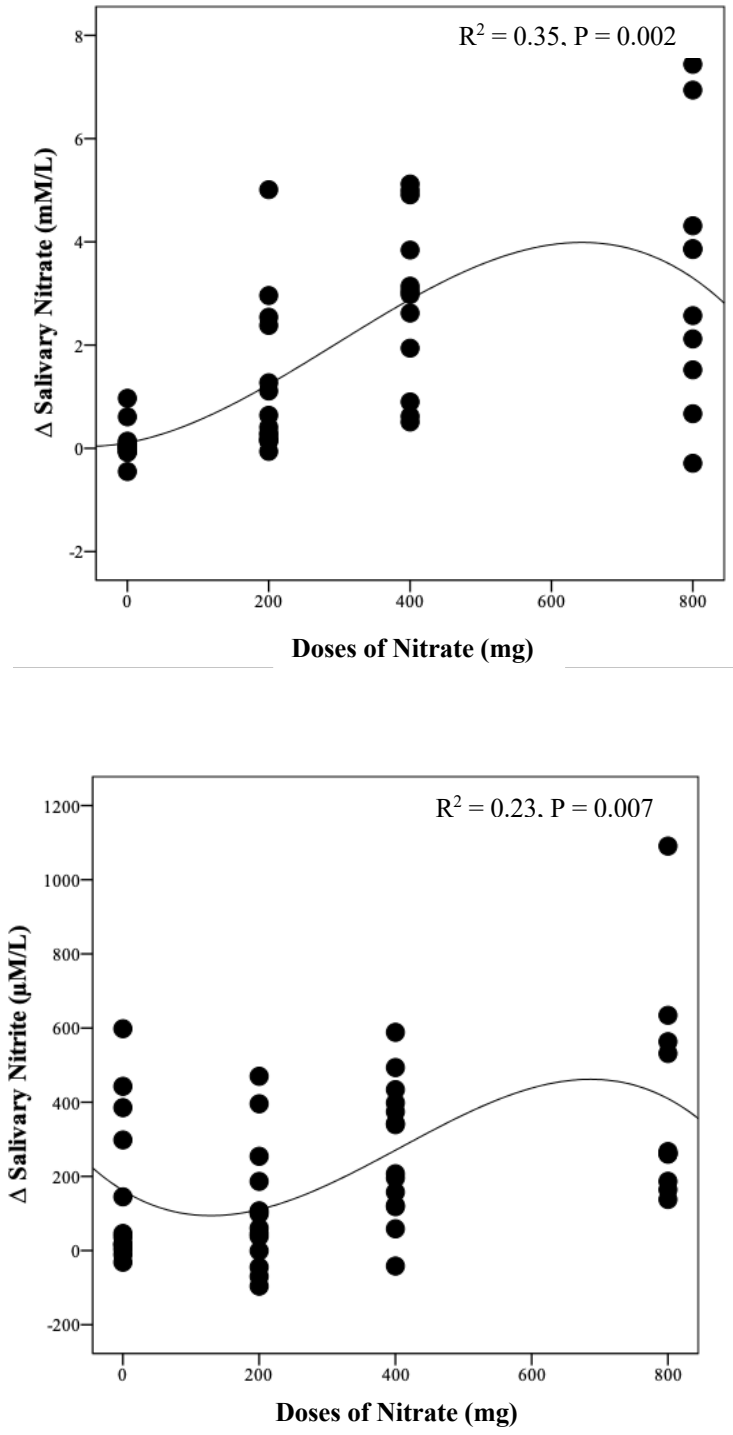

Figure 5.14: The relationship between the change in salivary NO_3^- (A) and NO_2^- (B) and daily doses of NO_3^- delivered during the intervention.
The line represents the line of best fit for each distribution, both are cubic, (n=50).

5.3.7. Urinary nitrate concentration

There was no significant difference in urinary NO_3^- concentrations between the two baselines (Visit 1 at Newcastle University, and Visit 2 at University of Northumbria) within and between intervention groups (P>0.05), as shown in **Figure 5.15 A**. A significant effect of time (P<0.001), intervention (P<0.001), and time*intervention interaction was found for urinary NO_3^- (P<0.001), and one-way ANOVA revealed a statistically significant difference between intervention groups in the change from baseline in urinary NO_3^- at 13-weeks There was no significant difference in urinary NO_3^- concentrations between the last two end visits (Visit 4 at Newcastle University and Visit 5 at University of Northumbria) within the intervention groups (P>0.05). Compared with PL, the change in urinary NO_3^- concentrations significantly increased after HN (Δ 6.5 µM/L, 95% CI: 3.8, 9.2, P<0.001) and MN (Δ 4.3 µM/L, 95% CI: 1.8, 6.8, P<0.001) (**Figure 5.15 B**). The mean of percentages of change (mean±SD) in urinary NO_3^- in HN, MN, LN and PL at 13- weeks relative to baseline are at 13- weeks were 2335±1145%, 1702±1571%, 504±114% and 19±59%, respectively. Overall, **Figure 5.15 A** shows that urinary NO_3^- concentrations were high and stable over time with HN and MN doses and urinary NO_3^- concentrations did not change in the PL group.

The pattern of change in urinary NO_3^- concentration for the LN group was similar to that for salivary NO_3^- concentrations with a significant difference between time points (P=0.001). (**Figure 5.16**). Compared with baseline, urinary NO_3^- concentrations increased significantly on the 1st and 3rd day (based on combined data obtained at week 4 and week 8) (Δ 3.9 mM/L, 95% CI: 1.5, 6.3 mM/L, P=0.001) and (Δ 4.3 mM/L, 95% CI: 1.9, 5.9 mM/L, P<0.001), respectively. No significant differences were found between concentrations measured at baseline and those measured on the 2nd day or at the end of the study (P>0.05). A significant linear relationship between NO_3^- doses and change in urinary NO_3^- concentrations was found (R^2 = 0.46, P<0.05, **Figure 5.17**).

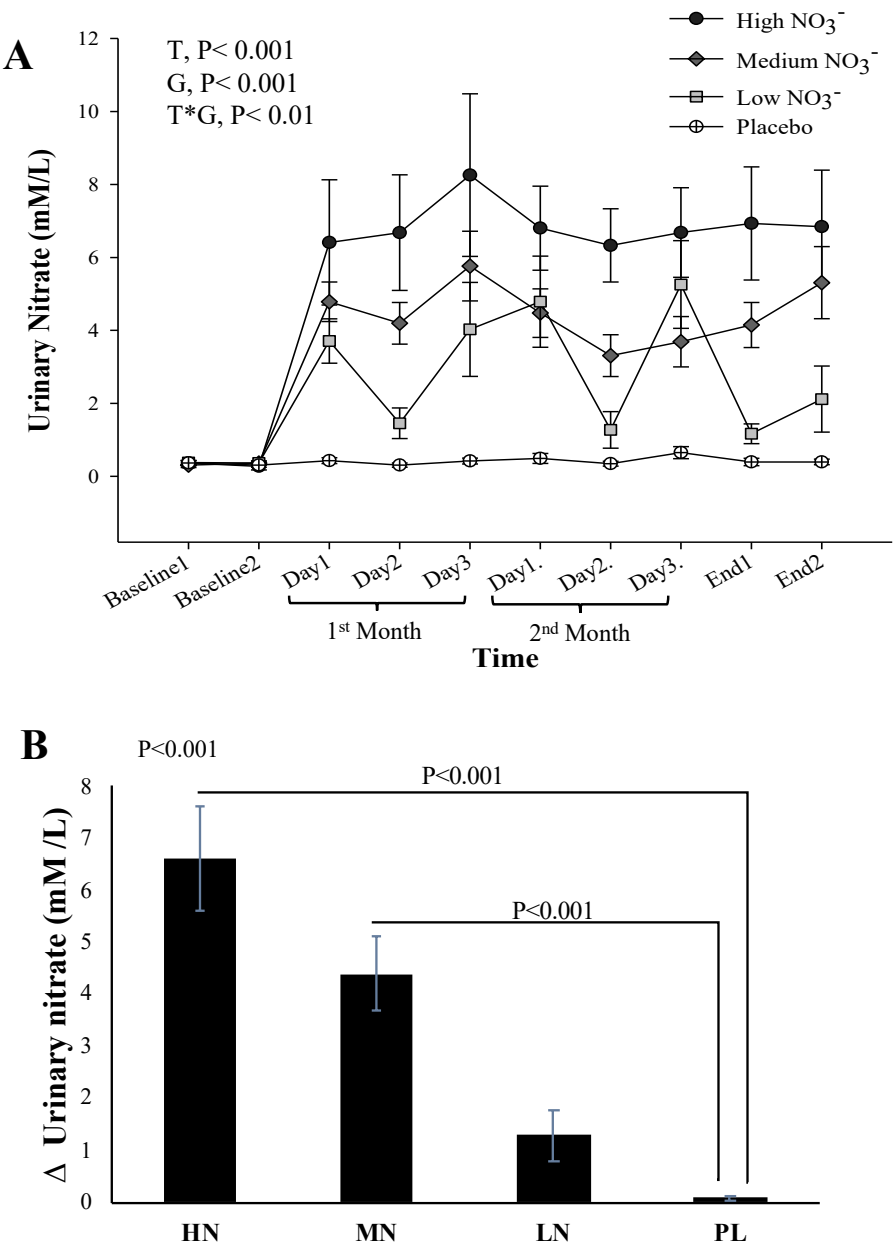

Figure 5.15: Urinary NO_3^- concentrations.
Mean urinary NO_3^- concentrations measured at baseline, 4 weeks, 8 weeks, and after 13-weeks for each of the intervention groups. HN (High NO_3^- ; two 70ml shots of BJ/day, morning and evening), MN (Medium NO_3^- ; 70 ml of BJ/day), LN (Low NO_3^-; 70 ml of BJ every alternate days) and PL (placebo; 70 ml of NO_3^- depleted BJ). Each shot of BJ contains 400 mg of NO_3^-. Data were analysed using a 2-factor repeated measures ANOVA (time*group of intervention). (B) The Mean of the change in urinary NO_3^- concentrations between baseline and week 13 for each of the intervention groups. These changes from baseline were analysed with one-way ANOVA. Data are expressed as mean ± SEM, (n = 50).

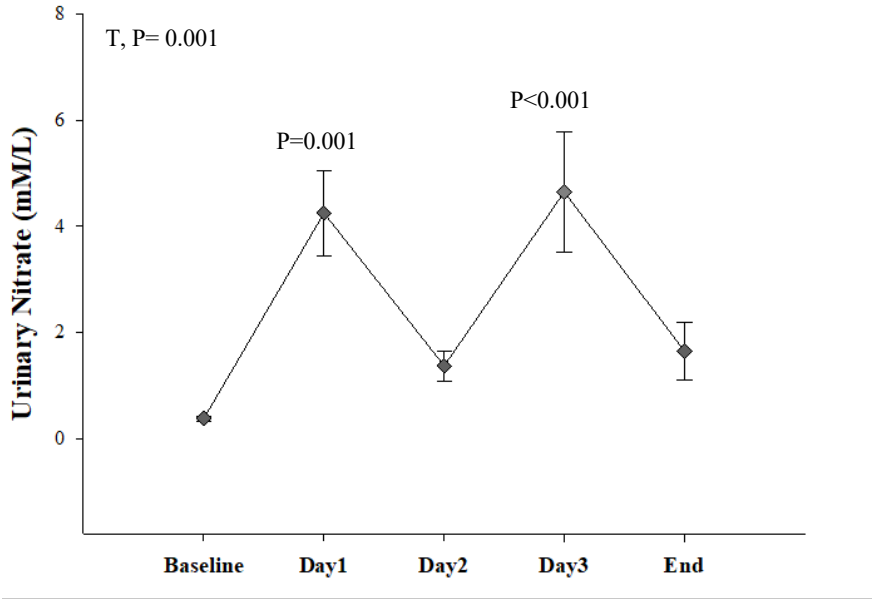

Figure 5.16: Mean of urinary NO_3^- concentrations in the LN group (low NO_3^- dose; 70 ml of BJ every alternate days).
Statistical analysis using one factor (time) repeated measure ANOVA. Saliva samples were collected on three consecutive days at week 4 and week 8. Data collected on the week 4 and week 8 were combined. Data are expressed as mean ± SEM, (n = 50).

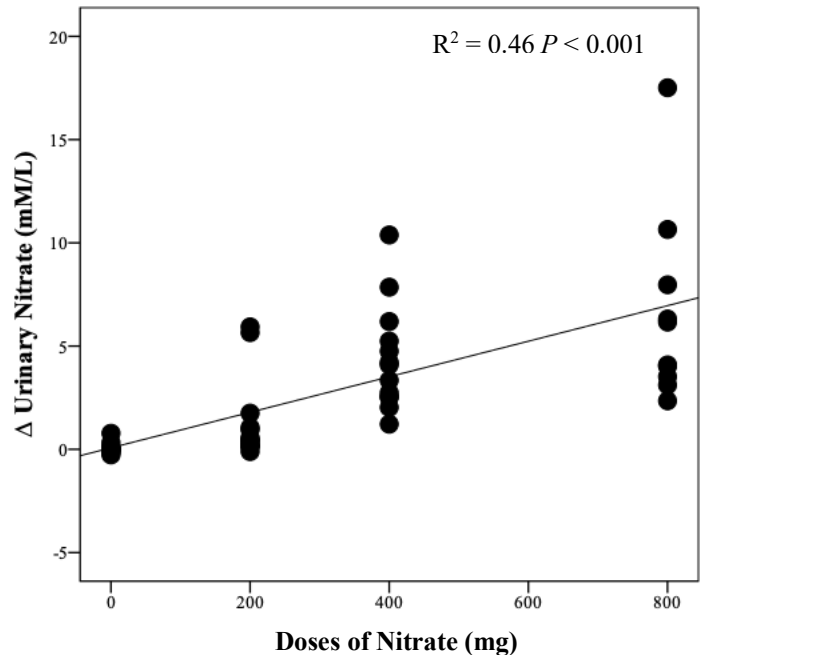

Figure 5.17: Linear relationship between changes in urinary NO_3^- and doses of NO_3^- (n=50).

5.3.8. Salivary nitrite strips (Berkeley)

Baseline salivary NO_2^- concentration, measured by the strips, were similar for all intervention groups (P>0.05). Repeated-measure analysis showed a significant effect of time (P<0.001), intervention (P<0.001), and time*intervention interaction (P<0.001). Overall, consumption of the HN and MN doses resulted in a significant elevation in Berkeley strip readings of salivary NO_2^- concentrations when compared with PL (P<0.001 and P=0.002, respectively). The LN group showed a more moderate increase in Berkeley strips readings (P=0.051) with day-to-day variation mirroring the alternate day provision of the BJ supplement (**Figure 5.18**). There was a significant correlation (r=0.41, p<0.001) between strips readings and salivary NO_2^- concentrations that measured by ozone-based chemiluminescence (**Figure 5.19**).

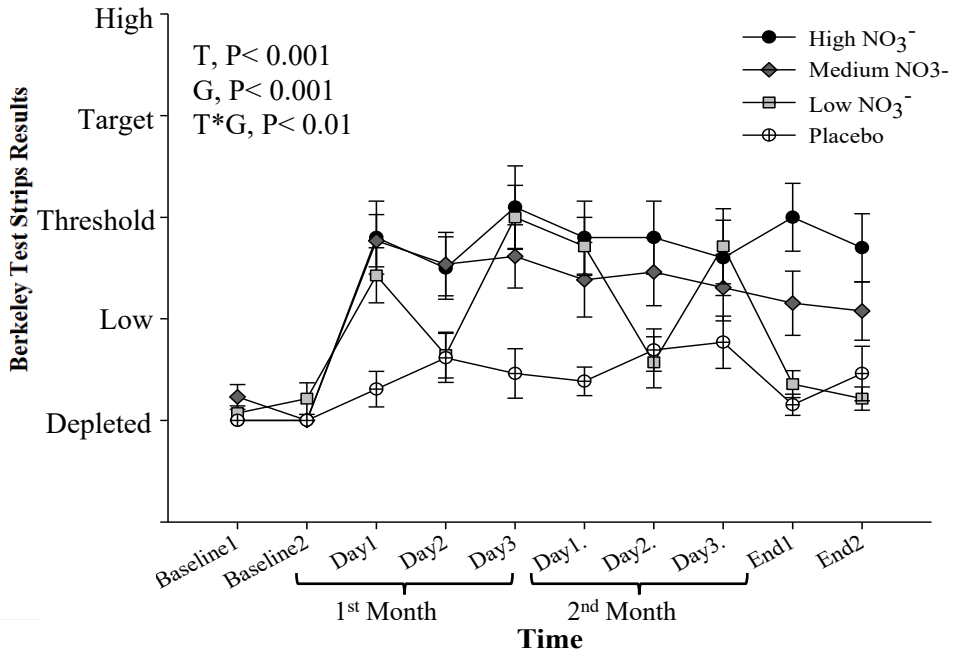

Figure 5.18: Salivary NO_2^- strips readings (Berkeley).
Mean salivary NO_2^- strips readings (Berkeley) measured at baseline, 4 weeks, 8 weeks, and after 13-weeks. HN (High NO_3^- ; two 70ml shots of BJ/day, morning and evening), MN (Medium NO_3^- ; 70 ml of BJ/day), LN (Low NO_3^-; 70 ml of BJ every alternate days) and PL (placebo; 70 ml of NO_3^- depleted BJ). Each shot of BJ contains 400 mg of NO_3^-. Data were analysed using a 2-factor repeated measures ANOVA (time*group of intervention). Data are expressed as mean ± SEM, (n = 50).

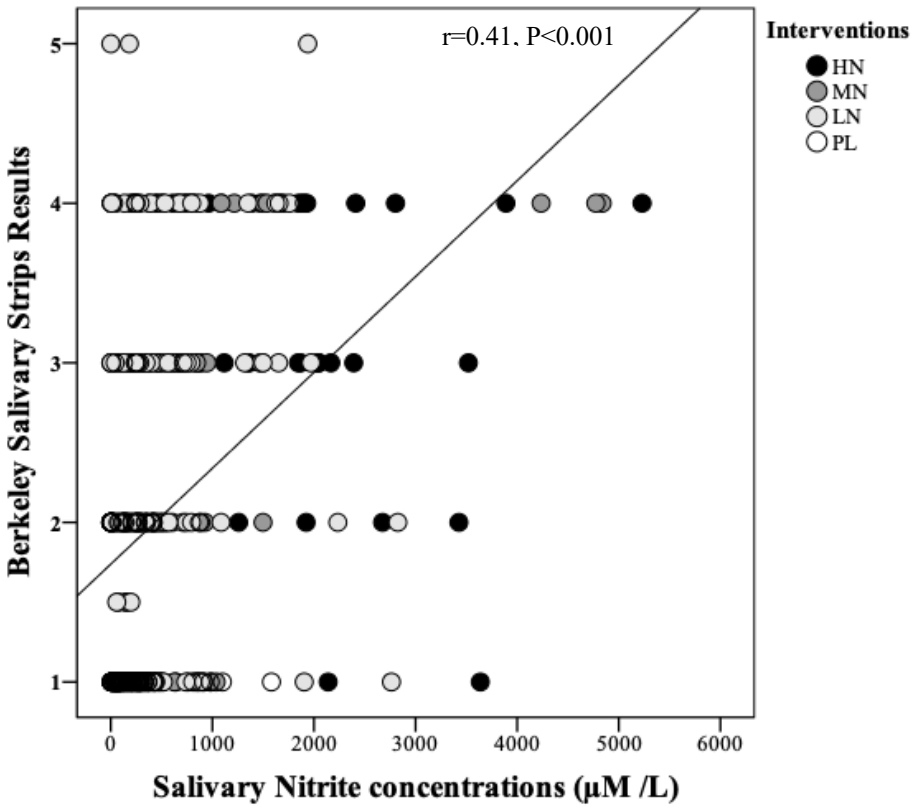

Figure 5.19: Scatterplot of Pearson correlation between Berkeley salivary NO_2^- strips readings and salivary NO_2^- concentrations.

Scatterplot showing Pearson correlation between Berkeley salivary NO_2^- strips readings and salivary NO_2^- concentrations measured by chemiluminescence; n=50. Berkeleysalivary strips results provided by the manufacturer (1=Depleted, 2=Low, 3=Threshold, 4=Target and 5=High).

5.3.9. The relationship between the duration of postal delivery time and the concentrations of salivary and urinary biomarkers

A sensitivity analysis was conducted to check whether the delay in receiving some samples affected the biomarkers concentrations. One-way ANOVA reveals that there was no statistically significant difference in salivary NO_2^- and NO_3^- and urinary concentrations of NO_3^- between the different days (P>0.05) as seen in **Figure 5.20 A, B and C,** respectively. However, **Figure 5.20 A and B** clearly show a reduction in NO_2^- and NO_3^- concentrations in saliva samples received after 3 or more days. Post hoc analysis showed a trend towards a lower salivary NO_2^- concentration in samples received after 3 or more days compared to 2 days or 1 day (P = 0.07 and P=0.08, respectively), but not in salivary NO_3^-.

A similar pattern was observed when the analyses were conducted separately for each intervention group (**Appendix 5.12**).

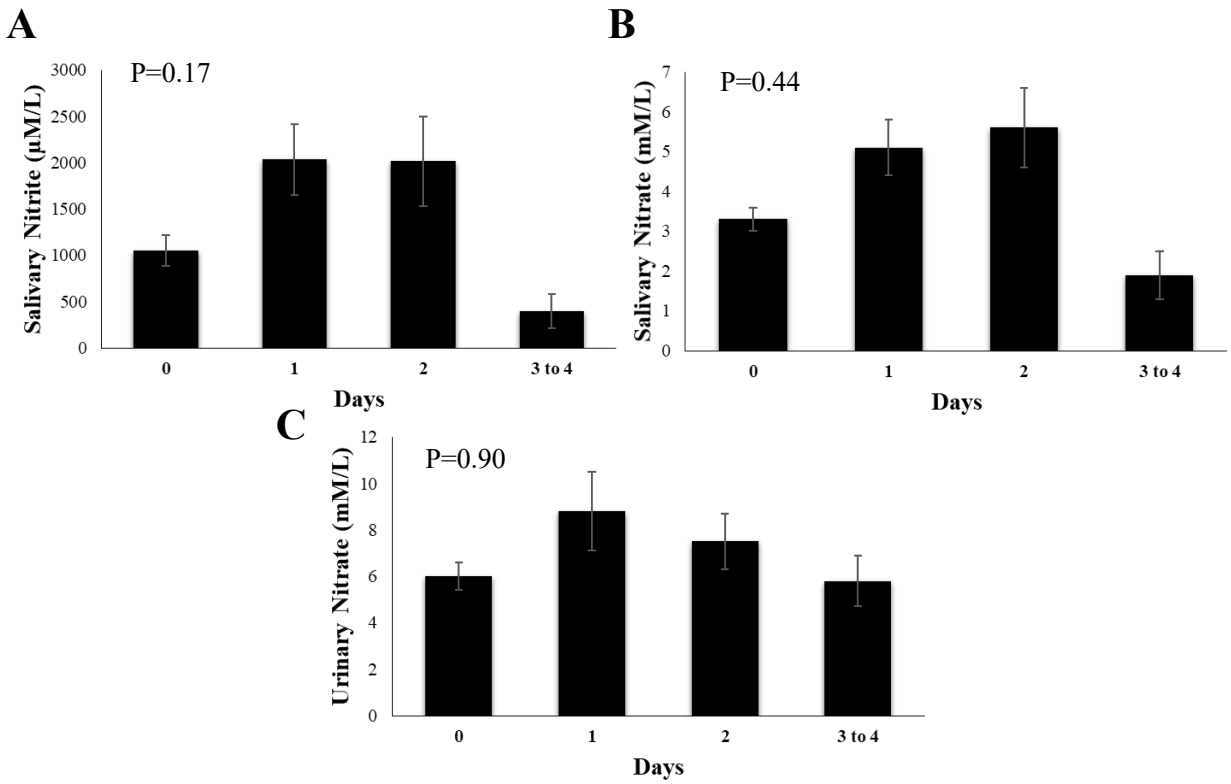

Figure 5.20: Salivary NO_2^-, NO_3^- and urinary NO_3^- (C) concentrations.
Mean salivary NO_2^- (A) and NO_3^- (B) and urinary NO_3^- (C) concentrations in samples received after a different number of days. Results presented in this analysis are based on combined data collected during the two interim monthly visits and across the 4 intervention groups. One-way ANOVA was used to test differences in biomarker concentrations between different days. Data are expressed as mean ± SEM.

5.3.10. Dietary nitrate intake assessed from Intake 24 reports and its association with baseline biomarkers

The repeated measures ANOVA analysis indicates that the habitual NO_3^- intakes over a period of 13-weeks were similar between and within the intervention groups (P>0.05), demonstrating that the dietary NO_3^- intake was stable throughout the study. Overall, the mean of NO_3^- intake for participants who completed and recorded their six intakes (n=42) was 102 mg with a range of 44-191 mg/day. **Figure 5.21** shows the average NO_3^- intake that was recorded every 2 weeks during the study for 42 participants.

The concentration of NO_3^- in urine is highly influenced by NO_3^- ingestion because about 60-70% of ingested NO_3^- is excreted in urine. Therefore, it has been suggested in Chapter 2 that collecting urine samples on multiple days may provide an objective and accurate assessment of NO_3^- intake in such a prolonged study as the current one. Interestingly, a highly significant, moderate positive correlation was observed between NO_3^- intake and baseline urinary NO_3^-

(r=0.38, P=0.01), as shown in **Appendix 5.13**. No association was observed between NO_3^- intake and plasma or salivary biomarkers, Figures are shown in **Appendix 5.13**.

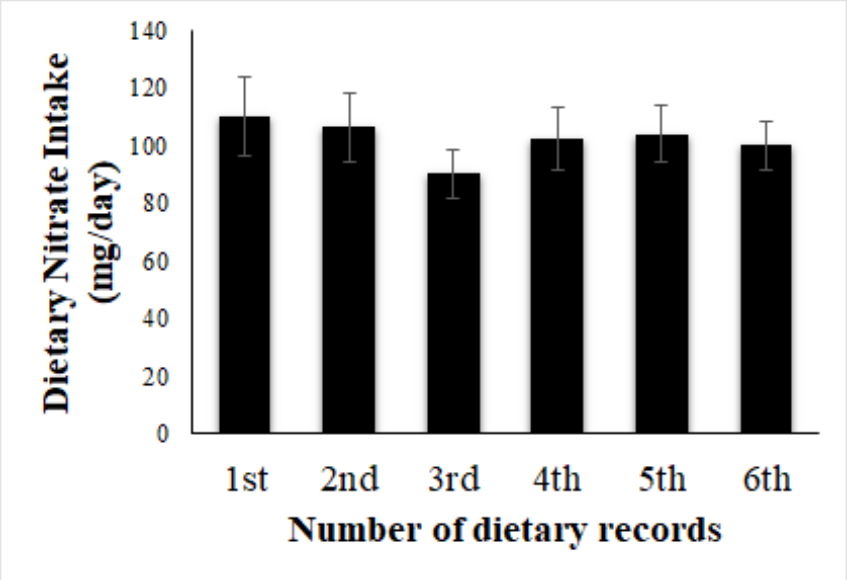

Figure 5.21: Daily dietary NO_3^- intake during each of the 6 assessment periods as recorded by participants using Intake 24.
Data are expressed as mean ± SEM, (n = 42).

5.3.11. Participant feedback on study

Completed questionnaires were returned by 52 participants (questionnaire with answers can be seen in **Appendix 5.13).** Of these, four were completed after six weeks as participants had withdrawn from the study. Most of the participants reported that the primary reasons for participating in the study were: 1) interest in nutritional research (n=25) and 2) health benefits of beetroot (n=18). Regarding their expectations from participating to the study, the majority of participants had no obvious expectations (n=28) while some expected that the BJ could improve their health and wellbeing (n=18). Interestingly, the majority of participants (n=38) expressed an interest in being approached for a similar future study, whereas only 13 said that they would not participate in another similar study, for which the primary reasons were 1) taste of BJ and 2) duration of the study. Other participants would not participate because of the side effects and/or the saliva collection procedure. One participant said: "*We would definitely taste the product first!"* (P25, Group 2) suggesting that a taste session may need to be introduced as part of the screening process. In addition, almost half of the participants reported that they would not join a longer study (i.e. of a duration longer than three months) (n=24).

Thirty-three participants reported that they did not eat or drink beetroot juice regularly and the most frequent reasons were the limited availability and its unpleasant taste: "*I don't like beetroot at all*" (P1, Placebo). "*I Like beetroot but it's not always available and convenient to prepare*" (P11, Group 1). When participants were asked whether or not they would recommend the BJ the overall responses (n=34) were supportive and some of the key reasons are outlined below:

"*I believe it provides benefits to blood circulation*" (P44, Group 1).

"*I feel my thoughts are clearer. My focus on things is better. I even took it to Amsterdam with me!"* (P41, Group 2).

"*It depends on research results; I would not recommend it due to the taste*" (P12, Group 2).

"*I believe it's good for health and it has a pleasant taste!"* (P13, Placebo)

"*I felt my memory slightly improved and my blood pressure was down*" (P19, Placebo)

"*It's a convenient way to take the beetroot, even though the taste is odd*" (P29, Group 2)

"*I don't feel that many people would tolerate the taste without a proven benefit!"* (P11, Group 1).

"*It has possibly improved my blood. Even the nurse was surprised from my blood results!"* (P 31, Group 3).

The majority of participants reported no major concerns or preferences with the consumption of BJ while others found it difficult due primarily to taste:

"*I hate the taste. The only way was to hold my nose whilst drinking*" (P31, Group 3).

"*Sometimes I had to hold my nose to gulp it down*" (P46, Placebo)
"*The smell, the syrupy texture, the Saltiness, all not good*" (P25, Group 2)
"*The taste made me gag*" (P56, Group 3).

Several participants reported that they felt some beneficial effects after the intervention:
"*Maybe an increase in my concentration levels*" (P22, Group 3).
"*My blood pressure reading has gone down, which is good*" (P14, Group 1).
"*Memory improvement"* (P8, Group 4).
"*Yes, I felt my cognition has improved, I am finding solutions to complicated situations!"* (P21, Group3).
"*I feel more alert*" (P2, Group 2).
"*Feel like I am thinking quicker*" (P41, Group 2).
"*I feel my memory improved*" (P19, Group 4).

Overall, feedback about the measurements undertaken as part of the study protocol was positive and participants found the procedures acceptable. The only exception was for use of Intake24 to record dietary intake, where the feedback was mixed. Although 31 participants did not have any difficulty using Intake24, 21 found that Intake24 was difficult and complex. The main problems were that it was time consuming and participants could not find all food items that they had eaten within the platform's database. **Appendix 5.13** summarizes the key results obtained from the participants' feedback questionnaire. **Appendix 5.14** shows some examples of participants' comments about the study.

5.4. Discussion

Over the past decade, there has been increased investigation of the beneficial effects of NO_3^- on health outcomes, including blood pressure, glucose control, insulin resistance, dyslipidaemia, cognition, heart failure and peripheral arterial diseases (Presley *et al.*, 2011; Allen *et al.*, 2012; Kelly *et al.*, 2013; Khalifi *et al.*, 2015; d'El-Rei *et al.*, 2016). However, most of these studies have been of short duration and there has been little investigation of the long-term effects of dietary NO_3^- supplementation on physiological functions, especially in older people. To the best of our knowledge, this pilot RCT is one of the longest that examined the feasibility and acceptability of different doses of NO_3^--rich BJ supplementation in overweight and obese older adults.

5.4.1. Recruitment

The most challenging aspects of this study included recruiting older participants who met our inclusion criteria and retaining these participants throughout the trial. The challenges associated with recruiting older people as participants for clinical trials have been documented (Ridda et al., 2010). We employed a variety of recruitment strategies over a 10-month period. Interestingly, of those strategies, advertising the study on social media (i.e. Facebook) was the most effective strategy and yielded the highest response rate within a short period compared with other traditional strategies. This suggests that this recruitment strategy is a viable method for recruiting older people into research studies despite the perception that they may be less likely to adopt newer technologies. Facebook advertising tools proposed a potential reach of 10,000; however, our advertisement reached 18,440, which exceeded that estimate by over 80%. Previous studies have shown that the use of social media is continuously increasing in older people (Greenwood et al., 2016). Moreover, a recent study indicated that the response rate of older people to social media advertising for recruiting participants into a clinical trial was higher than that found in younger adults (Cowie and Gurney, 2018). In the UK in 2017, 39% of those aged 65–74 years used a smartphone and 48% of internet users in this age group had a social media profile (Ofcom, 2017). Another recent study reported that the use of social media was a successful strategy for recruiting participants into a clinical trial investigating cardiovascular outcomes (Nash et al., 2017). Participants were also recruited from a database of participants who had participated in previous studies at Newcastle University and who had expressed an interested in being involved in future research. This approach was also effective in recruiting participants into the trial, but it was more time-consuming and had a lower response rate compared with Facebook. The RCT was also promoted in local newspapers, but the response rate was lower, and the advertising costs were considerably higher than the social media approach (£1,729.80 vs. £100). In general, Facebook can be considered a superior method of recruitment when there are limited resources for recruitment, including time and funding constraints. Our findings suggest that Facebook-targeted advertisements were a feasible and cost-effective strategy and offered the opportunity to reach a large number of older participants with potential for recruitment into clinical research. This approach is likely to be an efficient recruitment strategy for future definitive trials on the effects of BJ supplementation on cognitive and vascular functions in older people. Expanding the use of other social media (e.g. Twitter) to recruit older participants in clinical research could also help to make the recruitment easier and faster. Overall, under current condition, the use of social media (e.g. Facebook and Twitter) appears to offer a cost-effective

solution to facilitate the recruitment of older overweight and obese participants from the community into a RCT.

5.4.2. Attrition

While the recruitment target was met, 19% of the participants did not complete the trial. Losses to follow-up are common in long-term dietary interventions and other studies have reported attrition rates of up to 49% (Crichton *et al.*, 2012). The 3-month duration of our trial appeared to impact the attrition rate, as some participants found it difficult to maintain their adherence to the intervention for such a prolonged period. Reasons reported by participants for dropping out of the study included having adverse effects, such as gastrointestinal (GI) symptoms (n = 3), or an unwillingness to ingest the BJ due to its taste or smell (n = 3). However, these adverse events were reported mainly from participants in the HN groups who consumed two BJ shots per day. Mild GI symptoms have been reported within 2–2.5 hours after ingestion of similar BJ doses (140 ml) (Jonvik et al., 2018a; Jonvik et al., 2018b). This could indicate that the consumption of concentrated BJ for longer periods might have an adverse effect on GI function in some individuals. With regard to the unpleasant smell and taste of BJ, this is certainly an important aspect to consider in future studies using a similar BJ product. The unpleasant taste of the BJ was also reported by several participants who completed the study. One possible solution may be to introduce a taste session of the product at the screening visit so that participants can make an informed decision on the acceptability of the product. Although this could slow the recruitment process, it might decrease the attrition rate from studies with similar or longer intervention periods. The development of other dietary NO_3^- products providing equivalent doses of NO_3^- but without the adverse taste/ smell may also be valuable in facilitating sustained longer-term consumption of higher NO_3^- intake in those who are averse to the consumption of BJ (James et al., 2015).

A minor issue that should also be considered during chronic intervention studies using similar BJ products is the participants' holiday plans. Some of the participants complained about having difficulties carrying BJ bottles while travelling, especially those from Group 1 (two bottles/day). For such prolonged studies, high NO_3^- doses delivered via capsules would be more convenient and might improve their adherence, especially with long-term studies. However, from a physiological perspective, the NO_3^- content from various products might have a different impact on an outcome measure since other bioactive components are present in a natural product like BJ, but not in NO_3^- salts (James et al., 2015). Thus, it is worth investigating the prolonged effect of high NO_3^- doses via capsules and comparing the results with similar doses from natural

products. This type of experiment has previously been conducted acutely to compare different doses of NO_3^- from BJ and NO_3^- salts and the authors suggest that NO_3^- delivered from BJ might have greater beneficial effects (Flueck et al., 2015).

5.4.3. Compliance

The challenge of ensuring adequate compliance in dietary intervention studies is well known, especially when such studies take place over longer periods and in free-living settings (Crichton et al., 2012; Desroches et al., 2013). Although the present study was demanding, as reported by several participants, this study demonstrated a high degree of compliance with BJ consumption for all interventions (i.e. > 90%). This is an important indicator of the feasibility of this 13-week randomised trial. The incentive given at the end of the study, in conjunction with the support provided by the research team and the reminders sent during the trial, may have contributed to the high compliance. Only one participant had a low compliance; this participant stopped taking the BJ in the final two weeks due to GI problems.

5.4.4. Collection of biological samples at home and transfer to the research centre by post

To ensure compliance with the intervention, measuring nutritional intake biomarkers would help in monitoring and confirming compliance, and several studies have used NO_3^- and NO_2^- biomarkers as a measure of NO_3^- intake compliance in RCTs (Jajja et al., 2014; Blekkenhorst et al., 2018). Thus, biological samples, including urine, saliva and salivary NO_2^- strips, were collected at home by participants during the study. Previous studies showed that requiring participants to travel to research centre frequently to provide biological samples has reduced adherence with study requirements (Rockett *et al.*, 2004). The number of collected samples required can become unwieldy for participants, leading to loss of follow up. These barriers can be overcome by the use of at home collection biological samples protocols (Cox *et al.*, 2019). The present study showed that participants had excellent adherence in collecting biological samples at home, which indicate the acceptability of this procedure.

The process of the transportation of the samples from participants' homes to research centre may affect sample integrity in bioanalysis. The ideal approach would be to collect the samples from the participants and place them on dry ice on the day of collection, then store them within a few hours in -20 ºC freezers. However, the delivery of the samples by mail was considered a more pragmatic and realistic approach to reduce the burden on researchers and participants. Nonetheless, we predicted that the delivery of most of the samples would occur within one day, so ensuring the stability of the samples. This feasibility study demonstrated that this was not the

case and the delivery time of the samples varied from 1 to 5 days, with a few cases taking more than 5 days. This provided the opportunity to determine whether this delay in receiving the samples while they are being at room temperature (RT) affected the concentrations of the NO_3^- and NO_2^- biomarkers.

The present study indicated that salivary NO_3^- or NO_2^- concentrations were stable for at least 48 hours following their collection for samples maintained at RT, whereas urinary NO_3^- concentrations are stable for longer periods (3 to 4 days) at RT. This suggests that both saliva and urine samples may be used, with appropriate protocols for collection and delivery times, as biomarkers of NO_3^- intake. There is limited published evidence regarding the effect of storage conditions on the urinary and salivary NO_3^- and NO_2^- concentrations. To our knowledge, only two studies have investigated the effect of storage conditions on salivary and urinary NO_3^- or NO_2^- concentrations (Moshage et al., 1998; Sewwandi et al., 2016). These studies reported that salivary NO_2^- concentrations are significantly affected if samples are stored overnight at RT compared with samples analysed immediately after collection (Sewwandi et al., 2016). Urinary NOx (the sum of NO_3^- and NO_2^-) concentrations were markedly decreased in some urine samples (6 out of 17) when samples were stored at 37 °C for up to 24 hours. In addition, in the current study, NO_3^- levels were measured, whereas in Moshgash et al. (1998), the sum of concentrations of NO_3^- and NO_2^- ions was determined.

5.4.5. Biomarkers of nitrate intake after prolonged BJ consumption

Plasma nitrate and nitrite

There was a significant linear increase in plasma NO_3^- concentrations with increasing dose of BJ consumed. Since the time between the last BJ dose and the blood collection (~12–18 h) was similar for the two groups. This suggests that the consumption of two shots of BJ per day (morning and evening) for a long period may lead to a NO_3^- accumulation in the blood due to its long half-life (5–8 h). In contrast, increasing does of BJ did not result in a linear increase in plasma NO_2. Specifically, polynomial regression showed that the change in plasma NO_2^- increased gradually with the low doses and the highest increase was observed with MN dose with no additional changes detected after the HN dose. It might be expected that the HN dose would produce the highest levels of plasma NO_2^-, mirroring the finding with plasma NO_3^-. However, a significant difference was found in the change in plasma NO_2^- that followed the MN dose, when compared with after the PL dose. The explanation for these different findings between plasma NO_2^- and NO_3^- is not clear, but it is possible that the conversion efficiency decreased over a long period with the HN dose. The delay in processing the samples (10–12 minutes), due to the logistic

constraints, may also have contributed to this finding. It is known that NO_2^- is a highly degradable compound and oxidizes very quickly (within ~5 minutes). In the current study, plasma NO_3^- and NO_2^- concentrations were still elevated compared with baseline concentrations in the low NO_3^- dose group with ~2- and ~1.3-fold, respectively; however, the blood collection was done ~36 hours following the last shot of BJ. This suggests that blood concentration of NO_3^- and NO_2^- remained elevated for up to 36 hours following the last BJ dose. This is in agreement with Bondonno et al. (2015) who found that plasma NO_3^- and NO_2^- returned to baseline levels after 2 days following the final high NO_3^- diet intervention. Note, however, that the duration in their study was only 7 days.

Salivary nitrate and nitrite

Around 25% of the blood NO_3^- pool is actively taken up by the salivary glands and concentrated in saliva so that salivary NO_3^- concentrations are 10–20 times higher than plasma NO_3^- concentrations (Lundberg and Weitzberg, 2010). Overall, salivary NO_3^- concentrations were elevated throughout the study in the groups receiving NO_3^-, indicating that the entero-salivary circulation remained functional. Unlike plasma NO_3^-, a dose-dependent change in salivary NO_3^- was not apparent as high doses of BJ did not produce a greater increase in salivary NO_3^- concentration. Specifically, responses in salivary NO_3^- concentration to MN and HN doses were similar despite noticeable variations at some time points, suggesting no additional effects with the HN dose on salivary NO_3^- after 13 weeks. In further investigation of the data, we found that the relatively large increase in salivary NO_3^- concentration observed in the MN group was driven by only one participant who had a very high change value in salivary NO_3^- compared to the others. When data for that participant were removed, there was a clear linear dose-dependent response in salivary NO_3^- (**Appendix 5.12**).

A proportion of salivary NO_3^- is converted to NO_2^- by the action of oral facultative microflora (Doel et al., 2005), so that the concentration of NO_2^- in saliva is dependent on both the NO_3^- in saliva and the NO_3^- reductase activity of the oral microflora. The fundamental role of these bacteria has been confirmed in several studies testing the effects of antibacterial mouthwash on oral NO_3^- reductase activity, as illustrated in Chapter 4. The increase in salivary NO_2^- concentrations overtime was observed with HN and MN doses, compared with the PL. However, **Figure 5.12 A** shows a marked decrease in salivary NO_2^- concentration in the HN group at 13 weeks, and concentration at 13 weeks was significantly lower ($P = 0.006$) than the mean of values for the first and second months (Week 4 and week 8 data). A similar pattern of reduction, but to a lesser extent, was observed with the MN dose. The reason behind this reduction is unclear. It

is unlikely that this reduction can be related to the capacity of an active NO_3^- transport system into the salivary glands, as an increased concentration of salivary NO_3^- was observed at this time (13 weeks). In addition, it is unlikely that this finding is related to the time of collection, which was performed ~12–18 hours following the last ingestion, or due to overnight fasting before the collection, as suggested by Blekkenhorst et al. (2018). In this study, participants were asked to follow similar instructions at home when they collected samples during the first and second months of the study, and there were marked increases observed at these times, especially with the HN group. Recent studies have shown that dietary NO_3^- supplementation alters the oral microbiome (Vanhatalo *et al.*, 2018; Burleigh *et al.*, 2019b) and increases the oral pH (Li et al., 2007; Hohensinn et al., 2016; Vanhatalo et al., 2018). The rate of oral NO_3^- reductase activity is highest at a pH of ~7.0 to 8.0, and it falls with increasing acidity (Bojić et al., 2004). Previous studies are characterised by a short duration. Whether long-term NO_3^- supplementation has a different effect on the oral microbiota or on oral pH remains unknown. It would be interesting to test these hypotheses with a larger cohort in future studies.

Examination of salivary NO_3^- and NO_2^- concentrations from the LN dose group shows very interesting fluctuations in both salivary NO_3^- and NO_2^- concentrations when BJ was consumed on alternate days, and this becomes even more apparent when data from week 4 and week 8 were combined. When the saliva samples were collected at ~36 hours post BJ ingestion, the salivary NO_3^- and NO_2^- concentrations were much lower than those collected ~12–18 hours following the last BJ ingestion. This finding indicates that, following increased NO_3^- intake, salivary concentrations of NO_3^- and NO_2^- remain elevated for a relatively limited period of time (~up to 18 hours), even with prolonged intake. Thus frequent (daily) consumption of NO_3^- is required to maintain elevated concentrations of these biomarkers.

Salivary nitrite strips

Salivary NO_2^- strips are a reliable method to detect changes in salivary NO_2^- over a 5-hour period after a single shot of BJ, as reported in Chapter 4. The present study provided an opportunity to determine whether such strips can detect changes in salivary NO_2^- over a long period of supplementation with different doses of NO_3^-. We observed a significant moderate correlation between NO_2^- concentrations measured by the strips and those estimated by chemiluminescence for samples collected 12–18 hours following BJ ingestion. Our observation of no increase in salivary NO_2^- using the strips for samples taken 36 hours after the last BJ ingestion is consistent with our observation of no significant increase in salivary NO_2^- concentration in the same

samples when assayed by chemiluminescence .In conclusion, this evidence confirms that salivary NO_2^- strips are a useful tool for monitoring NO_3^- intake in chronic NO_3^- intervention studies.

Urinary nitrate

After ingested NO_3^- is absorbed from the GI system into the systemic circulation, approximately 60-70% is excreted in the urine. The current study showed a significant linear increase in urinary NO_3^-concentration with increasing BJ consumption. No higher excretion levels were observed with high or medium doses over time, and levels remained generally constant until the end of the study. This is consistent with results reported by Berends et al. (2019) and Jajja et al. (2015), who showed that urine excretion remained at similar elevated levels when urine samples were collected at different times during the supplementation period.

The majority of ingested NO_3^- is excreted in urine during 24-hour, which may suggest the lack of a further increase over time with consistent BJ doses (Berends *et al.*, 2019) . Previous studies have shown that urinary NO_3^- levels returned to baseline levels during 24 hours of NO_3^- ingestion (Bartholomew and Hill, 1984; Leach et al., 1987). Our findings are consistent with these studies, as the NO_3^- levels decreased markedly when samples were collected after 36 hours in the LN group. Thus, again regular consumption of NO_3^- is required to keep concentrations of these biomarkers elevated.

5.4.6. Estimation of dietary nitrate intake from estimates of food intake using Intake24

While there are still no universally agreed methods to assess NO_3^- intake, FFQ methods are used frequently to assess NO_3^- intake, as reported in Chapter 2.However, 24-hour recall methods require only short-term memory (Johnson, 2002), and could be a more accurate method than FFQ, especially in older adults. However, multiple recalls are needed to reliably assess food intake to address day-to-day variations. We asked participants to record their food intake once every two weeks, assuming that their usual intake would remain largely unchanged during the study (they were asked to maintain their usual dietary habits). Given that conventional approaches for dietary assessment are burdensome for both participants and researchers, recently, there has been an increased interest in the use of digital and other technologies to improve dietary assessment (Penn *et al.*, 2010). In the present study, Intake24, an online 24-hour dietary recall tool, was used to assess food intake and then these estimates of food intake were combined with data on NO_3^- content of foods from an Australian database (lead researcher Dr Michael Leveritt, Queensland University) to generate estimates of NO_3^- intake. The feedback on the use of Intake24 was mixed. From the feedback questionnaire, the majority of participants indicated that they did not have any difficulty recording their dietary recalls (63%), but a significant proportion of

participants found it complex (40%). This is in agreement with a recent Canadian study that used ASA24 online recalls and the majority of older participants who were able to access ASA24 (84%, 37 out of 44) found that the tool was easy to use without needing any support, while the rest found it a complex tool (Ettienne-Gittens et al., 2013). A recent study conducted in older adults tested the feasibility of Myfood24, another online dietary recall system. They asked participants to record their food on three occasions (once per month) and found that only 29% of the participants completed the three recalls (Ward *et al.*, 2019). However, in our study, 76% of participants completed all of the required recalls (six recalls). Therefore, the current findings support the use of Intake24 in older people.

5.4.7. The relationships between nitrate intake and concentrations of nitrate and nitrite in plasma, urine and saliva

There is a great variability in the NO_3^- content of food, which makes the estimation of NO_3^- intake challenging. In the current study, NO_3^- intake was assessed every two weeks. The primary reason for collecting NO_3^- intake data was to test whether a participant's NO_3^- intake was stable during the study, which was confirmed from the analysis. This helps to mitigate the limitation that dietary intake was not recorded at baseline. After this study was designed and started, testing the relationship between NO_3^- intake and biomarkers was added. Only baseline biomarker data were tested in relation to the average NO_3^- intake assessed by Intake24. The reason for this was that we relied on the typical values for inorganic NO_3^- as reported on the products and adding that amount of NO_3^- to the habitual intake would bias the analysis.

Our findings showed that urinary NO_3^- concentrations were significantly associated with NO_3^- intake assessed by a 24-hour recall (Intake24), whereas no such associations were found with salivary or plasma NO_3^- or NO_2^-. The repeated measurements of urinary NO_3^- helped reflect the long-term exposure. The levels of urinary NO_3^- concentrations remained similar until the end of the study when the NO_3^- dose was stable.

5.5. Conclusion

In summary, this feasibility study provided valuable information on how to overcome the challenges faced in recruiting and retaining older overweight/obese adults in a relatively long-term BJ supplementation study. The appropriate use of intensives and phone or email reminders are necessary to optimise participant retention, to maximize the collection of biological samples and to ensure overall compliance with the protocol of the study. In addition, this study demonstrated the feasibility of conducting a longer-term BJ supplementation intervention

(lasting 13 weeks) among older participants, which, overall, was safe and well tolerated. Finally, this study will help inform the design of larger and longer studies investigating the effects of NO_3^--rich BJ on health outcomes in older people.

Chapter 6: Effects of incremental doses of beetroot juice on blood pressure, cognitive function and cerebral blood flow in overweight and obese older adults

6.1. Introduction

Ageing is associated with a progressive decline in physiological functions including cognition, and metabolic and cardiovascular regulation (Deary *et al.*, 2009; Barzilai *et al.*, 2012; Strait and Lakatta, 2012). Vascular risk factors including hypertension and obesity are independently related to increased risk of cognitive decline and dementia in older adults (Reitz *et al.*, 2008). The association between cognitive decline and reduced CBF has been reported in several studies (Bangen *et al.*, 2014; Wolters *et al.*, 2016; Leeuwis *et al.*, 2017; Wolters *et al.*, 2017), and reduced CBF may eventually contribute to the development of dementia (Wolters *et al.*, 2017). A recent multi-ethnic observational study which included 452 participants over 65 years old showed that higher CBF was associated with better cognitive performance, particularly on executive functioning, attention and memory in the white European group (Leeuwis *et al.*, 2018).

NO is a neurotransmitter that plays essential roles in multiple neurobiological processes (Picón-Pagès *et al.*, 2019). Depletion of NO is likely to contribute to degenerative processes of the cardiovascular and central nervous systems (Venturelli *et al.*, 2018). NO production decreases with ageing as a result of a decreased efficiency of the enzymatic synthetic pathway, and is further reduced in older people with metabolic and cardiovascular impairment (Siervo *et al.*, 2011; Torregrossa *et al.*, 2011). The age-related decrease in NO production occurs gradually across the life-course and endothelium-derived NO may be ~75% lower in older individuals (70-80 years) compared with healthy, young individuals (20 years) (Egashira *et al.*, 1993). Nutritional and lifestyle interventions capable of maintaining, or restoring, normal NO production may reduce the risk of atherosclerosis and CVD. In turn, this might help to reduce the risk of cognitive dysfunction and dementia since better cardiovascular health is associated with lower rate of cognitive decline and lower risk of dementia (Samieri *et al.*, 2018). The two NO pathways can be influenced by nutritional interventions which can maintain or boost NO production via the enzymatic (i.e., arginine, citrulline, BH4) or non-enzymatic (NO_3^-, NO_2^-, vitamin C) pathways (Weitzberg and Lundberg, 2013). The non-enzymatic pathway is part of an elegant physiological system exploiting the action of oral commensal bacteria capable of

reducing NO_3^- into NO_2^-, which is further converted into NO in environments characterised by low pH (i.e., stomach) or low oxygen tension (i.e., peripheral microcirculation) (Weitzberg and Lundberg, 2013).

Recent studies have demonstrated that foods rich in NO_3^- can increase NO production and induce positive effects on BP, and brain function (Siervo *et al.*, 2013; Clifford *et al.*, 2015). In particular, some studies have reported improvements of cognitive function (executive performance) and motor skills after dietary NO_3^- supplementation, which appears to be mediated by augmented CBF and efficiency of cellular metabolism (Presley *et al.*, 2011; Wightman *et al.*, 2015b). However, I have shown in Chapter 3 of this thesis and in our recent publication (Clifford *et al.*, 2018) that there is currently no convincing evidence that NO_3^- supplementation improves cognitive function and CBF. This systematic review has identified several limitations including that all trials were characterized by small sample sizes (<30 subjects), short durations of intervention (only one study of effects of supplemental inorganic NO_2^- had a duration of 10 weeks (Justice *et al.*, 2015), whereas the remaining trials had a duration of less than 2 weeks). Moreover, most of the studies included participants with normal BMI. Hence, the current evidence regarding sustained effects of dietary NO_3^- on cognition and CBF is very limited, and studies of longer duration and larger sample size are needed. In addition, because it is highly unlikely that increased NO will have cognitive benefit in already healthy young people, potentially more informative studies conducted in at risk populations are also needed.

This pilot RCT was designed primarily to determine the feasibility and acceptability of the protocol for a 13-week intervention study in which overweight and obese older participants were asked to consume different doses of NO_3^--rich BJ for 13 weeks. Details of the study protocol and findings related to the feasibility aspects of the study have been described and discussed in Chapter 5. In this chapter, I report on the secondary aims of the study including testing whether the different doses of dietary NO_3^- result in change measures of cognitive function, BP and CBF. The findings from this study will inform the design of future definitive interventions to determine the prolonged effect of NO_3^- on vascular health, cognitive function and brain integrity.

6.2. Methods

Participants, exclusion criteria, study design and protocol are reported in Chapter 5. The study protocol is illustrated in **Figure 5.1** and shows that participants were randomised to one of four treatments that were administered for 13 weeks. The treatments were:

5- HN; two 70 ml shots of concentrated BJ per day (400 mg of NO_3^- per shot), one every morning (~8am) and one every evening (~9pm).
6- MN; one shot of concentrated BJ every evening (~9pm).
7- LN; one shot of concentrated BJ every other evening (~9pm).
8- PL; one shot of NO_3^- depleted BJ (0.001 mg of NO_3^-) every other evening (~9pm).

All included participants were cognitively healthy with no clinical diagnosis of cognitive impairment. As stated in Chapter 5, participants were asked to follow a low NO_3^- diet during the day before the visit, to limit their alcohol and caffeine consumption for 24h before the visit and to attend fasted (~ 12h).

6.2.1. Measurements

The measurements described below were applied at baseline and after 13 weeks:

6.2.1.1. Anthropometry and body composition

Height was measured at the screening visit using a stadiometer with an adjustable headpiece to the nearest 0.5cm. Body weight and body composition parameters (fat mass, fat free mass, body fat % and total body water) were assessed at baseline and end of the study by bioelectrical impedance analysis (Tanita BC420 MA, Tanita Corporation, Tokyo, Japan). Weight and height were subsequently used to calculate BMI. Waist circumference was measured at the midpoint between the lowest margin of the last rib and the top of the iliac crest.

6.2.1.2. Blood pressure

Resting clinic BP was measured in triplicate, with one-minute interval, in the supine position after resting for at least 10 minutes using an automated blood pressure monitor (model: Omron M3 [HEM-7200-E8(V)]). The mean of the three measurements was calculated.

6.2.1.3. Cognitive function

Participants were informed about the cognitive tasks and given three full practice runs at the screening visit to reduce learning effects and test anxiety prior to baseline testing day. This practical method has been described by Bell et al. (2018) and Goldberg et al. (2015) as a solution to the confounder practice effects in cognitive testing. The Computerised Mental Performance Assessment System software (COMPASS, University of Northumbria, www.cognitivetesting.co.uk), which has been shown to be sensitive to a range of nutritional interventions (Haskell *et al.*, 2010; Kennedy *et al.*, 2010) was used to assess cognitive function.

In addition, Trail Making Tasks A and B, which were shown to be improved after 10 weeks of NO_2^- supplementation in older adults (Justice *et al.*, 2015), were used. The main COMPASS test includes in the following order; word presentation, immediate word recall, numeric working memory, choice reaction time, stroop, digit vigilance, computerized corsi blocks, peg and ball, delayed word recall and words recognition. The scores from appropriate tasks were combined to deliver measures of the following cognitive domains: a) Accuracy of Attention, b) Speed of Attention, c) Working Memory, d) Episodic Memory, e) Speed of Memory and f) Overall Accuracy and g) Overall Speed using the method described recently by (Wightman *et al.*, 2018). **Figure 6.1** shows how these global cognitive measures were estimated from scores from the cognitive tasks included in the test battery. In addition, during the measurement of CBF, participants performed mainly executive function tasks including serial subtraction 3 and 7, Stroop and peg and ball tasks which are associated with an activation of the prefrontal cortex (Kazui *et al.*, 2000; Grandjean *et al.*, 2012; Ruocco *et al.*, 2014). The tasks were presented on a laptop PC with responses made either with the keyboard or using a mouse. These tasks are described below:

6.2.1.3.1. Word presentation and immediate word recall

At the beginning of the task battery, fifteen target words were presented on the screen, one at a time with inter-stimulus time of 1 sec. Participants were asked to remember as many words as they could. Immediately after that, a 60 second timer was displayed on the screen and participants were instructed to write down on paper as many of the words presented as they could remember within this time.

6.2.1.3.2. Numeric working memory

A set of five numbers were presented on the screen, separately, with an inter-stimulus time of 1 sec for the participant to memorise. Then, a series of numbers were displayed on the screen one at a time, and the participants were required to remember whether it had been in the original series or not, pressing YES or NO as quickly as possible. This task was repeated two further times with different numbers for memorising each time. Mean reaction times were measured in msec, and overall percentage of accuracy of responses to both original and novel (distractor) stimuli were recorded.

6.2.1.3.3. Choice reaction time

An arrow appeared on the screen pointing to the left or to the right. Participants were asked to press a left or right response pad button corresponding to the direction of the arrow, as quickly

and as accurate as possible. Fifty stimuli were presented with an inter-stimulus interval that varied randomly between 1 and 3 sec. Mean reaction time and percentage of accuracy were measured.

6.2.1.3.4. Stroop task

A series of colour names were presented on the computer screen, while 4 different coloured inks were constantly displayed to the right of the screen. The colour ink of the colour name could be identical with the name or different. Participants were required to respond to the colour of the ink using the peripheral mouse and corresponding colour response buttons. Fourty stimuli were presented in the main cognitive test (at NU food, Newcastle University), and 70 stimuli were presented during CBF measurement (at Brain Performance Research Facility, Northumbria University). Mean reaction time and percentage of accuracy were measured.

6.2.1.3.5. Digit vigilance

A target digit was randomly selected and constantly presented to the right of the screen. A series of changing numbers were presented in the left of the screen and the participants were required to press the centre button whenever the two numbers are matched as quickly as possible. The task had a duration of 3 minutes. Accuracy, reaction time and number of incorrect responses were recorded.

6.2.1.3.6. Computerised corsi blocks

Nine blue identical squares were presented on the screen in random positions. A set number of the squares randomly changed to red and back to blue. Participants were asked to remember and repeat the sequence by mouse clicking on blocks. The task was repeated five times at each level of difficulty starting from 4 level (four of the nine blue squares lighting up in a random sequence). The task ended when the participant no longer correctly recalled the sequence. Span score was calculated by averaging the last 3 correctly completed trials.

6.2.1.3.7. Peg and ball

Two configurations of three coloured balls (blue, green and red) on three pegs were shown on the screen, one was the target configuration and the other was the working configuration. The participants were required to rearrange the balls from the working configuration so that they matched the position of the balls in the target configuration using the mouse. Participants completed a total of 15 trials, in sets of 5 trials that could each be solved in 3, 4 and 5 moves,

respectively, in ascending order of difficulty. Each trial generated scores for planning times prior to moving, time to complete and number of moves made above the minimum required to complete the task (errors).

6.2.1.3.8. Delayed word recall

Each participant was given 60 seconds to write down as many as possible of the words presented at the beginning of the test. The number of words correctly recalled was recorded.

6.2.1.3.9. Word recognition

Thirty words including the 15 original ones presented at the beginning, together with another 15 distractor words, were displayed one at a time randomly. The participants were asked to respond using the "yes" button on the computer keyboard if the word had been presented previously and the "no" button if it had not. The word remained on the screen until the participant responded. Mean reaction time for correct response and accuracy were recorded.

6.2.1.3.10. Serial subtraction 3s & 7s

Participants were instructed to count backwards in 3s or 7s as accurately and as quickly as possible using the keyboard. A random number between 800 and 999 was displayed on the screen and it was cleared by the entry of first response. Each participant was informed verbally when they made any mistake and to carry on subtracting from the incorrect number, with the subsequent responses being scored as correct in relation to the new number. Number of responses, number of correct responses and erroneous responses were scored.

6.2.1.3.11. Trail making task A & B

Participants were instructed verbally how to perform the task. Before the test trial, a practice run was also administered to make sure the participants understood the task. In Part A, there were 25 circles numbered (1-25) distributed on the page, and participants were required to draw a line and join numbers consecutively as fast as possible. In Part B, there were circles including numbers (1-12) and others including letters (A-L) distributed on the page, and the participants were instructed to draw a line alternately between consecutive numbers and letters (e.g. 1-A-2-B−12-L) as quickly as possible (Examples of TMT A & B are shown in **Appendix 6.1**). Participants were asked to do the task without lifting the pen from the paper. Both in Part A and Part B, numbers or letters were pseudo randomly arranged on each page. In the case of any error that were made, the participants were instructed to return to the "circle" where the error

originated and continue. Time to completion represented the total time for each testing trial, including errors. The score corresponded to the time (in seconds) taken to complete trail-making tests.

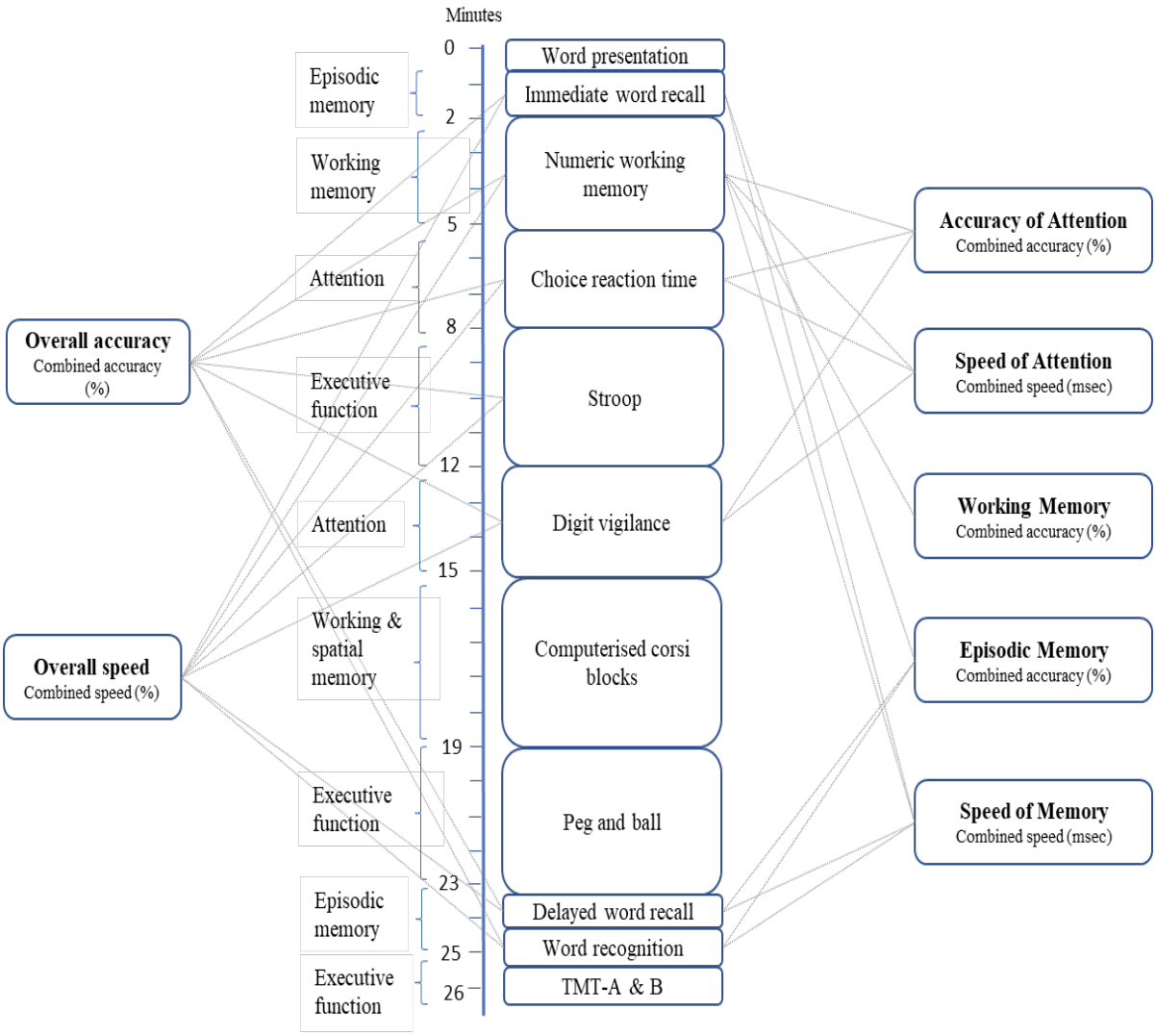

Figure 6.1: Cognitive task battery.
This shows the order in which each of the cognitive tasks was completed at baseline and after 13-weeks with the approximate task timings and predominant cognitive domain of each task. It also demonstrates how measures of each of the global cognitive domains were derived from scores from each of the individual tasks. TMT-A & B; Trail making tests A & B.

6.2.1.4. Quantitative Near-Infrared Spectroscopy (qNIRS)

A frequency domain "quantitative" NIRS system (OxiplexTS Frequency-Domain Near-Infrared Tissue Oximeter; ISS, Inc., Champaign, IL, USA) was used to estimate CBF. NIRS has been used widely in neuroscience research and has proven to detect changes in cerebral

blood oxygenation related to brain activity (Jackson and Kennedy, 2013). It allows for the quantification of oxygenated haemoglobin (HbO_2) and deoxygenated haemoglobin (HHb) by giving the absolute measurements of absorption of near-infarared light emitted at two different wavelengths. Total haemoglobin (THb) and oxygen saturation (Ox%) can be then determined using these values as follows: THb = HbO_2 + HHB and Ox%= HbO_2/THb. This system allows to quantify changes in CBF in a chronic context, as in the current study (comparing CBF between baseline and after 13-weeks).

Eight optical fibres that emit light at wavelengths 690 and 830 nm attached in pairs to four prisms that were separated from the collector bundle. Optical fibre emitters and collector bundle prisms were embedded into a flexible polyurethane resin to form the sensor. Two similar sensors were attached to either side of the forehead of the participants with self-adhering bandage. The data were collected at a rate of 5Hz.

After attaching the sensors to the participants' forehead, a 5-minute resting measurement of CBF parameters was recorded. Then, participants started to perform some cognitive tasks that are known to activate the prefrontal cortex of the brain (see 6.2.1.3. cognitive function).

As described in in Chapter 5 (data collection, section 5.1.3), CBF was measured at baseline and after 13-weeks' intervention. The qNIRS device was located in the Brain Performance Nutrition centre, Northumbria University and, therefore, this measurement was conducted on a separate day from other measurements that were conducted at Newcastle University. The measurement was performed between 3 and 4 PM and participants were not asked to fast before the assessments. Participants were asked to have their lunch around 12 Pm (low NO_3^- meal). As stated in Chapter 5, during the 24 hours before measurement of CBF, participants were instructed to reduce their intake of NO_3^- by avoiding consumption of green leafy vegetables, and to limit alcohol and caffeine consumption for 24h before the visit.

6.2.2. Statistical methods

The Statistical Package for Social Sciences (IBM SPSS, version 23, NY, USA) was used to perform the analysis. Data were checked for normality by visual inspections of histograms and by Shapiro-Wilks test. Summary data are presented as means ± SEM. One-way ANOVA were used to assess differences between the four intervention groups. To assess the effect of the 13-week intervention on cognitive performance and BP, the 13-week data were used to calculate change from baseline and analysed via one-way ANOVA.

Regarding NIRS data analysis, the data were averaged across the two hemispheres. To test the effect on the resting CBF, data recorded at resting time (5 minutes) were averaged and converted to the change from resting period at baseline, and one-way ANOVA then used to analyse the data. NIRS data recorded during performing cognitive tasks were converted to the change from the resting period (average of the 5 min) at baseline, and then averaged into 4 different epochs for analysis. An example of the analysis of NIRS data is provided in **Appendix 6.2**. Epoch 1, 2, 3 and 4 represent the average of the data collected during performing serial subtraction 3 (3:20 min), serial subtraction 7 (3:20 min), stroop (3 min) and peg and ball (2:30 min), respectively. Then, analysis was conducted via repeated measures ANOVA utilising the interventions and epochs as factors. Models were tested for sphericity using Mauchly's test and multivariate models were applied if these assumptions were violated. If significant main or interaction effects were observed, Dunnett test was conducted to compare active NO_3^- groups to PL control group. Statistical significance was set at $P<0.05$.

A sensitivity analysis was conducted to test whether the response to supplemental $NO_3^-$was influenced by habitual NO_3^- intake. NO_3^- intake data (the first measure of NO_3^- intake that was recorded) from all participants were pooled and dichotomised at the median to create two groups with higher and lower NO_3^- intake. As independent t-test was used to assess the difference in change in BP from baseline to end of intervention between the two groups.

Participants who dropped out from the study (and for whom I did not have end of intervention data) were excluded from the main analyses. To determine whether compliance with the intervention was restricted to individuals with particular characteristics, I compared the baseline characteristics of the dropouts with those of the other participants who completed the study using independent t-test. A Linear Mixed Model was also applied as an additional sensitivity analysis for BP and cognition data, considering all cases with missing data. Fixed factors include intervention groups and time. Participants were a random factor in this analysis. Results of this analysis can be seen in **Appendix 6.3 and 6.4**.

6.3. Results

6.3.1. Baseline characteristics

The baseline characteristics of participants have been described in Chapter 5 (**Table 5.1**). A total of 62 participants were randomised to one of four interventions, with an average age of 66±4 years. Sixteen participants were randomised to the HN dose group, 17 to the MN dose group, 14 to the LN dose group and 15 to the control group (PL). Participants in all groups were similar in height, weight, BMI, waist circumference, body composition, physical activity and

BP (P>0.05), as illustrated in Table 5.1. In addition, there were no significant differences between the cognitive scores or CBF parameters in all groups at baseline (all P>0.05). Finally, the baseline characteristics of the 12 participants who dropped out did not differ from those of the participants who completed the study (**Table 6.1)**.

Table 6.1: Comparison of baseline characteristics of the participants who dropped out with those of the participants who completed the study

	Participants completed the study (N=50)	**Participants who dropped out (N=12)**	**P value**
Main characteristics			
Age (years)	66.2±3.8	65.8±3.7	0.78
Education (years)	15.2±2.9	16±3.6	0.41
SBP	135.9±15.0	131.8±14.1	0.68
DBP	77.1±9.7	76.2±8.8	0.42
BMI (kg/m^2)	30.5±3.7	30.0±4.1	0.76
WC (cm)	103.6±9.1	98.0±9.0	0.18
FM (kg)	32.6±8.5	30.5±9.7	0.46
FM (%)	37.9±7.7	36.9±8.5	0.68
FFM (kg)	53.1±10.0	52.1±10.9	0.74
TBW (kg)	39.2±6.8	36.7±5.1	0.23
PA (METs/wk)	3686±6062	3434±2578	0.81
Global cognitive measures			
Accuracy of attention (%)	93.8±5.9	94.8±4.1	0.56
Speed of attention (msec)	761.3±143.1	736±111.0	0.57
Accuracy of working memory (%)	93.8±8.9	96.0±4.6	0.38
Accuracy of episodic memory (%)	50.0±9.5	48.5±9.3	0.61
Speed of memory (msec)	1191.9±243.5	1152.5±260.4	0.62
Overall speed (msec)	970.9±151.3	927.6±138.7	0.37
Overall accuracy (%)	75.4±4.9	75.1±3.6	0.85
Cerebral blood flow parameters			
Ox (%)	1.4±1.3	1.1±1.0	0.27
THb (µM/ml)	0.4±0.8	0.2±0.7	0.95
HBO (µM/ml)	0.8±0.8	0.5±0.6	0.59
HHb (µM/ml)	-0.3±0.4	-0.3±0.4	0.85

All data were analysed by independent t-test, BMI, body mass index; WC, waist circumference; FM, Fat mass; FFM, Fat free mass; SBP, systolic blood pressure; DBP, diastolic blood pressure; PA, physical activity; PA, physical activity; OX, Oxygen saturation; THb, Total haemoglobin; HBO, Oxyhaemoglobin; HHb, Deoxyhaemoglobin; G, group of intervention.

6.3.2. Body composition and physical activity

There were no detectable changes in body weight, BMI, waist circumference, or any body composition components including body fat percentage, fat free mass and total body water between baseline and the end of study in any of the intervention groups (P>0.05). Similarly, physical activity did not change from baseline to end of study in any intervention group (P>0.05).

6.3.3. Blood pressure

Resting clinic SBP and DBP did not differ between intervention groups at baseline, as illustrated in Table 5.1. ANOVA indicated no difference between treatment groups in the change in SBP from baseline (P=0.10). However, in comparison with PL, Dunnett's test indicated that the lower doses of NO_3^- used in the study (MN and LN) may be more effective in lowering SBP (Δ -10.5 mmHg, 95% CI: -21, 0.89 mmHg, P=0.07), (Δ -9.8 mmHg, 95% CI: -20.8, 1.3 mmHg, P=0.09), respectively. No effect on SBP was detected after the consumption of the HN dose (Δ -7.9 mmHg, 95% CI: -20.0, 4.2 mmHg, P=0.28). A sensitivity analysis was conducted after removing the data from the hypertensive participants who were taking anti-hypertensive medications (n=3; 2 and 1 participants from the MN and LN groups, respectively), and similar results were found; MN dose (Δ -10.9 mmHg, 95% CI: -22.8, 1.0 mmHg, P=0.08), LN dose (Δ -10.4 mmHg, 95% CI: -22.3, 1.6 mmHg, P=0.10). ANOVA did not reveal any significant difference between the intervention groups for the change from baseline in DBP (P=0.30) and Dunnett's test did not identify any changes in DBP with any NO_3^- dose compared with PL. **Figure 6.2 A** and **B** show the changes from baseline in SBP and DBP, respectively for each of the treatment groups. The Δ symbol in the results described above represents the mean difference between the NO_3^- dose and PL for the change in BP between baseline and end of intervention.

When data from the three NO_3^- interventions were pooled together changes in SBP in the NO_3^- group (n=37) were significantly different from the PL group (n=14) (Δ -9.7 mmHg, 95% CI: -16.4, -1.7 mmHg, P=0.02, **Figure 6.3 A**). There was no significant change in DBP between the two groups (Δ -4.7 mmHg, 95% CI: -10.5, 0.92 mmHg, P=0.09, **Figure 6.3 B**).

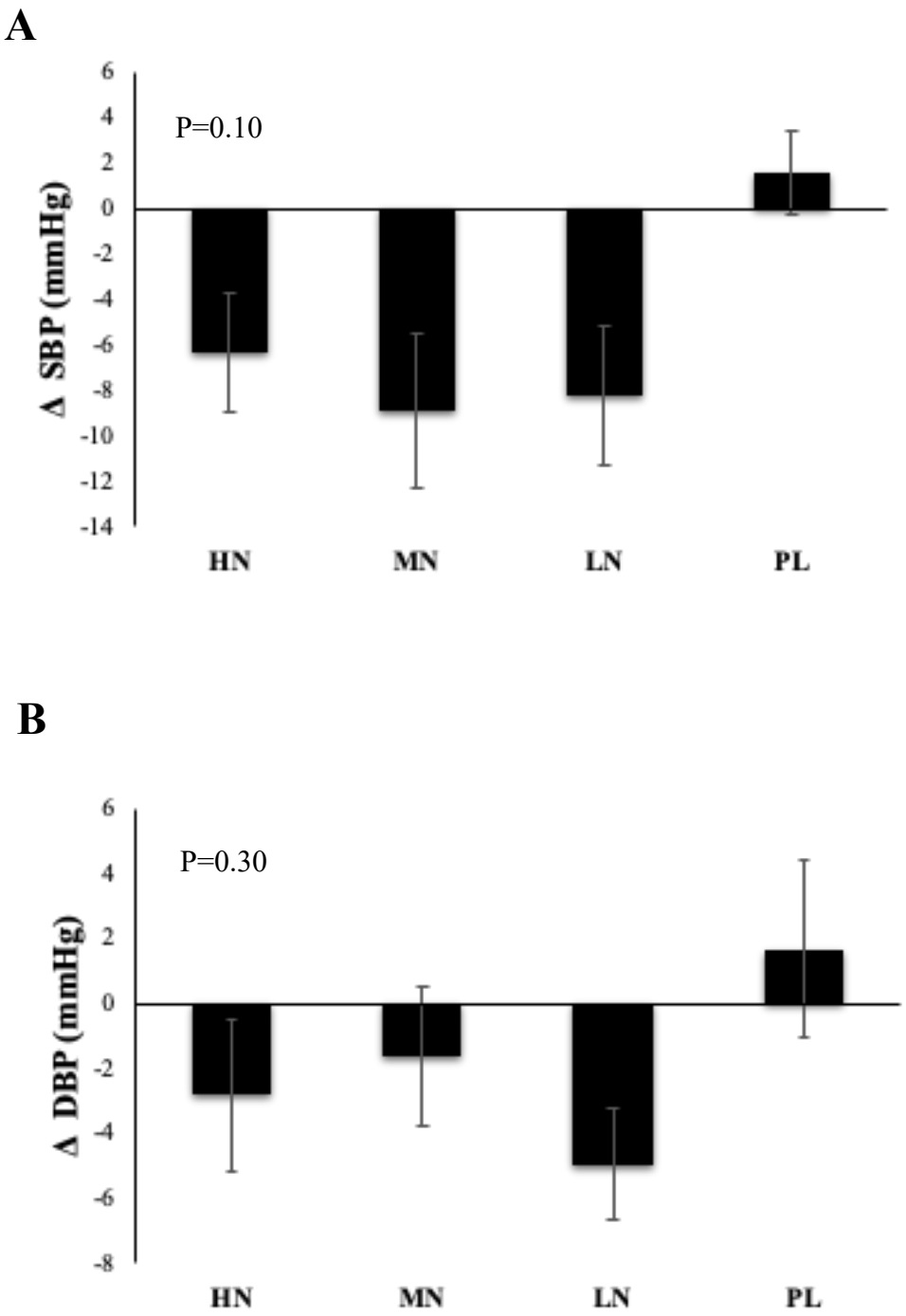

Figure 6.2: Changes from baseline in SBP (A) and DBP (B) for each of the intervention groups. Mean changes from baseline in SBP (A) and DBP (B) for each of the intervention groups. HN (High NO_3^- ; two 70ml shots of BJ/day, morning and evening), MN (Medium NO_3^- ; 70 ml of BJ/day), LN (Low NO_3^-; 70 ml of BJ every alternate days) and PL (placebo; 70 ml of NO_3^- depleted BJ every alternate days). Each shot of BJ contains 400 mg of NO_3^-. Analyses were conducted by one-way ANOVA. Data are expressed as mean ± SEM, (n = 50).

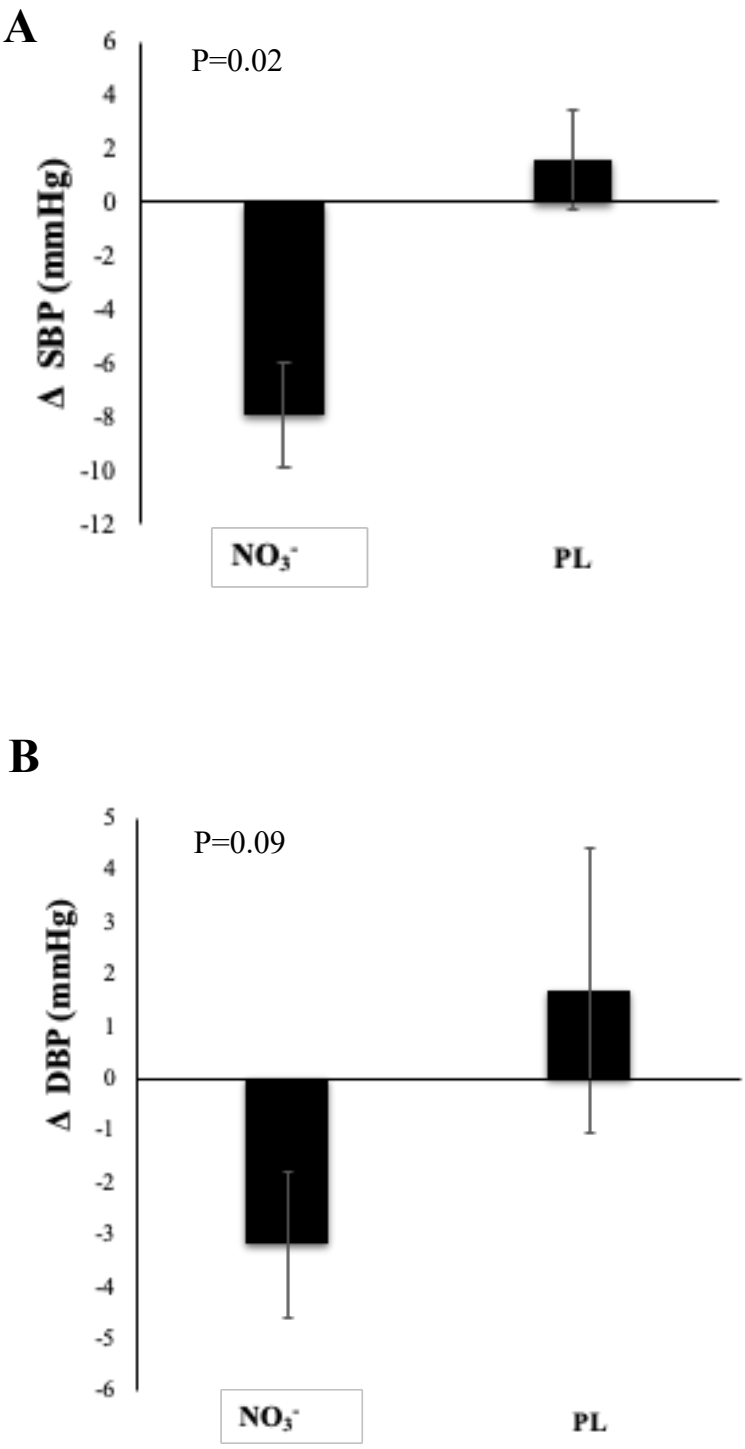

Figure 6.3: Changes from baseline of SBP (A) and DBP (B).
Mean changes from baseline of SBP (A) and DBP (B). NO_3^- group represents the average of pooled data from all NO_3^- doses (high, medium and low). These changes from baseline were analysed with independent t test. Data are expressed as mean ± SEM, (n = 50). PL; Placebo

6.3.3.1. Effect of habitual nitrate intake on blood pressure response after nitrate supplementation

When the data were dichotomised at the median habitual dietary NO_3^- intake, there was no evidence that the change in either SBP or in DBP in response to supplemental NO_3^- was influenced by habitual NO_3^- intake (P>0.05) (**Appendix 6.5**).

6.3.4. Effects of nitrate supplementation on measures of cognitive function

Examination of the change from baseline (using ANOVA) did not reveal any significant differences between intervention groups for any of the individual measures of cognitive function. In addition, when scores for the individual tests were combined to provide estimates of global cognitive measures, there were no significant treatment effects. The effects of NO_3^- dose on each cognitive measure and on the global cognitive domains are summarized in **Table 6.2 and 6.3**, respectively.

6.3.5. Effects of nitrate supplementation on measures of qNIRS parameters used to estimate cerebral blood flow

No significant difference was found between the two resting periods that were recorded at baseline and after 13-weeks for any parameters (P>0.05). Repeated measures ANOVA did not reveal any significant difference with epochs (Cognitive tasks used; serial sub3, serial sub7, stroop and peg and ball) (P>0.05), group of intervention (P>0.05) or the interaction (P>0.05) on any of CBF parameters. As no significant difference was found between epochs, thus, data from cognitive period were combined. **Figures 6.3 A, B, C and D** show the effect on oxygen saturation, total haemoglobin, oxyhaemoglobin and deoxyhaemoglobin.

Table 6.2: Mean values for baseline and for change from baseline after 13 weeks intervention on individual cognitive tasks for different doses of nitrate

Measure	HN (N=10)		MN (N=13)		LN (N=14)		PL (N=13)		P.value
	Baseline	13-weeks (Change)	Baseline	13-weeks (Change)	Baseline	13-weeks (Change)	Baseline	13-weeks (Change)	
IWR correct (number)	5.4±0.4	0.05±0.5	6.5±0.4	0.2±0.3	5.6±0.5	-0.07±0.4	6.8±0.5	-0.7±0.6	0.45
IWR error (number)	0.2±0.2	0.0	0.4±0.2	0.1±0.2	0.6±0.2	0.01±0.1	0.3±0.2	-0.1±0.1	0.83
NWM (% accuracy)	98.0±0.3	-0.23±1.5	93.8±1.7	1.2±0.7	96.5±1.4	-1.3±1.6	91.1±3.4	1.3±1.4	0.61
NWM-RT (msec)	1126±81	3±37	1288±179	-185±143	1087±77	63±61	1091±48	-78±46	0.76
CRT (% accuracy)	98.4±0.5	-0.6±0.6	98.0±0.5	0.8±0.6	98.7±0.5	-0.1±0.4	98.8±0.5	-0.6±0.5	0.49
CRT RT (msec)	570±37	-18±21	622±30	-31±27	641±46	-4±0.5	603±27	-6±21	0.21
Stroop %accuracy*	99.3±0.4	0.29±0.3	98.8±0.8	6.5±7.1	99.9±0.1	-0.2±0.2	99.8±0.1	-1.9±10	0.79
Stroop RT (msec)*	1304±28	-45±48	1434±70	128±195	1523±174	-40±74	1261±38	51±159	0.78
DV (%accuracy)	96.5±1.4	-2.5±1.7	90.4±2.2	-7.2±7.9	86.1±3.7	3.8±2.3	84.8±4.6	-0.89±2.0	0.49
DV RT (msec)	483±11	-2.8±7.7	469±10	-41.5±38	512±11	33±40	498±8	-0.03±4	0.36
DV false alarms (number)	1.3±0.3	0.7±1.0	4.2±0.7	-0.3±1.0	5.7±1.1	0.2±0.8	5.6±1.2	-0.4±0.8	0.79
Corsi blocks span	5.6±0.1	0.1±0.1	5.4±0.2	0.1±0.2	5.2±0.4	0.3±0.3	5.4±0.3	-0.3±0.4	0.29
P&B thinking time	4106±582	-367±349	5221±429	-1066±382	4600±317	-695±263	5092±541	-653±345	0.46
P&B working time (msec)	11640±747	-974±460	14311±980	-2325±893	14186±1675	-1882±144	12624±729	-963±641	0.59
P&B errors	3.7±2.5	0.8±1.5	2.2±1.9	-0.2±0.8	4.2±4.6	-1.3±1.6	3.3±3.3	-2.3±1.1	0.48
DWR correct (number)	3.8±0.5	0.3±0.5	5.3±0.4	-0.8±0.3	3.5±0.5	0.8±0.3	5.0±0.5	-0.1±0.6	0.03
DWR error (number)	0.6±0.2	0.4±0.2	0.8±0.2	0.4±0.1	0.6±0.2	0.1±0.1	0.6±0.2	0.1±0.3	0.65
WR (%accuracy)	77.0±7.9	-1.3±4.3	79.4±8.1	0.7±2.2	78.6±12.7	-0.7±3.6	82.4±7.8	-2.2±2.7	0.77
WR RT (msec)	1277±187	-14±103	1085±191	17±39	1226±316	6.1±52	1287±480	65±48	0.39
TMT-a (sec)	24.1±1.7	-2.4±1.5	28.1±1.8	-2.5±2.2	28.5±2.7	-0.1±1.8	24.6±1.5	-1.2±1.3	0.75
TMT-b (sec)	52.1±3.6	-8.1±3.0	52.2±4.2	2.2±4.9	56.3±5.5	-7.1±2.4	55.0±4.5	-9.7±3.9	0.11

Data are presented as means ± SEM. Data were analysed using one-way ANOVA.

*Due to non-compliance with the task instructions, data of some participants was removed: HN; High NO_3^- (Baseline n=13, 13-weeks n=9), MN; Medium NO_3^- (Baseline: n=15, 13-weeks: n=11), LN; Low NO_3^- (Baseline: n=13, 13-weeks: n=13), Placebo; PL (Baseline: n=14, 13-weeks: n=11).

IWR; Immediate word recall, NWM; Numeric working memory, CRT; Choice reaction time, DV; Digit vigilance, P&B; Peg and ball, DWR; Delayed word recall, WR; Word recognition, RT; Reaction time.

Table 6.3: Mean values for baseline and for change from baseline after 13 weeks intervention on global cognitive measures for different doses of nitrate

Measure	High NO_3^- (N=10)		Medium NO_3^- (N=13)		Low NO_3^- (N=14)		PL (N=13)		P.value
	Baseline	13-weeks (Change)	Baseline	13-weeks (Change)	Baseline	13-weeks (Change)	Baseline	13-weeks (Change)	
Accuracy of attention (%)	96.6±0.8	-1.8±0.8	93.2±1.3	-2.2±2.3	94.2±1.4	-0.8±1.0	91.7±2.2	7.2±7.2	0.88
Speed of attention (msec)	735±36	-6.1±13.5	788±54	-95.7±52.3	763±37	17.9±21.3	742±21	32±62.8	0.16
Accuracy of working memory (%)	96.3±1.7	-0.2±1.5	91.6±2.8	1.2±0.7	96.4±1.2	-1.3±1.7	91.3±3.2	8.0±6.8	0.49
Accuracy of episodic memory (%)	45.2±1.9	0.7±3.1	52.0±2.1	-1.2±1.8	46.3±2.5	1.4±1.3	54.8±2.9	2.5±6.1	0.42
Speed of memory (msec)	1205±51	-5.6±44.3	1186±72	-83.7±67.2	1155±66	34.7±32.1	1214±76	88.2±97.5	0.37
Overall speed (msec)	947±25	-15.8±15.2	969±37	-42.3±40.6	997±57	2.5±25.1	954±35	76.3±73.1	0.77
Overall accuracy (%)	74.6±1.2	0.7±1.6	75.7±1.3	-1.1±1.6	73.9±1.4	0.2±0.6	76.9±1.5	4.8±6.6	0.53

Data are presented as means ± SEM. Data were analysed using one-way ANOVA.

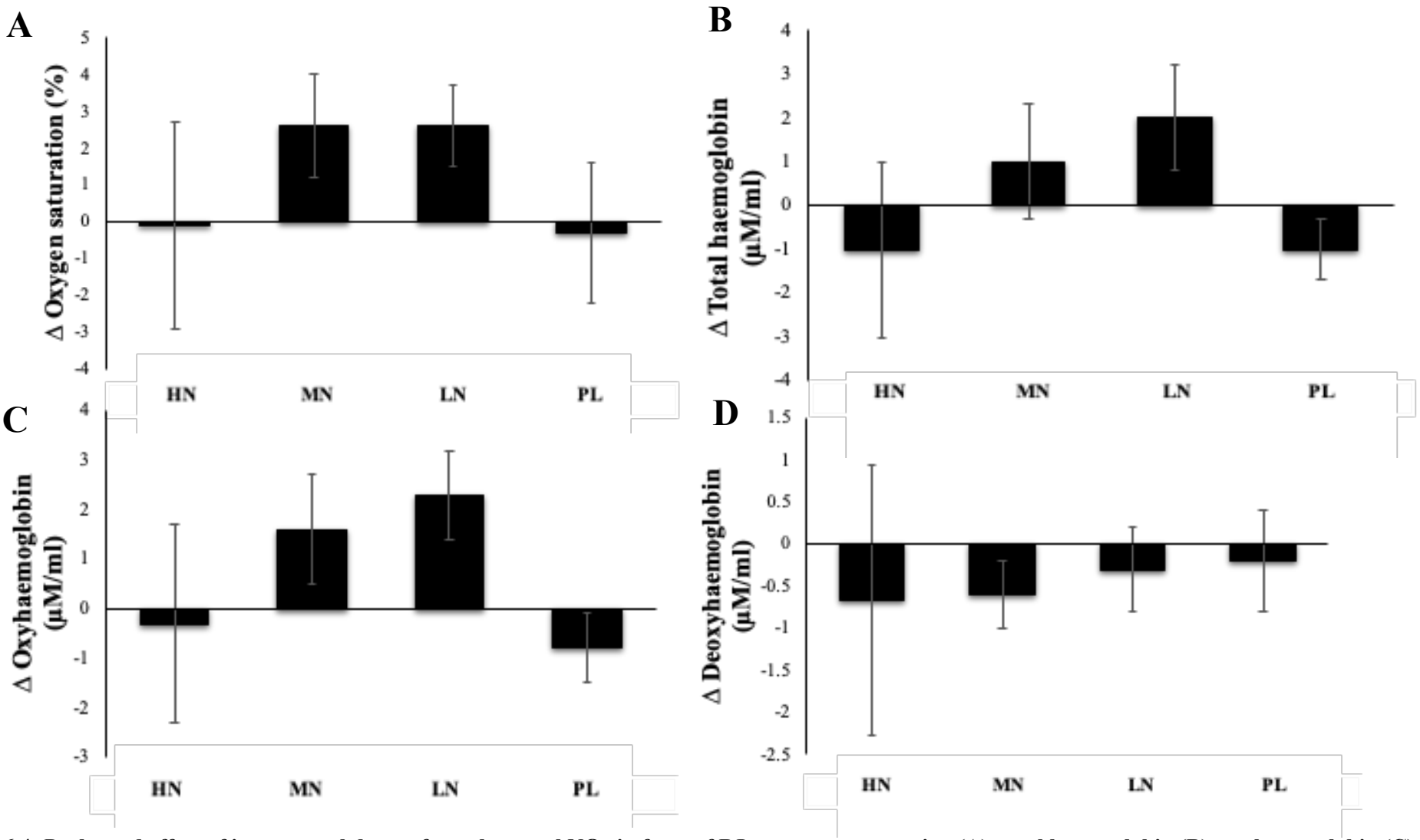

Figure 6.4: Prolonged effect of incremental doses of supplemental NO_3^- in form of BJ on oxygen saturation (A), total haemoglobin (B), oxyhaemoglobin (C) and deoxyhaemoglobin (D).

HN (High NO_3^- ; two 70ml shots of BJ/day, morning and evening), MN (Medium NO_3^- ; 70 ml of BJ/day), LN (Low NO_3^-; 70 ml of BJ every alternate days) and PL (placebo; 70 ml of NO_3^- depleted BJ every alternate days). Each shot of BJ contains 400 mg of NO_3^-. Data represent the change of CBF parameters recorded during performing cognitive tasks at 13-weeks from the resting period at baseline and were analysed by one-way ANOVA. Data are expressed as mean ± SEM, (n = 47).

6.4. Discussion

6.4.1. Summary of main findings

To date, this is one of the longest-duration RCT investigating the effects of incremental doses of supplemental NO_3^- in the form of BJ on BP, cognitive function and CBF in overweight and obese older adults. This study was designed primarily as a feasibility study and was not intended to provide a definitive investigation of the effects of supplemental NO_3^- on BP, CBF and cognitive function. The findings may indicate that 13-weeks supplementation with medium and, to some extent, low doses of NO_3^- had positive effects on BP in older overweight and obese adults. However, there was no evidence that cognitive function or CBF were influenced by NO_3^-supplementation at any dose.

6.4.2. Effects of prolonged beetroot juice consumption on blood pressure

A large number of clinical studies have assessed the effect of supplemental dietary NO_3^- intake on BP and these studies have been reviewed systematically by (Siervo *et al.*, 2013; Ashor *et al.*, 2017). Previous studies have provided inconsistent results; some studies have reported reduced BP with increased NO_3^- intake (Webb *et al.*, 2008; Kapil *et al.*, 2010), while others did not find such effects (Bondonno *et al.*, 2015; Blekkenhorst *et al.*, 2018). The duration of the studies, the doses of NO_3^- used and the characteristics of the participants that were included in various studies might contribute to such inconsistent results. As I have indicated in the introduction to this chapter, supplemental NO_3^- may be particularly effective in older, obese individuals in whom NO production is lower than in healthy younger people. However, to date, only three studies have investigated the effect of supplemental NO_3^- intake on BP in this population group (Gilchrist *et al.*, 2013; Jajja *et al.*, 2014; Ashor *et al.*, 2015). In addition, most intervention studies have been of short duration so that there is limited evidence regarding the prolonged effect of dietary NO_3^- supplementation in obese/overweight older adults on BP. The present study used three different doses of NO_3^- provided for 13-weeks and showed that the daily consumption of the medium dose of NO_3^- (400mg/day) may be effective in lowering SBP, although this effect was not statistically significant (P=0.07). Similarly, there was some evidence that BP may be reduced by the low dose (400 mg every alternate day) but, again, this effect was not statistically significant (P=0.09). This is possibly due to inadequate sample size. In the medium dose group, the SBP decreased from 135±16 mmHg to 126±9 mmHg (~ -9 mmHg) between the baseline and at the 13-weeks. This finding is consistent with Rammos *et*

al. (2014) who found that SBP in normal weight adults decreased significantly from 137±13 to 129±15 mmHg (- ~ 8 mmHg) after supplementation with sodium NO_3^- (150 µmol/kg body weight) for 4 weeks in older adults. Kapil *et al.* (2015) showed that daily consumption of unconcentrated BJ (~ 400 mg/day), which is similar to the medium NO_3^- dose used in the present study, reduced resting clinic SBP by 7.7 mmHg after 4 weeks.

The design of the current study is unique in that the lowest dose was achieved by asking participants to drink one shot of BJ on alternate days over 13-weeks. The SBP and DBP decreased by ~ 8 and 4 mmHg, respectively, in this group, although this reduction also was not significant in comparison with PL. Jajja *et al*. (2015) found that NO_3^- -induced reduction in SBP was maintained to some extent for two days after stopping BJ supplementation and that BP values returned to baseline levels 3 days after stopping BJ consumption. The acute BP lowering effects of supplemented NO_3^- in older people has been reported in several studies (Kenjale *et al.*, 2011; Hughes *et al.*, 2016; Raubenheimer *et al.*, 2017). Although the acute effect of BJ was not tested in the present study, my findings suggest that the acute BP lowering effects of NO_3^- supplementation may be maintained over a longer time period. It is known that the half –life of NO_3^- is about 5-6 h, suggesting that the repeated, and frequent, intake of dietary NO_3^- may be required to maintain its beneficial effects on BP**.** Together, these data suggest that frequent consumption of BJ at moderate doses might lead to sustained falls in BP, but this idea remains to be investigated in a future larger study.

Siervo *et al.* (2013) reported that there is a positive correlation between the dose of supplemental dietary NO_3^- and the observed hypotensive effect. Interestingly, there was no effect of the high dose of NO_3^- (2 shots of BJ/day, ~ 800 mg/day) on change in BP in this study. It might be suggested that consumption of such high doses of NO_3^- for this long period is associated with tachyphylaxis. Low doses of NO_3^- were associated with a reduction in BP, whereas prolonged administration of higher NO_3^- doses was associated with increased BP, in rats (Carlström *et al.*, 2015). This is due to down-regulation of vascular eNOS activity at higher doses of NO_3^- (Carlström *et al.*, 2015). It is possible that beyond a dose of 400 mg of NO_3^- consumed for prolonged period, the concentration of NO_3^- in tissues increases, and that this higher tissue concentration of NO_3^- may down regulate all aspects of the NO_3^- -NO pathway, including NO_3^- absorption and conversion to NO.

The response to supplemental NO_3^-could be influenced by habitual NO_3^- intake. In particular, it would be expected that participants with low basal NO_3^- intake are more responsive to supplemental NO_3^-. Therefore, I did a sensitivity analysis to test whether the BP lowering effect

of supplemental NO_3^- was greater in those participants with lower habitual NO_3^- intake. However, the present study did not provide evidence for such an influence of habitual NO_3^- intake. This hypothesis needs to be tested in a future larger study.

In the present study, after 13-weeks of different doses of NO_3^- in form of concentrated BJ, the range in reduction in SBP and DBP relative to baseline was ~ -6 to -9 mmHg and ~ -2 to -5 mmHg, respectively. These findings will need to be confirmed in a future larger study. If a reduction in BP between 5-12 mmHg was achieved following prolonged NO_3^- supplementation, this would have a potential benefit on cardiovascular diseases, including risk of stroke, heart failure and coronary heart disease (Ettehad *et al.*, 2016).

6.4.3. Effects of prolonged BJ consumption on cognition and on CBF

BJ is a rich source of NO_3^- which is converted to NO following consumption. NO plays an important role in regulation of CBF, neurotransmission and neurovascular coupling (Toda *et al.*, 2009; Dormanns *et al.*, 2016). Ageing is associated with endothelial dysfunction, which is characterised by lower release of endothelial NO. As a consequence, cerebral haemodynamic can be disturbed by reducing NO availability and increasing ROS production (Bor-Seng-Shu *et al.*, 2012). Lower CBF is broadly acknowledged as a vital contributor to cognitive decline (Bangen *et al.*, 2018; Korte *et al.*, 2020). The present study is the first to investigate the effect of 13-weeks consumption of BJ as a rich source of NO_3^- on cognition and CBF in individuals who are at greater risk of endothelial dysfunction and cognitive impairment (older overweight and obese individuals).

With regards to CBF, these findings did not show any effect of supplementation with BJ for 13-weeks on resting CBF or stimulated CBF (during the performance of cognitive tasks that activate the prefrontal cortex) following HN, MN or LN doses, in comparison with PL. However, the current evidence regarding the effect of dietary NO_3^- on CBF in older people is very limited and has provided inconsistent results. Acute dietary NO_3^-supplementation with BJ increased CBF in young healthy adults, when measured using a similar method of assessment (NIRS) to the one used in the current study (Wightman *et al.*, 2015b). Working with older adults, Presley et al., (2011) found that resting regional white matter perfusion (dorsolateral prefrontal cortex only), assessed by magnetic resonance imaging, was improved following 2 days of high NO_3^- diet supplementation. On the other hand, Kelly et al., (2013) did not detect any changes in apparent diffusion coefficients in the aforementioned regions of the brain following 3 days of NO_3^- supplementations in older adults, although a larger dose of $NO_3^-$was

used than that employed in the Presley et al. study. As to the reason for the discrepancy between the results of the two studies, Kelly et al. suggested that this is possibly related to the differences in participants' ages: those in the Presley et al. study were older by an average of 10 years, perhaps making them more responsive. This also might partially explain the lack of the effect in the present study compared with the Presley et al study (participants in the Presley et al. study were older by an average of 8 years). Both studies featured notably shorter intervention periods than the present study.

Alternatively, this inconsistency in results between studies may be explained by the reduction in NO formation due to long term NO_3^- supplementation. As is already known, NO can be synthesised via the enzymatic pathway (L-arginine-NOS pathway), and by the alternative pathway ($NO_3^- — NO_2^-$-NO). As mentioned earlier, long-term dietary NO_3^-supplementation in rats results in reversible down-regulation of eNOS activity, suggesting negative cross-talk between the two pathways (Carlström *et al.*, 2015). Perhaps after continuous NO_3^-intake for three months, as in the present study, the endogenous pathway was down-regulated. This idea could be tested by quantifying whole body NO production before and after long term NO_3^- supplementation.

Regarding cognitive performance, the results of the present study show that the 13-week ingestion of different doses of NO_3^- in the form of BJ did not reveal any beneficial modulation of cognitive function in our participants. The only statistically significant difference found was restricted to a single task outcome (delayed word recall), which most likely represents a chance finding. Gilchrist and co-authors (2013) were the first group to report the beneficial effects of BJ ingestion on cognitive performance, specifically in simple reaction time, in older adults with T2D following 14 days of BJ consumption. Recently, Justice et al., (2015) reported a 14% and 18% improvement in TMT-B following 10-weeks consumption of 80 mg/d and 160 mg/d NO_2^- , in older adults, respectively. Although the present study was significantly longer than the Gilchrist et al., (2014) study and 3 weeks longer than the Justice et al., (2015) study, no such beneficial effects were found with any of the cognitive outcomes. The lack of cognitive effects in the current study may be attributable to several factors including a lack of effect on CBF.

A recent study found an association between CBF and cognitive functioning in multi-ethnic older cohort, with higher CBF associated with improved executive function (Leeuwis *et al.*, 2018). However, an improvement in CBF is not always associated with an improvement in cognitive performance (Kennedy *et al.*, 2010). It is unknown whether improved CBF was the

key mediator in cognitive enhancement in the Gilchrist et al., (2014) and Justice et al., (2015) studies, as it was not measured. Again, the present study was a comparatively small pilot study with less than 15 participants per group, while Gilchrist et al included 27 patients using a crossover design and Justice et al recruited over 80 participants. Furthermore, it is possible that health status also has an impact on cognitive performance. The diabetic participants included in the Gilchrist et al., (2014) study had deficits in cognitive function compared with healthy participants, which may have amplified their sensitivity to cognitive testing. Although the participants in the present study were apparently healthy, it is known that high BMI is a risk factor for several chronic diseases, especially in later life. Obesity is associated with impaired NO availability (Toda and Okamura, 2013), and it has been shown that BMI is negatively correlated with global cognitive performance (Elias *et al.*, 2005; Hassing *et al.*, 2010).

6.4.4. Strengths and limitations of the study

Few studies have tested the effect of dietary NO_3^- in older overweight/obese people, and most studies to date regarding that age group have featured short duration intervention periods. The strength of the present study was the prolonged duration of the intervention, and this is one of the longest RCT, investigating the effect of NO_3^- in older overweight and obese adults. Thus, this study contributes to the small but growing body of research on the effects of dietary NO_3^- on brain and vascular functions, despite the absence of positive effects on brain function observed here. Further, the use of incremental doses of NO_3^- in the form of BJ during such a period, with the low dose being consumed on alternate days during the study, gave the study a unique design feature. Our participants were asked to maintain their habitual dietary habits, physical activity and medication throughout the study. Estimates of dietary NO_3^- intake (excluding that in the supplement) showed that NO_3^- intake remained stable during the study (see Chapter 5, section 5.3.10). Finally, the increase in concentrations of NO_3^- and NO_2^- biomarkers with increasing BJ dose (see Chapter 5, section 5.3.5, 5.3.6 and 5.3.7), provide assurance that the participants were complaint in consuming the BJ supplements.

On the other hand, this study has limitations. The first limitation is the lack of double blindness, which might introduce a potential bias. For example, the frequency and volume of the BJ given to each participant disclosed the nature of the dietary interventions to the researcher. The researcher was responsible for preparation and distribution of interventions to participants and also for collecting the data. This study was designed primarily to test the feasibility and

acceptability of the intervention in older adults and was not designed (or powered) to make definitive conclusions regarding the prolonged effect of NO_3^- on cognition and CBF.

In the present study, the CBF assessment was conducted on a separate study day from other measurements due to the different location of the NIRS apparatus. However, participants were asked to follow similar instructions to the previous study day that included following a low NO_3^- diet and avoiding alcohol and caffeine consumption for 24h before the visit. The only significant difference was that participants were not required to fast before the CBF assessment. Participants were asked to have their low NO_3^-lunch ~4 hours before the assessment. In addition, due to limited access to the NIRS equipment, only a short period was available to collect NIRS data compared with other studies that have used similar equipment (Wightman *et al.*, 2015a; Wightman *et al.*, 2015b; Wightman *et al.*, 2018), which might be not sufficient to see CBF parameters alterations. Furthermore, during the data collection period, NIRS equipment stopped working for 2 weeks, which led to loss of follow up data for 3 participants.

6.5. Conclusion

This feasibility study indicated that the repeated consumption of BJ at moderate doses showed promising results in BP reduction over a prolonged period of time in older overweight and obese adults. However, no beneficial effects were observed regarding cognitive performance or CBF. A larger definitive study would be needed to investigate the effect of prolonged dietary NO_3^- consumption on vascular and brain health in older population.

Chapter 7. General discussion and conclusions

7.1. Overview and summary of main findings

Ageing is associated with a progressive decline of physiological processes, including vascular and cognitive functions and it represents an important, non-modifiable risk factor for several chronic diseases (Niccoli and Partridge, 2012). Ageing is associated with reduced production of NO, endothelial dysfunction and reduced cerebral perfusion. Therefore, nutritional and lifestyle interventions aiming at maintaining or restoring normal NO production in older populations may represent viable strategies to promote a healthy brain and to reduce dementia risk. Recent meta-analyses of prospective cohort and cross-sectional studies have shown that a balanced diet rich in fruits and vegetables lowers CVD, cognitive impairment and dementia risk (Jiang *et al.*, 2017; Zhan *et al.*, 2017). Higher adherence to Mediterranean and DASH diets has been associated with slower cognitive decline and reduced risk of AD in older adults (Tangney *et al.*, 2014; Zhan *et al.*, 2017). These dietary patterns are characterised by high content of several nutrients associated with health benefits including essential fatty acids, dietary fibre, polyphenols and dietary NO_3^-. The importance of NO_3^- as a key dietary component linked to the health benefits of Mediterranean and DASH diets is increasingly recognised (Weitzberg and Lundberg, 2013). Previous studies have indicated that these benefits are likely to occur as a consequence of improved NO production via the NO_3^-–NO_2^-–NO pathway (Bondonno *et al.*, 2016). Therefore, there is growing interest in investigating the benefits of NO_3^- supplementation in older people.

Dietary NO_3^- is a substrate for NO production and therefore the association of higher NO_3^- intake with health outcomes, mainly with cancers, has been tested in several epidemiological studies (Xie *et al.*, 2016). However, the assessment of dietary NO_3^- intake is challenging due to use of different methods for assessing food and beverage intake and different data sources used to determine the NO_3^- content in foods and beverages. These issues have been extensively discussed in Chapter 2 that reports the results of a systematic review which showed that the median estimated daily NO_3^- intake for healthy and patient populations were similar (109 and 110 mg/day, respectively) and below the ADI of 3.7 mg of NO_3^- /kg of body weight. Recently, McMahon et al. (2017) have developed a food composition database for dietary NO_3^-which, to our knowledge, is the most comprehensive database collating published evidence on NO_3^-

content from various food sources. A collaborative agreement has been established between our groups and I have used this database to estimate the NO_3^- intake in participants recruited in the clinical trial described in Chapter 5.

The growing evidence that dietary NO_3^- may improve NO availability and enhance physiological functions has stimulated researchers to develop simple techniques to monitor NO_3^- intake. In particular, the design of salivary test strips for quick and easy measurement of NO_2^- concentration in saliva has been innovative. In Chapter 4, I conducted a small study to test the validity and reliability of these strips after acute consumption of BJ, with and without the use of mouthwash. I found that the strips can provide a reasonable surrogate marker for monitoring changes in salivary NO_2^- concentrations. Therefore, salivary strips were used to monitor NO_3^- intake during the prolonged dietary NO_3^- intervention with the aim to validate their utility under chronic BJ supplementations (Chapter 5).

In Chapter 3, I conducted a systematic review and meta-analysis to investigate the effects of dietary supplementation with NO_3^- or NO_2^- on cognitive functions and CBF, which found that cognition and CBF were not modified by NO_3^- or NO_2^- supplementation. However, these studies are characterised by short durations, small sample sizes and recruitment of healthy participants without evidence of cognitive impairment or risk factors for dementia. This meta-analysis helped to inform the design of the final study presented in Chapters 5 and 6, which was designed primarily to test the feasibility and acceptability of prolonged consumption (13 weeks) of incremental doses of dietary NO_3^- supplementation in the form of BJ in older, overweight and obese adults. The study confirmed the feasibility of longer-term BJ supplementation in older participants, which was safe and overall well tolerated. We hypothesised that prolonged supplementation with dietary NO_3^- would enhance brain health and reduce BP in older obese and overweight individuals, who are at greater risk of cognitive dysfunction. The current project provided only preliminary results and future larger studies are needed to further investigate this hypothesis. However, the present study provides a valuable addition to the evidence base for the role of dietary NO_3^- in vascular and brain functions among an older population at risk of cognitive decline.

7.2. Response to dietary nitrate supplementation

Current evidence shows that dietary sources rich in NO_3^- can reduce the risk of CVD (Jackson *et al.*, 2018b), as well as enhance physical performance (Jones *et al.*, 2018), although it remains to be determined whether higher NO_3^- intake *per se* explains these are causal relationships. The

ADI for NO_3^- was set by the WHO at 3.7 mg of NO_3^- ions/kg of body weight. However, this level can be easily exceeded in diets such as DASH or the Japanese diet (Weitzberg and Lundberg, 2013). Currently, there are no guidelines regarding the minimum NO_3^- intake required to produce a positive health effect or what is the optimal dietary NO_3^- intake associated with beneficial health outcomes. Some studies have tried to address this question by investigating the dose-dependent effects of NO_3^- supplementation (Kapil *et al.*, 2010; Hobbs *et al.*, 2012; Capper *et al.*, 2019; Coggan *et al.*, 2019) but all of these studies are characterised by short intervention periods (2-8 weeks). In Chapters 5 and 6, we tested the long-term effects of incremental doses of dietary NO_3^- supplementation and observed a non-significant trend for greater effects on BP reduction with the two lower doses of BJ. However, this effect becomes significant when data from the three NO_3^- interventions were pooled together and compared with the PL group. The responses to dietary NO_3^- supplementation may have been influenced by the habitual NO_3^- intake of the participants included in the trial. Chapter 2 indicated that the estimated dietary NO_3^- intake of healthy individuals was 109 mg/day and the average of NO_3^- intake measured in older participants in Chapter 5 was 102 mg/day. It is not known whether baseline or habitual NO_3^- intake influences the response to dietary NO_3^- supplementation. It might be expected that those with lower baseline NO_3^- intake would be more likely to respond to supplementation than those with higher baseline NO_3^- intake. Therefore, I tested that idea by comparing the BP response to BJ supplementation between those with higher and lower NO_3^- intake (see Chapter 6, section 6.3.3.1), and no difference was found in BP response between the two groups. However, data from only 32 participants were included in the analysis and therefore the power of the analysis was limited. Future dietary NO_3^- interventions should consider background dietary NO_3^- intakes, as this may be a confounding factor that could influence the efficacy of dietary NO_3^- interventions.

7.3. Sources of nitrate in RCTs

The source of NO_3^- influences the concentration, bioavailability route of administration and subject compliance. Food items contain varying concentrations of NO_3^-, with green leafy vegetables and BJ are relatively rich source. The most popular dietary NO_3^- supplements used in RCT to investigate the beneficial effect of NO_3^- is BJ, then NO_3^- salts such as sodium NO_3^-. Both sources have been shown to be effective in increasing plasma NO_3^- and NO_2^- (James *et al.*, 2015), and also in lowering BP (Larsen *et al.*, 2006; Webb *et al.*, 2008), in healthy adults. Other studies have also tried to assess the effect of various forms of dietary NO_3^-. For example, Jovnik et al (2016) assessed the acute effects of ingestion of various NO_3^- -rich sources

including BJ, rocket, spinach and sodium NO_3^- on plasma NO_3^- and NO_2^- concentrations and resting BP. They found that the time to peak in plasma NO_3^- was faster with BJ and sodium NO_3^- compared to rocket and spinach beverages, which is likely to be related to their higher fiber content and the larger volume ingested.

In terms of BP lowering effect, all vegetable natural sources were effective in lowering BP compared with nitrate salts. This could be because other compounds that work synergistically with NO_3^- like vitamin C which helps to increase reduction of NO_2^- to NO (Jonvik *et al.*, 2016). A very recent feasibility study conducted by Van de Avoort et al. (2020) compared two different intervention strategies (NO_3^- rich vegetables or BJ supplementation) that increasing NO_3^- intake on NO_3^- pharmacokinetics and BP. They showed that NO_3^- rich vegetables was effective, to the same extend as BJ supplementation to increase plasma $NO_3^-$and NO_2^- and to reduce BP. However, the duration of the study was only one week, and future study is needed to investigate the feasibility and acceptability of long-term NO_3^- rich vegetables.

7.4. Strengths and limitations of this project

***Strengths**:* The detailed assessment of quantitative and qualitative outcomes in a pilot RCT to evaluate the feasibility and acceptability of the effects of incremental doses of HN BJ in older overweight and obese subjects is a major strength of the study. The pilot study focused on older participants, over the age of 60 years, with higher BMI. Those inclusion criteria may increase the applicability of the research, because such population groups would be at a higher risk of physiological dysfunctions, such as cognitive impairment, than younger and leaner populations. The study also provided a comprehensive evaluation of the effectiveness of different recruitment strategies and it showed that the use of social media was a very effective strategy to recruit participants in our trial and offered a cost-effective solution to reach a large number of potential participants within a short period of time. The study also collected detailed information on participants' dietary NO_3^- intake which was obtained using a computerised dietary assessment software (Intake24). The software increased the accuracy of nutrient and food intake assessment via inclusion of food photographs to assist with portion size estimation. In addition, a comprehensive database on NO_3^- content in food and beverages was used to calculate NO_3^- intake.

The systematic review undertaken in this project provided evidence on how much dietary NO_3^- are people consuming globally. It also stipulated evidence-based evaluation of the methods used to assess NO_3^- intake in humans and emphasised the need for more evidence for longer-

term studies. The validity of salivary NO_2^- strips (Berkeley) to easily assess NO_3^- intake and enable researchers to utilise a quicker and less expensive method to monitor participants' compliance in long-term NO_3^- intervention studies.

Limitations: The limitations associated with the certain aspects of the research presented in this thesis have been discussed in detail in each chapter. Therefore, a summary of the main limitations has been provided here.

The relatively small sample size used in both experimental studies (Chapters 4, 5 and 6) may have limited the ability to detect significant changes in physiological functions (i.e., BP, cognition and CBF) in response to supplementation with BJ. However, the primary purpose of these studies was to test the the validity and reliability of salivary strips (Chapter 4) and to test feasibility and acceptability of the intervention in older adults (Chapter 5 & 6) and they were not designed to investigate the effects of the intervention on these health-related outcomes. Researcher blindness in Chapters 5 and 6 was difficult to achieve merely due to lack of resources. The researcher was responsible for preparation and distribution of interventions to participants and also for collecting the data. With additional staff, it would have been possible to blind those staff making the measurements to the nature of the intervention delivered to each individual participant. Such an approach would be important to implement in a future full-scale definitive trial. In addition, the study (Chapter 5) showed that the use by participants of conventional mail services to ship the biological samples would need to be reconsidered in designing future trials because of the large inconsistency in the time taken for delivery of the samples which affected NO_3^- and NO_2^- concentrations in urine and saliva samples. As with many long-term dietary-intervention RCTs, we had dropouts that could bias our findings. However, sensitivity analysis on missing data was performed using LMM analysis. In our study, the CBF assessment was conducted on a separate day from other measurements due to the different location of the NIRS apparatus, which could have had a potential impact on measurements considering the inability to fully standardise pre-testing conditions in terms of fasting.

7.5. Future research directions

Assessment of nitrate intake and links with human health

This thesis has highlighted some areas that warrant further investigation and replication. The systematic review in Chapter 2 provided an update on the heterogeneity of dietary assessment

methods and the use of food composition tables, which points to the need for consensus in development and use of food composition databases in future studies. The development of a comprehensive NO_3^- database is urgently needed to advance research in this area and allow for a more accurate assessment of NO_3^- intake in epidemiological studies. If available, information on NO_3^- content could be easily incorporated within computerised dietary assessment methods, such as Intake24, to facilitate the assessment of NO_3^- intake in larger studies. It could also help the relevant scientific and regulatory authorities to revise and update the recommended level of NO_3^- intake and re-evaluate the maximum limit of NO_3^- intake. Based on the fact that plant-based dietary patterns such as the DASH diets provide so much NO_3^- (up to 1222 mg/day) (Hord *et al.*, 2009), the current ADI of 3.7 mg of NO_3^- ions/kg of body weight (equivalent to 259 mg/day for a 70kg person) may require revision. In addition, my work has also highlighted that the epidemiological field is dominated by a "cancer-centric" view of NO_3^- as a risk factor whereas there is growing evidence that that NO_3^--rich dietary patterns are associated with lower risk of cardiovascular and metabolic diseases. There is a need for more focussed nutritional epidemiological studies, especially prospective cohort studies, that investigate the associations between dietary NO_3^- intake and risk of common non-communicable diseases including CVD, T2D and dementia. This would provide a basis for aligning epidemiological evidence with the evidence for potential beneficial effects of dietary NO_3^- supplementation on cardio-metabolic outcomes that have been reported in numerous small-scale randomised clinical trials.

Effects of dietary nitrate on sialin production in salivary glands

The mechanism by which NO_3^- is transported into, and accumulated in, saliva is still not fully understood. However, as mentioned earlier, Qin et al. (2012) identified sialin as an effective NO_3^- transporter and showed that NO_3^- transport was reduced when sialin expression was reduced. Chapter 4 of this thesis showed preliminary findings of the association between salivary sialin and NO_2^- concentrations after blocking NO_3^- conversion into NO_2^-. These findings suggest that there may be a feedback mechanism linking NO_3^- exposure to sialin expression. These findings could be verified in future studies with larger sample sizes. In addition, the hypothesis that dietary NO_3^- (or its derivative NO_2^-) regulates sialin expression could be tested in *in vitro* studies using primary cultures of human salivary acinar cells (Jang *et al.*, 2015). Such cells could be exposed to a range of NO_3^- (and/or NO_2^-) and expression of the *SLC17A5* gene (which encodes sialin) quantified using e.g. quantitative PCR.

Long-term effects of nitrate on the oral microbiota

The oral microbiome plays an important role in the conversion process from NO_3^- to NO_2^-, thus, regulating NO generation. In addition, there is evidence that dietary NO_3^- influences the oral microbiome. For example, seven days of dietary NO_3^- supplementation increased the abundance of bacteria expressing NO_3^- reductase in the rat mouth (Hyde *et al.*, 2014). Similar findings have been observed in hyperchloremic adults following six weeks of BJ supplementation at ~400 mg/day (Velmurugan *et al.*, 2016). In addition, the administration of BJ for 10 days in both young and older people increased the abundance of bacteria expressing NO_3^- reductase (Vanhatalo *et al.*, 2018). These studies indicate that increased dietary NO_3^- intake may alter the oral microbiome. Investigation of the oral microbiota after prolonged BJ supplementation was outside the scope of this thesis, but our study showed that salivary NO_2^- concentrations increased in the first 8 weeks of supplementation followed by a great reduction at 13 weeks of supplementation, especially with the highest BJ dose. Future research is needed to establish whether this salivary NO_2^- reduction was due to the oral microbiota adapting to prolonged NO_3^- supplementation at higher doses. In future work, it would be possible to produce preliminary findings using the saliva samples from the present study and to investigate the changes in salivary microbiota to elucidate the mechanisms behind the lower saliva NO_2^- concentration following prolonged different doses of NO_3^- supplementation. Given the suggestion that higher abundance of NO_3^- -reducing oral bacteria may be associated with lower cardiometabolic risk (Goh *et al.*, 2019), there are opportunities for further investigation of changes in oral microbiota and their relationship with cardiometabolic outcomes following long-term BJ supplementation.

Long-term effects of nitrate supplementation on blood pressure, cognitive function and cerebral blood flow

The findings in Chapter 6 are preliminary and showed a non-significant trend for greater effects on BP reduction for lower BJ doses, whereas CBF and cognitive function were not affected by 13 weeks BJ supplementation. After completing this project, a recent paper emerged which reported a longer duration (24 weeks) trial (Mills *et al.*, 2020). However, this study differed from my study in a number of important aspects. First, the NO_3^- intervention was combined with a diabetic drug (spironolactone). In addition, Mills et al. investigated people with high risk of T2D, specifically, whereas I investigated older individuals who were overweight or obese.

Our study was designed primarily as a feasibility study and was not designed to provide a definitive test of the effects of supplemental NO_3^- on BP, CBF and cognitive function. However,

the findings from this study will help to inform the design of future definitive trials to investigate the long-term effects of dietary NO_3^- supplementation on cognitive and vascular functions and CBF, including by providing data for sample size calculations. For example, for SBP, although the overall test of significance of differences between the groups was not significant, *post hoc* analysis suggested a trend for an effect with the MN dose compared with placebo (P=0.07). Based on this result, 54 participants would be needed to demonstrate a difference between two independent groups (MN vs PL) of effect size 0.8 at a significance level of 0.05 and power of 80%.

7.6. Effects of nitrate on clinical populations

Although the study in chapter 6 was conducted on an at-risk population (i.e., older overweight and obese individuals), studies in those with mild cognitive impairment, hypertension may be more sensitive in detecting beneficial effects of prolonged BJ consumption on cognitive function and cardiometabolic health.

A lack of endogenously derived NO has been suggested as one proposed mechanism responsible for other health complications such as metabolic syndrome, which is characterised with a combination of cardiovascular risk factors of metabolic origin, including dyslipidaemia, high BP, visceral obesity, glucose intolerance and insulin resistance (McDonagh *et al.*, 2019). Experimental studies showed consumption of low NO_3^- and NO_2^- diet for 3 to 18 months, demonstrated characteristics typically associated with metabolic syndrome (Kina-Tanada *et al.*, 2017). Whereas the same study showed that supplementation of sodium NO_3^- for 18 months inhibited the development of the metabolic and endothelial abnormalities induced by a low NO_3^- and NO_2^- diet. This is suggesting the potential importance of exogenous NO_3^- ingestion in maintaining health. However, the studies that investigated the effect of dietary NO_3^- on human population with metabolic syndrome are very limited.

Cardiopulmonary conditions were also shown to be associated with reduced endogenous NO production. For example, pulmonary hypertension pathogenesis, which is a multi-factorial disorder manifested by elevated pulmonary resistance vasculature and right ventricular failure, is thought to be associated with reduced biological activity and responsiveness of NO. A recent RCT, double-blind and crossover study investigated the effect of BJ (~ 1000 mg/day for 7 days) in 15 pulmonary hypertension patients. The study showed that BJ increased levels of exhaled NO, including FeNO and improved right ventricular systolic function.

Overall, the studies that investigated the effect of NO_3^- on clinical population are limited and characterised by short duration. Further studies with longer duration, to explore the benefits of dietary NO_3^- in patients with various clinical conditions are warranted. The outcomes of such studies would provide valuable evidence for future public health interventions targeting those at-risk groups within the population.

7.7. Conclusions

In conclusion, this thesis has provided estimates of median NO_3^- intakes globally and shown that intakes are similar for healthy individuals and those with chronic diseases. This work has highlighted the need of the development of a comprehensive NO_3^- database that would help future epidemiologic research to assess NO_3^- intake more accurately. For the first time, we also conducted a rigorous systematic review and meta-analysis of the effects of supplemental NO_3^- and NO_2^- on cognition and CBF which found no effect of supplementation on either outcome. This systematic review identified the lack of evidence of the long-term effects of supplemental NO_3^- on these aspects of brain function. This provided the background for the design one of the longest duration feasibility studies to test of the effect of supplemented NO_3^- on BP (as a marker of cardiometabolic health), cognitive function and CBF in older people with overweight or obesity. The findings presented in this thesis have made a contribution to the understanding of the long-term effects of supplemented NO_3^- intake on vascular and brain health outcomes in older overweight and obese participants. This thesis provided evidence that the repeated consumption of low to moderate doses of BJ for prolonged period is an acceptable intervention in older overweight and obese participants. Moreover, it broadly supports the notion that dietary NO_3^- intakes at low to moderate doses may lower BP and so could have a role in the prevention of CVD. However, a larger definitive RCT is needed to confirm these findings. In addition, further research is needed to establish whether the prolonged consumption of NO_3^- has benefits on brain and vascular health in older people with cognitive impairment or hypertension.

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Appendix

Appendix 2.1: Published paper (Assessment of dietary nitrate intake in humans: a systematic review)

Assessment of dietary nitrate intake in humans: a systematic review

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ABSTRACT

Background: The nitrate content of foods and water is highly variable, which has implications for the compilation of food-composition databases and assessment of dietary nitrate intake.

Objective: A systematic review was conducted to ascertain the dietary assessment methods used and to provide estimates of daily nitrate intake in humans.

Design: Relevant articles were identified by a systematic search of 3 electronic databases (PubMed, Web of Science, and Embase) from inception until February 2018. Observational studies conducted in adult populations and reporting information on dietary assessment methods and daily nitrate intake were included. Ecological analyses were conducted to explore the association of nitrate intake with indexes of economic development [Gross Domestic Product (GDP) and KOF Index of Globalization].

Results: A total of 55 articles were included. Forty-two studies investigated associations between nitrate intake and disease risk; 36 (87%) of these studies examined the association between nitrate intake and cancer risk, whereas only 6 studies explored the association of nitrate intake with the risk of diabetes, glaucoma, kidney failure, hypertension, and atherosclerotic vascular disease. The majority of studies used food-frequency questionnaires to assess nitrate intake ($n = 43$). The median daily nitrate intakes in healthy and patient populations were 108 and 110 mg/d, respectively. We found a significant inverse correlation of nitrate intake with GDP ($r = -0.46$, $P < 0.001$) and KOF index ($r = -0.31$, $P = 0.002$).

Conclusions: The median estimated daily nitrate intakes by healthy and patient populations were similar, and these values were below the safe upper intake of daily intake (3.7 mg nitrate ion/kg body weight). However, there is considerable heterogeneity in the application of food-composition tables, which may have implications for the accuracy of estimated daily nitrate intake. The association between nitrate intake and risk of cardiometabolic diseases needs further investigation. The protocol for this systematic review has been registered in the PROSPERO database (https://www.crd.york.ac.uk/prospero; CRD number: 42017060354). *Am J Clin Nutr* 2018;108:878–888.

Keywords: inorganic nitrate, observational studies, food intake, health status, Gross Domestic Product, dietary assessment methods

INTRODUCTION

Inorganic nitrate is a water-soluble ion that occurs naturally in water and soil (1). Nitrates and nitrites are commonly used as food additives in processed meats to increase shelf life and to avoid bacterial growth, which, in addition, causes the pink coloration of processed meats (2). High consumption of processed meats has been linked with greater risk of cancer of the upper gastrointestinal tract, which may be due to the formation of the highly reactive and mutagenic N-nitrosamines (3–6). Consequently, after reviewing the toxicologic effects of nitrate and nitrite, the WHO established the Acceptable Daily Intake of nitrate as 0–3.7 mg/kg body weight (7), and this estimate was confirmed recently by the European Food Safety Authority (8). However, diets with a high nitrate content, such as vegetarian or Mediterranean dietary patterns, are associated with beneficial effects on cancer risk and other health outcomes (9, 10), and current reviews have confirmed the lack of association between dietary nitrate consumption and cancer risk (11, 12). In addition, several clinical trials have reported beneficial effects of dietary nitrate supplementation on oxidative stress, blood pressure, endothelial function (13), and exercise performance (14). Furthermore, longitudinal studies have reported a lower risk of cardiovascular mortality and lower incidence of coronary artery disease and stroke in individuals with higher intakes of nitrate-rich vegetables (11). The health benefits of higher dietary nitrate consumption appear to be related to increase nitric oxide (NO) generation via the nitrate-nitrite-NO pathway (12). NO is a free-radical gas and a highly reactive

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Supplemental Tables 1–6 and Supplemental Figures 1 and 2 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: FFQ, food-frequency questionnaire; GDP, Gross Domestic Product; NO, nitric oxide.

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molecule involved in various physiologic functions, including vascular regulation, host defense, nerve transmission, and cellular energetics (15). Two distinct pathways are responsible for NO synthesis in humans. The first pathway utilizes arginine as a precursor, which is converted into equimolar amounts of NO and citrulline by NO synthases (16). The second pathway involves the progressive reduction of nitrate into nitrite and NO in a series of reactions involving oral bacterial reductases, gastric acidic environment, and molybdenum-containing enzymes including as xanthine-oxido reductases and aldehyde dehydrogenase [reviewed in detail in Weizberg and Lundberg (15)]. These 2 pathways are not independent, and cross-talk between them may help to maintain normal NO production (17). Green leafy vegetables are the main source of nitrate in most human diets (60–80% of total nitrate intake) (11, 15). However, the nitrate content in vegetables is influenced by environmental and agronomic conditions (18). Furthermore, nitrate occurs in widely differing concentrations in drinking water, which can contribute ≤15–20% of total ingested nitrate (15). These factors, combined with the lack of reliable nitrate data in most food-, beverage-, and water-composition databases, contribute to difficulties in estimating nitrate intake in humans (15). After the ingestion of inorganic nitrate, urinary nitrate concentration peaks after 4–6 h and returns to baseline within 24–36 h (19, 20). Nitrate output in urine is influenced strongly by nitrate ingestion, but only 60–70% of consumed nitrate is excreted in urine, so that 24-h urinary nitrate output may not quantitatively reflect long-term dietary nitrate intake (21, 22).

In dietary surveys and surveillance programs, the ability to assess the safety of nitrate intakes depends on having reliable standards (i.e., the WHO Acceptable Daily Intake noted above) coupled with reference methods for assessment of nitrate intake that require valid and widely available databases of food nitrate and nitrite composition (23). Currently, there is no consensus on a reference method for assessing dietary nitrate intake, which could explain some of the differences in daily nitrate intake observed between studies (23). We conducted a systematic review of observational studies to identify these sources of heterogeneity and to quantify daily nitrate intake in adult populations. Specifically, we evaluated the compilation and application of different food-composition databases and dietary assessment methods that were used to calculate daily nitrate intake. Secondary aims included investigation of factors influencing nitrate intake, such as health and disease status and stage of economic development of the countries included in the systematic review.

METHODS

The protocol for this systematic review has been registered in the Prospero database (https://www.crd.york.ac.uk/prospero; CRD number: 42017060354). This systematic review was conducted according to Cochrane and the Center for Reviews and Dissemination guidelines (24) and is reported according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (25).

Literature search

Studies to be included were identified by searching 3 databases— Medline (https://www.ncbi.nlm.nih.gov/pubmed/), Web of Science (http://wok.mimas.ac.uk/), and Embase (https://www.elsevier.com/en-gb/solutions/embase-biomedical-research)—from inception to February 2018. The search was restricted to human studies. To maximize the inclusiveness of the search and to ensure the inclusion of articles in press, these databases were searched for titles and abstracts and without any restriction for type of studies. Hand-searching was performed for relevant articles and reviews to retrieve potential, eligible studies not found in the primary search.

The following keywords were entered in the databases to conduct the searches: nitrate, inorganic, diet, dietary, food content, food frequency, dietary recall, 24-h recall, dietary record, food composition, ingestion, intake, consumption, exposure, effect, cross sectional, case control, cohort, longitudinal, prospective, ecological, and epidemiology. We used Boolean terms (i.e., AND) to build different algorithms to increase the sensitivity of the search strategy. A summary of the specific search algorithms is reported in **Supplemental Table 1.**

The results for each individual search were exported to a reference manager software (ENDNOTE X7.7.1, Clarivate Analytics, Philadelphia, United States). A master file was created to contain all of the results of the search. Duplicates were subsequently removed. All of the stages of the search process were logged in detail and a summary is provided in **Figure 1**.

Study selection

Articles were selected for inclusion in the systematic review according to the following criteria: *1*) observational study (cohort, case-control, cross-sectional), *2*) populations with a mean age ≥18 y, *3*) quantification of dietary nitrate consumption (in milligrams per day or millimoles per day), and *4*) nitrate consumption was reported for the whole diet. Studies were excluded if they were intervention studies, conducted in nonadult populations, and if nitrate intake was not reported or was assessed only for selected foods or beverages (e.g., vegetables, water, or meat products). Only articles published in English were included. Two investigators (AMB and GF) screened the titles and abstracts of the articles independently to assess eligibility for inclusion. If agreement was reached, articles were either excluded or moved to the next stage (full-text). If agreement was not reached, the article was moved to the full-text stage. The full texts of the selected articles were critically evaluated by 3 reviewers (AMB, GF and MS) to determine final eligibility for inclusion in the systematic review. Disagreements were resolved by discussion between the reviewers until consensus was reached.

Data extraction and quality assessment

The following information was extracted from eligible articles: author names, journal and year of publication, country where study was conducted, sample size, age, sex, disease status, dietary assessment method, daily nitrate intake, methods used to quantify nitrate content in foods, and measurement of nitrate in biological fluids. If articles included populations with different age groups, only data for those with a mean age >18 y were extracted and included in the systematic review.

The quality assessment of the studies included in the present review was assessed according to specific criteria taking into account the study design, description of the study objective or objectives, results, and study limitations. These criteria

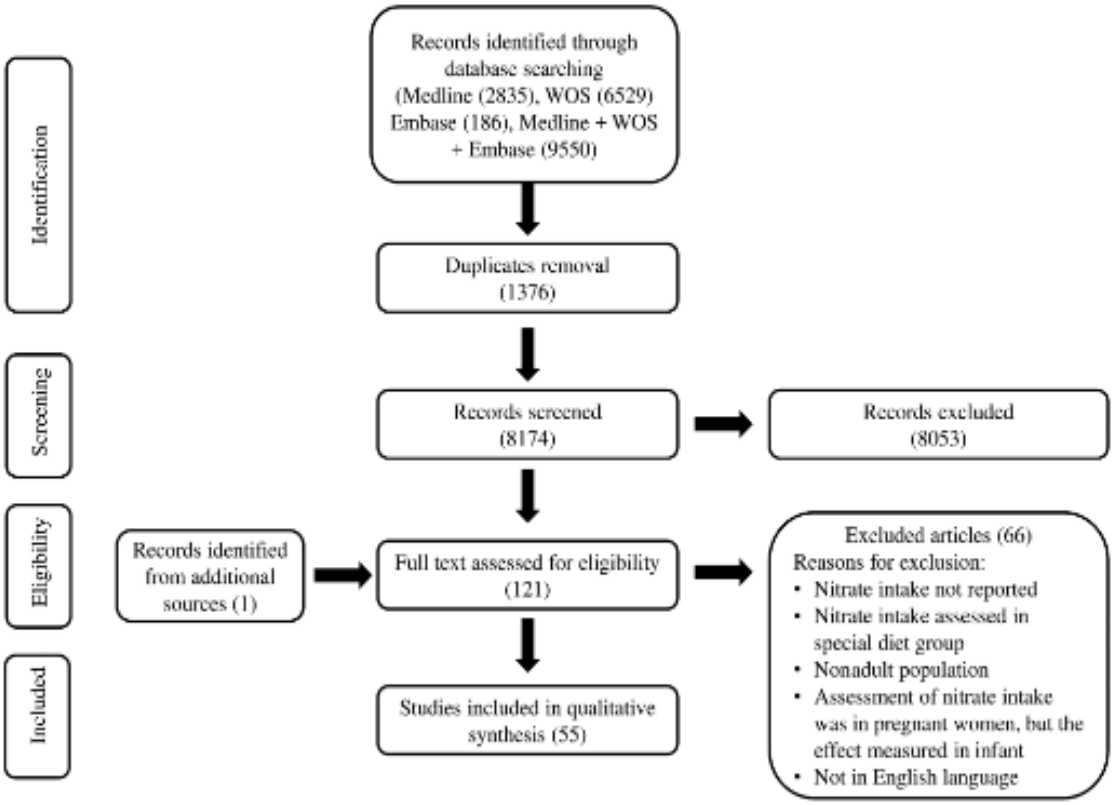

FIGURE 1 Flow diagram of the selection process of the studies. WOS, Web of Science.

were adapted from previous quality-assessment checklists (26, 27). We also developed additional criteria assessing dietary methods, approaches used to quantify nitrate content in foods and beverages, and measurement of nitrate in biological samples. A description of the study quality checklist and quality assessment of studies is provided in **Supplemental Table 2**. The appraisal of the quality of the studies was a carried out by one researcher (AMB) and reviewed by a second researcher (MS). Discrepancies in the assessment of study quality were resolved by consensus. Low-quality studies were not excluded from the systematic review to enhance the representativeness of the findings, to inform discussion of the strengths and limitations of the current evidence, and as a basis for developing proposals for the conduct of future investigations of nitrate intake.

Calculation of nitrate intake

Most included studies reported nitrate intake as means or medians for the study cohort. However, a few articles reported nitrate intake after stratification of the population into separate groups (e.g., quintiles of nitrate intake) and did not report nitrate intake for the total population. For the latter studies, we calculated total nitrate intake as the weighted average of nitrate intake by each group (see **Supplemental Table 3** for details). If data were not available or incompletely reported, authors were contacted directly to obtain the missing information. When it was not possible to use the available data to adjust nitrate intake, the article was excluded. A sensitivity analysis was conducted to evaluate the contribution of water consumption to total nitrate intake, and we investigated possible differences in nitrate intake according to study design (cross-sectional, case-control, cohort).

Nitrate intake and disease outcomes

Phenotypic characteristics of the populations including disease status at baseline and incident disease cases were extracted. This information offered the opportunity to describe the frequency and distribution of articles that reported associations of nitrate intake with disease risk. In addition, we calculated the median nitrate intake for patient populations (i.e., disease cases reported in cohort and case-control studies) and compared it with the median nitrate intake derived from healthy populations.

Gross Domestic Product, globalization index, and nitrate intake

An ecological analysis was conducted to assess the relations between nitrate intake and Gross Domestic Product (GDP) per capita of respective countries, as a measure of the economic development of each included country. Relevant GDP data were obtained from www.data.worldbank.org/indicator/NY.GDP.PCAP.CD (in US dollars). Because globalization

TABLE 2
Characteristics of case-control studies[1]

First author, year (ref)	Country	Cases	Sample size (cases/controls), *n/n*	Age, y	Sex (M/F), *n/n*	Duration, y	Dietary assessment
Barbone, 1993 (28)	USA	EC	103/236	63–64	All F	4	116-item FFQ
Chen, 2002 (29)	USA	Glioma	236/449	≥21	390/295	3	Modified 48-item FFQ
Coss, 2004 (30)	USA	PC	189/1244	40–85	877/556	27	55-item FFQ
Espejo-Herrera, 2016 (32)	Spain	BC	1245/1520	58 (mean)	All F	7	Validated 140-item FFQ
Espejo-Herrera, 2016 (31)	Spain/Italy	CC	1869/3530	20–85	4190/3051	6	Validated 140-item FFQ
Gonzalez, 1994 (33)	Spain	GC	354/354	65 (mean)	Available only for cases: 235/119	2	Diet history
Hansson, 1994 (34)	Sweden	GC	338/479	40–79	NR	20 before the interview	45-item FFQ
Hernández-Ramírez, 2009 (35)	Mexico	GC	257/478	49–70	NR	2	Validated 127-item FFQ
Kilfoy, 2012 (37)	USA	NHL	586/NA	21–84	All F	4	120-item FFQ
Kilfoy, 2010 (38)	USA	NHL	594/710	67 (mean)	All F	4	120-item FFQ
Kilfoy, 2013 (36)	USA	NHL	348/470	20–75	NR	3	FFQ
Kim, 2007 (39)	Korea	GC	136/136	57 (mean)	190/88	1	Validated 109-item FFQ
La Vecchia, 1994 (40)	Italy	GC	103/236	63–64	All F	7	29-item FFQ
Palli, 2001 (41)	Italy	GC	382/561	50–64	567/376	2	181-item FFQ
Pobel, 1995 (42)	France	GC	92/128	66 (mean)	133/87	3	Diet history
Rogers, 1995 (43)	USA	UATC	645/458	NR	NR	3.5	125-item FFQ
Virtanen, 1994 (44)	Finland	T1D	1168/1168	NR	1096/1240	3	FFQ
Ward, 2006 (45)	USA	NHL	361/276	63 (mean)	329/309	2	Modified 117-item FFQ
Yang, 2010 (46)	Korea	BC	362/362	30–65	All F	1	121-item FFQ

[1]BC, breast cancer; CC, colorectal cancer; EC, endometrial cancer; FFQ, food-frequency questionnaire; GC, gastric cancer; NHL, non-Hodgkin lymphoma; NR, not reported; PC, pancreatic cancer; ref, reference; T1D, type 1 diabetes; UATC, upper aerodigestive tract cancer.

and Italy (31), 3 studies from Finland (44, 69, 75) and the United Kingdom (73, 80), and 1 study each from Belgium (78), Sweden (34), France (42), and Poland (82)], 5 studies were conducted in Asia [2 studies from each of China (50, 59) and Korea (39, 46) and 1 study from Thailand (76)], 2 studies were conducted in the Middle East [both studies in Iran (47, 48)], and 1 study was from Australia (67). The study-specific quality assessment scores are shown in **Supplemental Table 5.**

Participants and health status

Thirteen studies assessed nitrate intake in healthy individuals and 42 studies investigated the association between nitrate intake and disease risk. The majority of these studies (88%) investigated the association between nitrate intake and various types of cancer. Single studies were found for 6 other conditions, including type 1 and type 2 diabetes (44, 48), glaucoma (56), kidney failure (47), hypertension (47), and atherosclerotic vascular disease (67).

Dietary assessment methods

Studies were characterized by substantial variation in the type of dietary assessment methods used to estimate nitrate intake. The majority of the studies (43 studies) used food-frequency questionnaires (FFQs) (28–32, 34–41, 43–68, 71, 73, 74, 80). Diet history was used in 3 studies (33, 42, 69). Two studies combined both FFQs and 24-h recall methods (72, 78), 3 studies used dietary records (70, 77, 79), 1 study used 48-h recall (75), and another used 24-h recall (81). The method of dietary assessment was not reported in 1 study (82). Six studies reported the measurement of nitrate concentrations in biological samples (plasma, urine, or saliva) as an objective measure of exposure to dietary nitrate (47, 48, 73, 74, 77, 80).

Nitrate data in food-composition tables

Different methods were used to quantify the nitrate contents in foods, which were then used to compile food-composition databases and applied to calculate daily nitrate consumption. The majority of the included studies used data from the literature as the primary source of information to estimate nitrate content of foods [34 studies (28–30, 33, 34, 36–39, 42–46, 49–55, 58–62, 69, 71–75, 81)]. Study-specific databases or food-composition tables containing information on nitrate content were used in 11 studies (31, 32, 35, 41, 47, 48, 56, 57, 63–65). Three studies used the duplicate food portion method combined with 24-h recall to measure nitrate content in the consumed foods and then to calculate daily nitrate intake (70, 77, 79). Two studies measured nitrate content in food and also used compositional information reported in the literature (76, 78). One study did not report the approach used to estimate nitrate content in foods (82) (see **Supplemental Table 6** for more details).

Daily nitrate intake

Estimated nitrate intake reported in each article is summarized in Supplemental Table 6. The estimated median (IQR) nitrate intake in 51 studies including healthy participants was

TABLE 3
Characteristics of cohort studies[1]

First author,[2] year (ref)	Country	Cases	Sample size, *n*	Age, y	Sex (M/F), *n*/*n*	Duration, y	Dietary assessment
Bahadoran, 2017 (48)	Iran	T2D	Total: 2139; cases: 143	20–70	971/1168	6	Validated 168-item FFQ
Bahadoran, 2016 (47)	Iran	CKD and HT	Total: 2799; cases of CKD: 1780; cases of HT: 1878	20–70	806/1072	6	Validated 168-item FFQ
Blekkenhorst, 2017 (67)	Australia	ASVD	Total: 1226; died: 238	70–85	All F	15	74-item FFQ
DellaValle, 2013 (49)	USA	RCC	Total: 491,841; cases: 1816	50–71	292,125/198,069	9	Validated 124-item FFQ
DellaValle, 2014 (50)	China	CC	Total: 73,118; cases: 619	40–70	All F	5	Validated 77-item FFQ
Dubrow, 2010 (51)	USA	Glioma	Total: 545,770; cases: 585	50–71	322,347/223,423	1	124-item FFQ
Inoue-Choi, 2015 (52)	USA	OVC	Total: 28,555; cases: 315	55–69	All F	1	Validated 126-item FFQ
Inoue-Choi, 2012 (53)	USA	BC	Total: 41,836; cases: 2875	55–69	All F	1	127-item FFQ
Jones, 2016 (54)	USA	BLC	Total: 33,964; cases: 258	55–70	All F	21	Validated 127-item FFQ
Jones, 2017 (55)	USA	KC	Total: 33,964; cases: 256	55–69	All F	21	Validated FFQ
Kang, 2016 (56)	USA	Glaucoma	Total: 104,987; cases: 1483	>40	63,893/41,094	2	Validated 116-item FFQ
Keszei, 2013 (57)	Netherlands	EC and GC	Total: 4959; cases: 927	55–69	1947/2085	16.3	Validated 150-item FFQ
Keszei, 2014 (66)	Netherlands	BE	Total: 3717; cases: 433	55–69	2074/2076	16.3	150-items FFQ
Kilfoy, 2012 (60)	USA	OVC	Total: 151,316; cases: 709	50–71	All F	10	Validated 124-item FFQ
Kilfoy, 2013 (59)	China	TC	Total: 73,317; cases: 164	40–70	All F	11	Validated 77-item FFQ
Kilfoy, 2011 (58)	USA	PC	Total: 492,226; cases: 1728	50–71	293,491/198,735	10	Validated 124-item FFQ
Kilfoy, 2011 (61)	USA	TC	Total: 490,194; cases: 370	50–71	292,125/198,069	7	Validated 124-item FFQ
Michaud, 2009 (62)	USA	Glaucoma	From 3 studies: 49,935 (HPFS), 92,468 (NHS I), 95,391 (NHS II); cases: 335	25–75	49,935/187,856	>24	131-item FFQ
Quist, 2018 (68)	USA	PC	Total: 32,242; cases: 313	55–69	All F	25	127-items FFQ
van Loon, 1998 (63)	Netherlands	GC	Total: 3405; cases: 282	55–96	1525/1598	6.3	Validated 150-item FFQ
Ward, 2010 (64)	USA	TC	Total: 20,651; cases: 40	61 (mean)	All F	19	126-item FFQ
Zeegers, 2006 (65)	Netherlands	BLC	Total: 5230; cases: 871	62 (mean)	2391/330	9.3	Validated 150-item FFQ

[1] ASVD, atherosclerotic vascular disease; BC, breast cancer; BLC, bladder cancer; BE, Barrett's esophagus; CC, colorectal cancer; CKD, chronic kidney disease; EC, esophageal cancer; FFQ, food-frequency questionnaire; GC, gastric cancer; HPFS, Health Professionals Follow-Up Study; HT, hypertension; KC, kidney cancer; NHS, Nurses' Health Study; OVC, ovarian cancer; PC, pancreatic cancer; RCC, renal cell carcinoma; ref, reference; TC, thyroid cancer; T2D, type 2 diabetes.
[2] The 2 studies each by Bahadoran, Inoue-Choi, and Kilfoy each used the same cohort but studied different outcomes.

108 mg/d (87–145 mg/d) (**Figure 2**A). The estimated median nitrate intake in 41 studies including patients was 110 mg/d (89–153 mg/d) (Figure 2B). There was no significant difference in daily nitrate intake between healthy participants and patients ($P = 0.6$). Total nitrate intake was significantly higher in studies that did not account for the contribution of nitrate from water intake compared with those that included nitrate from water ($P = 0.005$). (**Supplemental Figure 1**). Dietary nitrate intake was similar in studies that used different study designs ($P > 0.30$) (**Supplemental Figure 2**).

Dietary nitrate intake, GDP, and KOF Globalization Index

We found a significant inverse association between daily nitrate intake and GDP ($n = 53$; β coefficient $\pm$ SE $= -0.44 \pm 0.12$ mg/d, $r = -0.46$, $P < 0.001$) (**Figure 3**A). There was a similar inverse association between nitrate intake and the KOF Globalization Index ($n = 53$; β coefficient $\pm$ SE $= 1.73 \pm 0.73$ mg/d, $r = -0.31$, $P = 0.02$) (Figure 3B). Daily nitrate intake was higher in low- or middle-income countries such as Mexico, China, or Thailand than in countries with a higher per capita income such as the United States or the Netherlands.

DISCUSSION

Main findings

The estimated nitrate intakes for healthy individuals and patients were very similar (i.e., 108 and 110 mg/d, respectively). There was much heterogeneity in types of dietary assessment methods and in data sources used for the nitrate composition of foods and beverages. The results also indicated that the majority of epidemiologic studies that considered health outcomes focused on the association between nitrate intake and cancer risk and very few articles assessed associations between nitrate intake and the risk of cardiovascular and metabolic diseases.

Assessment method used for estimating dietary nitrate intake

The accurate assessment of daily nitrate intake is important for studies of relations between nitrate exposure and human health. This review shows that several methods have been used to estimate nitrate intake with the FFQ as the most commonly used tool (84). However, there are several specific issues when using FFQs for assessing nitrate intake. One of these issues is

TABLE 4
Characteristics of cross-sectional studies[1]

First author, year (ref)	Country	Health status	Sample size, *n*	Age, y	Sex (M/F), *n*/*n*	Dietary assessment
Anyzewska, 2014 (82)[2]	Poland	Healthy	NR	NR	NR	NR
Dich, 1996 (69)	Finland	Healthy	10,054	>15	5304/4750	Diet history
Ellen, 1990 (70)	Netherlands	Healthy	110	42 (mean)	57/53	DR
Griesenbeck, 2010 (71)	USA	Healthy	5958	NR	All F	58-item FFQ
Inoue-Choi, 2016 (72)	USA	Healthy	490,194	50–71	292,125/198,069	124-item FFQ and 24-h recall
Jonvik, 2017 (81)	Netherlands	Healthy (Athletes)	553	NR	226/327	24-h recall
Knight, 1987 (73)	United Kingdom	Healthy	747	15–74	284/463	16-item FFQ
Knight, 1990 (74)	Italy	Healthy	313	16–60	159/172	22-item FFQ
Laitinen, 1993 (75)	Finland	Healthy	747	15–24	582/632	48-h recall
Mitacek, 2008 (76)	Thailand	Healthy	467	19 to <60	212/255	97-item FFQ and analyzing real sample
Stephany, 1980 (77)	Netherlands	Healthy	100	NR	70/30	DR
Temme, 2011 (78)	Belgium	Healthy	3083	<15	1546/1537	FFQ and two 24-h recalls
Vaessen, 1999 (79)	Netherlands	Healthy	123	18–74	60/63	DR
Vandenbrandt, 1989 (80)	United Kingdom	Healthy	59	29.7 (mean)	35/24	120-item FFQ

[1]DR, dietary record; FFQ, food-frequency questionnaire; NR, not reported; ref, reference.
[2]Unclear study design.

the grouping together within the FFQ of multiple foods rich in nitrate (e.g., green vegetables). Because the nitrate content of green vegetables can vary greatly and range from 1 to 4800 mg/kg (i.e., Brussels sprouts and arugula, respectively) (85), failure to quantify intakes of individual green vegetables could lead to significant under- or overestimation of nitrate intake and this could be exacerbated by decisions made by investigators in specifying the nitrate content of green vegetables used in their calculations. Moreover, because of optimistic bias and the structure of questions used, FFQs can overestimate intakes of fruit and vegetables (86, 87), which may lead to overreporting of nitrate intake.

Currently, there is no standard database that contains detailed and accurate information on nitrate content. The majority of the epidemiologic studies included in this review relied on estimates of food nitrate content obtained from various sources, such as peer-reviewed articles, official reports from government bodies, or position statements from scientific working groups. Some studies used databases from other countries because of the lack of information on nitrate content in their local foods, which could affect the reliability of the results. Factors such as seasonality, temperature, light exposure, and use of fertilizers contribute to the variable nitrate content of foods and complicate the comparison of daily nitrate intake between countries (15, 88). In addition, the majority of the databases contain data on nitrate contents of raw foods and do not take into account effects of preservation and cooking processes on nitrate in foods as eaten. More recently, a group of researchers from Australia developed a reference database for assessing dietary nitrate from large variety of vegetables (89), which considers some of the processing factors such as cooking and preservation methods that could influence the nitrate concentration in vegetables. There is a need to apply similar approaches to establish the concentrations of nitrate in other food types and in other countries. The measurement of

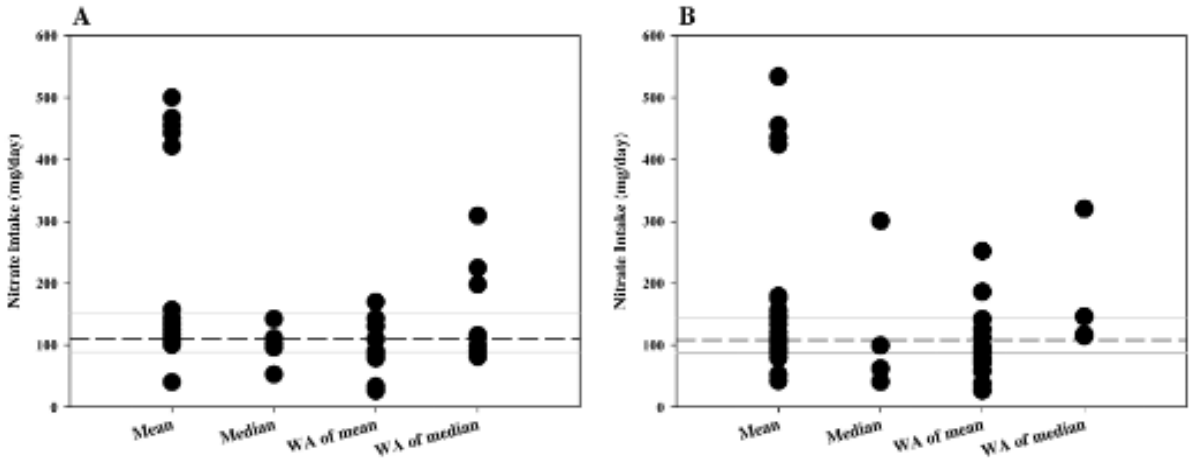

FIGURE 2 Nitrate intake assessment in unhealthy and healthy individuals. Daily nitrate intake reported by each of the included articles in patients (A) [disease cases from case-control studies or individuals who developed diseases during follow-up; $n = 42$; median (IQR) = 110 (89–153) mg/d] and in healthy individuals (B) [$n = 52$; median (IQR) = 108 (87–145 mg/d)]. WA, weighted average.

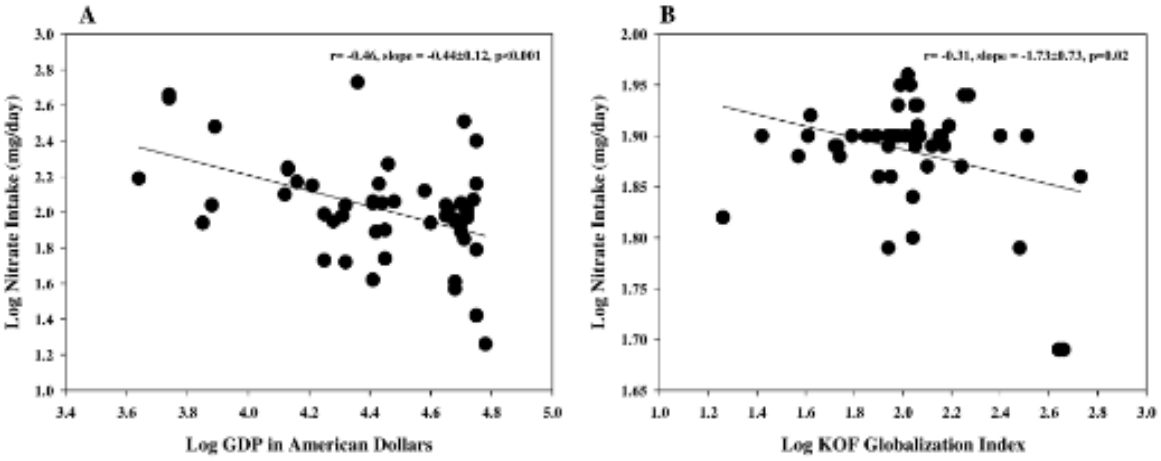

FIGURE 3 Association between daily nitrate intake and GDP and KOF Globalization Index. Nitrate intake was inversely associated with GDP (A) ($n = 53$; slope = -0.44 ± 0.12 mg/d; $r = -0.46$, $P < 0.001$) and with KOF Globalization Index (B) ($n = 53$; slope = 1.73 ± 0.73 mg/d; $r = -0.316$, $P = 0.02$). Data were log transformed before analysis ($n = 53$ studies). Pearson's correlation was used to test the association between variables. GDP, Gross Domestic Product.

nitrate concentrations in biological fluids such as plasma, saliva, or urine could be useful to assess the validity of estimated nitrate intake. However, only 6 studies reported the associations between dietary intake and nitrate concentrations in saliva (73, 74, 77) and urine (47, 48, 80) (Supplemental Table 2).

Two studies reported analyses conducted in the same cohort and collected a single urine sample, but they did not provide a description of the collection protocols and timing of urine collection (47, 48). The studies reported significant correlations between dietary intake of nitrate and nitrite with their corresponding urinary concentrations ($r = 0.59$, $P < 0.001$; $r = 0.29$, $P < 0.001$). The other study collected total overnight urine specimens on 2 occasions and separated by 2 wk (80). Such overnight urinary nitrate outputs are likely to provide an indication of short-term nitrate intake only. Given the day-to-day heterogeneity in food intake and the high variability in the nitrate content of foods, it is probable that urine sampling on multiple days would be needed to provide an objective and accurate assessment of long-term typical exposure to dietary nitrate. Hence, there is a clear research gap in developing and validating objective biomarkers of nitrate intake in humans.

Three studies estimated nitrate intake by using the duplicate-portion sampling method, which combines dietary assessment with the determination of nitrate content from similar food samples collected by the participants. However, this approach is expensive and time-consuming and therefore unsuitable for use when estimating nitrate intakes by large numbers of people.

Estimated nitrate intake

By using data on nitrate content of foods from the United Kingdom, the total nitrate intake for the UK population was estimated to be 95 mg/d (73). The International Agency for Research on Cancer (IARC) Monograph on the Evaluation of Carcinogenic Risks to Humans report (90) estimated that total dietary nitrate intake globally ranged from 58 to 218 mg/d. In the present study, the estimated intake of dietary nitrate from the included studies was within this range, with median intakes of 108 and 110 mg/d for healthy individuals and for those with disease, respectively. Regardless of the dietary assessment method or food-composition database used, estimated nitrate intakes were below the Acceptable Daily Intake of 3.7 mg nitrate ion/kg body weight (~250 mg nitrate/d) established by WHO and confirmed recently by the European Food Safety Authority (8). Using information on cultural meal patterns, a recent study estimated nitrate intake from 4 countries (United States, China, Japan, and India) and found values similar to those reported here (91). We found that studies that did not include water in their estimates of ingested nitrate reported significantly higher nitrate intake than studies that included water in their calculations. These results need careful interpretation, because water is an important, if variable, source of ingested nitrate. Therefore, our comparison is likely to be confounded by differences in the characteristics of studies in each group (with and without inclusion of water), such as geography or contamination of water sources with nitrate from fertilizer run-off or leaching from organic sources. Five studies that did not include nitrate from water [3 from Iran (2 articles) (47, 48) and 2 from China (50, 59)] reported very high daily nitrate intakes (>300 mg/d). The removal of these studies from the analyses significantly modified the results and showed that total daily nitrate intake was comparable between studies that did (104 mg/d) and did not (115 mg/d) include nitrate intake from ingested water.

Associations between GDP, KOF Globalization Index, and nitrate intake

Ecological analysis showed that nitrate intake was inversely associated with both GDP and KOF Globalization Index of the countries in which the studies were conducted. These analyses are speculative because they are based on the assumption that the dietary nitrate intake of each study is representative of the entire county. A large proportion of the studies included in the analysis were conducted in the United States (20 out of 53

studies), and this might have confounded the association by inflating the numbers of countries with a high GDP and high KOF Globalization Index. However, the removal of the studies from the United States from the analysis did not modify the findings and nitrate intake remained inversely associated with GDP ($n = 33$; β coefficient $\pm$ SE $= -0.57 \pm 0.15$ mg/d, $r = -0.57$, $P < 0.001$) and with the KOF Globalization Index ($n = 33$; β coefficient $\pm$ SE $= -2.29 \pm 0.85$ mg/d, $r = -0.57$, $P < 0.001$). The significant inverse association that we observed suggests that nitrate intake may decrease as countries become more developed economically. Because the KOF Globalization Index attempts to assess characteristics that affect food supplies, food intake patterns, or both, this may capture dietary changes associated with the nutrition transition (92), such as higher intakes of animal products and reduced intakes of nitrate-rich fruit and vegetables (93). In addition, ethnicity, religion, and cultural food patterns are key determinants of the diversity of food choices (94), and thus may affect nitrate intake.

Strength and limitations

A major strength of this review is the inclusion of data from >3 million participants from 15 different nations. Additional strengths include the sensitivity analyses performed to evaluate possible confounding effects of study design or water intake on estimates of nitrate intake. Limitations of the study include the exclusion of non-English articles, which could result in selection bias. In addition, we did not stratify nitrate intake by dietary assessment methods because the majority of studies used FFQs to estimate nitrate intake and therefore the results were described narratively.

Conclusions

This review provides an evidence-based evaluation of the methods used to assess nitrate intake in humans and estimates the median daily nitrate consumption in both healthy and patient populations. An important finding of the review is the high heterogeneity in dietary assessment methods and in the use of food-composition tables. In addition, few studies attempted to validate their dietary nitrate intake data using biomarkers such as nitrate excretion in urine. As a consequence, the accuracy of published estimates of nitrate intake remains uncertain. In summary, the outcomes of this systematic review highlight the need for a consensus initiative to delineate guidelines to improve and standardize the assessment of nitrate intake in humans.

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Appendix 2.2: Checklist for quality assessment of studies (higher number, higher quality)

Criteria	Scores
1-Are the objectives clearly described?	Yes (1), No (0)
2-Are the characteristics of participants clearly described?	Yes (1), No (0)
3-Is sample size adequate?	≥100 (1), < 100 (0)
4-Dietary assessment method used	Dietary record OR combined two different methods (3), FFQ (2), 24-hour recall (1)
5-Is dietary assessment method clearly described?	Yes (1), No (0)
6-Method used to quantify nitrate in food declared by author	Measured during study and also used published literature (3), Measuring nitrate in food OR using duplicate portion sampling technique (2), Food composition table OR Databases OR Published literature (1)
7-Is the method of quantifying nitrate content clearly described?	Yes (1), No (0)
8-Is nitrate level measured in a biological sample?	Yes (1), No (0)
9-Was the representation of results clear?	Very clear (2), Moderately clear (1), Unclear (0)
10-Are the limitations of the study discussed?	Yes (1), No (0)

Appendix 2.3: Quality assessment scores of studies (Low quality: < 5, Medium: 5-9, High: 10-14)

Study	Total score	Reference	Total score
(Anyzewska and Wawrzyniak, 2014)	2	(Hernandez-Ramirez *et al.*, 2009)	11
(Bahadoran *et al.*, 2017)	14	(Inoue-Choi *et al.*, 2012)	10
(Bahadoran *et al.*, 2016a)	14	(Inoue-Choi *et al.*, 2015)	10
(Barbone *et al.*, 1993)	9	(Inoue-Choi *et al.*, 2016)	10
(Blekkenhorst *et al.*, 2017a)	11	(Jones *et al.*, 2016)	9
(Chen *et al.*, 2002)	11	(Jones *et al.*, 2017)	10
(Coss *et al.*, 2004)	10	(Jonvik *et al.*, 2017)	7
(DellaValle *et al.*, 2013)	10	(Kang *et al.*, 2016)	10
(Dellavalle *et al.*, 2014)	10	(Keszei *et al.*, 2013)	11
(Dich *et al.*, 1996)	9	(Keszei *et al.*, 2014)	10
(Dubrow *et al.*, 2010)	10	(Kilfoy *et al.*, 2012)	10
(Ellen *et al.*, 1990)	12	(Kilfoy *et al.*, 2010)	11
(Espejo-Herrera *et al.*, 2016a)	11	(Kilfoy A *et al.*, 2013)	11
(Espejo-Herrera *et al.*, 2016b)	11	(Kilfoy *et al.*, 2013)	9
(Gonzalez *et al.*, 1994)	9	(Kilfoy *et al.*, 2011a)	11
(Griesenbeck *et al.*, 2010)	11	(Kilfoy *et al.*, 2011b)	10
(Hansson *et al.*, 1994)	9	(Kilfoy A *et al.*, 2012)	10
(Knight *et al.*, 1987b)	12	(Kim *et al.*, 2007)	11
(Knight *et al.*, 1990)	10	(Temme *et al.*, 2011)	11
(La Vecchia *et al.*, 1994)	10	(Vaessen and Schothorst, 1999)	10
(Laitinen *et al.*, 1993)	8	(van Loon *et al.*, 1998)	11
(Michaud *et al.*, 2009)	10	(Vandenbrandt *et al.*, 1989)	9
(Mitacek *et al.*, 2008)	12	(Virtanen *et al.*, 1994)	11
(Palli *et al.*, 2001)	9	(Ward *et al.*, 2006)	10
(Pobel *et al.*, 1995)	9	(Ward *et al.*, 2010)	9
(Quist *et al.*, 2018)	9	(Yang *et al.*, 2010)	11
(Rogers *et al.*, 1995)	9	(Zeegers *et al.*, 2006)	11
(Stephany and Schuller, 1980)	8		

Appendix 2.4: Examples of calculation of total nitrate intake in studies.

Two examples have been provided to clarify the steps used to calculate total NO_3^- intake in studies that reporting NO_3^- intake in individual, separate groups but failed to report the total NO_3^- intake.

EXAMPLE 1: IF NITRATE INTAKE WAS REPORTED IN TERTILE, QUARTILE OR QUANTILE (STUDY OF WARD ET AL, 2006)

Step 1: Identification of relevant data

TABLE 5. Association of Dietary Intake of Nitrate and Nitrate With NHL*

Dietary Nitrate and Nitrate (mg/d)	No. of Cases	No. of Controls	OR† (95% CI)
Nitrate			
<76‡	159	98	1.0
76–113.9	116	98	0.75 (0.51–1.10)
114–169.9	111	98	0.71 (0.47–1.07)
170+	80	97	0.54 (0.34–0.86)
Nitrite			
<0.71‡	82	98	1.0
0.71–0.909	108	98	1.5 (1.0–2.3)
0.91–1.209	110	98	1.7 (1.1–2.7)
1.21+	166	97	3.1 (1.7–5.5)
From animal sources			
<0.16‡	101	98	1.0
0.16–0.259	106	98	1.0 (0.7–1.5)
0.26–0.419	123	98	1.0 (0.7–1.6)
0.42+	136	97	1.0 (0.6–1.7)
From plant sources			
<0.48‡	90	98	1.0
0.48–0.639	104	98	1.3 (0.8–1.9)
0.64–0.859	128	98	1.7 (1.1–2.6)
0.86+	144	97	2.0 (1.2–3.5)

*Participants from all 4 centers in the diet arm of the study.
†Adjusted for age group (deciles), education, sex, study center, race, dietary vitamin C, and total energy.
‡Reference category.

Step 2: We considered 0 as a lowest value in the first range (0 – 76), and the difference of this range (76-0=76) was added to last range (170+76=246) to calculate the upper limit of the last quartile; hence the last quartile range was 170 – 246 mg/d.

Step 3: We then calculated the mean of each range:

Range (mg/day)	Mean (mg/day)
0– 76	35.5
76 – 113.9	94.95
114 – 169.9	141.95
170 – 246	205.5

Step 4: The Weighted Average (WA) was then calculated:

WA= [(mean of 1st quartile range × number of individuals in the quartile range) + (mean 2nd quartile range × number of individuals in the quartile range) + (mean of 3ed quartile range × number of individuals included the quartile range) + (mean of 4th quartile range × number of individuals included the quartile range]/ the total number of individuals)

WA= (35.5 ×159) + (94.95 ×116) + (141.95 × 111) + (205.5 × 80) / 466 = 108.6 mg/d

Note: Several studies reported median values directly for each quantile without reporting the range of values, in these cases we have calculated the WA of the median (WA of median) using the same approach described above.

EXAMPLE 2: IF NITRATE INTAKE WAS REPORTED IN MEDIAN MG/1000KCAL (ENERGY ADJUSTED) AND STRATIFIED BY GENDER (STUDY OF KILFOY ET AL., 2011)

Step 1: Identification of relevant data for the calculation of nitrate intake for the female and male groups and total population

Table 3. Multivariate Hazard Ratios[a] for Pancreatic Cancer According to Quintile of Nitrate or Nitrite Intake in the NIH-AARP Diet and Health Study, 1995–2006

Quintile	Median Value, mg/1,000 kcal	Total (*n* = 1,728)			Men (*n* = 1,103)			Women (*n* = 628)		
		No. of Cases	HR	95% CI	No. of Cases	HR	95% CI	No. of Cases	HR	95% CI
Nitrate										
Quintile 1	19.3	370	1.00	Reference	282	1.00	Reference	88	1.00	Reference
Quintile 2	29.9	330	0.91	0.78, 1.06	229	0.91	0.76, 1.09	101	0.89	0.67, 1.19
Quintile 3	40.9	360	1.02	0.88, 1.18	232	1.05	0.88, 1.25	128	0.93	0.71, 1.23
Quintile 4	57.4	340	0.99	0.85, 1.16	204	1.07	0.89, 1.30	136	0.84	0.64, 1.11
Quintile 5	94.8	322	1.01	0.85, 1.20	151	1.07	0.86, 1.33	171	0.88	0.66, 1.17
P for trend			0.58			0.27			0.49	

Step 2: We calculated the WA of total energy intake per day stratified by quintiles (Table 1 in the original paper) reported for the **female group**. The mean for each quintile was calculated which was then used to calculate the WA by taking into account the number of participants in each group and the sample size of the total population. The WA of energy intake for the female group as 1595.4 kcal/d.

Step 3: We then converted the median value of nitrate intake in mg/1000kcal for each quintile group into mg/d (by using the following formula:

Nitrate Intake (mg/day): [Total Energy Intake (kcal/day)/1000] X energy-adjusted median nitrate intake for each quintile group (mg/1000kcal)

Step 4: The results from these calculations are described in the table below

Nitrate Intake	
Median value (mg/1000 kcal)	mg/day
1595.9/1000× 19.3	30.8
1595.9/1000× 29.9	47.7
1595.9/1000× 40.9	65.25
1595.9/1000× 57.4	91.5
1595.9/1000× 94.8	154.2

Step 5: Finally, we calculated the WA of quintiles of daily nitrate intake for the female group as described in point 3 of example 1. The WA of daily nitrate intake was 70.9 mg/d.

Step 6: We calculated first the WA of total energy intake per day stratified by quintiles (Table 1 in the original paper) reported for the **male group**. The mean for each quintile was calculated which was then used to calculate the WA by taking into account the number of participants in each group and the sample size of the total population. The WA of energy intake for the male group was 1988.8 kcal/d.

Step 7: We then converted the median value of nitrate intake in mg/1000kcal for each quintile group into mg/d by using the following formula:

Nitrate Intake (mg/day): [Total Energy Intake (kcal/day)/1000] X energy-adjusted median nitrate intake for each quintile group (mg/1000kcal)

Step 8: The results are described in the table below

Nitrate Intake	
Median value (mg/1000 kcal)	mg/day
1988.8/1000× 19.3	38.4
1988.8/1000× 29.9	59.4
1988.8/1000× 40.9	81.3
1988.8/1000× 57.4	114.2
1988.8/1000× 94.8	188.5

Step 9**:** Finally, we calculated the WA of quintiles of daily nitrate intake for the female group as described in point 3 of example 1. The WA of daily nitrate intake was 80.8 mg/d.

Step 10: The WA of the nitrate intake for the male and female groups was calculated to estimate the total daily nitrate intake in the **total population**. See below for details of the calculations based on the values used in this example.

Step 11: WA for daily nitrate intake of the whole population (mg/day): (70.9×628) + (80.8×1103)/1728 = 76.8 mg/day

Appendix 2.5: A list of the countries with GDP and Globalization Index[1-5]

Reference	Country	GDP	Globalization Index
Anyzewska et al., 2014	Poland	14,341.9	78.19
Bahadoran et al., 2016	Iran	5,442.9 [1]	48.66 [4]
Bahadoran et al., 2016	Iran	5,442.9 [1]	48.66 [4]
Blekkenhors et al., 2017	Australia	49,927	79.29 [4]
Chen et al., 2002	USA	38,166	77.89
Coss et al., 2003	USA	39,677.2	78.39
DellaValle et al., 2013	China	7,683.5	61.99
DellaValle et al., 2014	USA	52,749.9	79.4
Dichet et al., 1995	Finland	26,273.5	78.98
Dubrow et al., 2010	USA	48,374.1	79.4
Ellen et al., 1990	Netherland	21,019.1	78.51
Espejo-Herrera et al., 2016	Spain	25,684.7	84.53 [4]
[2] Espejo-Herrera et al., 2016	Italy	29,993.1	82.15 [4]
[2] Espejo-Herrera et al., 2016	Spain	25,684.7	84.53 [4]
Gonzalez et al., 1994	Spain	13,378.8	74.51
Griesenbeck et al., 2010	USA	48,374.1	79.41
Hansson et al., 1994	Sweden	25,747.2	82.5
Hernández-Ramírez et al., 2009	Mexico	7,661.2	62.73
Inoue-Choi et al., 2012	USA	51,433	79.56
Inoue-Choi et al., 2014	USA	54,539.7	79.95
Inoue-Choi et al., 2015	USA	56,115.7	79.95
Jones et al., 2016	USA	57,466.8	79.95 [4]
Jones et al., 2017	USA	57,466.8 [3]	79.95 [4]
Jonvik et al., 2017	Netherlands	45,637.9	90.24 [4]
Kang et al., 2016	USA	57,466.8	79.95 [4]
Keszei et al., 2013	Netherlands	51,574.5	88.99
Keszei et al., 2014	Netherlands	52,157.7	90.06
Kilfoy et al., 2013	USA	52,749.9	79.4
Kilfoy et al., 2010	USA	48,374.1	79.4
Kilfoy et al., 2012	USA	51,433	79.56
Kilfoy et al., 2013	China	7,077.8	61.88
Kilfoy et al., 2011	USA	49,781.8	79.79
Kilfoy et al., 2011	USA	49,781.8	79.79
Kim et al., 2007	Korea	23,101.5	72.81
Knight et al., 1987	Italy	20,757.1	73.52
Knight et al., 1990	UK	13,118.6	69.95
La Vecchia et al., 1994	Italy	19,273.8	73

Reference	Country	GDP	Globalization Index
Laitinen et al., 1993	Finland	17,617	77.47
Mitacek et al., 2008	Thailand	4,384.8	66.58
Palli et al., 2001	Italy	20,400.8	81.1
Pobel et al., 1995	France	27,038	78.54
Quist et al., 2018	USA	57,466.8 [3]	79.95 [4]
Rogers et al., 1995	USA	28,782.2	75.25
Stephany et al., 1980	Netherlands	13,615.8	78.64
Temme et al., 2010	Belgium	44,382.9	87.09
Vaessen et al., 1999	Netherlands	27,951.7	86.26
Van Loon et al., 1998	Netherlands	27,533.6	85.36
Vandenbrant et al., 1989	UK	16,239.3	72.61
Virtanen et al., 1993	Finland	17,617	77.47
Ward et al., 2006	USA	21,515.2	79.93
Ward et al., 2010	USA	48,374.1	79.41
Yang et al., 2010	Korea	46,437.1	75.08
Zeeger et al., 2006	Netherlands	44,454	88.17

[1]GDP and Globalization index data are for year of publication, except as indicated below.
[2]GDP data were available only for 2014.
[3] These are one paper, but because the study conducted in Italy and Spain, we treated it separately for this analysis.
[4] GDP data were available only for 2016.
[5] Globalization Index data were available only for 2015.

Appendix 2.6: Nitrate intake and methods used for its assessment for the studies included in the systematic review

Reference	Country	Nitrate Intake Unhealthy (mg/day)	Nitrate Intake Healthy (mg/day)	Nitrate Food Content Method Declared by Authors (Reference as in original paper)	Measurement of Nitrate in Biological Fluids (concentration)	Primary Outcome of Study	Main Findings
Anyzewska, 2014	Poland	---	(2006-2012) 147 (mean)	NR	NR	To determine whether the level of domestic nitrate consumption is safe in polish household during 2006-12	Domestic nitrate consumption is found to be safe in polish household during 2006-12
Bahadoran, 2016	Iran	467 (mean)	435 (mean)	Recent survey conducted on frequently consumed food items among Iranians that used Validated spectrophotometric methods (39)	In urine (NR)	The aim of this study was to examine the potential association of dietary nitrate on the occurrence of type II diabetes	No significant association between nitrate in overall, and plant- and animal sources as well, with the risk of type II diabetes
Bahadoran, 2016	Iran	1: HTN patients 463 (mean) 2: CKD patients 443 (mean)	1: 455 (mean) 2: 467 (mean)	Recent survey conducted on frequently consumed food items among Iranians that used Validated spectrophotometric methods (39)	In urine (NR)	To evaluate the association between dietary intakes of nitrate and the risk of hypertension and chronic kidney disease	Dietary intake of nitrate had no significant association with the risk of hypertension or chronic kidney disease
Barbone, 1993	USA	NR	134.0 (mean)	Published Literature (16)	NR	Association between nitrate and other nutrients intake and risk of endometrial cancer	High intake of protein, carotene, nitrate, and yellow and green vegetables was associated with a decreased risk of endometrial cancer in this study
Blekkenhorst, 2017	Australia	31.1 (mean)	79.4 (mean)	Published literature (27-29)	NR	To investigate the association of nitrate intake with atherosclerotic vascular disease mortality	Nitrate intake from vegetables was inversely associated with atherosclerotic vascular disease mortality independent of lifestyle and cardiovascular disease risk factors
Chen, 2002	USA	125 (mean)	133 (mean)	Nitrate content was determined from published literature (29-34)	NR	To investigate potential associations between diet and adult glioma	No associations were seen for dietary nitrate vegetables with risk of adult glioma

Cross, 2003)	USA	87 (WA of mean)	87.05 (WA of mean)	Nitrate content was determined from published literature (20-24)	NR	To investigate association between nitrate intake from diet, water and neural pancreatic cancer	No association between nitrate and pancreatic cancer
DellaValle, 2013	USA	224.4 (WA of median)	93.4 (mean)	Published literature (20,32,33,34,35)	NR	To evaluate the association between nitrite and nitrate intake and renal cell carcinoma	Total intake of nitrate and nitrite from processed meat sources was positively associated with renal cell carcinoma risk. No associations for nitrate intake overall from plant sources and renal cell carcinoma risk
DellaValle, 2014	China	197.6 (WA of median)	300.7 (median)	Published literature (26-30)	NR	To evaluate the association between nitrate and nitrite from diet and colorectal cancer in woman	High nitrate intake associated with an increased risk of colorectal cancer
Dich, 1995	Finland	---	77.5 (WA of mean)	In vegetables was mainly estimated based on analysed values on Finnish (32) or Swedish foods (17). Nitrate concentrations in salted and cured meat products were obtained from an official food quality control study completed in Finland in 1976 (27).	NR	To assess nitrate intake in Finnish population	Mean daily dietary intake of nitrate was 77 mg. More than 90% of dietary nitrate was derived from vegetables, including potatoes
Dubrow, 2010	USA	89.93 (WA of median)	89.2 (WA of mean)	Published literature(CSFII) (29,30)	NR	To evaluate the association between nutrients and glioma risk	Consumption of processed or red meat, nitrite, or nitrate does not meaningfully increase adult glioma risk and that consumption of fruit and vegetables, fruit and vegetable sub-groups, vitamin C, or vitamin E does not meaningfully protect against adult glioma risk
Ellen, 1990	Netherland	---	52 m (mean)	Duplicate portion method during 24h period. Samples were collected in two periods of one week: October 1948 and March 1985	NR	To determine the amount of nitrite and nitrate and other components in the duplicates of 24-h diets to establish the oral daily intake of these analyses	Average nitrate intake was 52 mg /day, about 25% of the ADI
Espejo-Herrera, 2016	Italy	110.2 (WA of mean)	112.0 (WA of mean)	Nitrate content (milligrams/100 grams) was calculated in food items including vegetables from [European Food Safety Authority	NR	To investigate the association between dietary nitrate and nitrite intake and breast cancer	Dietary nitrate was not associated with breast cancer regardless of the animal or vegetable source or of menopausal status

				(7)], animal products, and others from (12,17)			
Espejo-Herrera, 2016	Spain/Italy	112.2 (WA of median)	114.3 (WA of median)	Published food composition databases (26,27)	NR	To investigate the association between nitrate intake from diet and water and colorectal cancer	Dietary nitrate from animal sources increased rectal cancer risk, but high intake from vegetables seems to decrease it
Gonzalez, 1994	Spain	156.7 (mean)	175 (mean)	Published literature (10)	NR	To investigate the association between nutrients and gastric cancer risk	An inverse association with the risk for gastric cancer was seen for high intake of fibre, vitamin C, folate, carotene, and nitrates
Griesenbeck, 2010	USA	---	40.4 (median)	Multible reference databases were searched for published literature reflecting nitrate, nitrite, and nitrosamine values in foods and alcoholic beverages, steps of data collection in (34)	NR	To investigate the relationship between various maternal characteristics and intake of nitrates, nitrites, and nitrosamines from dietary sources	Median intake per day for nitrates was estimated at 40.4 mg. Results of this study indicate that intake of nitrates vary considerably by race/ethnicity and other characteristics
Hansson, 1994	Sweden	40 (mean)	42 (mean)	Published literature (23,25)	NR	To investigate the effect of dietary intake of polyphenols, nitrate and nitrite and other micro and macronutrients with gastric cancer risk	A negative association was seen between nitrates and risk of gastric cancer
Hernández-Ramírez, 2009	Mexico	101.9 (median)	108.91 (median)	By using Food Intake Analysis System computerized program version 3.0 (FIAS)	NR	To estimate the risk of gastric cancer in relation to the individual and combined consumption of polyphenols nitrate and nitrite	High consumption of nitrate was associated with an increased risk of gastric cancer
Inoue-Choi, 2012	USA	116.8 (WA of median)	116 (WA of median)	Published literature (32,33,34)	NR	To investigate the interaction of nitrate and folate on the risk of breast cancer among postmenopausal women.	Nitrate intake was not associated with breast cancer.
Inoue-Choi, 2014	USA	115 (WA of median)	145.9 (WA of median)	Published literature (18)	NR	To investigate the interaction of nitrate and nitrite on the risk of ovarian cancer among postmenopausal women	Dietary nitrate was inversely associated with ovarian cancer risk
Inoue-Choi, 2015	USA	---	Male: 68.9 Female: 74.1 (WA of mean:71)	Published literature (1,23,25,26,35–56)	NR	To describe the development of a dietary nitrate and nitrite database and its calibration	The performance of the FFQ in assessing dietary nitrate intakes is comparable to that for many other macro- and micronutrients

Jones, 2016	USA	24.22 (WA of mean)	24.9 (WA of mean)	Published literature (37)	NR	To investigate the ingestion of nitrate and nitrite from drinking water and diet and bladder cancer risk in women	Dietary nitrate intakes was not associated with bladder cancer. Long-term ingestion of elevated nitrate in drinking water was associated with an increased risk of bladder cancer among postmenopausal women
Jones, 2017	USA	26.22 (WA of mean)	26.6 (WA of mean)	Published literature (37)	NR	To evaluate exposure nitrate and disinfection byproducts in relation to kidney cancer risk	No association between dietary nitrate intake and kidney cancer risk
Jonvik, 2017	Netherland	---	106 (median)	Published literature (NR)	NR	To assess the habitual dietary nitrate intake and identify the main contributing food sources in a large group of highly trained athletes	Dietary nitrate intake was strongly associated with the intake of vegetable
Kang, 2016	USA	152 (WA of median)	251.8 (WA of mean)	US Department of Agriculture food composition was used (50)	NR	To evaluate the association between dietary nitrate intake, derived mainly from green leafy vegetables and glaucoma	Higher dietary nitrate and green leafy vegetable intake was associated with a lower glaucoma risk
Keszei, 2013	Netherland	103.75 (mean)	107.2 (mean)	Food-composition values for nitrate were derived from the databank on contaminants in food from the State Institute for Quality Control of Agricultural Products (RIKILT; Wageningen, Netherlands) (23-25).	NR	To evaluate the association between N-nitroso compounds and the risk of esophageal and gastric cancer subtypes	There was no significant association between any esophageal of gastric cancer subtype and dietary nitrate intake
Keszei, 2014	Netherland	96.9 (median)	99.1 (median)	Food composition values for nitrate were derived from the databank on contaminants in food from the State Institute for Quality Control of Agricultural Products (RIKILT). Estimations were based on the mean nitrate contents between 1985 and 1989.	NR	To investigate the association of nitrate intake with Barrett's oesophagus risk	Total nitrate intake was inversely associated with Barrett's disease risk in men and positively associated with it in women
Kilfoy, 2012	USA	95.9 (median)	NR	Published literature (14,15)	NR	To test the hypothesis that nitrate and nitrite intake affects Non-Hodgkin Lymphoma survival	No significant increasing trend of mortality for Non-Hodgkin Lymphoma survival for nitrate intake

Kilfoy, 2010	USA	116.5 (mean)	112.1 (mean)	Published literature (22,23)	NR	To investigate Non-Hodgkin Lymphoma risk overall and by histologic type in relation to dietary nitrate and nitrite intake	No association between risk of Non-Hodgkin Lymphoma and nitrate intake
Kilfoy, 2011	USA	80.8 (WA of median)	88 (mean)	The nitrate and nitrite contents for 29 vegetables, 19 meats, six preserved foods (including two processed meat items), rice and noodles, 13 dessert and bean items and eight fruits were detected using values from the published literature (44,45)	NR	To evaluate the relationship between nitrate intake and risk of pancreatic cancer	Nitrate intake was not associated with pancreatic cancer risk
Kilfoy, 2011	USA	53.9 (WA of median)	88 (mean)	Published literature (25,26)	NR	To evaluate the relationship between dietary nitrate and risk of thyroid cancer	Higher intake of dietary nitrate was associated with an increased risk of thyroid cancer in man and no association with dietary nitrate and cancer in women
Kilfoy, 2012	USA	98.5 (WA of median)	91.9 (mean)	Published literature (30)	NR	To evaluate the association between nitrate intake and ovarian cancer	Women in the highest intake quintile of dietary nitrate had a 31% increased risk of epithelial ovarian cancer, compared with those in the lowest intake quintile
Kilfoy, 2013	China	320.2 (WA of median)	309 (median)	Published literature (25-29)	NR	To evaluate the relationship between nitrate intake and risk of thyroid cancer in Shanghai	Nitrate intake was not associated with thyroid cancer risk
Kilfoy, 2013	USA	100.3 (mean)	103 (mean)	Published literature (24,25)	NR	To evaluate dietary sources of nitrate as risk factor for non-Hodgkin lymphoma overall and for non-Hodgkin lymphoma subtypes	No association found between nitrate intake and risk of non-Hodgkin lymphoma.
Kim, 2007	Korea	499.8 (mean)	533.5 (mean)	Published literature (32)	NR	To investigate the association between nitrate intake and risk of gastric cancer	A high nitrate intake combined with a lower folate consumption was associated with a high risk of gastric cancer
Knight, 1987	USA	-	109 (mean)	Published literature (3,11,49,55)	In saliva (133.91 nmol/ml)	To estimate total daily intake of nitrate	Vegetables contributed over 90% of the nitrate intake

Knight, 1990	UK	-	125.4 (WA of mean)	Published literature (17) with the help of an Italian nutritionist	In saliva (76.8 nmol/ml)	To evaluate the association between gastric cancer mortality rates and estimated dietary nitrate intake	No association was found between nitrate and gastric cancer mortality rates
La Vecchia, 1994	Italy	78.4 (WA of mean)	89.22 (WA of mean)	Italian tables of food composition (22)	NR	To investigate the relationship between nutrients intake and gastric cancer	Consumption of nitrate found to be protective
Laitinen, 1993	Finland	---	57.82 (WA of mean)	Database: NBTCA, National Board of Trade and Consumer Affairs in Finland and published literature (7,18,31,36,37,38)	NR	To estimate total daily intake of nitrate in Finnish	Vegetables including potatoes contributed 86% of nitrate intake
Michaud, 2009	USA	NR	HPFS = 142.6 NHS I = 85 NHS II = 130.2 (WA of mean)	Published literature (15-39)	NR	To examine the relation between intakes of meats, nitrate, nitrite, and 2 nitrosamines [nitrosodimethyl-amine (NDMA) and nitrosopyrolidine (NPYR)] and glioma risk	No association between nitrate and glioma
Mitacek, 2008	Thailand	---	155.7 (mean)	1-Analytical studies of food composition in specific Thai communities (5,7, 8–10). 2- In real food samples, nitrate concentration were Griess method	NR	To determine and to report on the mean daily dietary intake of (nitrate, nitrite, nitrosodimethyl-amine) in Thailand	Significant differences in dietary nitrate, nitrite, and nitrosodimethyl-amine intakes were seen between various Thai regions
Palli, 2001	Italy	NR	96.4 (WA of mean)	Italian tables of food composition (14 as in original paper)	NR	To better understand the role of overall dietary patterns and major energy-providing components in gastric cancer etiology	Significant inverse associations with gastric cancer risk were shown for nitrate
Pobel, 1995	France	143 (mean)	143.19 (mean)	Food composition table based on literature data (10)	NR	To investigate the association between nitrate, nitrite, nitrosodimethyl-amine intakes with the risk of gastric cancer	Nitrate intake was not associated with an increased risk of stomach cancer
Quist, 2018	USA	26.88 (WA of mean)	61.8 (median)	Published literature (39,40)	NR	To investigate the association between pancreatic cancer and nitrate intake	No association between nitrate intake and pancreatic cancer

Rogers, 1995	USA	Nitrate intake in Larynx cancer: 157.2 (WA of mean) Nitrate intake in Oesophagus cancer : 178.7 (WA of mean) Nitrate intake in oral cavity cancer: 173.3 (WA of mean) WA of mean all patients = 169.6	185.7 (WA of mean)	Published literature (4)	NR	To evaluate whether dietary sources of nitrate and drinking water as increase risk of laryngeal, esophageal and oral cancer	Nitrate intake was associated with a reduction in cancer risk at all three sites
Stephany, 1980	Netherland	---	179 (mean)	Duplicate all food and drinks during 24h sampling period, Griess method was used to measure nitrate	In saliva (NR)	To assess nitrate intake	Mean nitrate intake was equivalent to 179 mg/day
Temme, 2010	Belgium	---	96 (mean)	Already existing data on nitrate concentrations in fresh vegetables and fruits from Belgium were used from (6). The average nitrate content of bottled water, as communicated by the industry to the Federal Public Service of Health. Also they collected food samples to ensure representative concentration data, and they used HPLC system to quantify nitrate.	NR	To re-estimate the nitrate and nitrite intake via the diet in Belgium by use of actual concentrations and individual food consumption data	Exposure of the Belgian population to nitrate at a mean intake corresponded to 38% of the ADI
Vaessen, 1999	Netherland	---	80 (mean)	Duplicate 24 h diet samples were collected in 2 different periods; 1 week in March and 1 week in September, 1994. High performance Ion Chromatography	NR	To determine the amount of nitrite and nitrate in the duplicates of 24-h diets to establish the oral daily intake of these analyses.	The daily intake for nitrate was higher than that found in the duplicate diet study carried out in 1984/1985, when an average daily intake of 52mg/person was measured.

				separation and UV detection to determine nitrate in food samples.			
van Loon, 1998	Netherland	108 (mean)	111 (mean)	Databank on contaminants in food from the State Institute for Quality Control of Agricultural Products (RIKILT. Wageningen). (23)	NR	To examine the association between nitrate intake and gastric cancer	This study does not support a positive association between the intake of nitrate and gastric cancer risk
Vandenbran, 1989	UK	---	141.9 (WA of mean)	Published literature (6)	In urine (M: 0.08±0.04 F:0.05±0.02 (mmol/hr))	To investigate the relationship between dietary nitrate intake and urinary excretion of nitrate	This study suggest that a simple self-administered questionnaire may provide useful information on usual nitrate intake
Virtanen, 1993	Finland	Fathers (F): 78 (mean) Mothers (M): 101 (mean) WA of mean (F+M) = 90.2	Fathers (F): 85 (mean) Mothers (M): 108 (mean) WA of mean (F+M)= 97.2	Published literature (19)	NR	To evaluate whether child's or parent's intakes of nitrate and nitrite from food and drinking water were associated with the risk for Type 1 diabetes in a large population-based Finnish case-control series	The nitrate intake of the children and fathers was not associated with the risk for Type 1 diabetes in children. Mothers' nitrate intake was associated with a decreased risk for Type 1 diabetes in children
Ward, 2006	USA	108.6 (WA of mean)	120.5 (WA of mean)	Published literature (18,19)	NR	To evaluate whether dietary sources of nitrate and drinking water as a risk factors for non-Hodgkin lymphoma	Dietary nitrate was inversely associated with risk of non-Hodgkin lymphoma
Ward, 2010	USA	33.0 (WA of mean)	37.4 (WA of mean)	Harvard nutrient database updated in 2008 for specific food (25,27)	NR	To study the association between nitrate intake and risk of thyroid cancer	Increase intake of nitrate is associated with increased risk of thyroid cancer
Yang, 2010	Korea	421 (mean)	424 (mean)	Database reported in (29) (137 food items) and Lee. (30) (16 food items).	NR	To evaluate the association between nitrate intake relative to antioxidant vitamins with the risk of breast cancer	A high nitrate intake combined with a lower folate consumption was associated with a higher risk of breast cancer
Zeegers, 2006	Netherland	109.8 (mean)	109.4 (mean)	Databank on contaminants in food from the State Institute for Quality Control of Agricultural Products (RIKILT; Wageningen, the Netherlands) (van Loon 1997 et al. 1998). (8)	NR	To study the association between nitrate intake and bladder cancer incidence in more detail in the Netherlands Cohort Study.	There is no association between nitrate intake and bladder cancer

FFQ; Food frequency questionnaire, 24h recall; 24 hour recall, NR; Not reported

Appendix 2.7: Characteristics of the 9 identified studies (after the systematic review was published) including the nitrate intake and methods used for its assessment

Study	Study design	Country	Sample size and cases (if present)	Duration	Age	Sex(m/f)	Dietary assessment	Nitrate intake (mg/day)	Method Declared by Authors to calculate nitrate intake
(Barry *et al.*, 2020)	Case-control	USA	Cases of BC: 987 Control: 1180	3 years	30–79	1737/595	124-FFQ	Cases: 34 Control: 35	A database developed by (Inoue-Choi *et al.*, 2016).
(Bahadoran *et al.*, 2019)	Cross-sectional	Iran	Total: 250	-	20-70	NR	168-FFQ	505	A database (Bahadoran *et al.*, 2016b)
(Gopinath *et al.*, 2018)	Cross-sectional	Australia	Total: 3654	-	65 (mean)	1236/2418	145-FFQ	187	3 databases (Griesenbeck *et al.*, 2009; Inoue-Choi *et al.*, 2016; Blekkenhorst *et al.*, 2017b).
(Gopinath *et al.*, 2018)	Cohort	Australia	Total: 2856 Early AMD: 4% Late AMD:15%	15 years	64 (mean)	881/1156	145-FFQ	Reported in quartile without number of participants in each quartile	3 databases (Griesenbeck *et al.*, 2009; Inoue-Choi *et al.*, 2016; Blekkenhorst *et al.*, 2017b).
(Jackson *et al.*, 2018a)	Cohort	Australia	Total: 8161	12 years	62-67	All f	FFQ	65-70	3 databases (Griesenbeck *et al.*, 2009; Inoue-Choi *et al.*, 2016; Blekkenhorst *et al.*, 2017b).
(Jackson *et al.*, 2019)	Cohort	USA	Total: 62,536 CHD: 2267	26 years	30-55	NR	126-FFQ	Total:152 CHD:155	US Department of Agriculture food composition tables.
(Jones *et al.*, 2019a)	Cohort	USA	Total:15,910 CC: 624 RC: 158	14 years	61 (mean)	NR	127-FFQ	Total: 60.5 Cases: NR	Published literature.
(Liu *et al.*, 2019)	Cohort	Australia	Total: 2229 Mortality (CVD, CHD and Stroke): 610	14 years	64 (mean)	915/1314	145-FFQ	Total: 128.8	3 databases (Griesenbeck *et al.*, 2009; Inoue-Choi *et al.*, 2016; Blekkenhorst *et al.*, 2017b).

Study	Study design	Country	Sample size and cases (if present)	Duration	Age	Sex(m/f)	Dietary assessment	Nitrate intake (mg/day)	Method Declared by Authors to calculate nitrate intake
(Sim *et al.*, 2019)	Cross-sectional	Australia	Total: 1420	-	75 (mean)	NR	74-FFQ	79.5	A databases developed by (Blekkenhorst *et al.*, 2017b) and published literature.
(Zheng *et al.*, 2019)	Case-control	USA	Cases of PC: 957 Control: 938	7 years	50 - ≥70	1102/793	86 and 131-FFQs	Cases: 44.21 Control: 43.69	N-nitroso database consisting of 21different N-nitroso compounds as well as nitrate and nitrite (i.e., a total of 23 items) for 500 foods from 39 different food groups (Stuff *et al.*, 2009).

AMD; Age-related macular degeneration, BC; Bladder cancer, CC; Colorectal Cancer, FFQ; Food frequency questionnaire, RC; Rectal cancer, PC; Pancreatic cancer, CVD; Cardiovascular disease, CHD; Coronary heart disease

Appendix 3.1: Published paper (Effects of inorganic nitrate and nitrite consumption on cognitive function and cerebral blood flow: A systematic review and meta-analysis of randomized clinical trials

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Effects of inorganic nitrate and nitrite consumption on cognitive function and cerebral blood flow: A systematic review and meta-analysis of randomized clinical trials

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ABSTRACT

We conducted a systematic review and meta-analysis of randomized clinical trials examining the effect of inorganic nitrate or nitrite supplementation on cognitive function (CF) and cerebral blood flow (CBF). Two databases (PubMed, Embase) were searched for articles from inception until May 2017. Inclusion criteria were: randomized clinical trials; participants >18 years old; trials comparing a nitrate/nitrite intervention with a control. Thirteen and nine trials were included in the meta-analysis to assess CF and CBF, respectively. Random-effects models were used and the effect size described as standardized mean differences (SMDs). A total of 297 participants (median of 23 per trial) were included for CF; 163 participants (median of 16 per trial) were included for CBF. Nitrate/nitrite supplementation did not influence CF (SMD +0.06, 95% CI: -0.06, 0.18, P = 0.32) or CBF under resting (SMD +0.14, 95% CI: -0.13, 0.41, P = 0.31), or stimulated conditions (SMD + 0.23, 95% CI: -0.11, 0.56, P = 0.19). The meta-regression showed an inverse association between duration of the intervention and CBF (P = 0.02) but no influence of age, BMI or dose ($P < 0.05$). Nitrate and nitrite supplementation did not modify CBF or CF. Further trials employing larger samples sizes and interventions with longer duration are warranted.

KEYWORDS

Cerebral blood flow; cognitive function; inorganic nitrate; nitrite; nitric oxide

Introduction

Cognitive impairment and dementia are global health challenges because of the costs associated with management and treatment, severity of symptoms for the affected individual and impact on patients' families, carers and communities (Wortmann, 2012). Furthermore, the prevalence of people diagnosed with dementia is rising at an alarming rate, with a recent report estimating that by 2050 the total number of individuals living with dementia worldwide will increase from 47 to 131 million (Prince et al. 2016). Therefore, effective interventions to prevent cognitive decline and dementia onset are a global research priority.

A major risk factor for cognitive decline is thought to be inadequate nitric oxide (NO) availability (de la Torre and Stefano 2000; Toda, Ayajiki, and Okamura 2009). NO is a free radical soluble gasotransmitter with pleiotropic actions, of which several are integral to normal cognitive function (CF), including regional blood flow, immune-surveillance, metabolic efficiency, glucose homeostasis, and neurotransmission (Toda, Ayajiki, and Okamura 2009; Weitzberg and Lundberg 2013). NO availability is determined by the activity of NO synthases (NOS), which are widely distributed across tissues in different isoforms (endothelial, inducible, neuronal) (Lundberg, Weitzberg, and Gladwin 2008; Weitzberg and Lundberg 2013). In cognitive decline, NO generation via these pathways becomes dysregulated resulting in chronic hypo-perfusion, neurodegeneration and impaired cognitive ability (de la Torre and Stefano 2000; Toda, Ayajiki, and Okamura 2009).

NO can also be produced by a distinct alternative pathway involving the conversion of nitrate into nitrite and NO via a series of reducing reactions (Lundberg, Weitzberg, and Gladwin 2008; Zweier, Wang, Samouilov, and Kuppusamy 1995). Both nitrate and nitrite are present in a wide range of concentrations in a variety of foods with the higher content found in green leafy vegetables, beetroot, or meat products that have had nitrite salts added as preservatives (Lidder and Webb 2013). In the past decade, it has emerged that increasing nitrate and nitrite ingestion may improve vascular and metabolic outcomes via increased generation of NO (Weitzberg and Lundberg 2013). More recent evidence also indicates potentially beneficial effects of both compounds, administered as ionic

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salts or nitrate-rich food products, on cognition and brain metabolic and vascular health (Clifford, Willett and Ding 2015; Gilchrist et al. 2014; Justice et al. 2015; Presley et al. 2011; Wightman et al. 2015). Such effects could be due to improved NO-mediated synaptic activity and/or as a consequence of increased cerebral blood flow (CBF) and thus a better coupling of blood flow to metabolism (Presley et al. 2011; Toda, Ayajiki, and Okamura 2009; Aamand et al. 2013).

Mechanistic support for the latter hypothesis in humans has been provided by Presley and colleagues (2011), who observed that a diet rich in nitrate-containing foods (e.g., green leafy vegetables) stimulated cerebral perfusion in the prefrontal cortex of elderly adults, the region of the brain associated with executive function, working memory, and other processes reliant on cognitive ability. However, subsequent studies measuring CBF, or directly measuring CF, after inorganic nitrate or nitrite ingestion have produced mixed findings, possibly because of the small size and diversity of study designs employed (Clifford et al. 2015; Kelly et al. 2013). Thus, despite the therapeutic potential, it remains unclear whether augmenting NO bioavailability with either nitrite salts or nitrate-rich foods is an effective strategy for increasing CBF and/or mitigating cognitive deficits.

Consequently, we undertook a systematic review and meta-analysis of randomised clinical trials (RCTs) examining the efficacy of inorganic nitrate and nitrite supplementation on CBF and CF in adult participants with and without medical conditions. We set out to determine whether the ingestion of nitrite-salts or nitrate-rich foods (i.e., beetroot, spinach, rocket, lettuce, cabbage; Lidder and Webb 2013) augments CBF and improves CF and to estimate effect sizes. We also examined whether test conditions (e.g., exercise vs. rest), age, body mass index (BMI), supplement dose, quality of the studies and intervention duration modified the effects of inorganic nitrate or nitrite on CBF and CF. These results will help to inform whether nitrate or nitrite supplementation holds promise as a relatively inexpensive strategy for augmenting CBF and combatting cognitive decline.

Methods

The present systematic review was conducted according to the Cochrane guidelines and it is reported according to PRISMA guidelines (Higgins and Green 2011; Liberati et al. 2009). The protocol of the systematic review is available on request.

Literature search

Two databases (PubMed, Embase) were searched for articles from inception until May 2017. In addition, included reviews and eligible full text articles were searched manually to identify other suitable articles to be included in the systematic review. The following terms and keywords were entered and Boolean terms were used to increase sensitivity of the search strategy: nitrate, nitrite, beetroot, rocket, cabbage, lettuce, spinach, green leafy vegetables, cognition, brain, dementia, cerebral, memory, executive, attention, motor skills, blood flow, vascular flow, perfusion. A summary of the specific search algorithms is reported in the **Online Supplementary Material (Box 1).**

Study selection

Titles and abstracts were screened using pre-defined eligibility criteria in accordance with the PICOS (population, intervention, comparator, outcome, study design) framework (Table S1 of the Online Supplementary Material) before retrieval of the full-text articles. The following inclusion criteria were used to assess the eligibility of articles for inclusion in this systematic review: 1) randomized controlled trials (no exclusion criteria were used for study design, or blinding); 2) trials recruiting adult participants (≥18 years) and no exclusion criteria were applied in relation to participants' health status; 3) trials based on nitrate or nitrite supplementation were included if they provide information on the type of nitrate salt (potassium or sodium), dose, formulation, frequency and route of administration. A list of .the inclusion and exclusion criteria is provided in the Online Supplementary Material (Box 2). Trials based on beet root juice supplementation or ingestion of nitrate-rich foods were included in the analyses if they provided information on the frequency and amount of nitrate-containing food provided; 4) trials reporting effects of nitrate or nitrite on global and domain-specific CF and CBF measured by different techniques including magnetic resonance imaging (MRI), ultrasound or near infrared spectroscopy (NIRS); 5) English-language restriction but not time restriction was applied in searching the databases; 6) Full text papers and abstracts were included (if they contained sufficient information to complete qualitative and quantitative analysis). Two investigators (TC, OS) independently evaluated the titles and abstracts to check eligibility for inclusion. If the reviewers agreed, each article was either excluded or moved to the next stage (full-text). If agreement was not achieved, the article was moved for evaluation after retrieval of the full-text. The selected full-texts were then reviewed to confirm their inclusion in the systematic review. Disagreements were discussed with a third reviewer (MS) and resolved by consensus.

Data extraction

Relevant information was extracted and tabulated separately for CF and CBF. If information was not available from the full text, authors were contacted to obtain the relevant data.

Cognitive function

The following information was extracted independently by two investigators (AB, TC) from eligible articles: 1) authors and year of publication; 2) study characteristics (design, sample size); 3) participant characteristics (age, male/female ratio, health status and baseline values for BMI; 4) route, dose and duration of inorganic nitrate and nitrite supplementation; and 5) cognitive tests and exercise condition. Any disagreements in data extraction were resolved through discussion until consensus was reached.

Cerebral blood flow

Two independent reviewers (AB, MS) extracted relevant information from the eligible articles: 1) authors and year

of publication; 2) study characteristics (design, sample size); 3) participant characteristics (age, male/female ratio, health status and baseline values for BMI, and 4) route, dose and duration of inorganic nitrate/ nitrite supplementation 5) method to assess cerebral blood flow (CBF) and testing conditions (i.e., exercise, mental stimulation). Any disagreements in data extraction were resolved through discussion until consensus was reached.

Quality assessment

The modified Jadad score was applied to evaluate the risk of bias of the trials. Specific questions linked to randomization procedure, blinding and description of dropout or attrition rates were used rank the quality of the trials (Jadad et al. 1996). Scores ranged from 0 to 5; a score less than 3 indicates a low quality trial where a score greater or equal to 3 indicates high quality trial.

Statistical analysis

The primary outcomes of the meta-analysis were changes in CF and CBF after inorganic nitrate or nitrite supplementation. Random effect models were applied to address the heterogeneity related to differences in study design and application of different and concomitant methods for the evaluation of CF and CBF. In addition, some trials used several cognitive tests to assess domain-specific changes in CF and CBF, as shown in Table 1 and 2. This may lead to reduced independence of measurements and to consequential over-estimation of the effect size derived from the meta-analysis. These methodological aspects were taken into account into the analysis by averaging the standardised effect sizes for each trial with the aim of providing a more conservative estimate of the effect size. Forest plots were created to summarise and illustrate the individual and overall effects of inorganic nitrate and nitrite supplementation on CF and CBF. The meta-analysis was conducted using Comprehensive Meta-Analysis software (Biostat, Engelwood, New Jersey). Results are described as standardized mean differences (SMDs) and 95% confidence intervals (95%CI). If data were not available in the main text or in tables, figures were used to extract the information.

Sensitivity analyses were performed to investigate whether the effects of inorganic nitrate and nitrite supplementation on CF and CBF were influenced by testing conditions (i.e., exercise or mental stimulation). A random-effect meta-regression model was applied to examine the associations between effect sizes for CF and for CBF and age, BMI, dose of nitrate/nitrite supplementation, duration of the trial and Jadad score. Funnel plots and Egger's regression tests were performed to evaluate the publication bias (Egger, Smith, Schneider, and Minder 1997). Heterogeneity was assessed by using Cochrane Q statistic; $P > 0.1$ indicates significant heterogeneity. The I2 test was utilised to assess heterogeneity across trials where a value < 25% indicates low risk, 25–75% indicates moderate risk, and >75% indicates a high risk (Higgins, Thompson, Deeks, and Altman 2003).

Results

Search results

The screening process and the number of the studies included in the systematic review are described in Figure 1. The initial search of the two electronic databases produced 12865 articles which was reduced to 5387 after the deletion of duplicates. No relevant studies were found by manual search of relevant reviews and studies. After the first title and abstract selection phase, 23 full-text articles were identified for further assessment and, from these, 18 trials were included in the systematic review. Thirteen trials and nine trials were included in the meta-analysis to investigate effects of nitrate and nitrite supplementation on CF and CBF, respectively.

Cognitive function

Studies characteristics

The trials included in the systematic review reported on a total of 297 participants with a median of 23 (range 10–48) participants per trial. The median age of the participants was 36 (range 21 – 73) years. The systematic review includes 2 parallel and 11 crossover trials and 12 of them were double-blind. Six of these studies included an exercise component as part of the protocol to evaluate the effects of dietary nitrate and nitrite on CF at rest and during exercise. The large majority (12 of 13 studies) supplemented with nitrate or nitrate-rich foods; eleven trials used beetroot and one trial used spinach as sources of inorganic nitrate, and one study supplemented with sodium nitrite (see Table 1). As placebo, eight trials used nitrate-depleted beetroot juice (Kelly et al. 2013; Gilchrist et al. 2014; Lefferts et al. 2015; Rattray et al. 2015; Thompson et al. 2015; Thompson et al. 2016; Vanhatalo et al. 2016; Shannon et al. 2017), one studied employed nitrite-free capsules (Justice et al. 2015), two trials combined apple and blackcurrant juice (Thompson et al. 2014; Whitman et al. 2015) and one study did not report information on the control group (Bondonno et al. 2014).

Participant health status and intervention duration

Two trials included patients with type 2 diabetes (T2DM) (Gilchrist et al. 2014; Shepherd et al. 2015), four trials included middle-aged and older healthy participants (Kelly et al. 2013; Bondonno et al. 2014; Justice et al. 2015; Vanhatalo et al. 2016) and the remaining seven trials recruited young healthy participants (Table 1). The median BMI of the adults included in the trials was 24.6 kg/m^2 (range: 24.0 – 30.8 kg/m^2). The duration of interventions ranged from 90 minutes to 10 weeks but ten trials (out of 13) had a duration less than 7 days. For nitrate supplementation studies, the median dose of inorganic nitrate provided was 7.2 mmol/day (range: 2.9 – 12.8 mmol/day); the trial using nitrite supplemented with 2.4 mmol/day of sodium nitrite (Justice et al. 2015).

The greatest source of heterogeneity in the CF trials was the type of cognitive assessment with 23 different tests being reported. Three trials used a single CF test (Rattray et al. 2015; Thompson et al. 2015; Vanhatalo et al. 2016) whereas one trial employed eight different CF tests (Lefferts et al. 2015). A summary of the distribution of cognitive tests per trial is provided

Table 1. Characteristics of the studies included in the systematic review and meta-analysis of the effects of dietary nitrate/nitrite on cognitive function.

Author (year)	Country	Study Design	Sample Size	Health Status	Age (years)	Males	Nitrate Dose (mmol/day)	Type of Intervention	Placebo	Duration of intervention	Baseline BMI (Kg/m^2)	Cognitive Tests	Exercise Testing?	Jadad Score
Bondonno et al. 2014	Australia	CO, R, UB	30	Healthy Middle-Aged	47.3	6	2.9	SP	—	150 min	23.6	SRT, DV, CRT, SM, NWM, DWR	NO	2
Gilchrist et al. 2014	UK	DB, CO, R, PI	27	T2DM	67.2	18	7.5	BJ	ND-BJ	14 days	30.8	SRT, SM, RVIP, DRT, SPM	NO	3
Justice et al. 2015	USA	DB, P, PI, R	30	Healthy Older	62	16	1.2/2.4	SN	NF-C	10 weeks	24.9	TMT-A TMT-B	NO	4
Kelly et al. 2013	UK	DB, CO, R, PI	12	Healthy Older	64	6	9.6	BJ	ND-BJ	3 days	24.1	RVIP, SS, NR	NO	3
Lefferts et al. 2015	USA	DB, CO, R, PI	20	Healthy, Young	23	20	6.5-7.0	BJ	ND-BJ	120 min	24.6	MR, ER, DV, AST, CRT, MZ, CPT, GNG	YES	3
Rattray et al. 2015[a]	Australia	DB, CO, R, PI	12	Healthy, Young	—	—	12	BJ	ND-BJ	120 min	—	CST	YES	—
Shannon et al. 2017	UK	DB, CO, R, PI	10	Healthy, Young	23	10	12.5	BJ	ND-BJ	175 min	23.9	SST, AST, RVIP	YES	3
Shepherd et al. 2015[a]	UK	DB, CO, R, PI	48	T2DM	63.3	35	6.4	BJ	ND-BJ	4 days	30.1	SRT, SM, CST	NO	
Thompson et al. 2015	UK	DB, CO, R, PI	16	Healthy, Young	24	16	12.8	BJ	ND-BJ	7 days	24.6	CST, DRT	YES	3
Thompson et al. 2014	UK	DB, CO, R, PI	16	Healthy, Young	24	16	5	BJ	BCJ+AJ	90 min	24.1	RVIP, CST	YES	3
Thompson et al. 2016	UK	DB, CO, R, PI	36	Healthy, Young	24	36	6.4	BJ	ND-BJ	5 days	24.6	CST	YES	3
Vanhatalo et al. 2016[a]	UK	DB, CO, R, PI	30	Healthy Older	73	10	12	BJ	ND-BJ	10 days	25	RVIP	NO	—
Wightman et al. 2015	UK	P, DB, R, PI	40	Healthy, Young	21	12	5.5	BJ	BCJ+AJ	90 min	24	SS, RVIP, MFT	NO	3

BCJ+AJ, blackcurrant cordial JUICE and apple juice; BMI, body mass index; CAD, coronary artery diseases; CO, crossover; Conc, concentration; DB, double-blind; NF-C, nitrite free capsules; P, Parallel; PI, placebo-controlled; R, Randomized SB, single-blind; SN, Sodium Nitrite; SP, spinach T2DM, type 2 diabetes; UB, non-blind. SS, Serial Subtractions, RVIP, Rapid Visual Information Processing; MFT, Mental Fatigue Test; CST, Colour Stroop Test; SRT, Simple Reaction Time; SM, Shape Memory; DRT, Decision Reaction Time; SM, Spatial Memory; DV, Digit Vigilance; CRT, Choice Reaction Time; NWM, Numeric Working Memory; DWR, Delayed Work Recognition; AST, Attention Switching Task; SST, Spatial Span Task; TMT-A, Trail Making Tests A; TMT-B, Trail Making Test-B; NR, Number Recall; MR, memory recognition; ER, Emotion Recognition; VS-1, Visual Interference; VB-1, Verbal Interference; MZ, Maze, CPT, Continuous Performance Test; GNG, Go/No-Go. [a] These are abstracts and the quality assessment was not performed.

Table 2. Characteristics of the studies included in the systematic review and meta-analysis of the effects of dietary nitrate/nitrite on cerebral blood flow.

Author (year)	Country	Study Design	Sample Size	Health Status	Age (years)	Males	Nitrate Dose (mmol/day)	Type of Intervention	Placebo	Duration of intervention	Baseline BMI (Kg/m^2)	CBF Assessment	Exercise Testing?	Effect at resting	Effect in stimulated conditions	Jadad Score
Aamand et al. 2013	Denmark	DB, PL, CO, R	20	Healthy, Young	25	20	7.7	SNA	NF-S	3 days	—	ASL	NO	No change	—	2
Bond et al. 2013	USA	PL, CO, R	12	Healthy, Young	20	—	5-6	BJ	OJ	120 min	24.4	CVRI, MCAV	YES	Positive	Positive	1
Chirinos, 2017	USA	DB, CO, R, PI	17	HFpEF	65	14	12.9	BJ	ND-BJ	150 min	34.4	CCID, CCSA, CBVRD	NO	Positive	—	4
Curry et al. 2016	USA	PL, CO, R	10	Healthy, Young	20	—	24.2	BJ	OJ	120 min	23.5	MCAV	YES	Positive	Positive	1
Lefferts et al. 2015	USA	DB, CO, R, PI	20	Healthy, Young	23	20	6.5-7.0	BJ	ND-BJ	120 min	24.6	MCAV	YES	No change	No change	3
Presley et al. 2011	USA	R,CO	16	Healthy, Old	≥ 70	NR	12.4	High nitrate diet	Low nitrate diet	2 days	—	ASL	NO	Positive (reginal cerebral perfusion)	—	2
Rattray et al. 2015[a]	Australia	DB, CO, R, PI	12	Healthy, Young	—	—	13	BJ	ND-BJ	120 min	—	MCAV	YES	Positive	—	—
Thompson et al. 2014	UK	DB, CO, R, PI	16	Healthy, Young	24	16	5	BJ	BCJ+AJ	90 min	24.1	NIRS	YES	Positive	Positive	3
Wightman et al. 2015	UK	P, DB, R, PI	40	Healthy, Young	21	12	5.5	BJ	BCJ+AJ	90 min	24	NIRS	NO	Positive	Negative	3

BCJ+AJ, blackcurrant cordial JUICE and apple juice; OJ, Orange juice; SN, Sodium Nitrate; NF-S, Nitrate free solution; BJ, Beetroot juice; ND-BJ, Nitrate depleted beetroot juice BMI, body mass index; CO, crossover; DB, double-blind; NF-C, nitrite free capsules; P, Parallel; PI, placebo-controlled; R, Randomized SB, single-blind; ASL, Arterial spin labelling; CVRI, Cerebrovascular resistance index; SBP, Systolic blood pressure; TVR, Total vascular resistance; MCAV, Middle cerebral artery blood flow velocity; HFpEF, Heart failure preserved left ventricular ejection fraction; CCID, Carotid characteristic impedance, dynes; CCSA, Carotid cross-sectional area; CBVRD, Carotid bed vascular resistance, dynes.
[a]This is an abstract and the quality assessment was not performed.

Table 3. Meta-regression analysis to evaluate whether age, BMI, dose of nitrate and duration of the intervention modified the effects of nitrate/ nitrite supplementation on cognitive and cerebral blood flow.

	Slope (β)	SE	Q (df)	*P*
Cognitive function (n = 13)				
Age (years)	-0.002	0.003	0.92 (1)	0.33
BMI (kg/m^2)	-0.02	0.02	1.38 (1)	0.23
Dose (mg/day)	0.002	0.004	0.22 (1)	0.63
Duration (hours)	0.0005	0.0002	3.20 (1)	0.07
Jadad	0.30	0.18	2.85 (1)	0.09
Resting CBF (n = 9)				
Age (years)	0.001	0.006	0.09 (1)	0.98
BMI (kg/m^2)	0.02	0.032	0.58 (1)	0.45
Dose (mg/day)	-0.02	0.023	0.43(1)	0.51
Duration (hours)	-0.001	0.0006	5.07 (1)	0.02
Jadad	0.03	0.13	0.06 (1)	0.79

BMI, Body Mass Index; SE, standard error; CBF, cerebral blood flow.

in Table 1 whereas the frequency of application of each test across all the trials is summarised in **Figure S1** of the **Online Supplementary Material.**

Meta-analysis

Overall, inorganic nitrate or nitrite supplementation did not improve CF (SMD +0.06, 95% CI: -0.06, 0.18, P = 0.32) and we observed no significant heterogeneity between studies (I2 = 0%; P = 0.68) (Figure 2). However, the only study which supplemented healthy older individuals with inorganic nitrite for 10 weeks reported a significant improvement in CF (Justice et al. 2015). When stratified by inclusion of exercise testing in the protocols, there was no significant effect of inorganic nitrate supplementation in either the exercise (N = 6, SMD +0.13, 95% CI: -0.05, 0.32, P = 0.16) or non-exercise (N = 7, SMD +0.02, 95% CI: -0.15, 0.21, P = 0.76) trials. Meta-regression analysis did not reveal any significant association between CF effect size and age (β: -0.002, SE: 0.003, P = 0.33), BMI, (β: -0.02, SE: 0.02, P = 0.23), dose (β: 0.002, SE: 0.004, P = 0.63), study duration (β: 0.0005, SE: 0.0002, P = 0.07) or Jadad score (β: 0.09, SE: 0.18, P = 0.09) (Table 3).

Study quality and publication bias

The quality of the trials ranged from 2 to 5 (median: 3) on the Jadad score and only one study had a score < 3 (Bondonno et al. 2014), indicating the overall high quality of the trials (Table 1). Visual inspection of the Funnel Plot revealed a study with a large positive effect size and the presence of publication bias was also confirmed by the Egger's Regression test (p = 0.01; **Figure S3** of the **Online Supplementary Material**).

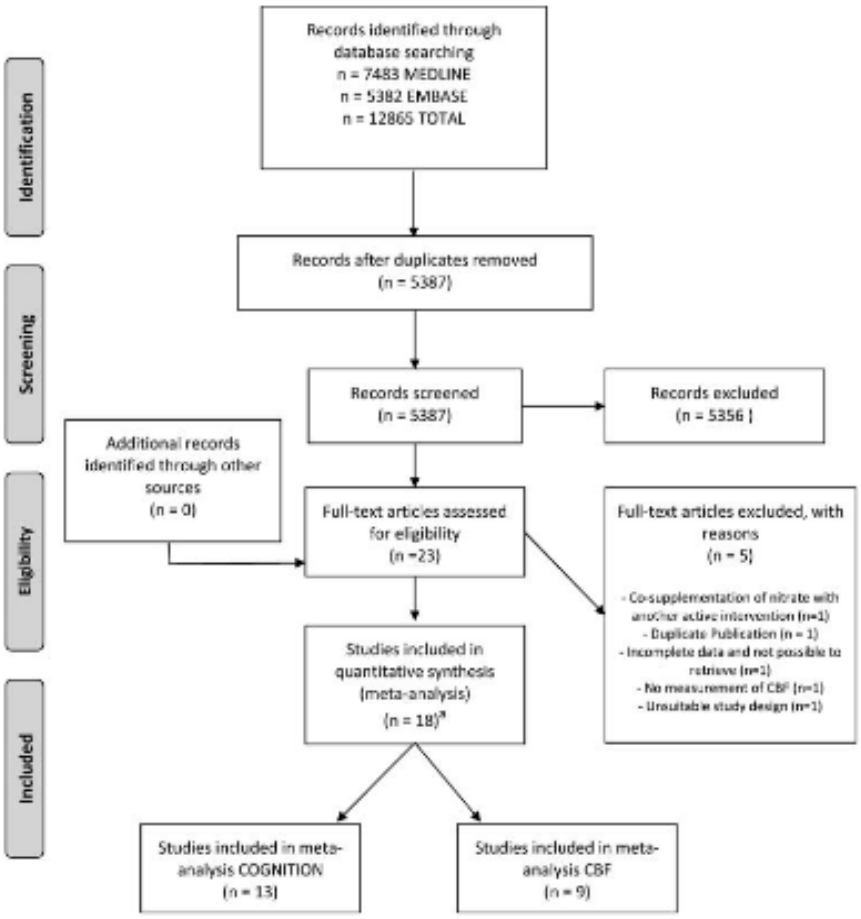

Figure 1. Flow diagram of the process used in selection of the randomised controlled trials included in this systematic review and meta-analysis

Exclusion of the study (Justice et al. 2015) with the largest positive effect size removed the publication bias (N = 12, Egger's test, P = 0.13).

Resting and stimulated CBF

Studies characteristics

Nine trials assessed changes in CBF in resting conditions and included a total of 163 participants (sample size range: 10 – 40); the overall median age of the participants was 22 years (range 20 – 70). Five of these studies also assessed CBF under stimulated conditions (i.e., exercise (Bond et al. 2013; Curry et al. 2016; Lefferts et al. 2015; Thompson et al. 2014), or mental stimulation (Wightman et al. 2015).

One study employed a parallel study design (Whitman et al. 2015) whereas all remaining eight trials used a cross-over design (Aamand et al. 2013; Bond et al. 2013; Chirinos et al. 2017; Curry et al. 2016; Lefferts et al. 2015; Presley et al. 2011; Rattray et al. 2015; Thompson et al, 2014) (Table 2). Most studies (seven) used beetroot juice as a source of inorganic nitrate but high nitrate foods or sodium nitrite were also used in some studies (Table 2).

Cerebral blood flow tests

Four studies reported the effect of inorganic nitrate supplementation on middle cerebral artery blood flow velocity (MCAV) (Aamand et al. 2013; Curry et al. 2016; Lefferts et al. 2015; Rattray et al. 2015) and two reported the effect of inorganic nitrate on CBF measured by arterial spin labelling (Presley et al. 2011; Aamand et al. 2013). Additional measurements used to assess CBF included Near Infrared Spectroscopy (Thompson et al, 2014; Whitman et al. 2015), cerebrovascular resistance index by Transcranial Doppler Ultrasonography (Bond et al. 2013) and evaluation of changes in Carotid Characteristic Impedance, Carotid Cross-Sectional Area and Carotid Bed Vascular Resistance (Chirinos et al. 2017). The frequency of application of each method across all the trials is summarised in **Figure S2** of the **Online Supplementary Material.**

Participant health status and intervention duration

Eight trials recruited healthy individuals and one trial recruited patients with heart failure (Chirinos et al. 2017) (Table 2). The duration of the inorganic nitrate supplementation ranged from 3 hours to 3 days. The dose of inorganic nitrate ranged from 5.5 to 24 mmol/day (median dose: 9.8 mmol/day).

Meta-analysis

Overall, inorganic nitrate did not improve CBF under either resting (SMD +0.14, 95% CI: -0.13, 0.41, P = 0.31), or under stimulated conditions (SMD +0.23, 95% CI: -0.11, 0.56, P = 0.19). We observed moderate heterogeneity between studies testing the effect of inorganic nitrate on CBF at rest and stimulated conditions (I2 = 56.7%; P = 0.01; I2 = 44.1%; P = 0.12, respectively) (Figure 3). Meta-regression analysis produced no evidence for significant associations of resting CBF effect size with age (β: 0.001, SE: 0.006, P = 0.98), BMI, (β: 0.016, SE: 0.019, P = 0.41), dose (β: -0.01, SE: 0.019, P = 0.58), or Jadad score (β: 0.03, SE: 0.13, P = 0.79). However, there was a significant negative association between CBF effect size and study duration (β: -0.001, SE: 0.0006, P = 0.02) (Table 3).

Study quality and publication bias

The quality of the trials ranged from 2 to 4 (median: 2) according to the Jadad score. On this scoring system, 4 studies showed a score ≥ 3 (Chirinos et al. 2017; Lefferts et al. 2015; Thompson et al. 2014; Wighman et al. 2015) (Table 2). We could not assess the quality of one study (Rattary et al. 2015), as it was an abstract. Visual inspection of the Funnel Plot revealed no evidence of publication bias and this was confirmed by the Egger's Regression test for both resting (p = 0.43) and stimulated (p = 0.58) CBF; **Figure S4** and **S5** of the **Online Supplementary Material).**

Discussion

Our meta-analysis revealed that inorganic nitrate or nitrite supplementation was not associated with improved CF or

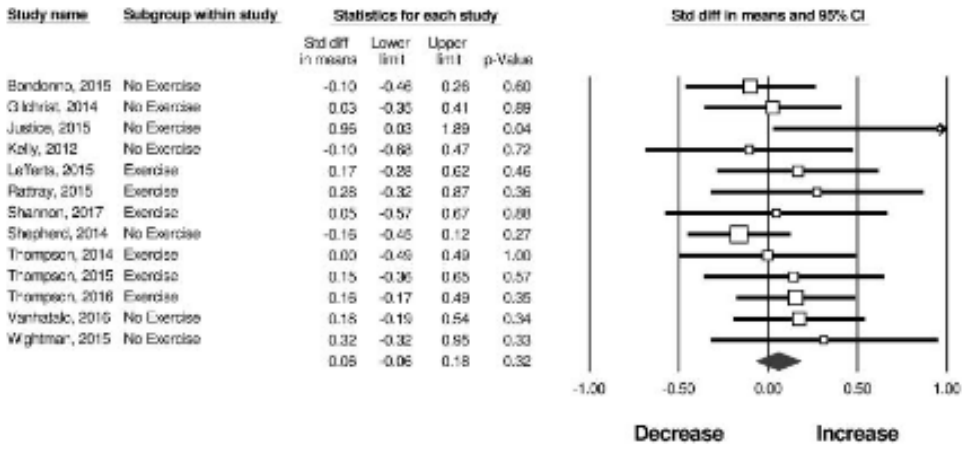

Figure 2. Forest plots showing the effect of dietary nitrate and nitrite supplementation on cognitive function.

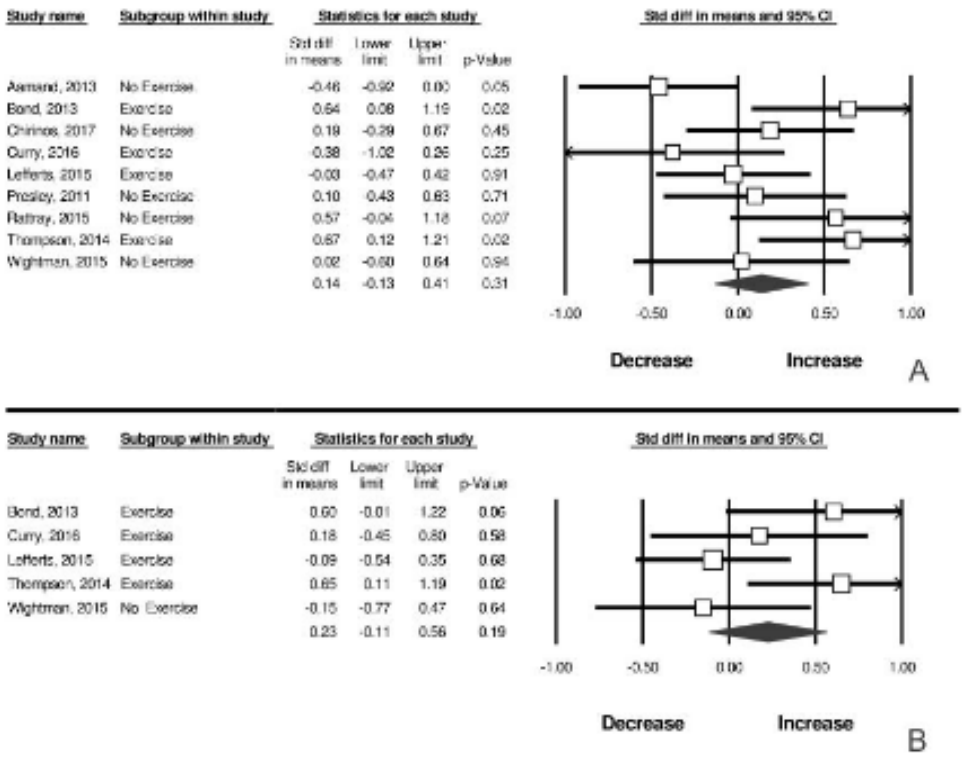

Figure 3. Forest plots showing the effect of dietary nitrate and nitrite supplementation on cognitive function, cerebral blood flow at rest (A) and in stimulated conditions (B).

increased CBF. The combined standardized mean difference (placebo vs. intervention) was +0.06 for CF and +0.14 and +0.23 for CBF at rest and in simulated conditions, respectively. These findings were not influenced by whether the tests were performed at rest, during exercise or with mental stimulation, by the age or health status of the participants or by the dose of inorganic nitrate or nitrite. Overall, the studies had small sample sizes and were of short duration, making it difficult to draw definitive conclusions about the efficacy of inorganic nitrate or nitrite in modulating CF and CBF.

The quality of the studies assessing CF was generally high; all studies employed a randomized design, used appropriate interventions, and in all but one of these studies (Bondonno et al. 2014) the intervention agent was provided in a double-blind fashion. Similarly, with the exception of one study, those assessing effects on CBF were all randomized, crossover trials. However, only 6 of the 9 trials were double-blind so that (along with other factors) meant they were generally of lower quality than those assessing effects on CF (Table 2). The overall utility of all the included trials was severely limited by the small sample sizes. Indeed, only 2 of the 21 studies reported that they had conducted an *a priori* power analysis to determine if they had an adequate sample size to detect a treatment effect for CF or CBF. One of these studies (Bondonno et al. 2014), suggested that at 80% power, 30 participants was sufficient to detect subtle treatment effects (e.g., 27 ms in simple reaction time) in various cognitive tasks. Given that the median sample size in the studies that assessed CF was only 23, it would be reasonable to assume that many of the studies were not adequately powered to detect potential effects of the nitrate or nitrite interventions, and that the risk of type 2 errors was high. In view of this, it is vitally important that future studies include larger sample sizes and ensure they are sufficiently powered to detect anticipated nitrate/nitrite-induced changes in CBF or CF.

The participants in most studies were of normal BMI, male, healthy, and not suffering from a cognitive-related disease. Of the two studies that examined effects of inorganic nitrate or nitrite on CF in a non-healthy cohort (T2DM patients), one observed improvements following the intervention (Gilchrist et al. 2014) and one did not (Shepherd et al. 2015). All remaining 11 trials that investigated effects of nitrate/ nitrite supplementation on CF were performed in participants with a BMI $\leq$25 kg·m^2. Because obesity is associated with impaired NO availability, it could be argued that individuals with a BMI $\geq$30 kg·m^2 might be more responsive to nitrate or nitrite induced vascular or metabolic effects (Ashor et al. 2016). Conversely, consequent to their greater body mass, it is possible that obese individuals will require a larger absolute nitrate/nitrite dose to manifest meaningful physiological changes. Prescribing a nitrate dose relative to body mass could help ameliorate this issue. Future studies should compare the effects of nitrate or nitrite supplementation on CF in both normal weight and obese individuals. As for studies that assessed effects on CF, only one study assessed effects of inorganic nitrate supplementation on CBF in non-healthy, obese participants. Chirinos and colleagues (2017) examined the effects of nitrate-rich beetroot juice in heart failure patients, and observed no significant changes in carotid artery hemodynamics. Arguably, older individuals suffering from a disease — especially a diagnosed cognitive disorder — are more likely to benefit from an intervention

attempting to re-establish a dysfunctional pathway than young, healthy individuals, in whom NO availability is less likely to be impaired. Thus, it would seem prudent that future research prioritizes studying the effects of inorganic nitrate and nitrite supplementation on CF and CBF with older individuals with some cognitive dysfunction e.g. mild cognitive impairment or subjective memory complaints. In addition, few studies were carried out using female participants. Although there is no strong *a priori* rationale to anticipate that the impact of such supplementation would differ by sex, future studies should address potential effects in women.

Our meta-regression showed that the duration of the nitrate or nitrite supplementation had a modest influence on CBF. More specifically, the longer the duration of the supplementation, the smaller was the improvement in CBF. However, this observation should be interpreted with caution because the majority of the trials had a very short duration. Of the nine studies assessing effects on CBF, only two provided the supplement for >150 min pre-assessment — and both displayed positive effects. The first, by Presley et al. (2011), was a randomized crossover trial in which healthy older adults received either a low nitrate or high nitrate diet for 2 days prior to measurements of cerebral perfusion using magnetic resonance imaging (MRI). Those in the high nitrate diet (12.6 mmol/day) group had a substantial and preferential increase in frontal cortex perfusion compared to those in the low nitrate diet group (0.9 mmol/day). The other study, by Aamand and colleagues, (2013) found that 3 days of sodium nitrate (vs. nitrate-free saline) decreased the haemodynamic lag of the blood oxygenation level dependent (BOLD) response in the visual cortex of healthy, young males (Table 2). However, CBF, as measured by MRI, was unchanged. Clearly, more studies with longer supplementation periods are required before we can establish whether duration moderates the efficacy of nitrate/nitrite on CBF.

Most of the studies provided nitrate in the form of beetroot juice or nitrate-rich foods such as green leafy vegetables. Given only two studies assessed the effects of nitrate/ nitrite salts on CF or CBF, it was not possible to examine whether the vehicle for nitrate delivery (i.e., nitrate salts or nitrate-rich vegetable products) influenced the efficacy of supplementation. Interestingly, however, compared with nitrate salts, recent studies have reported greater effects of nitrate-rich vegetable products on blood pressure (Jonvik et al. 2016), the oxygen cost of exercise (Flueck, Bogdanova, Mettler, and Perret 2015), and post-exercise recovery (Clifford, Howatson, West, and Stevenson 2017). This suggests possible additive or synergistic effects between nitrate and other plant-based compounds. Indeed, several plant-based compounds other than nitrate have potential benefits on CF and CBF (Ide et al. 2014; Desideri et al. 2012; Macready et al. 2009). These compounds include polyphenols, such as catechins, anthocyanins, and other flavonoids, and carotenoids (Macready et al. 2009; Gómez-Pinilla 2008) that are purported, at least in part, to exert their beneficial effects on CBF and CF through NO-dependent mechanisms, namely increased vasodilative effects (Sokolov, Pavlova, Klosterhalfen, and Enck 2013). To our knowledge, there is no evidence to suggest that beetroot, the main vehicle used in the RCTs included in this analysis, contains high quantities of the polyphenolic compounds showing potential for cognitive modulation. Indeed, the most abundant bioactive compound in beetroot, other than nitrate, is betanin and, to date, its effects on cognitive function are unknown. Notwithstanding, we acknowledge that the current evidence makes it impossible to differentiate the effects of nitrate/nitrite salts and nitrate-rich plants on cognitive function, the latter of which contains additional bioactive compounds. The independent effects of the bioactive compounds and the nitrate/nitrite in these foods is an important question for future research.

Our study also has a number of other limitations. Firstly, because such a wide range of assessments and methods were used to evaluate CF, several of which were domain-specific (e.g., reaction time vs. working memory), pooling the average effect size for all tests overlooks potential changes for isolated tests. This is illustrated by the fact that when each cognitive test was modelled as an independent outcome in the meta-analysis, nitrate supplementation showed a modest benefit for CF (data not shown). Nonetheless, this latter finding, in which all tests are considered independently, can overestimate the effect size; hence, to provide a more conservative estimate, we chose to use the average effect size from each study as our main outcome measure. Secondly, we observed moderate heterogeneity between studies for CBF, likely because of the wide variability in participant age and health status, CBF measures used, and the dose and duration of the nitrate/nitrite interventions used in each study. As outlined in a recent commentary (Barnard, Willett and Ding 2017), heterogeneity between studies may disguise the benefits observed in single, well-controlled studies that, under specific conditions (e.g., dose, duration, population) demonstrated *real* effects. This possibility needs to be taken into consideration when interpreting our findings.

Conclusions

In conclusion, there is no robust evidence that inorganic nitrate or nitrate supplementation influences CBF or CF. However, these findings might not be generalizable to older people, those with higher adiposity or and those with reduced cognitive ability; all of the included studies were performed in individuals <75 years old. In addition, all available trials were characterized by small sample sizes and short intervention durations and, thus, most of the studies may not have been designed optimally to observe any potential benefits. Consequently, the main conclusion of this study is that there is insufficient evidence to know whether supplemental inorganic nitrate or nitrite could improve CF or enhance CBF. Given the interest in use of non-pharmacological approaches for maintenance and improvement of cognitive function during ageing and the mechanistic rational for potential benefits of enhanced NO availability, further well-controlled and sufficiently powered trials, especially in more at-risk populations, with longer duration of nitrate/nitrate supplementation, need to be conducted.

Acknowledgments

We would like to thank Dr Rattray for sharing the data from his studies.

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Acknowledgments

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Appendix 3.2: Summary of the 12 identified studies (after the meta-analysis was published)

Reference	Study Design	Sample Size	Health Status	Baseline BMI (Kg/m^2)	Age	Gender (M/F)	Nitrate Dose (mmol/day)	Type of Intervention	Placebo/ control	Duration	Cognitive Tests	Effects on cognition/CBF
Cognitive function												
Dobashi *et al.*, (2019)	RCT, crossover, double-blind	8	Healthy	21	21	All M	8.4	BJ	ND-BJ	4 days	Stroop	Cognitive performance did not improve at rest and during exercise, under hypoxic condition
Stanaway *et al.*, (2019)	RCT, crossover, double-blind	O:11 Y:13	Healthy	-	O: 50-70 Y: 18-30	12/12	10.5	BJ	ND-BJ	145 min	CRT, RVIP, Stroop	Reaction time was improved in the Stroop test for both groups.
White *et al.*, (2019)	RCT, parallel	102	Healthy	25	34	56/46	1.36	BB	WB	12 weeks	FBD, FBS, Simon, Pro, Anti, and Pro/Anti	Cognitive performance did not improve, possibly due to poor compliance and other dietary changes.
Woodward *et al.*, (2019)[1]	RCT, parallel, double-blind	49	Healthy	-	68	-	1.7	SNi	NaCl	12 weeks	NIH Toolbox	Cognitive performance did not improve.
Fascia *et al.*, (2020)[1]	RCT, crossover, double-blind	5	Healthy with history of concussions	-	21	All M	-	BJ	PCJ	One prior to sleep, and one prior to visit	Stroop	Preliminary findings suggested that BJ does not influence cognitive function in young adults with a history of concussion
Jackson *et al.*, (2020)	RCT, crossover,	32	Healthy	23	18-49	6/26	-	BE	CFB	60, 180 and 360 min	SS, RVIP, Stroop, P&B, IWR,	The combination of beetroot, sage, ginseng and phenolics from different

	double-blind										DWR, WR, PR	structural groups did not improve cognitive function.
Cerebral blood flow												
Petrie *et al.*, (2017)	RCT, crossover, double-blind	26	Hypertensive	34.5	65.5	14/12	9	BJ+Exercise	ND-BJ +Exercise	6 weeks	MRI	The combination of exercise with BJ showed the potential enhanced neuroplasticity in older asults
Fan *et al.*, (2018)	RCT, crossover, singe-blind	12	Healthy cyclists	-	31	All M	~7.3	SNA	SCL	3 days	MCAV, CVR, NIRS	SN marginally lowered MCAV and moderately elevated prefrontal tissue oxygenation during exercise in normoxia. However, It had no effect on CVR during exercise in normoxia or hypoxia
Franko *et al.*, (2019)	RCT, crossover, singe-blind	21	Healthy	-	46.4		0.6 mg/kg/h (infusion)	SNi	NaCL	60 min	EEG, TCD	Brain activity was not influenced by nitrite infusion.
Fascia *et al.*, (2020)[1]	RCT, crossover, double-blind	5	Healthy with history of concussions	-	21	All M	-	BJ	PCJ	-	MCA	Preliminary findings suggested BJ does not influence CBF in young adults with a history of concussion
Fan *et al.*, (2020)	RCT, parallel, single-blind	30	TIA	28	67.5	20/10	~ 13.3	SNA	MC	7 days	MCAV	Dietary nitrate reduced CBF fluctuations and improved CA.
Fan *et al.*, (2019)	RCT, crossover, single-blind	17	Healthy	23	24.4	7/10	~ 11.7	SNA	MC	7 days	MCAV	Modest increases in the MCAV response was observed.

Jackson *et al.*, (2020)	RCT, crossover, double-blind	32	Healthy	23	22.2	6/26	-	BE	CFB	60, 180 and 360 min	NIRS	The combination of beetroot, sage, ginseng and phenolics from different structural groups did not improve CBF parameters.
Horiuchi *et al.*, (2020)	RCT, crossover, double-blind	12	Healthy	20	21	All M	-	BJ	ND-BJ	4 days	ICA, CVC	Cerebrovascular responses or dynamic CA were not influenced by BJ in hypoxia or normoxia.

BJ; Beetroot juice, BE; Beetroot extraction, BMI; Body mass index, CFB; Cherry-flavoured beverage, CA; Cerebral autoregulation, CBF; Cerebral blood flow, CVC; Cerebrovascular conductance, CVR; cerebrovascular resistance, CRT; Choice Reaction Time, CPT; Continuous performance test, DRT; Decision reaction time, DWR; Delayed word recognition, DV; Digit vigilance, ER; Emotion Recognition, EEG; Electroencephalographic, F; Female, FBD; Forward and Backward, IWR; Immediate word recall, ICA; Internal carotid artery, MC; microcrystalline cellulose, MCAV, Middle cerebral artery blood flow velocity; NIRS; Near-infrared spectroscopy, NaCl; Sodium chloride, ND-BJ; Nitrate depleted beetroot juice, , O; Old, OJ, Orange juice; PCJ; Purple carrot juice, PR; Picture recognition, P&B; Peg and ball, RCT, Randomised clinical trial, RVIP; Rapid Visual Information Processing, SS; Serial subtractions, SNA, Sodium Nitrate; SBP, SNi; Sodium nitrite, TCD; Transcranial Doppler,.TVR; Total vascular resistance, WR; Word recognition, WB; White bread, Y; Young.

[1] Abstract.

Appendix 4.1: Published paper (Validity and reliability of test strips for the measurement of salivary nitrite concentration with and without the use of mouthwash in healthy adults)

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Validity and reliability of test strips for the measurement of salivary nitrite concentration with and without the use of mouthwash in healthy adults

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ARTICLE INFO

The material presented in this manuscript is original and it has not been submitted for publication elsewhere while under consideration by **Nitric Oxide**

ABSTRACT

The nitrate (NO_3^-)-nitrite (NO_2^-)-nitric oxide (NO) pathway has received considerable interest in recent years as a potential target for nutritional interventions designed to increase NO production, and elicit therapeutic effects in humans. In particular, studies have evaluated the effects of supplemental dietary NO_3^-, which serves as a 'substrate' for this pathway, on numerous different health outcomes. One challenge has been to evaluate compliance with the NO_3^- interventions. A recent advance in this field has been the development of a non-invasive, simple and rapid method to measure nitrite concentrations in saliva using small test salivary strips.

In the present study, ten healthy adults were recruited to a randomised, crossover study and received an acute dose of NO_3^--rich beetroot juice (BJ) after rinsing their mouth with either water or commercially available antibacterial mouthwash. Salivary NO_3^- and NO_2^- concentrations were measured at baseline and up to 5 h after BJ consumption using the gold-standard chemiluminescence and a colorimetric Griess assay. In addition, two salivary test strips (Berkeley Test strips, CA, USA) were used to measure NO_2^-concentrations at the same time points. Five observers read the strips and inter- and intra-observer reliability was measured. The Bland-Altman method was used to provide a visual representation of the agreement between the methods used to evaluate salivary NO_3^-/NO_2^-concentration. Sialin concentrations were measured at baseline and up to 5 h after BJ consumption.

BJ elevated salivary NO_3^- and NO_2^- concentrations when the mouth was rinsed with water (both $P < 0.01$), as assessed via both chemiluminescence and Griess methods. Rinsing the mouth with antibacterial mouthwash attenuated markedly the increase in NO_2^- ($P < 0.001$), while NO_3^-concentrations were unaffected ($P > 0.05$). The Intra-Class Coefficients of Correlation (ICC) showed a high inter- and intra-observer reliability ($r > 0.8$). A significant positive correlation was found between absolute salivary NO_2^- concentrations measured by strips and Griess and chemiluminescence methods (rho = 0.83 and 0.77, respectively) and also when expressed as changes in salivary NO_2^- concentrations (rho = 0.80 and 0.79, respectively). Bland Altman analysis indicated a poor agreement for absolute NO_2^- concentrations between salivary strips and the chemiluminescence and Griess methods. A small significant negative correlation was found between changes in salivary sialin and salivary NO_2^- concentrations ($r = -0.20$, $P = 0.04$). A non-significant positive correlation was observed between the change in salivary sialin and salivary NO_3^- concentrations ($r = 0.18$, $P = 0.06$).

This study suggests that commercially available salivary NO_3^- test strips provide a reasonable surrogate marker for monitoring changes in salivary NO_2^- concentrations in humans. However, the strips do not provide accurate estimates of absolute NO_2^- concentrations.

1. Introduction

Nitric Oxide (NO) is a reactive gas which is involved in numerous physiological processes, including blood flow regulation, immune defence and neurotransmission [2]. NO can be synthesised endogenously from L-arginine in a reaction catalysed by the NO synthase (NOS) enzymes [3]. Additionally, NO can be generated via an alternative pathway that depends on the entero-salivary circulation of NO_3^- – an inorganic anion which is present in a range of commonly consumed foods [3]. Ingested NO_3^- is absorbed rapidly from the upper

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gastrointestinal tract into the blood. Approximately 25% of circulating NO_3^- is taken up by the salivary glands and concentrated, prior to being excreted into the mouth in saliva [4]. The protein sialin (SLC17A5) was recently identified as the principal NO_3^- transporter in the salivary glands and knockdown of sialin expression reduced NO_3^- transport [5]. Once in the mouth, a portion of the NO_3^- is reduced to nitrite (NO_2^-) by commensal facultative anaerobic bacteria which reside predominantly on the dorsal surface of the tongue [6]. The resulting NO_2^- is then swallowed in saliva and may be further reduced to NO via enzymatic and non-enzymatic pathways to help support or maintain NO-signaling, especially in acidic [4] and ischemic [3] conditions.

Dietary supplementation with NO_3^-, which increases NO production via the NO_3^--NO_2^--NO pathway, elicits multiple beneficial effects on physiological outcomes. Effects include a significant reduction in blood pressure (BP) [7], improved exercise performance [8,9], and enhanced cognitive function [10], although this latter finding was not confirmed in a recent meta-analysis [11]. The NO_3^- reducing bacteria which reside in the oral cavity play a fundamental role in facilitating these beneficial effects following dietary NO_3^- ingestion [6]. Indeed, several studies have shown that using antibacterial mouthwash diminishes considerably the colony size of the bacteria [1,12]. This reduction affects the production of NO_2^- in the oral cavity, concomitantly lowering the concentration of NO_2^- in saliva and plasma, and diminishes the physiological effects that may otherwise manifest following NO_3^- supplementation [1,12,13]. Thus, whilst antibacterial mouthwash could help to maintain oral health, its regular use may interfere adversely with the beneficial effects of NO_3^- on cardiovascular health. Indeed, short term (3–7 days) mouthwash use has been shown to abolish the BP lowering effects of dietary NO_3^- supplementation [1,14,15]. The effects of long term mouthwash use on oral and cardiovascular health remains to be fully elucidated, although, interestingly, a recent study found that frequent mouthwash use is associated with an increased risk of diabetes [16].

Evidence that dietary NO_3^- may improve NO bioavailability and thus enhance a range of physiological functions has attracted researchers to develop a range of simple techniques to monitor systemic NO-bioavailability, including, amongst others, NO salivary test strips. These strips allow the estimation of salivary NO_2^- concentration, which can be used as a surrogate for NO bioavailability [17]. These strips could also help to provide information on compliance with dietary NO_3^- interventions. The strips have been validated in non-supplemented [18] and supplemented subjects [19] and correlate significantly with salivary NO_2^- measured via gold-standard techniques (i.e. ozone-based chemiluminescence). However, to our knowledge, no study has investigated in a randomised study whether antiseptic mouthwash could affect the sensitivity of the salivary strips by inhibiting the conversion of NO_3^- into NO_2^- [14]. In addition, previous studies validated these strips using simple correlation analyses and adjusting analyses for mouthwash use [18], and have not applied the Bland-Altman method to evaluate the magnitude, variability and direction of the measurement bias [20].

Therefore, the purpose of this study was to test the validity of these strips against reference standard laboratory measures (i.e. ozone-based chemiluminescence) of salivary NO_2^- and NO_3^- concentrations with and without the use of mouthwash, using the Bland-Altman method. In addition, as these strips are based on a modified Griess reagent reaction, we also measured salivary NO_2^- using the Griess method for further comparison. Finally, we also took the opportunity to investigate the effect of both NO_3^- supplementation and mouthwash on salivary sialin concentrations, which functions as a NO_3^- transporter in the plasma membrane of salivary glands.

2. Methods

2.1. Subjects

Ten healthy, non-smoking, normal weight or overweight (body mass index (BMI) range: 20–29.9 kg/m^2) participants aged ≥ 20 years were recruited via email from Newcastle University staff and students to take part in this study. Exclusion criteria included: smoking, history of clinical conditions and medical treatments likely to interfere with the study outcome, pregnancy and breastfeeding. All participants were fasting for at least 12 h prior to participating in the experiment. All participants provided written, informed consent and the study was approved by the Faculty of Medical Sciences, Newcastle University (1459/3414/2018).

2.2. Experimental protocol

This study was a cross-over, randomised, validation study consisting of two experimental trials (without or with mouthwash) conducted on two separate visits and with a washout period of 24 h. At present, there is limited *in vivo* evidence on the minimum time taken for the oral NO_3^--reducing microbiome to recover following administration of antibacterial mouthwash [21,22]. For practical reasons, a washout period of 24 h was selected and this also allowed us to elucidate whether the oral microbiome remained compromised a day after mouthwash use. Eligible participants were invited for their first experimental visit early in the morning (−8.30–9.00 a.m.) after an −12-h overnight fast and having avoided consumption of high NO_3^- foods for the previous 24 h. Body weight was measured, and participants were asked to collect a baseline saliva sample followed by the application of two NO Test Strips (Berkeley Test®, CA, USA), as per the manufacturer's instructions. Baseline resting BP was then measured, and participants were randomised to either rinse their mouth with 20 ml of low NO_3^- water (Buxton water) or 20 ml of antiseptic mouthwash (Corsodyl, Chlorhexidine Digluconate 0.2%, UK) for 2 min. After 15 min, participants consumed one 70 ml 'shot' of concentrated BJ (Beet-it, James White Company). This juice contains approximately 400 mg (−6.5 mmol) of NO_3^-, which is roughly equivalent to eating a large portion 200–300 g of lettuce or rocket. Participants were asked to collect saliva samples, apply the salivary strips, and measure their resting BP at 1, 2, 3, 4 and 5 h post-consumption. During this period, participants were asked not to eat any food, except for consumption of a low NO_3^- chocolate bar after collection of the third saliva sample. The consumption of low NO_3^- water was allowed *ad libitum*, but was prohibited in the 15 min prior to the collection of each saliva sample. An overview of the protocol is shown in Fig. 1. Dietary instructions and necessary materials for the collection of saliva samples were provided. Participants were asked to refrain from using mouthwash in the 24 h period before each trial, and throughout each experimental trial.

2.3. Blood pressure measurements

Baseline resting BP was measured in duplicate using automated BP monitor (Omron M3, Omron Healthcare Ltd., Kyoto, Japan). The mean of the two records was taken as the baseline BP. At 1, 2, 3, 4 and 5 h post administration of BJ, participants measured their own BP in duplicate.

2.4. Saliva samples collection

For the measurement of NO_3^-, NO_2^- and sialin concentrations, saliva samples were collected by chewing a cotton ball for 1–2 min. The cotton ball was then placed in a 20 ml syringe, which was used to squeeze the saliva into a 1.5 ml Eppendorf tube. Samples were stored at −20 °C within 30 min from collection for further analyses.

Fig. 1. Overview of the study protocol.

2.5. Salivary NO_2^- assessment using strips

Salivary NO_2^- strips (Berkeley Test strips, CA, USA) were used as per the manufacturer's guidelines. Specifically, the test strip with the 'saliva here' side was placed on the tongue and swabbed over a 10 s period covering different areas including the dorsal surface of the tongue. The two ends of the strip were folded and pressed gently for 10 s. The colour of the NO test pad was then allowed to develop over a 45 s period. The intensity of the colour was compared with a colour chart using a mobile phone based application developed by the manufacturers (Berkeley Test Application, CA, USA). The application has a long colour chart and each colour is associated with a quantitative value for NO_2^- concentration, with darker colours corresponding to higher NO_2^- concentrations. To evaluate the repeatability of this method, participants estimated their salivary NO_2^- concentration using two, separate test strips, with a 1-min interval between them. In addition, five observers read each of the strips independently to quantify inter-observer reproducibility.

2.6. Salivary nitrate and nitrite analyses

Salivary NO_3^- and NO_2^- concentrations were quantified using gas-phase chemiluminescence and a colorimetric Griess assay as described below:

Chemiluminescence: Salivary NO_3^- and NO_2^- concentrations were analysed using a Sievers gas-phase chemiluminescence NO analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). Sodium iodide in acetic acid was used as a reductant for NO_2^- to NO, while vanadium chloride in hydrochloric acid at 95 °C was used to determine NO_3^- concentrations by the reduction of NO metabolites to NO and subsequent subtraction of NO_2^- concentration. The concentrations of NO_3^- and NO_2^- were determined by plotting signal area (mV) against a calibration plot of known concentration NO_3^- and NO_2^- standards and data were analysed using the Sievers® NOAnalysis™ Software Version 3.2.

Griess method: A commercial kit (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical, Ann Arbor, MI, US) was used to measure NO_3^- and NO_2^- concentrations using the Griess method.

2.7. Sialin (SLC17A5) analysis

Sialin (SLC17A5) concentrations in saliva were quantified using a commercial BioAssay™ ELISA Kit (Human) from Stratech Scientific Ltd, in a 96-well format.

2.8. Statistics

A two factor ANOVA for conditions (water and mouthwash) with repeated measures for sampling time was applied to determine the effects of the BJ intervention on BP, salivary NO_3^- and NO_2^-,and salivary sialin. Bland-Altman analysis was applied [20] to provide a visual representation of the agreement between methods used to analyse salivary NO_3^- and NO_2^- concentrations. Normal distribution was checked via the Shapiro-Wilk test, and data were log transformed when necessary. Spearman's correlation analysis was performed to evaluate whether changes in NO_3^- and NO_2^- concentrations were associated with changes in BP and sialin concentrations. In addition, we evaluated whether changes in salivary NO_2^- concentrations measured by salivary strips and Griess and chemiluminescence methods were significantly associated. To evaluate the effects of mouthwash/water use on recovery time of the NO_3^- reducing capacity of oral bacteria the areas under the curve (AUC) of NO_2^- concentrations for participants receiving the mouthwash on the first day (n = 5) was compared with the AUC derived from participants receiving the mouthwash on the second day (n = 5) (independent sample *t*-test). All data are presented as mean ± SEM unless otherwise indicated. Statistical significance was accepted when $P < 0.05$. The Statistical Package for Social Sciences (IBM SPSS, version 23, NY, USA) was used to perform the analysis.

3. Results

3.1. Participants' baseline characteristics

Ten healthy young participants were recruited (6 females and 4 males) with an age range of 20–45 years and a BMI range of 21.1–29.8 kg/m^2 (Table 1). Baseline systolic and diastolic blood pressure (SBP and DBP) were not different between the water and mouthwash experiments ($P = 0.91$ and $P = 0.60$ for SBP and DBP, respectively).

3.2. Salivary NO_3^- concentration

There was no significant difference in salivary NO_3^- concentration at baseline between the mouthwash and water conditions, as determined by both chemiluminescence ($P = 0.34$) and Griess methods ($P = 0.43$). Following BJ ingestion, salivary NO_3^- concentration rose rapidly to peak within 1–3 h after which concentrations declined. Time to peak appeared to be delayed after use of the mouthwash. This pattern of response in salivary NO_3^- concentrations was similar when measurements were made by the chemiluminescence and Griess methods

Table 1
Baseline characteristics of the participants (n = 10).

Characteristic	Mean	SD
Age (years)	31.2	8.7
Height (cm)	163.0	12.9
Weight (kg)	65.5	13.8
Body mass index (kg/m^2)	24.3	2.7
SBP (mmHg):		
Water experiment	115.4	8.3
Mouthwash experiment	115.0	7.9
DBP (mmHg):		
Water experiment	71.5	9.7
Mouthwash experiment	73.6	9.3

SBP, Systolic blood pressure; DBP, Diastolic blood pressure.

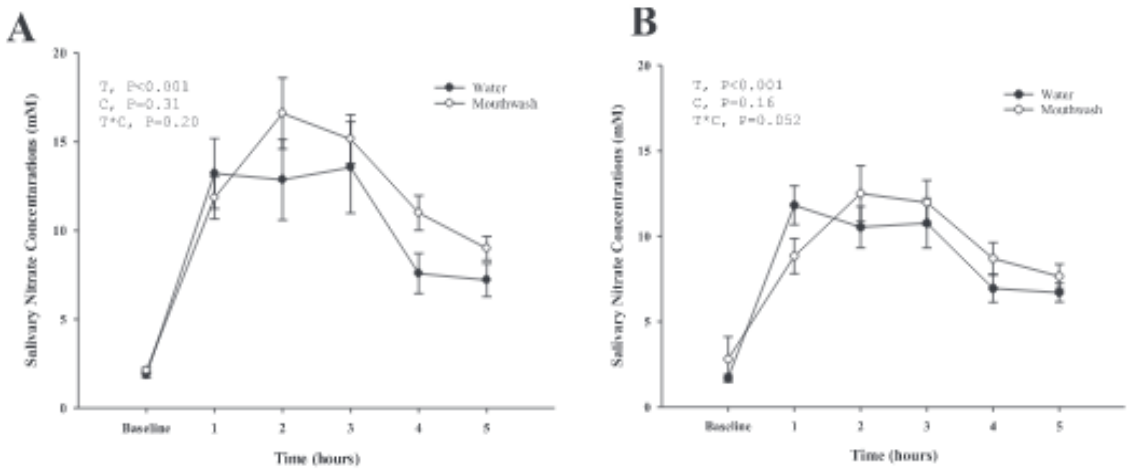

Fig. 2. Mean salivary nitrate concentrations measured by chemiluminescence (A) and Griess (B) methods after acute ingestion of BJ (70 ml). Filled circles represent times when individuals rinsed their mouth with water 15 min before the ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time*condition) ANOVA. Data are expressed as mean ± SEM, n = 10.

but, overall, concentrations determined by the Griess method were −12% lower (Fig. 2). Salivary NO_3^- concentration was higher than baseline at all-time points after BJ ingestion ($P < 0.001$).

3.3. Salivary NO_2^- concentration

There was a significant main effect for time on salivary NO_2^- concentration (chemiluminescence: $P < 0.001$; Griess: $P = 0.004$). In addition, a significant effect for condition (both $P < 0.001$) and interaction between time*condition (both $P < 0.001$) was observed. Following BJ ingestion, salivary NO_2^- concentration rose significantly to peak within 2–3 h in participants drinking water, and it remained elevated until the end of the observation period. However, this increase in salivary NO_2^- concentration vanished after using anti-bacterial mouthwash (Fig. 3). This pattern of response in salivary NO_2^- concentration was similar when measurements were made by the chemiluminescence and Griess methods but, overall, measurement derived from the Griess method were −38% and −27% lower for the water and mouthwash conditions, respectively (Fig. 3). There was no significant difference ($P = 0.32$) in the AUC for NO_2^- concentration measured in participants receiving the mouthwash on the first day compared with participants receiving it on the second day (Figure S1 of the Online Supplementary Material).

3.4. Salivary NO_2^- strips

The effects of BJ on salivary NO_2^-, as determined by the salivary strips, are presented in Fig. 4. Overall, the salivary NO_2^- strips detected changes in salivary NO_2 concentration following to BJ supplementation similar to those measured by the chemiluminescence and Griess methods. The response was virtually abolished when participants rinsed their mouth with antibacterial mouthwash before consuming the BJ. There were significant main effects for time, conditions and for their interaction (time*condition) ($P < 0.01$). Overall, in the water experiment, the strips underestimated NO_2^- concentration by more than 50% and by −27% compared with chemiluminescence and Griess methods, respectively.

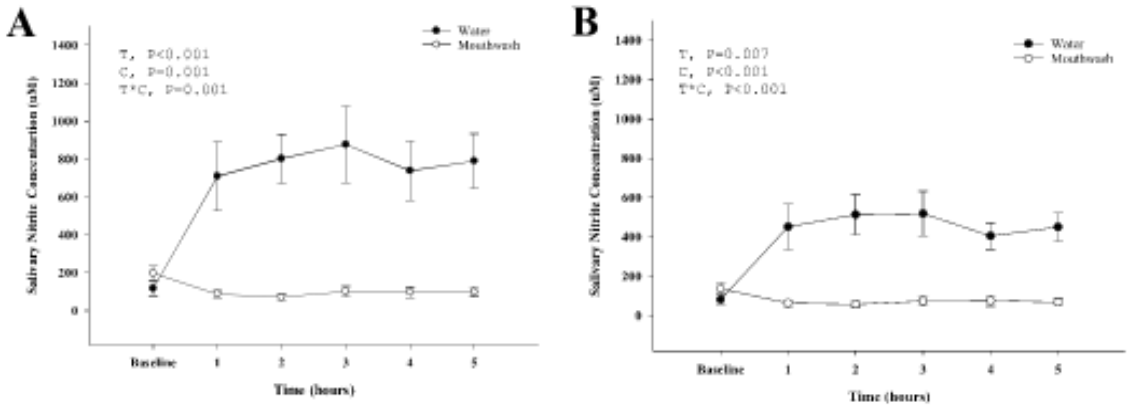

Fig. 3. Mean salivary nitrite concentrations measured by chemiluminescence (A) and Griess (B) methods after acute ingestion of BJ (70 ml). Filled circles represent times when individuals rinsed their mouth with water 15 min before beetroot ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time *condition) ANOVA. Data are expressed as mean ± SEM, n = 10.

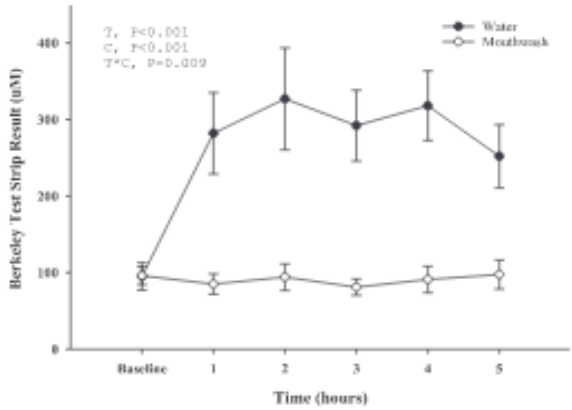

Fig. 4. Mean salivary nitrite concentrations measured by salivary NO_2^- strips after acute ingestion of BJ (70 ml). Filled circles represent times when individuals rinsed their mouth with water 15 min before beetroot ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time *condition) ANOVA. Data are expressed as mean ± SEM, n = 10.

3.5. Agreement analysis (Bland & Altman method)

Concentrations of salivary NO_2^- estimated using the salivary strips were significantly and strongly correlated with measurements obtained using the chemiluminescence (rho = 0.77, $P < 0.001$) and Griess (rho = 0.83, $P < 0.001$) methods. Similarly, changes in salivary NO_2^- measured by the strips were significantly correlated with changes in concentration measured by the chemiluminescence (rho = 0.79, $P < 0.001$) and Griess (rho = 0.80, $P < 0.001$) methods. As expected there was a significant, strong correlation between salivary NO_3^- concentration measured by the chemiluminescence and Griess methods ($P < 0.001$, r = 0. 86). However, despite these statistically significant correlations, the limits of agreement between methods illustrated in the Bland Altman analysis (Fig. 5) were wide, indicating a lack of accuracy of the Griess method. In addition, the Griess and salivary strips methods showed a significant differential bias as magnitude of the differences became larger with increasing concentrations. For salivary NO_2^- measured by Griess and chemiluminescence, the estimated bias was −150 μM (95% CI −193 to −107, P = 0.0001) and the 95% limits of agreement were fairly wide (−618, 318 μM). For salivary NO_2^- measured by the salivary strips and chemiluminescence the estimated bias was −201 μM (95% CI −266 to −136, p = 0.0001) and the 95% limits of agreement were also wide (−909, 506 μM). For salivary NO_2^- measured by the salivary strips and Griess the estimated bias was −64 μM (95% CI -97 to −32, p = 0.0001) and the 95% limits of agreement ranged from −418 to 289 μM. The differences between measurements increased with higher NO_2^- concentrations. For salivary NO_3^- measured by Griess and chemiluminescence, the estimated bias was −2 mM (95% CI− 2 to −1, p = 0.0001) and 95% limits of agreement were between −8 and 5 mM).

3.6. Reliability of salivary NO_2^- strips

3.6.1. Reproducibility

Table 2 shows the mean concentrations of salivary NO_2^- estimated using the salivary strips for all 10 participants at each time-point obtained from five different observers. The intra-class correlation coefficient (ICC) showed a high reproducibility between the five observers.

3.6.2. Repeatability

Table 3 shows the results of NO_2^- measurements by salivary strips performed by five observers on two different occasions. The high ICCs indicated a high repeatability of the salivary NO_2^- strips.

3.6.3. Salivary sialin

There was no significant difference between salivary sialin concentrations measured at baseline between the water and mouthwash conditions (P = 18). There were no significant effects of condition (water v. mouthwash; P = 0.54) or time (P = 0.49), or time*condition (P = 0.41) interaction. We found a trend for an increase in sialin in the mouthwash experiment compared to water experiment but this increment was not significant at any of the time points ($P > 0.05$) (Fig. 6). A weak but significant correlation was found between the change in salivary sialin and the change in salivary NO_2^- concentration ($r = -0.20$, P = 0.04). Conversely, there was a weak positive correlation between the change in salivary sialin and the change in salivary NO_3^- concentration (r = 0.18, P = 0.06) (data not shown).

3.6.4. Blood pressure

There was no significant difference in SBP and DBP at baseline between the mouthwash and water conditions (P = 0.91 and P = 0.60, respectively). Over the 5 h following ingestion of BJ, there were no significant changes in SBP and DBP ($P > 0.05$) with, or without, use of the mouthwash (Fig. 7).

4. Discussion

This study investigated for the first time the validity of salivary NO_2^- strips against the gold standard chemiluminescence technique and Griess methods after acute consumption of NO_3^- rich BJ consumption with and without the use of mouthwash. Furthermore, this study evaluated for the first time whether acute NO_3^- supplementation, with and without mouthwash, altered sialin concentrations in saliva. Overall, salivary strips provide a simple and user-friendly method to detect changes in salivary NO_2^- concentrations after the consumption of high-NO_3^- foods, which could be useful for the monitoring of compliance in longer-term high-NO_3^- nutritional interventions. The strips are also characterised by a high repeatability and reproducibility, but they underestimated NO_2^- concentration compared with other laboratory methods (Griess and chemiluminescence) especially at higher salivary NO_2^- concentrations. In addition, when study participants used the mouthwash, salivary sialin concentration tended to increase following the ingestion of BJ and salivary sialin concentrations correlated with salivary NO_2^- concentration.

Clodfelter et al., tested different brands of NO saliva test strips and found that they reacted with solution containing sodium nitrite ($NaNO_2^-$) but not with sodium NO_3^-, indicating that these strips can be utilised for the selective detection of NO_2^- in biological fluids [23]. The study found that the colour intensity of the strips increased with greater concentrations of $NaNO_2^-$ and that the lowest limit of detection was 10 μM. However, the Clodfelter et al., study was performed *ex-vivo*. When the strips were applied on the tongue, we observed that colour intensity increased after the consumption of high NO_3^- BJ. However, there was no increase in colour on the strips after the use of mouthwash which confirmed the lack change in salivary NO_2^- concentration when measured by standard laboratory methods (chemilunimesence and Griess). This finding clearly indicates the sensitivity of the strips in detecting the effect of the antibacterial mouthwash, which is known to block the activity of the oral bacterial NO_3^- reductase and inhibit the conversion of NO_3^- into NO_2^- [1]. This study also demonstrated a high level of repeatability and reproducibility of the strips. In addition, our study revealed the capacity of the strips to detect changes in salivary NO_2^- concentrations following an acute oral dose of NO_3^- rich BJ (400 mg). This is in agreement with McDonagh and colleagues who found that the strips measured changes in salivary NO_2^- concentrations following the ingestion of a range of doses of NO_3^- (−5.76 and −1.40 mmol of NO_3^-) [19].

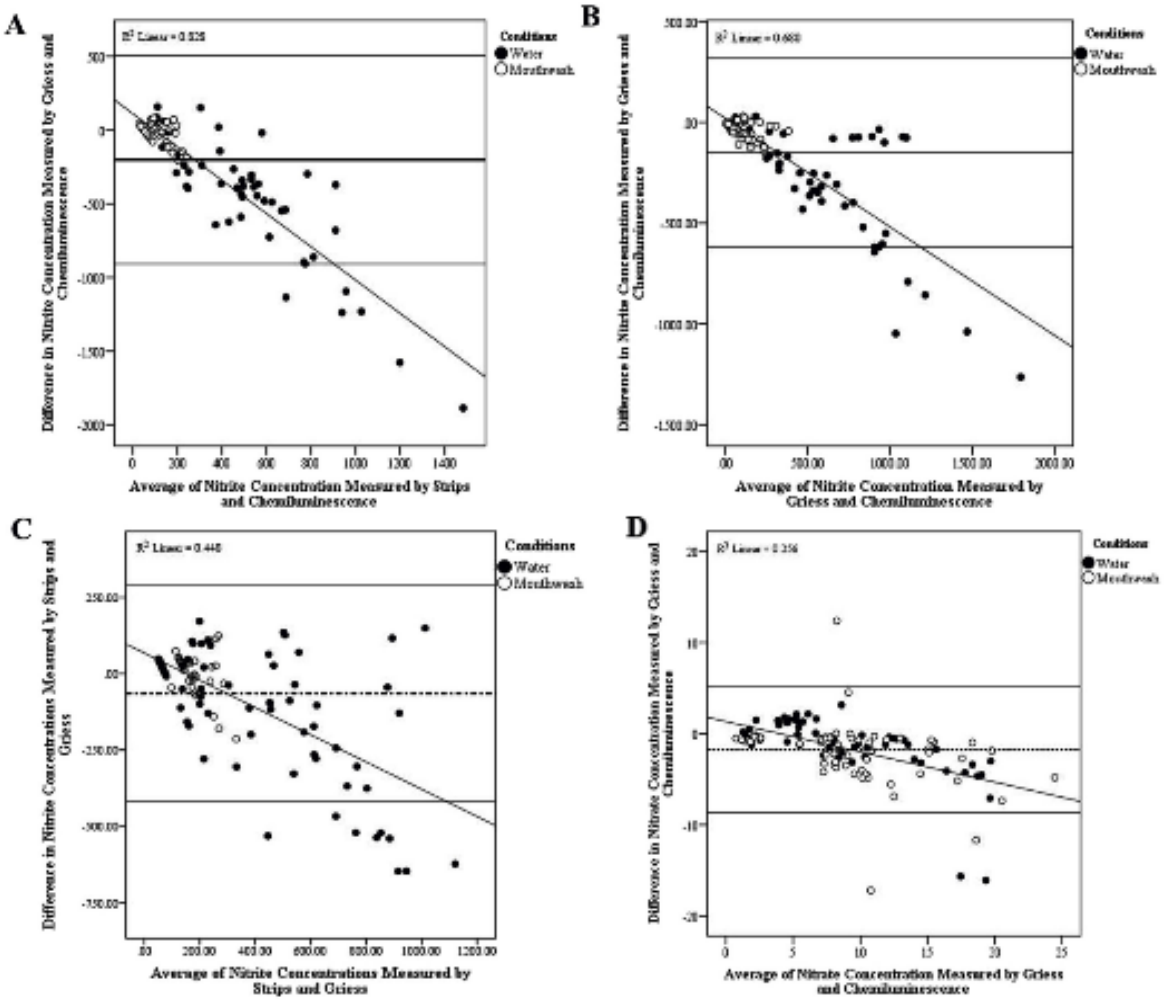

Fig. 5. Comparison of mean salivary nitrite (A, B and C) and nitrate (D) concentrations measured by two different methods. Black dash horizontal line shows the mean difference and the ± 2S.D. range (fine, black line). A regression line was fitted to the points to evaluate differential bias.

The current study revealed significant strong correlations between the strips and other laboratory methods for the measurement of absolute salivary NO_2^- concentrations and also for the measurement of changes in salivary NO_2^- concentrations. Overall, the strength of the correlations found in this study (rho = 0.80 and 0.79 for the Griess and chemiluminescence methods, respectively) was greater than the correlation (r = 0.57) reported by McDonagh et al., [16]. The different strength of the associations reported in the two studies may be explained by the use in our study of a mobile phone based application that provides a more detailed colour chart providing an assigned, quantitative value of NO_2^- concentration to each colour. McDonagh et al. used a simple colour chart which simply classified NO_2^- concentrations as depleted, low, threshold, target and high. McDonagh et al. [16]

Table 3
Intra-observer repeatability of the two strips used at each time point.

Observers	First strip	Second strip	ICC	P-value
Observer 1	173 ± 145	179 ± 153	0.938	< 0.001
Observer 2	200 ± 147	200 ± 146	0.813	< 0.001
Observer 3	185 ± 127	174 ± 103	0.833	< 0.001
Observer 4	158 ± 76	156 ± 72	0.918	< 0.001
Observer 5	76 ± 141	73 ± 137	0.720	< 0.001

ICC, intraclass correlation coefficient (absolute agreement). Salivary nitrite values are presented as mean ± SD. P-values indicate that the readings of the two strips used at each time point are significantly correlated between observers.

Table 2
Inter-observer reproducibility of strips.

Observer 1	Observer 2	Observer 3	Observer 4	Observer 5	ICC	P-value
176 ± 147	200 ± 140	193 ± 117	157 ± 72	75 ± 129	0.91	< 0.001

ICC, intraclass correlation coefficient (absolute agreement). Salivary nitrite values are presented as mean ± SD. The average of two strips' readings of salivary nitrite strips was reported. P-value indicate that the strips reading are significantly correlated between observers.

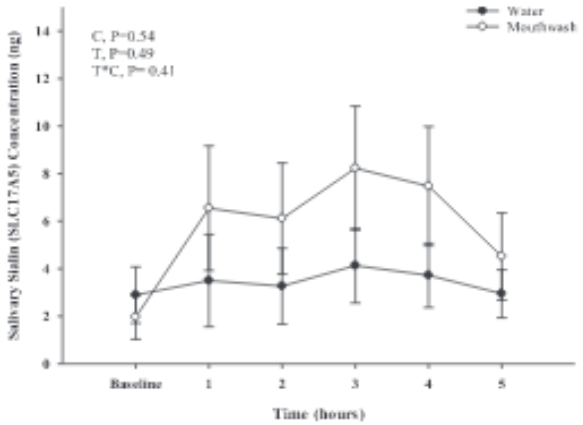

Fig. 6. Mean salivary sialin concentrations. Filled circles represent times when individuals rinsed their mouth with water 15 min before beetroot ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time*condition) ANOVA. Data are expressed as mean ± SEM, n = 10.

concluded that salivary strips are a practical method to estimate salivary NO_2^- concentrations after the consumption of dietary NO_3^-. However, the poor level of agreement and the significant bias between the methods may limit the application of the strips for absolute measurement of salivary nitrite concentrations [24]. We used the Bland Altman method to assess the agreement between the strips and Griess and chemiluminescence methods and found that the limits of agreement between salivary strips and other, more precise, laboratory methods are wide, suggesting that salivary strips may not provide accurate estimates of salivary NO_2^- concentrations. However, the strips detected changes to acute ingestion of high doses of NO_3^- and therefore they may be useful for monitoring the compliance in nutritional interventions testing the effects of inorganic NO_3^-. This could be a simple cost-effective method of monitoring for NO_3^- intake as the results can be seen almost instantaneously and this method can also provide an indication of NO bioavailability, without requiring access to expensive laboratory equipment. Further, this method could represent a convenient and effective solution to research studies conducted in situations where the collection and storage of saliva samples for later analysis may be problematic (e.g. studies conducted in rural areas and/or developing countries).

In pigs, Qin and co-authors identified sialin as the primary NO_3^-transporter in salivary glands and observed inhibition of NO_3^-transport after sialin expression was knocked down [5]. To our knowledge, this is the first study to examine the association between salivary sialin concentrations and changes in salivary NO_2^- and NO_3^- concentrations in humans. The acute dose of NO_3^- rich BJ did not affect sialin concentrations in saliva as concentrations remained similar to baseline levels during the experimental period. Sialin concentrations tended to increase, however, after blocking the NO_3^- conversion into NO_2^- via mouthwash and a weak but significant correlation between salivary sialin and salivary NO_2^- concentrations was observed. Tentatively, these results may suggest the existence of a feedback mechanism linking NO_3^-transport and conversion to sialin expression, but mechanistic studies with larger sample size are needed to confirm this.

Previous studies have showed that SBP can be reduced after 3 h of BJ consumption [7,25]. In addition, in a recent study, Woessner and coworkers found a significant difference in SBP changes between water and mouthwash over a 4-h period post BJ consumption [12]. In our study, we found no effect of BJ on SBP or on DBP and no effect of use of mouthwash. A possible explanation for this difference is the higher SBP baseline values in the study by Woessner et al. compared with our study (124 vs 115 mmgH), which may make individuals more responsive to the BP lowering effect of NO_3^- [26].

In this study we administered mouthwash on one occasion, which contrasts with several previous investigations where mouthwash has been administered two or more times daily over several days [14,15,27]. We found that acute mouthwash use abolished the increase in salivary NO_2^- concentration consequent to beetroot juice ingestion, suggesting that one-time use of mouthwash is sufficient to blunt, at least temporarily, the NO_3^--reducing capacity of the oral microbiome. However, in contrast with some (e.g. Ref. [14]) but not all (e.g. Ref. [27]) prolonged mouthwash studies, we did not observe a mouthwash-induced increase in BP. Future studies are warranted to determine potential differential effects of acute versus chronic mouthwash use on markers of NO bioavailability and physiological responses.

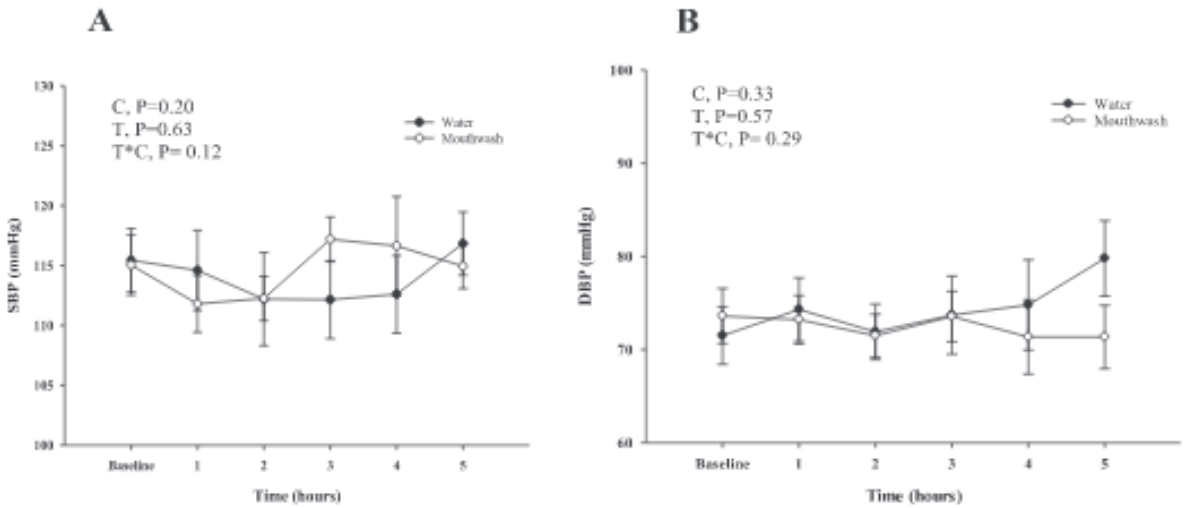

Fig. 7. Mean systolic blood pressure (SBP) and diastolic blood pressure (DBP). Filled circles represent times when individuals rinsed their mouth with water 15 min before beetroot ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time*condition) ANOVA. Data are expressed as mean ± SEM, n = 10.

A limitation of the current study is the small sample size, which reduced the power to detect significant changes in BP and sialin concentrations after BJ ingestion. However, the primary purpose of the study was to test the validity of the salivary strips for which the study size was deemed as adequate based on the sample size of previous studies with a similar study design [13]. The short washout period between the two experiments (mouthwash and water) could be considered a potential limitation if the oral microbiota had not recovered from the acute use of mouthwash. However, there was no significant difference in the AUC for salivary NO_2^- ($P = 0.34$) between the participants who used mouthwash on the first experimental day and those who used mouthwash on the second day. This indicates that the NO_3^--reducing capacity of the oral bacteria may recover within 24 h following the use of mouthwash. It is challenging to reconcile this finding with the currently limited evidence on the effects of antiseptic mouthwash on oral bacteria. *Ex vivo* studies have shown that, after exposure to chlorhexidine digluconate (0.2%) for 3 min, the proportion of viable bacteria is reduced by approximately 30% within a few hours and that full recovery of the bacteria requires up to 5 weeks [21]. The vitality of plaque bacteria after treatment with chlorhexidine digluconate (0.2%) was investigated in 6 volunteers studied over four days [22]. At 24 h after the last exposure to the mouthwash, plaque bacteria vitality was 60%. Our observation of no increase in NO_2^- concentrations over the 5 h following use of mouthwash indicates immediate, and total, suppression of the NO_3^--reducing capacity of the bacteria. However, 24 h after mouthwash use, the capacity of oral NO_3^--reducing bacteria has been re-established as we observed no difference in the AUCs of salivary NO_2^- concentrations measured during the two water experiments. These findings suggest differential kinetics after mouthwash use for total plaque bacteria viability and for the specific bacteria responsible for NO_3^--reduction. The effects of frequent mouthwash treatment on NO_3^--reducing oral bacteria and its impact on the recovery the bacterial flora after stopping the treatment are very relevant research questions that should be explored in future studies.

In conclusion, the commercially available salivary NO_2^- strips applied in this study showed a high level of reproducibility and repeatability in detection of changes in salivary NO_2^- concentrations following acute ingestion of inorganic NO_3^-. However, Bland Altman plots indicated that there is a poor agreement between salivary strips, chemiluminescence and Griessmethods, which means that these strips are not sufficiently accurate for the measurement of absolute concentrations of NO_2^- in saliva. Salivary strips may be a cost effective and simple method for monitoring changes in salivary NO_2^- concentrations and for monitoring compliance in intervention studies focussed on increasing dietary NO_3^- intake. The preliminary findings suggesting an association between salivary NO_2^- concentrations with the salivary NO_3^- transporter (sialin) are intriguing and should be explored in future mechanistic studies.

Conflicts of interest

The authors have no conflict of interest to declare.

Funding

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.07.002.

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Appendix 4.2: Ethical Approval

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

Abrar Babateen
Institute of Cellular Medicine

FACULTY OF MEDICAL SCIENCES: ETHICS COMMITTEE

Dear Abrar,

Title: Validation of nitric oxide saliva test strips against standard laboratory techniques to measure nitrite concentrations in saliva with and without mouthwash
Application No: 1459/3414/2018
Start date to end date: 01/02/2018 to 30/01/2019

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: **1459/3414/2018**. 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
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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Appendix 4.3: A standard curve for the calibration for nitrate and nitrite and examples of peak in analysing salivary nitrate and nitrite using chemiluminescence

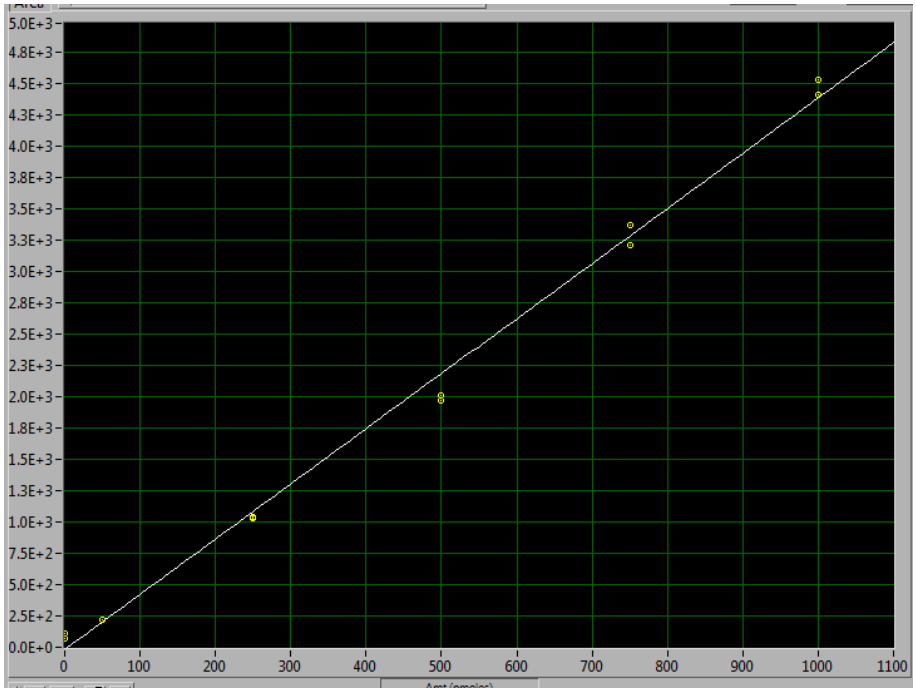

Figure 4.3 A: Examples of NO_3^- calibration curves.

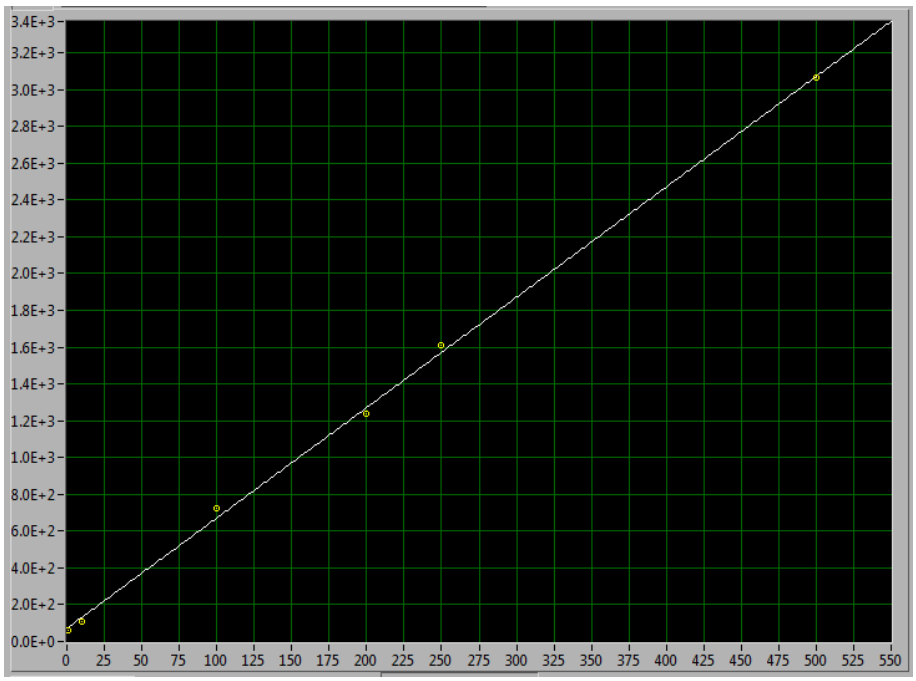

Figure 4.3 B: Examples of NO_2^- calibration curves.

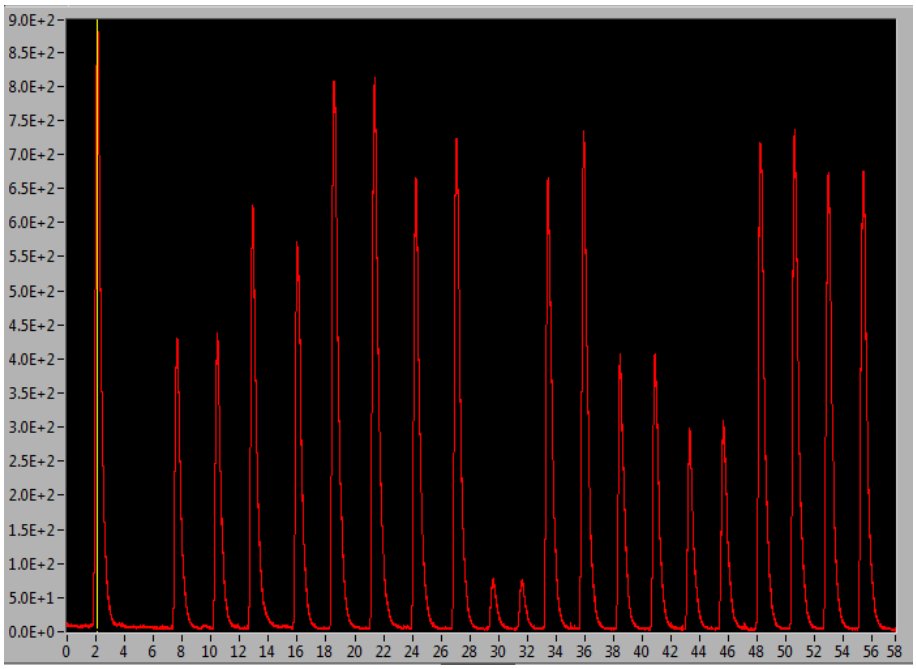

Figure 4.3 C: Examples of salivary NO_3^- sample analysis with and without mouthwash

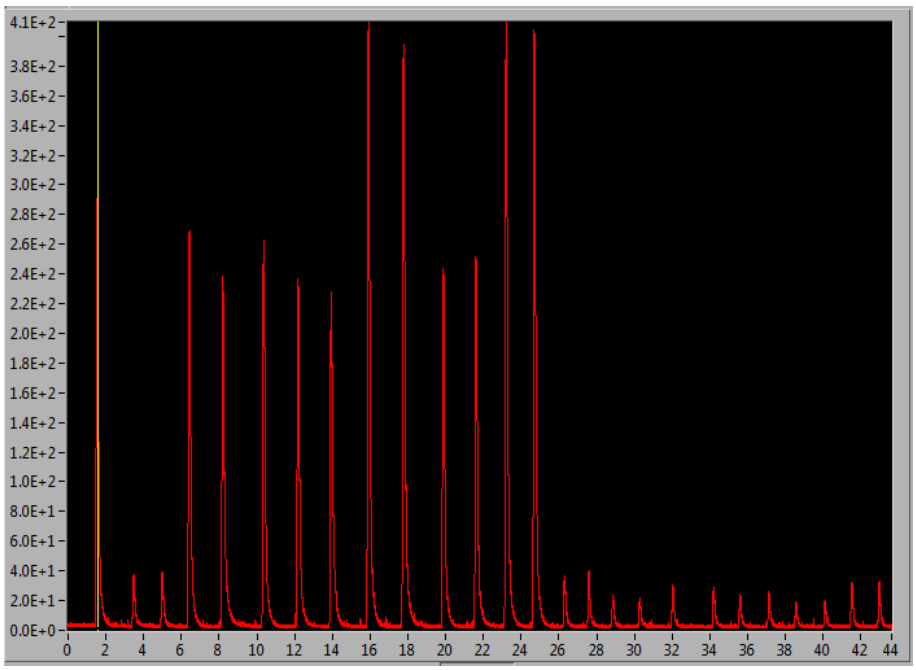

Figure 4.3 D: Examples of salivary NO_2^- sample analysis with and without mouthwash

Appendix 4.4: Performing NO_3^- and NO_2^- analyses using chemiluminescence

1-Preparation of NO_3^- Standard solutions

- A standard/stock solution (100mM NO_3^-) was prepared by adding 85mg of $NaNO_3$ into 10ml Mini Q H_2O to obtain the standard curve by conducting a serial dilution.
- Seven tubes were marked with the following: 10mM, 1mM, 100µM, 10µM, 1µM, 100nM, 10nM, and 900µl MiniQ H20 was added to each tube.
- Three more tubes were marked with 50µM, 5µM, 50nM, and 300µl MiniQ H_20 was added to each tube.
- 10nM tube was prepared by adding 100µl of stock solution and was vortexed thoroughly. 100µl of the 10mM standard was transferred to 1mM tube by new pipette. These processes were repeated to prepare 10nM.
- The following tubes were prepared as follows: 50µM tube (adding 300µl of the 100uM standard and vortexed thoroughly), 5µM tube (adding 300µl of the 10µM standard, and vortexed thoroughly) and 50nM tube (adding 300µl of the 100µM standard and vortexed thoroughly).
- 10mM and 1mM were discarded (most concentrated).
- To produce NO_3^- standard curves, 20 µL standards were injected (in duplicate) into the purge vessels, starting from the most diluted standard, and the peak area of each standards were measured.

Preparation of Nitrate Reducing Agent

- 160 mg of VCL_3 was dissolved in 10ml MiniQ H_2O, then 10ml of HCL was added to mix and solve the pellet, then the solution became blue. Filter paper was used (Whatman #1) to filter the solution.
- 100µL of diluted antifoaming agent (1:30) was added into 3ml MiniQ H_2O.
- 50% of NaOH was prepared by adding1ml of NaOH into 19ml of MiniQ H_2O, then 15ml of this solution was added to the bubble base. This solution was used to prevent HCL vapors from entering the NOA.

General Operation Procedures

- Firstly, gas supplies (O_2 and N_2) to the NOA were switched on.
- The chiller and water bath were turned on in advance until temperature of water reach 95 ºC.
- The machine was allowed to cool down to -12ºC.
- The supply pressure was 6psig and the cell pressure is >300 torr, the ENTER was pressed to returned to the main menu.
- 15ml of 1M NaOH was added to the bubble base after opening the gas bubbler.
- After preparing the machine, 3ml of the filtered VCL_3/HCL reagent and 200µl of the diluted antifoaming agent were added and the screw cap was leaved off while adjusting the gas flow into the purge vessel.
- By opening the gas inlet stopcock on the purge vessel and slowly opening the needle valve to start the flow of gas into the purge vessel, then a slow, gentle bubbling of gas was obtained through the reagent.
- The gas flow into the purge vessel was adjusted using the needle valve so that the cell pressure with the purge vessel were connected the same as recorded when the frit restrictor was open to the air (4 -7 torr).

2-Preparation of NO_2^- Standard solutions

- A standard/stock solution (100mM NO_2^-) was prepared by adding 69mg of $NaNO_2$ into 10ml MiniQ H_2O to obtain the standard curve by conducting a serial dilution.
- The remaining steps are exactly similar to (*Preparation of NO_3^- Standard solutions*).

Preparation of NO_2^- Reducing Agent

- 50mg of Nal was dissolved in 2 ml MiniQ H2O.
- 2ml of acetic acid, 500µl of Nal and 100µl diluted antifoaming were added to the purge vessel.

General Operation Procedures

- N2 and O2) gas supplies were connected to the NOA machine and switched on.
- NOA machine and the connected computer were switched on. Then, the temperature for NOA machine was allowed to cool down up to -12ºC.

- Then, the pressure and the cell pressure were adjusted to 6 psig and >300 torr, respectively.
- Once the machine was adjusted, ENTER was then pushed to show the main menu.
- The drain stopcock, gas inlet stopcock and outlet stopcock on the purge vessel were closed. In addition, the septum and cap on the top of the purge vessel was detached. Then, the needle valve on the purge vessel was completely screwed to prevent the gas flow.
- After adding 2mL of concentrated acetic acid (2 mL) to the purge vessel, the gas inlet stopcock (on the purge vessel) was opened. The needle valve was also carefully unlocked to allow gas flow and any remain oxygen was removed by gentle bubbling of the gas into the acid.
- Nal (500μL) was then added to the purge vessel and the screw cap and septum on the purge vessel were gently closed.
- Once the IFD filter line was connected to the frit restrictor and the nut was checked using finger tighten, the outlet stopcock was opened. The cell pressure was kept between 4 to 7 torr by adjusting the gas flow into the purge vessel.
- Once the experiment was completed, all stopcocks, power supply (for the water bath and chiller) and gas supplies were switched off.

Appendix 4.5: Performing NO_3^- and NO_2^- Colorimetric Assay

Performing the Griess Reaction

A commercial kit (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical, Ann Arbor, MI, US) was used to measure salivary NO_3^- and NO_2^- concentrations.

- The following components were required in order to perform the NO_3^- analysis: Nitrate Assay Buffer, Nitrate Reductase Cofactors Preparation, Nitrate Reductase Enzyme Preparation, Nitrate Standard and Griess Reagent Reagents.
- The following components were required in order to perform the NO_2^- analysis: Nitrite Assay Buffer, Nitrite Standard and Griess Reagent Reagents.

Table 4.5 A: Preparation of the standards for NO_3^- and NO_2^-

Standard	NO_3^- or NO_2^- standard (200µM) (µL)	Assay Buffer (µL)	Final NO_3^-or NO_2^- concentration (µM)
1	0	80	0
2	5	75	5
3	10	70	10
4	15	65	15
5	20	60	20
6	25	55	25
7	30	50	30
8	35	45	35

Measurement of Nitrate: Assay procedure

In this procedure, all samples and standards were assayed in duplicate. Location of standards and samples were mapped. Standard curve for NO_3^- can be seen in **Figure 4.5 A**

The following steps were followed to perform the assay:

1) 200 µL of assay buffer were added to the blank wells.
2) 80 µL of diluted samples was added to each of the sample wells.
3) 10 µL of the enzyme cofactor and reductase mixture were added to each of the wells.
4) The plate was covered and incubated at room temperature for one hour.
5) After the required incubation time, 50 µL Griess reagents 1 and 2 were added to each well.

6) The colour in the plate was allowed to develop for 10 minutes before the absorbance was read at 540 nm using plate reader (Multiskan Go Plate Reader, Thermo Scientific).

Measurement of Nitrite: Assay procedure

Again, all samples and standards were assayed in duplicate. Location of standards and samples were mapped. Standard curve for NO_2^- can be seen in **Figure 4.5 B**

The following steps were followed to perform the assay:

1) 200 µL of assay buffer were added to the blank wells.
2) 100 µL of diluted samples was added to each of the sample wells.
3) 50 µL of Griess reagent 1 and 2 were added to each well
4) The colour in the plate was allowed to develop for 10 minutes before the absorbance was read at 540 nm using plate reader (Multiskan Go Plate Reader, Thermo Scientific).

Determination of sample concentrations

The last 2 wells were left for absorbance blanks (containing 200 µL of DW). The absorbance of these wells was then subtracted from the absorbance measured in all other wells. Standard curves for NO_2^- and NO_3^- were obtained for the calculation of the concentrations. A plot of absorbance at 540-550 nm as a function of NO_3^- concentration was made. The NO_3^- standard curve was used for the determination of total NO_3^- + NO_2^- concentration.

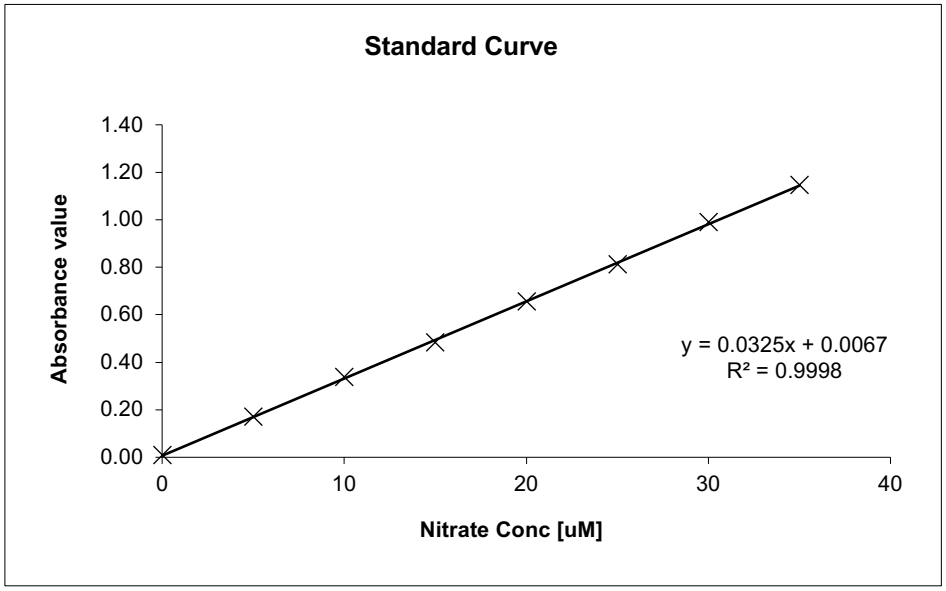

Figure 4.5 A: Standard curve for nitrate analysis using Griess

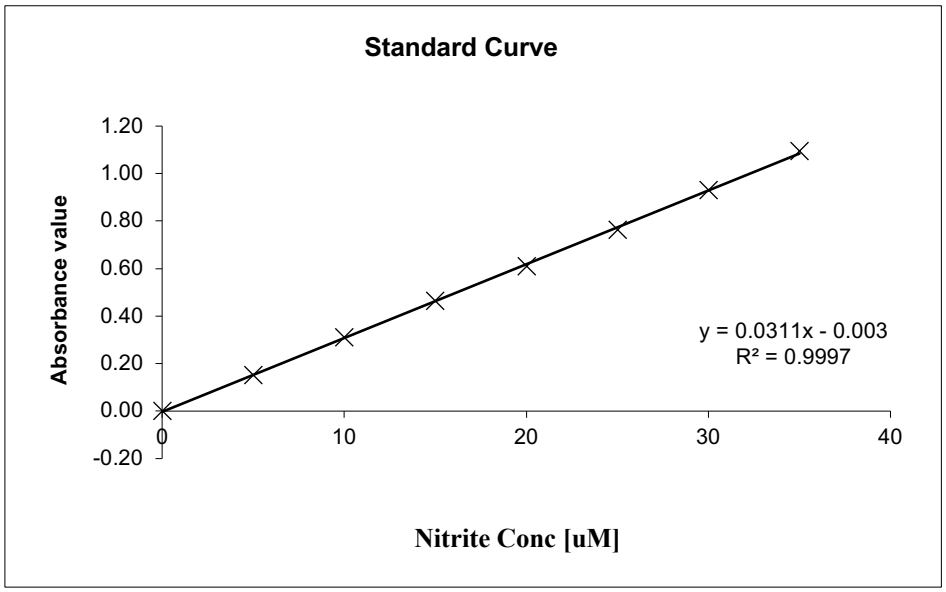

Figure 4.5 B: Standard curve for nitrite analysis using Griess

Appendix 4.6: Performing sialin (SLC17A5) assay

Preparation of sialin standard

Standard	Volume standard (µL)	Volume of standard dilutant (µL)	Final concentration (ng/ml)
1	10ng SD	1ml	10
2	300	300	5
3	300	300	2.5
4	300	300	1.25
5	300	300	0.625
6	300	300	0.312
7	300	300	0.156
8	0	300	0

Preparation of Antibody-Biotin working solution: the total volume of the solution (100 µL/well). The volume needed was diluted in antibody dilution buffer at 1:100. This solution was prepared within 1 hour before the experiment.

Preparation of streptavidin-HRP (SABC) working solution: the total volume of the solution (100 µL/well). The volume needed was diluted in SABC dilution buffer at 1:100. This solution was prepared within 30 min before the experiment.

Assay procedure

The microtiter plate precoated with antibody specific to sialin. After washing the plate 2 times, , standards and samples were added to appropriate wells and then incubated at room temperature for 1.5 hours. The plate was washed again for 3 times. The antibody (biotin) was then added to each well. After which, the plate was inculpated for 1 hour at room temperature, then plate was washed for 3 times. Streptavidin (HRP) was added and plate was incubated for 30 min and then washed. To develop the colourin those wells that contain sialin, TMB substrate solution was added and incubated for 15-20 min. The reaction is terminated by adding stop solution and the colour changed immediately to yellow and it was measured spectrophotometrically at wavelength 450nm. The concentration of sialin is then determined by comparing the O.D. of samples to the standard curve.

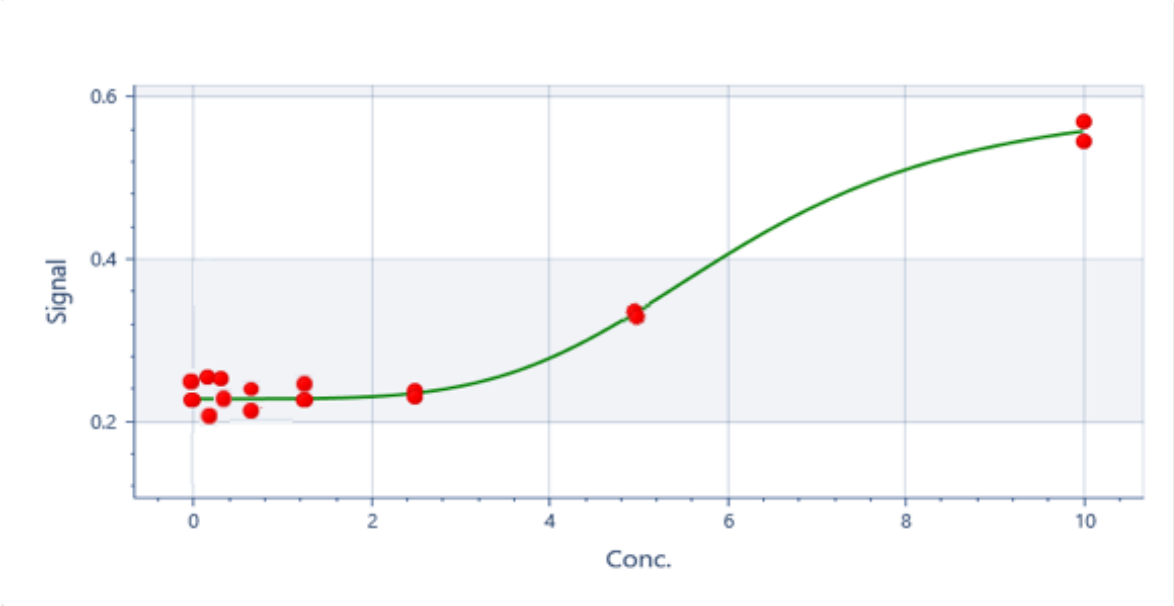

Figure 4.7 A: Standard curve for human sialin using ELISA

Appendix 4.7: Chapter 4 supplementary figures

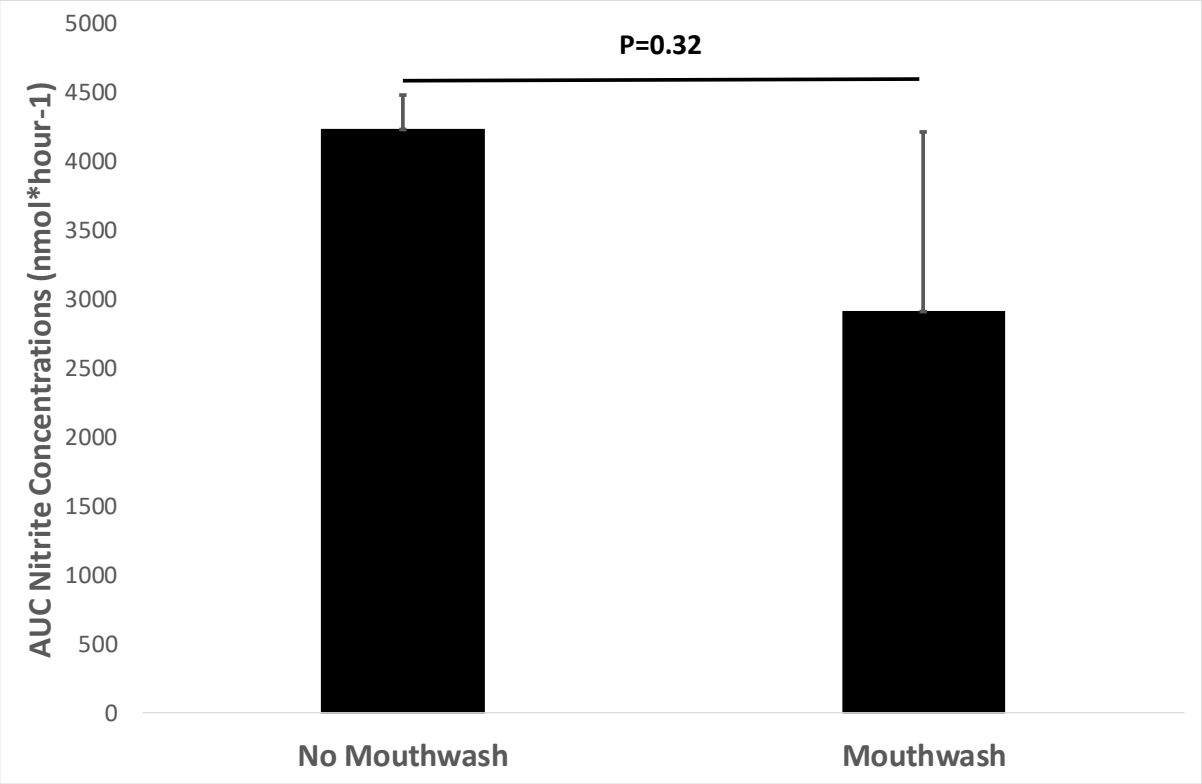

Figure 1: Area under the curve (AUC) for nitrite concentrations measured in five participants receiving no mouthwash prior to water and beetroot ingestion (1st day) compared to five participants who received the mouthwash 24-hour prior to water and beetroot ingestion (2nd day). AUCs were calculated using the trapezoidal method and compared using an independent t test.

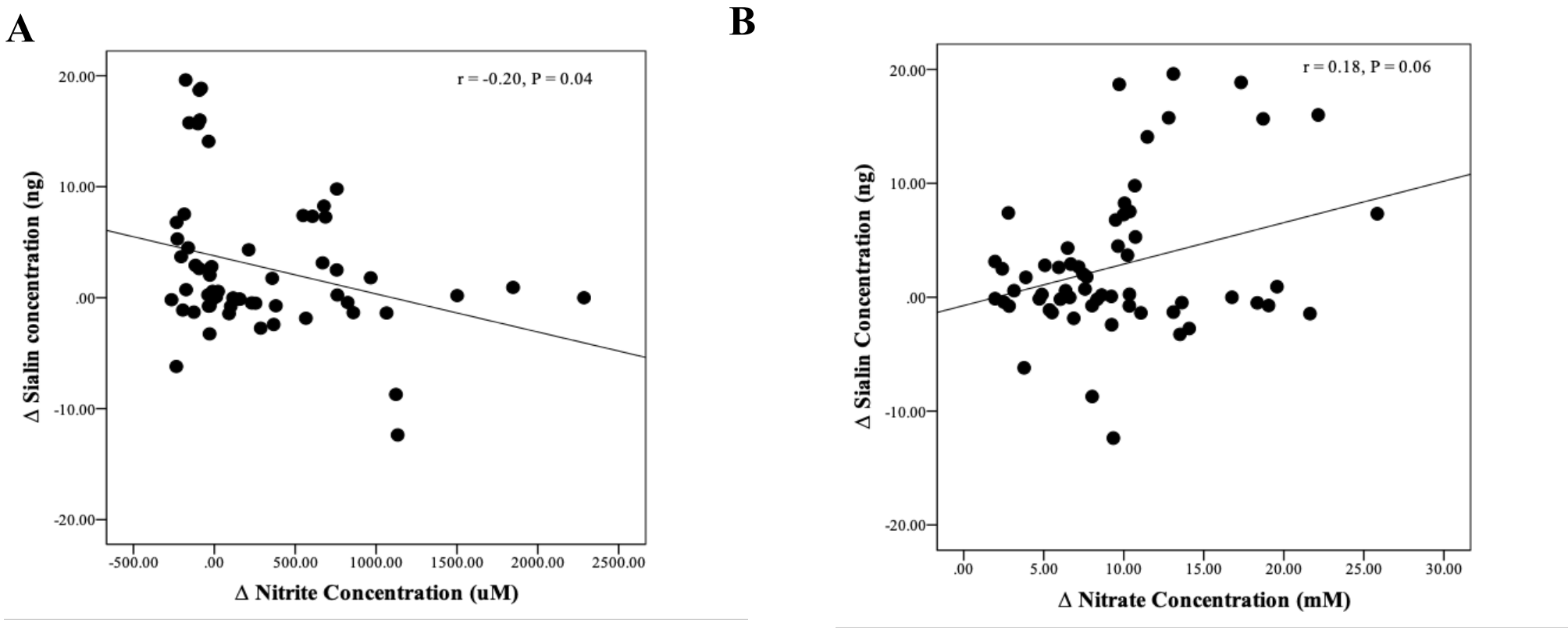

Figure 2: Scattor plot showing correlation between the change in salivary silain and salivary nitrite concentrations (A), and between salivary sialin and nitrate concentrations (B), n=10, Statistical analysis using Pearson's coefficient.

Appendix 5.1: Published paper (Protocol and recruitment results from a 13-week randomized controlled trial comparing the effects of different doses of nitrate-rich beetroot juice on cognition, cerebral blood flow and peripheral vascular function in overweight and obese older people)

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Protocol and recruitment results from a 13-week randomized controlled trial comparing the effects of different doses of nitrate-rich beetroot juice on cognition, cerebral blood flow and peripheral vascular function in overweight and obese older people

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ABSTRACT

Background: Nitrate-rich food can increase NO production and may induce positive effects on brain function. This study examined the feasibility of a randomized clinical trial (RCT) testing the effects of prolonged consumption of incremental doses of dietary nitrate (NO_3^-) in overweight and obese older participants. Secondary aims tested dose-dependent changes in cognitive, vascular and pulmonary functions and cerebral blood flow (CBF).

Methods: This was a single blind, four-arm parallel RCT conducted in 60 overweight and obese older participants. Eligible participants were randomized to:1) high NO_3^- (140 ml of beetroot juice (BJ) per day, ~800 mg of NO_3^-/day), 2) moderate NO_3^- (70 ml of BJ per day, ~400 mg of NO_3^-/day), 3) low NO_3^- (70 ml on alternate days, ~400 mg of NO_3^-) or 4) NO_3^- depleted (70 ml on alternate days, ~0.001 mg of NO_3^-). Measurements of cognitive, vascular and pulmonary functions and CBF were conducted at baseline and 13-weeks NO_3^- intake was assessed by six 24-h recalls, and by measuring NO_3^- intake biomarkers. Feasibility was assessed by obtaining qualitative feedback and evaluating trial recruitment, retention, compliance with study visits and measurement protocols.

Results: Participant recruitment started in July 2018 and ended in April 2019. Of all the recruitment strategies that were used, advertisement of the study via Facebook generated the highest response rate. Sixty-two participants consented and were enrolled. Overall, characteristics of included participants matched our recruitment criteria.

Conclusion: The findings from this study provide evidence of the acceptability and feasibility of an intervention investigating the effects of incremental doses of high-nitrate BJ over a prolonged period.

Trial registration: The intervention study was registered with clinical trial ISRCTN registry (ISRCTN14746723) on 27 December 2018.

1. Introduction

Nitric oxide (NO) is a gaseous signalling molecule which is produced primarily via the activity of inter-connected enzymatic and non-enzymatic pathways [1]. Reduced NO synthesis is associated with learning and memory impairment in rats which is improved by L-arginine supplementation [2]. In humans, reduced NO availability is associated with increased risk of neurodegenerative diseases such as

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Alzheimer Disease [3]. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NO synthase (NOS) and higher plasma ADMA concentrations are associated with reduced NO synthesis, endothelial dysfunction and increased risk for cardiovascular diseases [4,5].

NO production decreases with age as a result of decreased efficiency of the enzymatic synthetic pathway, and is reduced further in older people with metabolic and cardiovascular impairment [6,7]. Key characteristics of vascular ageing include decreased NO production, reduced endothelial integrity and increased arterial stiffness; established risk factors for the development of atherosclerosis [8]. Nutritional and lifestyle interventions capable of maintaining, or restoring, normal NO production may reduce the risk of atherosclerosis and cardiovascular diseases. In turn, this might help to reduce the risk of cognitive dysfunction and dementia since, among older people, better cardiovascular health is associated with a lower incidence of cognitive decline and lower risk of dementia [9].

Recent studies have demonstrated that foods rich in inorganic nitrate (NO_3^-) can increase NO production and induce positive effects on blood pressure, muscular performance and brain function [10,11]. In particular, some studies have reported improvements in cognitive function (memory and executive performance) and motor skills after dietary NO_3^- supplementation, which appears to be mediated by augmented cerebral blood flow (CBF) and efficiency of cellular metabolism [12,13]. However, our recent systematic review and meta-analysis found no evidence that NO_3^- supplementation improves cognitive function and CBF [14]. This systematic review identified several limitations in the current evidence base including that the majority of trials were of short duration and conducted in healthy, normal weight participants (only one study supplemented inorganic nitrite had a duration of 10 weeks whereas the remaining trials had a duration of less than 2 weeks). Hence, the current evidence on the sustained effects of dietary NO_3^- and nitrite (NO_2^-) on cognition, brain function and CBF is limited, and studies of longer duration and larger sample size are needed in individuals with reduced NO synthesis and at greater risk of endothelial dysfunction and cognitive impairment.

This pilot RCT was designed primarily to determine the feasibility and acceptability of the protocol for a 13 weeks intervention study in which overweight and obese older participants were asked to consume different doses of NO_3^--rich beetroot juice (BJ). Secondary aims of the study included testing whether the different doses of dietary NO_3^- result in different changes in cognitive, vascular and pulmonary functions and CBF.

2. Methods

2.1. Study design and randomization

This feasibility study was designed as a randomized, single-blind, placebo-controlled, four-arm parallel trial with a duration of 13 weeks. After a screening assessment for the evaluation of the inclusion and exclusion criteria, eligible participants were randomized to one of the four intervention groups. The first intervention group (Group 1) was asked to consume two 70 ml shots of concentrated BJ per day (400 mg of NO_3^- per shot, Beet-It, James White Company), one every morning (~8am) and one each evening (~9pm). Groups 2 and 3 were asked to consume one shot every evening (~9pm) and every other evening (~9pm), respectively. Group 4 (the control group) received the placebo (NO_3^- depleted BJ, 0.003 mg of NO_3^-) and were asked to consume a bottle every other evening (~9pm). The randomization pattern was generated using the RAND function in Excel (Excel Microsoft software, Microsoft corp, Redmond, WA, USA). The study adhered to the SPIRIT guidelines (see Table 1).

2.2. Study setting

The study visits were conducted at the NU-Food research facility at Newcastle University and the CBF measurements were performed at the Brain Performance Nutrition research centre at Northumbria University.

2.3. Blinding

This was a single-blind study as participants were not informed about whether they were allocated to the NO_3^--rich BJ or NO_3^--depleted BJ (placebo). Blinding of the researchers to the intervention was not possible due to the nature of the dietary interventions and study design as the frequency and volume of the BJ given to each participant revealed the nature of the interventions.

2.4. Recruitment strategies

Participant recruitment occurred between July 2018 and April 2019. Potential participants were identified through several recruitment strategies:

- Flyers and posters distributed in local communities
- Advertisements in local newspapers and newsletters
- Emails circulated to Newcastle University members of staff
- Search of existing databases held by Newcastle University of participants in previous studies who had consented to be contacted to take part in future research studies
- Advertisement of the study via social media (i.e., Facebook)
- Invitation from recruited participants to friends and relatives

2.5. Retention

Several strategies were adopted to minimize the drop-out of participants during the study. All participants were provided with contact details of the lead investigator who replied promptly to participants' queries. Regular emails were sent to participants to remind them to complete the scheduled dietary assessments using Intake24 every two weeks and to collect the urine and saliva samples every 4 weeks. All appointments were scheduled at the participants' convenience but careful planning was required to ensure that time intervals between visits were similar between participants (deviation from scheduled appointments: ±2 days). Participants received a £60 shopping voucher on completion of the study.

3. Participants

3.1. Inclusion criteria

We aimed to recruit 60 healthy, overweight and obese (BMI) range: 25–40 kg/m^2) male and female older participants (60–75 years).

3.2. Exclusion criteria

- Current participation in other clinical research studies.
- Vegetarian.
- Smokers.
- Systolic BP lower than 115 mmHg and greater than 160 mmHg; diastolic BP lower than 70 mmHg and greater than 100 mmHg.
- Active cancer and any diagnosis of malignant cancer in the last 5 years
- Excessive alcohol intake (>21 units per week) (Alcohol unit = Total alcohol volume (ml) * Alcohol by volume (%)/1000).
- Allergy or intolerance to the intervention food (BJ).
- Diagnosis of chronic or acute metabolic and inflammatory conditions that may interfere with the study outcomes.
- Major surgical operations.
- Use of prescribed psychiatric drugs (antidepressants, sedatives, antipsychotics), diuretics, organic nitrates and proton pump inhibitors.

Table 1
Standard protocol items: recommendations for interventional trials (SPIRIT).

	Study period																
Time point (weeks)	Enrolment	Baseline	Post allocation														Follow up
	W0	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W23
Enrolment														
Eligibility screen	X													
Informed consent	X													
Randomisation	X													
Allocation	X													
Interventions														
High RJ dose			↔	↔	↔	↔	↔	↔	↔	↔	↔	↔		
Medium RJ dose			↔	↔	↔	↔	↔	↔	↔	↔	↔	↔		
Low RJ dose			↔	↔	↔	↔	↔	↔	↔	↔	↔	↔		
Placebo (control)			↔	↔	↔	↔	↔	↔	↔	↔	↔	↔		
Assessments														
Demographic data	X													
Anthropometric measurement		X					X						X	X
Physical activity questionnaire		X											X	X
Blood pressure		X												X
Endothelial function ind		X												X
Pulmonary function ind		X												X
Cognitive function ind		X												X
Cerebral blood flow ind		X												X
Dietary intake			X		X		X		X		X		X	X
Blood samples		X										X		
Saliva samples		X			X				X					X
Urine samples		X			X				X					X
Saliva strips		X			X				X					X
Participant feedback		X			X				X					X

W = week of the study.

- Use of prescribed hormonal therapies (oestrogens, thyroxin, and progesterone), anti-hypertensive (Ca++ channel blockers, beta-blockers, and angiotensin-converting-enzyme (ACE) inhibitors), statins and any other anti-dyslipidaemic agent, if the prescription had started, or the dose had been started/changed, in the previous three months.
- Non-prescribed dietary supplements were stopped at least for 2 weeks before starting the trial.
- Use of the mouthwash during the study was not allowed as it interferes with the conversion of oral NO_3^- into NO_2^-.

3.3. Screening and consent

Individuals who contacted the research team and expressed an interest to participate received detailed information about the study either by email or by post. A telephone screening was then arranged to assess eligibility based on date of birth, medical history and medication use and commitment and availability to participate to the study over a period of three months. If potential participants met the eligibility criteria at the telephone screening, they were invited to an onsite screening visit for further assessments to confirm the eligibility.

During the on-site screening visit, a member of the research team explained the study protocol and measurement procedures and answered any queries from participants. Potential participants were asked to read and sign the consent form. Next, body weight and height were measured to calculate BMI and resting clinic BP was measured in triplicate. Participants were included in the study if BMI and BP were within the range specified in the study protocol. At the end of the screening visit, eligible participants started a familiarization session for the computerised cognition tasks which included the completion of three consecutive cognitive tasks. This aimed to minimize the risk of a learning effect between baseline and end of study assessments.

4. Outcome measures

4.1. Primary

The primary aim of the study was to assess the acceptability and feasibility of the proposed intervention trial in terms of recruitment rate and time taken to complete the recruitment, adherence, adverse effects and process evaluation. This was achieved through documentation of adherence to the intervention and measurement protocol, recording of any adverse events, as well as measurements of changes in health outcomes that may occur during the intervention period.

4.2. Secondary

The study protocol includes the investigation of changes in:

- Cognitive function
- Cerebral blood flow
- Resting clinic and home BP
- Nitric oxide biomarkers (urinary, plasma and salivary NO_2^- and NO_3^-, NO exhaled and whole-body NO production using stable isotopic methods)
- Validation of salivary NO strips for the long-term compliance to NO_3^- -based nutritional interventions
- Metabolic risk factors (glucose, insulin)
- Oxidative stress biomarkers (3-Nitrotyrosine)
- Inflammatory biomarkers (IL-6)
- Endothelial-dependent and independent microvascular blood flow, measured by Laser Doppler Iontophoresis (LDI).
- Pulmonary function measured by portable spirometer

4.3. Compliance with the intervention

All participants were provided with verbal and written instructions on the tasks to be completed at home during the trial. Participant compliance was checked primarily by a daily compliance log which was used to record the time they consumed the BJ. In addition, they were asked to return any unopened bottles to the study team. After six weeks, participants returned to the research centre for a mid-study assessment visit to check their compliance, to collect information on safety and adverse events that may have occurred during the first part of the study and to provide participants with the final batch of BJ bottles to complete the study.

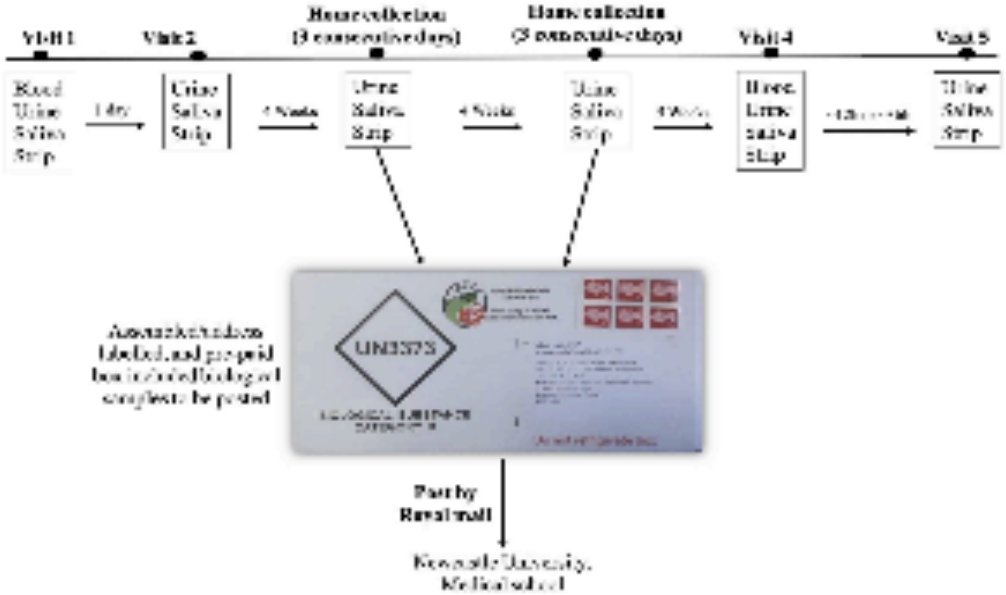

Fig. 1. Overview of the protocol for the collection of the biological samples during the study.

Compliance with the intervention was also assessed objectively by measuring dietary NO_3^- and NO_2^- concentrations in plasma, saliva and urine samples and using salivary strips. Plasma samples were collected at baseline and 13 weeks. Salivary strips and urine and saliva samples were collected for 3 consecutive days every 4 weeks. The protocol for the collection of the biological samples is described in Fig. 1. The measurement of NO_3^-/NO_2^- concentrations were taken as a measure of the adherence to the intervention and it is expected to find greater concentrations in these biomarkers in the samples collected from high NO_3^- dose group compared to lower NO_3^- dose groups and placebo.

Salivary NO strips were also collected concomitantly with the collection of the saliva samples as part of a validation sub-study to assess the utility of this non-invasive method for the assessment of compliance to prolonged NO_3^- -based nutritional interventions. The colour of the salivary strips was read immediately after receiving them to determine salivary NO_2^- concentrations. All participants were instructed not to change their dietary habits or physical activity and to avoid using mouthwash during the study.

4.4. Data collection

Socio-demographic, lifestyle and medical history: A questionnaire was used to collect detailed information on any previous or current illnesses, prescribed and non-prescribed medication use, smoking habits, education and alcohol intake.

Feasibility and acceptability: Feasibility was evaluated by assessing recruitment and retention rates, time taken for recruitment, and number of dropouts, with reasons. In addition, adherence to the intervention and compliance with other components of the protocol to be completed at home were evaluated. Completeness of recording of the outcome measures was reported. Acceptability was evaluated by obtaining feedback from participants on the nutritional interventions and measurement protocols as well as reporting the reasons for discontinuation of the intervention.

Anthropometry and body composition: Height was measured to the nearest 0.5 cm at the screening visit using a stadiometer with an adjustable headpiece. Body weight and body composition parameters (fat mass, fat free mass, body fat % and total body water) were assessed at baseline and at the end of the study by bioelectrical impedance analysis (Tanita BC420 MA, Tanita Corporation, Tokyo, Japan). Weight and height were used to calculate Body Mass Index (BMI). Waist circumference at the midpoint between the lowest margin of the last rib and the top of the iliac crest was measured at baseline and end of the study.

Blood pressure: Resting clinic blood pressure was measured using an automated monitor (model: Omron M3 [HEM-7200-E3(V)]). Participants were in a supine position and measurements were performed after participants had rested for at least 10 min. Three blood pressure measurements were taken and the average of the three readings was calculated. Home resting blood pressure was also measured using the same monitor at baseline and end of the study as part of the protocol for the measurement of whole-body NO production. Each participant was asked to measure blood pressure after they had collected a saliva sample (eight measurements in total). All participants were asked to rest for about 10 min before each measurement (see below for more details).

Peripheral vascular function: Vascular function data were collected at baseline and the end of study using Laser Doppler iontophoresis (LDI, Moor Instruments, Axminster, UK). LDI has been shown to provide a valid measure of skin blood flow [15,16]. One % acetylcholine (Ach, Sigma-Aldrich) and 1% sodium nitroprusside (SNP, Sigma-Aldrich) solution (v/v) were prepared fresh before each visit. One mL of each solution was applied into the iontophoresis chambers placed on the forearm of the participants to quantify changes in endothelial-dependent and independent microvascular blood flow, respectively. Forearm skin erythrocyte flux was measured for 3 min prior to the start of the iontophoresis (baseline), for 5 min during Ach and SNP delivery by iontophoresis (stimulation, current set at 30 μA) and for 10 min after the Ach and SNP delivery (recovery). All assessments were performed with the participant lying supine on a bed.

Cerebral blood flow: Haemodynamic responses were monitored using a frequency domain 'quantitative' NIRS system (OxiplexTS Frequency-Domain Near-Infrared Tissue Oximeter, ISS Science) at rest and stimulated conditions (i.e. while participants performed computerized cognitive function tests including serial subtraction 3 and 7, Stroop (congruous & incongruous) and peg and ball tasks, which were mainly executive function tasks). NIRS has been used extensively in neuroscience research as it provides measurements of changes in cerebral blood oxygenation related to brain activity [17]. These measurements were performed at baseline and end of the study. CBF data were collected at a rate of 5 Hz.

Cognitive function: The Computerised Mental Performance Assessment System software (COMPASS, University of Northumbria), which has been shown to be sensitive to a range of nutritional interventions [18,19], and the Trail Making Tasks A and B, were used to assess cognition at baseline and the end of the study. The COMPASS test includes word presentation, immediate word recall, digit vigilance, numeric working memory, choice reaction time, computerized corsi blocks, Stroop, peg and ball, delayed word recalls and words recognition. A description of these cognitive tasks is provided in Supplemental Table 1.

Lung function: Pulmonary function was assessed using a portable spirometer (Micro Spirometer®, Micro Medical Instruments Ltd, UK), at baseline and the end of the study. The following variables were recorded: forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC) and the ratio of the two volumes (FEV1/FVC). In addition, the real-time fractional exhaled nitric oxide (FeNO) was assessed using a portable, non-invasive, automated device (NIOX VERO®, Circassia AB, UK).

Dietary Intake: Dietary NO_3^- intake during the study was assessed using a 24-hr dietary recall every two weeks (6 dietary assessments were performed in total) using an online validated platform (Intake 24) [20]. Dietary NO_3^- intake was calculated by assigning the specific NO_3^- content for each reported food (from a comprehensive database including the NO_3^- concentrations from 3498 individual foods and beverages estimated from sixty different countries [21]) and taking into account the amount consumed. Dietary NO_3^- intake was reported in mg/day.

5. Collection of biological samples

5.1. Blood

Blood samples were collected after an overnight fast (at least 12 h) at baseline and at 13 weeks post-dose. Blood samples were collected by venepuncture by trained phlebotomists in 3 tubes (4 ml each), containing (LH) lithium heparin, EDTA (Ethylenediaminetetraacetic acid) and sodium fluoride and potassium oxalate, respectively.

The samples were processed within 10 min of collection to minimize nitrite degradation. Samples were spun at 4000 rpm for 10 min at room temperature and plasma aliquots were stored at −80 °C until further analysis. Samples were used to measure the concentrations of NO_3^-, NO_2^-, cGMP, glucose, insulin, 3-nitrotyrosine (a marker of oxidative stress) and Interleukin-6 (a pro-inflammatory marker).

5.2. Urine and saliva samples and salivary nitrite strips

Salivary nitrite strips, urine and saliva samples were collected at baseline and then again at 4 weeks (3 consecutive days), 8 weeks (3 consecutive days), and 13 weeks post-dose (see Fig. 1).

5.3. Urine

A mid-stream urine sample was collected at the baseline and end of study visits using a sterile, collection kit (Mid-stream urine collection

set, UN252, Sherwood, UK). After 4 and 8 weeks, urine samples were collected at home by each participant for three consecutive days using a urine collection kit. A pre-paid box with sealed envelopes for the storage of biological samples was provided and each participant was asked to mail the samples to the research team using a fast delivery service. During each three-day collection period, participants were asked to keep urine samples in the fridge. All urine samples were stored at −20 °C until further analyses. Urine samples were used for the measurement of NO_3^- concentrations using ozone-based chemiluminescence.

5.4. Saliva

Participants were asked to chew a cotton ball for approximately 2 min, which was then placed in the barrel of a 20 mL syringe. The plunger was then inserted back into the syringe and used to squeeze the saliva into a 2 mL Eppendorf tube. Participants were asked to repeat this saliva collection procedure at home as required by the study protocol. Samples collected after 4 and 8 weeks were posted [together with urine samples (see above)], and participants were instructed to keep the saliva samples in the freezer until put in the post. After arrival in the laboratory, saliva samples were stored at −20 °C until required for analysis of NO_3^- and NO_2^- concentrations using ozone-based chemiluminescence and nitrite concentrations using an ELISA kit (BioAssay™ (Human), Stratech Scientific Ltd, Cambridge, UK).

5.5. Salivary nitrite strips

Berkeley strips (Berkeley Test®, CA, USA) were used as per the manufacturer's guidelines. Specifically, participants were asked to place the test strip with the 'saliva here' side on the tongue and swab it over a 10 s period covering different areas including the dorsal surface of the tongue. The two ends of the strip were folded and pressed gently for 10 s. The colour of the NO test pad was then allowed to develop over a 45 s period. The intensity of the colour was compared with a colour chart included in the product package (Depleted, Low, Threshold, Target and High), with darker colours corresponding to higher NO_2^- concentrations. For the purpose of quantitative analysis, categorical colours were given a categorical number from 1 to 5, where 1 corresponding to "Depleted" and 5 corresponding to "High". Participants were asked to repeat a similar procedure at home as required by the study protocol. The strips collected at 4 and 8 weeks were posted (with the urine and saliva samples) to the research centre. Participants were asked to keep the strips in a dry place until put in the post. The colour of the strips was recorded as soon as they were received in the laboratory.

5.6. Whole-body NO production

The measurements of whole-body NO production were performed at baseline and at the end of the study. Participant were asked to consume a controlled low NO_3^- meal at lunch time. Four hours later, a baseline saliva sample was collected followed by ingestion of 4 mg of $Na^{15}NO_3$ dissolved in 100 mL of distilled water. Further saliva samples were collected at 6, 7, 8, 9, 18, 19 and 20 h post-meal. NO_3^- enrichment in saliva was measured using Gas Chromatography Mass Spectrometry [22] and NO production will be estimated using a validated model proposed by Refs. [6].

5.7. Adverse events reporting

Adverse events are symptoms or signs that may occur during the trial and may or may not be causally related to the intervention. All adverse events potentially related to the BJ product were recorded.

5.8. Sample size calculation

This is a pilot study designed to assess the feasibility and acceptability of the proposed intervention. A sample size of 15 per group was based on the predicted effect size provided by the study and based on the guidelines indicated by Whitehead et al. [23]. This study provides guidance on sample size calculation for pilot studies with the aim of maximising use of resources and to avoid occurrence of a type II error. Specifically, a sample size of 15 individuals per group would provide a 90% power to detect a medium effect size (δ) between 0.3 and 0.7.

5.9. Statistical analysis

Baseline characteristics are presented as mean ± SD. Feasibility was evaluated by the effectiveness of the screening process and recruitment of participants who met the eligibility criteria for the study, together with retention of participants. Retention was evaluated by collection of information on the proportion of participants lost after randomization in each arm of the study. Compliance with the interventions was estimated by calculating the proportion of BJ bottles returned unused relative to the dispensed bottles.

5.10. Confidentiality

Every effort was made to ensure confidentiality. In this study, paper forms are stored in locked filing cabinets and transferred to password secured computer databases accessible only to the researchers. Participant data were anonymised by assigning a unique identification code which was used on all documents and electronic databases used in the study.

6. Results

6.1. Recruitment

In total, 250 responses were received from use of our various recruitment methods. As shown in Fig. 2, advertisement via Facebook generated the highest response rate with 77 contacts (31%) made within a period of 10 days, followed by 68 contacts (28%) established from recruitment emails that were sent to participants in previous studies who were identified from our databases, 61 responses (24%) from local newspapers, 13 responses (5%) received through the advertisement on the Voice website (https://www.ucl.ac.uk/nica/voice/), 12 responses (5%) from advertisement in the Cathedral church of St Mary newsletter, 11 responses (4%) received after referral by current participants. The least effective strategy was the distribution of flyers, from which only 7 responses (3%) were received.

Fig. 3 summarises the recruitment and retention of participants in the study. A hundred and six participants were either unreachable or declined to participate. The initial telephone screening was conducted with 144 individuals. At this stage, 68 potential participants were not

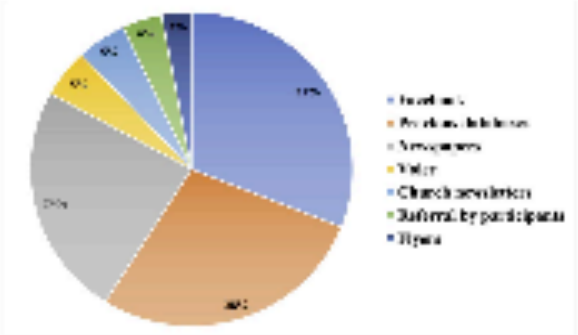

Fig. 2. Response rate from each recruitment strategy.

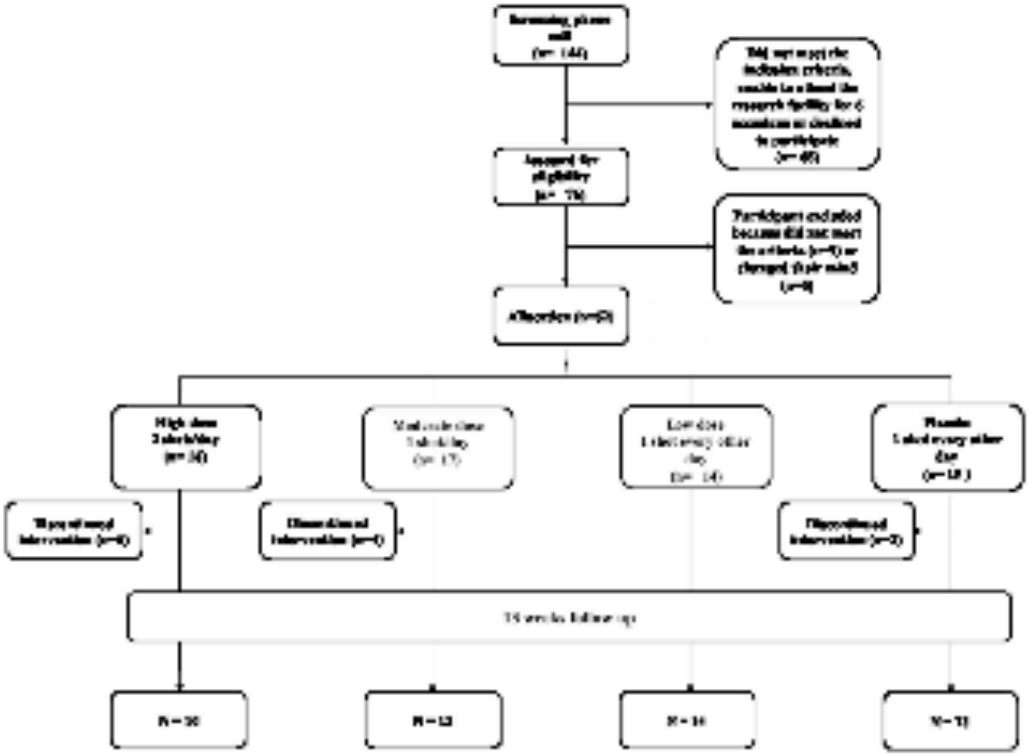

Fig. 3. Flowchart describing the recruitment of participants into the feasibility study.

included for the following reasons: 1) taking medications such as antacids, antidepressants or diuretics (54%), 2) co-existent health conditions including cancers, cardiovascular disease, kidney disease, type 1 diabetes or epilepsy (19%), 3) declined to take part after reading the information sheet (12%), 4) unreachable (6%), 5) smokers (4%) and 6) age over 75 years old (4%).

A total of 76 participants attended the on-site screening visit to confirm their eligibility. Seventy of these persons were eligible and 62 participants were consented and enrolled. Nine participants (11%) were excluded at the screening visit, and the reasons included high BP (55%), normal BMI < 24.9 kg/m^2 (33%) and BMI > 40 mg/m^2 (11%). Additional reasons to not participate included: 1) no longer interested to take part (4%), 2) personal bereavement (1%) and 3) being unwell (1%). The recruitment target was 60 participants, but two participants who dropped out the study immediately after the start of the intervention were replaced and, therefore, 62 participants were randomized and had a baseline visit assessment.

The sixty-two participants were randomized to one of the four intervention groups as follows: 16 participants were allocated to Group 1 (2 shots of BJ/day, every morning and evening), 17 participants to Group 2 (1 shots of BJ/day, every evening), 14 participants to Group 3 (1 shot of BJ every other evening) and 15 participants were allocated to Group 4 (Placebo, 1 shot of BJ depleted NO_3^- every other evening).

6.2. Attrition

Of those who were randomly assigned to Group 1 ($n = 16$), 10 participants completed their 13 weeks assessment. Similarly, 13, 14, and 13 participants from Groups 2, 3 and 4 completed their 13 weeks assessment. The overall attrition rate for the study was 19% with 12 participants dropping from the study.

6.3. Baseline characteristics of participants

Baseline characteristics of the participants are reported in Table 2. The age of participants ranged from 60 to 73 years (mean ± SD, 66 ± 4 years) and 62% were men ($n = 38$). All included participants were either overweight ($n = 35$) or obese ($n = 27$). BMI for all randomized participants ranged from 25 to 39 kg/m^2 (30.4 ± 4 kg/m^2). The range of SBP was from 110 to 167 mmHg (mean 135 ± 15 mmHg). The range of DBP was from 60 to 100 mmHg (mean 77 ± 10 mmHg).

7. Discussion

There has been a growing research interest into the investigation of the potential beneficial effects of NO_3^- on cardio-metabolic health outcomes including blood pressure, glucose control, dyslipidaemia, cognition, heart failure and peripheral arterial diseases [12,24–27]. However, these studies had limited power due to the small sample sizes and the duration of the studies was short (< 8 weeks). To the best our knowledge, this is the longest RCT evaluating the feasibility and potential beneficial effects of different doses of NO_3^--rich BJ supplementation on cognitive and vascular functions, CBF and pulmonary functions in overweight and obese older adults. The study involved a mixed-method approach where the primary outcomes on feasibility and adherence were evaluated by asking participants to complete detailed questionnaires with closed and open questions. Questions focussed on process evaluation, adherence to nutritional interventions and measurement protocols, safety of the intervention and procedures and suggestions for future studies. An exit questionnaire was also administered to participants who dropped out of the study asking to explain the reasons for their withdrawal and to provide feedback and suggestions for future studies. Objective measures of adherence were number of dropouts and completion of daily logs to

Table 2
Baseline characteristics of the study participants including use of medications.

	Total (n = 62)
Characteristics	
Gender, M/F	24/38
Age (years)	66 ± 4
Education (years)	15 ± 3
Body weight (kg)	85 ± 13
BMI (kg/m^2)	30 ± 4
WC (cm)	103 ± 9
Fat Mass (kg)	32 ± 9
Fat Mass (%)	38 ± 8
Fat free mass (kg)	53 ± 10
SBP (mm Hg)	135 ± 15
DBP (mm Hg)	77 ± 10
PA (METs/wk)	3667 ± 3684
Energy intake (Kcal)	2440 ± 992
Medication use	
Antihypertensive	6 (9.8%)
Hormonal therapy	
Thyroxin	9 (14.5%)
Testosterone	1 (1.6%)
Antihistamine	1 (1.6%)
Lipid lowering agents	10 (16%)
Vitamin D	3 (5%)
Aspirin	1 (1.6%)
Corticosteroid inhaler	2 (3%)
No therapy	35 (56%)

M/F, male/female; BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; PA, physical activity; PA, physical activity; Data are expressed as mean ± SD. Medications are presented as n (% of group).

record BJ consumption. In addition, we asked participants to complete online 24-hr dietary recalls every 4 weeks and to collect biological samples (urine, saliva and salivary strips) to measure changes in NO_3^- concentrations to provide objective evaluation of compliance with the interventions. Additional strengths of the study include a detailed assessment of physiological outcomes using advanced methods for the measurement of cognitive function, peripheral and central vascular function, clinic and home measurements of resting blood pressure and measurement of nitrate and nitrite concentrations using ozone-based chemiluminescence. In addition, this is the first study to employ a non-invasive stable isotopic method for the evaluation of changes in systemic NO production following prolonged NO_3^- supplementation.

The most challenging aspects of the study included recruiting older participants who met our inclusion criteria and in retaining them in the trial. Some of the difficulties associated with recruiting older people in clinical trials have been documented [28]. We employed a variety of recruitment strategies over a 10-month period and we found that advertising the study on social media (i.e. Facebook) was the most effective strategy. This approach yielded a high response rate within a short period of time and we believe that this recruitment strategy may represent a viable approach to enrol older participants from the community in future nutritional and clinical studies. The use of social media by older people is increasing [29]. In the UK in 2017, 39% of those aged 65–74years used a smartphone and 48% of internet users in this age group have a social media profile [30]. A recent study indicated that the response rate from older people to a social media advertisement for recruiting participants into a clinical trial was higher than for younger adults [31]. Another recent study reported that the use of social media was a successful strategy for recruiting older participants into a clinical trial investigating cardiovascular outcomes [32]. We also recruited participants from a database of participants who had participated previously in other studies at Newcastle University and who had expressed an interest to be involved in future research. This approach was also effective in recruiting participants into the trial, but it was more time-consuming, and it had a lower response rate compared with the social-media approach. The RCT was also promoted in local newspapers, but the response rate was lower, and the costs of the advertisement were considerably higher than the social media approach. Overall, the use of social media (i.e., Facebook, Twitter) appears to offer a cost-effective solution to facilitate the recruitment of older overweight and obese participants from the community into a RCT.

There is limited evidence on the long-term effect of dietary NO_3^- on physiological functions in older populations. This feasibility study will inform the design and conduct of definitive, larger and longer studies investigating the effects of nitrate-rich BJ on cognitive and vascular outcomes in older people.

Funding

This study was funded by Newcastle University core budget.

Trial status

Data collection in the trial ended in July 2019.

Declaration of competing interest

The authors have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.conctc.2020.100571.

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Appendix 5.2: Ethical approval

22 March 2019

Abrar Babateen
Institute of Cellular Medicine

Faculty of Medical Sciences
Newcastle University
Medical School
Framlington Place
Newcastle upon Tyne
NE2 4HH

FACULTY OF MEDICAL SCIENCES: ETHICS COMMITTEE

Dear Abrar Babateen,

Title: Effects of different doses of beetroot juice on cognitive function and cerebral blood flow in obese/overweight older subjects
Application No: 1503_2/4477/2018 (Amendment)
Start date to end date: 15/05/2018 to 31/09/2019

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: **1503_2/4477/2018 (Amendment).** 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

M Holbrough

Marjorie Holbrough
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.

Appendix 5.3: Examples of flyer that have been distributed to recruit older participants

VOLUNTEERS NEEDED FOR NUTRITION RESEARCH

Effect of Beetroot juice on cognitive and vascular function and cerebral blood flow

We are currently recruiting volunteers for a nutrition study at the Human Nutrition Research Centre, Newcastle University and Brain, Performance and Nutrition Research Centre, Northumbria University.

Preliminary animal and human investigations have reported an improvement of cognitive function (memory and executive performance) after dietary nitrate supplementation. This study will investigate the effects of dietary nitrate supplementation in form of beetroot juice on cognitive function and cerebral blood flow in humans, the duration of intervention is 3 months.

We are looking for a total of 60 overweight or obese participants for this study. Each volunteer will be required to attend the Human Nutrition Research Centre clinical facilities at Newcastle University on four occasions, and two occasions at Brain, Performance and Nutrition Research Centre at Northumbria University. The screening visit might take approximately 2 hours. Two visits will have a duration of approximately 2:30 hours. One visit will have a duration of approximately 1 hour. Finally, the two visits at Northumbria University will have a duration of approximately 1 hour.

If:

- **Your age is between 60 and 75 years old**
- **You do not smoke**
- **You are not vegetarian**
- **You are willing to attend the research centre on six occasions**

If you are interested in taking part please contact us on 07465615915 or 0191 208 2004 or 0191 208 1140 or email either
Abrar Babateen or
(A.M.O.Babateen2@ncl.ac.uk)
or Dr Mario Siervo
(mario.siervo@ncl.a[redacted]
[redacted]on [redacted]me
commitment, you will receive an honorarium of **£60** at the end of the study. Reasonable travel expenses will also be reimbursed.

Kind regards

Ms Abrar Babateen / Dr Mario Siervo
Human Nutrition Research Centre
Institute of Cellular Medicine
Newcastle University
Newcastle on Tyne
NE2 4HH, UK
Tel: 07465615915/ 0191 208 2008 and 0191 208 1140
Email: A.M.O.Babateen2@ncl.ac.uk or
mario.siervo@ncl.ac.uk

Appendix 5.4: Newspaper advertisement

Appendix 5.5: Information sheet

Beetroot, cognitive function and cerebral blood flow

Information Sheet for Study Participants

This study was approved by the Faculty of Medical Sciences Research Ethics Committee, part of Newcastle University's Research Ethics Committee. This committee contains members who are internal to the Faculty, as well as one external member. This study was reviewed by members of the committee, who must provide impartial advice and avoid significant conflicts of interests.

Chief Investigator: Ms Abrar Babateen

Human Nutrition Research Centre
Newcastle University
Medical School Newcastle upon Tyne,
Framlington Place, NE2 4HH, UK

For further information contact the study team (Ms Abrar Babateen, Dr Mario Siervo)
Telephone: 0191 208 2004 and 0191 2081140
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mario.siervo@ncl.ac.uk
http://www.ncl.ac.uk/hnrc

You are being invited to take part in a research study. Before you decide whether or not you wish to take part it is important that you understand why the research is being done and what it will involve. Please read this information carefully and discuss it with others if you wish. Please do not hesitate to contact us if anything is unclear, or if you require more information. Take time to decide whether or not you wish to take part. Details about the conduct of the study are also detailed on this document; lease read this sections carefully before deciding whether or not you wish to take part.

What is the purpose of this study?

Nitric oxide (NO) is a small molecule produced in the body which is involved in the control of several functions such as blood pressure (BP), blood clotting, energy metabolism and inflammation. NO is also involved in enhancing cognitive function, which usually declines as we age and with increased body weight. This can happen by increasing blood flow to brain. Recent human and animal studies conducted in our group have showed that we can modify the amount of NO produced in the body by using nutritional interventions with a high nitrate content. These could be achieved by ingestion of water solutions containing potassium nitrate or ingesting food sources with high nitrate content such as beetroot, spinach or rocket. This study will ask participants to drink beetroot juice. We will then investigate whether ingestion of beetroot for three months would increase the concentrations of NO in the body and understand the effects on cognitive function and cerebral blood flow in overweight and obese participants.

Why have I been chosen?

You have been contacted because you might an interest in our research. We are looking for overweight and obese men or women between the ages of 60-75 years to take part in this study. We will be recruiting 60 volunteers in total.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to do so, you will be asked to sign a consent form. However, you will still be free to withdraw at any time and without giving a reason.

What will happen to me if I take part?

You will be required to attend the research centre on 6 occasions:

Telephone screening: 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 and we will establish whether you can participate. You will NOT be included in the study if you have any medical conditions or if you are taking medications that will affect the measurements in the study. Then, we will ask you to come for your screening visit to check and confirm your eligibility.

Screening Visit (NU Food research facilities, Newcastle University, approx. 2 hours) If eligible at the telephone interview, we will invite you for a screening visit at the NU Food Research Facility at Newcastle University to confirm your eligibility. The visit can be arranged between 9.00am and 2.00pm and we will find a time that is most convenient for you. First you will have the opportunity to ask again questions about the study and, if you still decide to take part, you will be asked to sign a consent form. We will measure your height, weight, waist

circumference and blood pressure. Procedures are safe, not invasive and induce minimal discomfort. You will then be trained on the cognitive tasks. Please note that you do not need any prior experience is using a computer to complete these tasks. These tasks will be conducted three times (around 20-30 minutes each).

At the end of this visit, we will provide you with an appointment for the next visit. Also, a labelled urine container will be provided to collect the first urine in the morning at home in the day of next visit and you should bring the sample with you. Before this visit you will be asked to follow a simple dietary plan (avoid high nitrate food) and fast for at least 8 hours (only water allowed). It is also important to note that you will be asked to avoid the use of mouth wash for the duration of the study period.

Study design and study visits

The study includes five visits in total which will be conducted at the NU Food Research Facilities at Newcastle University (Visit 1, 3 and 4) and at Brain, Performance and Nutrition Research Centre at Northumbria University (Visit 2 and 5).

Visit 1 and Visit 4 (NU Food research facilities, Newcastle University, approx. 2 hours). Visit 1 appointment will take place within 7 days of your screening visit. You will arrive at the research facility early in the morning at 9:00 having been fasted at least 8 hours previous evening and avoided high nitrate food. You will have avoided caffeinated products for 12 hours and alcohol for 24 hours prior the visits.

First we will take the urine sample that you collected at home before you arrive research centre. You will have the opportunity to ask again questions about the study. Then, we will check whether there has been any change in your health status since your last screening visit. Then, we will measure your body composition using a non-invasive technique called bioimpedance analysis; this is routinely used in clinical practice and research and assessment takes less than 2 minutes. This will be followed by the collection of a small blood (~16 ml), and saliva (~1ml) samples. In addition, we will also ask you to place a small paper strip on your tongue for about 10 seconds. This is to measure the amount of nitrate present in the saliva in your mouth. The procedure is safe and tasteless. This is important as you will be performing these measurements at home every four weeks for 3 consecutive days **(this will be done only twice during the study duration).**

Then, pulmonary function will be assessed using a portable spirometer. You will be asked to inhale as deeply as possible, seal your lips around the mouthpiece and exhale as hard and fast as possible until no more air can be exhaled. Measurements will be performed three times. After that, the amount of NO in your breath will be measured using a portable, non-invasive, automated device. You will be asked to sit in upright position and wear a nose clip during the measurements. You will be guided to breathe at the right flow rate, and the first acceptable measurement will be used for our analysis. All these procedures are safe and non-invasive. These measurements will last approximately 15-20 minutes.

Then, we will measure your blood pressure and vascular function while you are sitting comfortably on a reclining chair. BP will be measured three times separated by 1-minute rest between measurements. This will be followed by the assessment of vascular health. The procedures are safe and performed by trained operators and the total duration of the procedures should be approximately of 15-20 minutes. After this assessment, you will be asked to move to another room where you will complete a series of computer-generated tasks which will

assess your cognitive performance (same as the one in the screening visit, but you will do it only once this time). The assessment of cognitive function will take approximately 20-30 minutes. After that you will do a quick cognitive test using pen and paper.

At the end of these measurements you will be provided with a light snack (orange juice and option of muffin or muesli bar) and you will be provided with instruction on what to eat on your lunch. Afterwards we will provide you with all the information and material (dietary instructions, provision of nutritional supplements and low nitrate bottled water) to continue the study at home you will be asked to collect several saliva samples and measure your resting BP every time you take a saliva sample. A recording sheet will be provided to you to write down the BP measurements at each time point. You will be also reminded that you will continue the measurements until the next morning (approximately 16-18 hours) and you will be allowed to drink nitrate free water (specific bottled water will be provided). In addition, we will provide you a bottle containing a 100-ml solution of distilled water containing a very small amount of inorganic nitrate (4mg) labelled with stable isotopes, a kit for the collection of saliva samples and detailed instructions on how to perform the measurements. We will also provide you with a small snack (two muesli bars) to eat at home between 7 and 8pm, you are allowed also to eat banana or apple with the two bars. The samples should be stored in a cool, dry place or, ideally, in the fridge and you will return them to the research team when you attend your second visit (Visit 2) at Northumbria University. Finally, we will provide you with urine container to collect urine next day at the same time in the morning, and you should bring this with you as well at Northumbria University. We will ask you to complete food frequency questionnaire (only at this visit) and physical activity questionnaire (visit 1 and 4) at home and retain them with you next visit. These baseline measurements will be repeated in visit 4 in the same order and using the exactly the same protocols.

Visit 2 and 5 (Brain, Performance and Nutrition Research Centre of Northumbria University, approx. 1 hour) Next day, you will be asked to come to Northumbria University in the afternoon to complete the rest of the baseline measurements (cerebral blood flow measurements). You will be asked to follow the same dietary plan before this visit (low nitrate diet), but you will not need to be fast. However, you should still continue avoiding drinking alcohol and caffeinated drinking before the visit, you can only drink coffee if you want in morning, after collecting last saliva sample at 9:00 am. Firstly, we will take the saliva samples that you collected and resting blood pressure device. Then, we will measure your cerebral blood flow (CBF) by device called the quantitative near-infrared spectroscopy (qNIRS) headband which will be fitted across the forehead and a 5 min baseline measure of CBF will be recorded, and another 15-20 minutes will be recorded while you are performing some cognitive tasks on computer. The procedures are safe and commonly applied across different populations (i.e., children, healthy adults and patients) to assess brain blood flow.

After that, you will be randomised to a specific intervention and you will be receiving a different amount of beetroot juice (two bottles of beetroot juice /day, one bottle/day or one bottle every two days. One of the groups will receive a beetroot juice that has no nitrate in it which will function as control for the intervention. However, we will be able to disclose this information only at the end of the study. This beetroot has the same characteristics in terms of taste and smell compared to the beetroot containing nitrate.

You will leave the research unit with the number beetroot juice bottles you will need over the next six weeks. In addition, 10 ml urine containers, necessary materials for saliva collection, saliva test strips, all to be collected for 3 consecutive days every four weeks as mentioned

earlier, you will be asked to post them in closed box to us and you will be provided with all the material to perform the shipping without incurring in any additional costs. You will be asked as well to complete a 24h recall every two weeks using a user-friendly online software called INTAKE-24. We will provide you with your specific account and password to access the program to complete it. At the end of the questionnaire, your answers will be securely stored and your dietary data will be automatically analysed.

These baseline measurements will be repeated on the last visit (visit 5) in the same order and using the exactly the same protocols. At the end of last visit (visit 5), you will be asked to complete Caffeine Intake Tool, which is a questionnaire to assess you habitual caffeine intake during the study and feedback questionnaire as your opinion is valuable to us.

Visit 3 (middle visit, 6 weeks, NU Food research facilities, Newcastle University, approx. 1 hour).. A member of the research team will be in touch with you before the visit to remind you of the appointment. First, we will check whether you have had any problem during the intervention. Then we will measure your body weight and resting BP. Then, you will be asked to perform same cognitive tasks that you did in visit 1. We will ask you as well to complete physical activity questionnaire. We will provide you with the rest of bottles of juice that you are going to drink over the next 6 weeks. Finally, we will also provide you with the urine containers, necessary materials for saliva collection and salivary strips. This will be done after two weeks from this visit for three consecutive days as mentioned earlier. Again, you will be asked to post them to us. You will be also reminded as well to complete INTAKE-24 for the dietary assessment every two weeks.

A description of the nutritional interventions and dietary and lifestyle advice is provided below:

Diet and lifestyle advice: We aim to minimize changes in dietary and lifestyle habits during the study and therefore we encourage you to maintain your habitual physical activity and dietary practices during the study. We will also ask you to maintain your habitual consumption of alcoholic and caffeinated drinks, just limit them before study visits as mentioned earlier.

Beetroot juice: We will provide you with the bottles of beetroot juice and you will be asked to drink at the same time of the day during the study. The amount of nitrate contained in the beetroot juice is similar to the amount in a normal portion of lettuce or cabbage. It is important to highlight again that the assignment of the intervention to each participant is randomised and participants will not be aware of the type of beetroot they will be taking during the study (nitrate-rich or nitrate-depleted). Both the juice rich in nitrate and the placebo will be provided by James White Drinks Ltd, UK. It is important that you keep the empty bottles and that you return them to the research team at the end of the study. Also, we will give a form for you to record the time you have drunk the beetroot juice every day.

Labelled Nitrate with Stable Isotopes: In this study you will be given a very small amount of sodium nitrate containing stable isotopes. Stable isotopes are harmless, they are not radioactive and exist normally in nature including everything you eat and drink. However, by enriching substances like sodium nitrate with stable isotopes and subsequently measuring their concentrations in saliva we can measure the amount of NO produced by the body. This is a safe protocol which has been used without any harm or side effects in more than 300 subjects including children, diabetics and older subjects.

The measurements: You may have noticed that we are performing various measurements to assess vascular health and cerebral blood flow and cognitive function. We would like again to emphasize that these measurements are non-invasive, safe and routinely used in clinical research practice. However, in order to obtain accurate measurements they require standardised procedures and therefore we will require your cooperation in performing them. We will explain them to you and we will provide leaflets with the instructions on how they will be performed and what you will have to do.

What do I have to do?

It is critical for the study that you follow the instructions we will give you for the day before the study and during the study. You will also be asked to arrive fasted (not had anything to eat for 8hr) on the day of the study. We will ask you to maintain your normal physical activity level. If the dose of your medications change or you are prescribed a new medication during the study we may need to discuss your participation in the study. If you are a blood donor you will be asked not to donate blood during the study and for at least 2 months after the completion of the study.

What are the possible benefits of taking part?

This is a nutritional intervention and there may be some direct benefit to you. The knowledge gained from this study will help our research into the understanding of the effects of dietary nitrate on cognitive function and help us to clarify some of the mechanisms related to brain function. However we will measure a number of blood tests (glucose), your weight, BMI and BP and these will be of interest to you.

What are the possible disadvantages and risks of taking part?

There is a minor risk of bruising, bleeding or infection (very rare) when we take blood samples. In a small number of subjects (<15% of the population) the consumption of beetroot juice could be associated with a red or pink urine colour. This is related to a pigment present in beetroot (betalain), and does not have any effect on your health.

What will happen if anything goes wrong?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. If you wish to make a complaint you can contact Dr Mario Siervo (Mario.siervo@ncl.ac.uk) Tel: 01912081140

Will my taking part in this study be kept confidential?

Yes. All information that is collected about you during the course of this research will be kept strictly confidential and stored in locked cabinets in a secure office. In addition, we will use codes to label the tubes used to collect the blood, urine and saliva samples to ensure that no personal information can be disclosed. All forms and samples will be destroyed at the end of the study.

What will happen to the study results?

We will inform you of your study results (BMI, waist circumference blood pressure and relevant results from blood tests). The overall results of the study may be presented at scientific

meetings or published in a scientific journal. You will not be identified in any of these presentations or publications. We will be happy to discuss the results with you when the study is completed and will let you know where you can obtain a copy of the published results.

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

You will be free to withdraw from the study without giving a reason anytime up to the end of your final visit. If you decide to withdraw from the study, with your consent, samples and data obtained may be kept and used to contribute to study results or, with your consent, for future studies. However, should you request your samples and data to be destroyed along with any other information relating to you; we will ensure that this takes place.

Will I be reimbursed for my time?

In recognition of your time commitment, you will be paid an honorarium of £60. Reasonable travel expenses will also be paid.

Contact for further information

If you have any further questions then please contact either Ms Abrar Babateen or Dr Mario Siervo. Telephone: 01912082004 and 01912081140. Email A.M.O.Babateen2@newcastle.ac.uk or mario.siervo@ncl.ac.uk.

And finally…

Thank you for having taken the time to read this information sheet and your interest in the study. If you do decide to take part in the study, you will be given a copy of the information sheets and a signed consent form for you to keep.

Appendix 5.6: Consent form

Dietary nitrate, cognition and Cerebral blood flow

CONSENT FORM

Ethics Reference Number: 1503/4477/2018
Name of Chief Investigator: Abrar Babateen

	Please Initialise
1. I confirm that I have read and understand the information sheet dated _____ for the above study and have had the opportunity to ask questions.	
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected.	
3. I give permission that my samples (blood, urine, saliva) taken as part of the protocol of this study may be stored and used in further research studies. Ethical approval will be sought before such analyses will be carried out.	
4. I agree to take the beetroot juice for the duration of the study	
5. I give permission that my samples taken as part of this study will be analysed at the Human Nutrition Research Centre at Newcastle University. All samples will be linked-anonymised and no personal information will be disclosed.	
6. I agree to take part in the above study.	

_____	_____	_____
Name of Volunteer *(Please print)*	Date	Signature
_____	_____	_____
Name of Research Team Member	Date	Signature

3 copies required: top copy for researcher; one copy for volunteer; one copy to be kept with volunteer's notes.

Appendix 5.7: List of high nitrate items

Low nitrate diet

In order to increase the precision and validity of our measurements during the study we need to control the amount of nitrate in your diet. Therefore, you should try to follow the dietary guidelines provided below when we ask you to limit the dietary nitrate intake as indicated in your study plan. The dietary plan will be thoroughly explained to you and if you will have any other questions about the diet during the study please let us know and we will be very happy to clarify them for you.

A list of food to limit the consumption prior to the study visits is shown below.

LIMIT CONSUMPTION	
• Beet • Beetroot • Rocket • Chinese Leaf • Broccoli • Cabbage • Celery • Kale	• Leek • Lettuce • Rhubarb • Spinach

We would also like to remind you that while you are on the study you should try to drink and cook with the same **water**. Different sources of water may contain different amount of nitrate and in this way we will ensure that the amount of nitrate you will be consuming will be constant during the week.

Appendix 5.8: International physical activity questionnaire

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

   _____ **days per week**

   ☐ No vigorous physical activities → ***Skip to question 3***

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

   _____ **hours per day**

   _____ **minutes per day**

   ☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

   _____ **days per week**

   ☐ No moderate physical activities → ***Skip to question 5***

1. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

Appendix 5.9: Examples of daily compliance log for BJ consumption for each intervention groups

1)For High NO_3^- group

PARTICIPANT'S CHECKLIST Code: ----------

		Week1							Week2						
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14
Beetroot juice	Morning Time														
	Evening Time														

Dietary assessment: Intake 24
Date: ----------
You will be reminded

		Week3							Week4						
		D15	D16	D17	D18	D19	D20	D21	D22	D23	D24	D25	D26	D27	D28
Beetroot juice	Morning Time														
	Evening Time														

- Dietary assessment: Intake 24
- Urine, saliva and strips (in the morning before drinking BJ)

Date: ----------
You will be reminded

2) For Medium NO_3^- group

PARTICIPANT'S CHECKLIST Code: ----------

		Week1							Week2						
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14
Beetroot juice	Evening Time														

Dietary assessment: Intake 24
Date: ----------
You will be reminded

		Week3							Week4						
		D15	D16	D17	D18	D19	D20	D21	D22	D23	D24	D25	D26	D27	D28
Beetroot juice	Evening Time														

- Dietary assessment: Intake 24
- Urine, saliva and strips (in the morning before drinking BJ)

Date: ----------
You will be reminded

3) For Low NO_3^- and placebo groups

PARTICIPANT'S CHECKLIST Please drink on alternate days Code: ----------

		Week1							Week2						
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14
Beetroot juice	Evening Time		■		■		■		■		■		■		■

Dietary assessment: Intake 24
Date: ----------
You will be reminded

		Week3							Week4						
		D15	D16	D17	D18	D19	D20	D21	D22	D23	D24	D25	D26	D27	D28
Beetroot juice	Evening Time		■		■		■		■		■		■		■

- Dietary assessment: Intake 24
- Urine, saliva and strips (in the morning before eating or drinking)

Date: ----------
You will be reminded

Appendix 5.10: Some tips that used to calculate nitrate intake from the database

There are some tips followed to calculate NO_3^- intake:

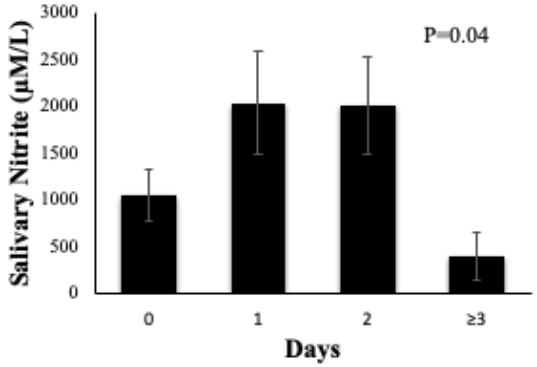
- Food items such as rice, any kind of meet have been considered as a cooked item from the database.
- Any kind of bread reported by a participant (white, brown bread or toast), "Bread" from database was used for all (NO_3^- content is 25.132 mg).
- If participant inserted any prepared dishes, we searched online for the main ingredients of that dish, then, total NO_3^- content has been calculated from the averages of nitrate content of the main ingredients. For example, if someone inserted "scotch broth", we found that the main ingredients are barely, beef and root vegetables potato and carrots. Nitrate contents for those ingredients have been searched from database as cooked items as followed: Barely (51.5491 mg), Cooked beef (32.094 mg), cooked carrot (17.8946 mg) and cooked potato (10.6177 mg). Then, the average of nitrate contents was calculated (28.038 mg).

Appendix 5.11: Colour chart of Berkeley strips

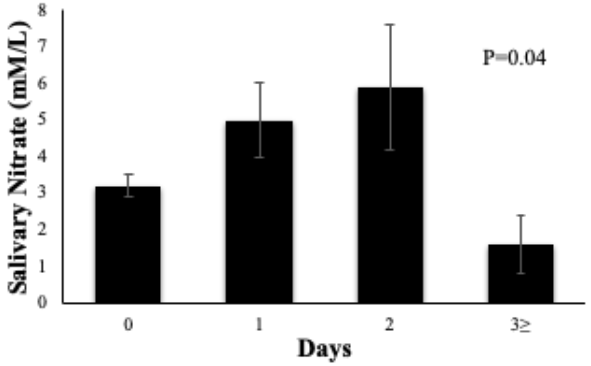
Appendix 5.12: Chapter 5 supplementary figures

A) HN dose

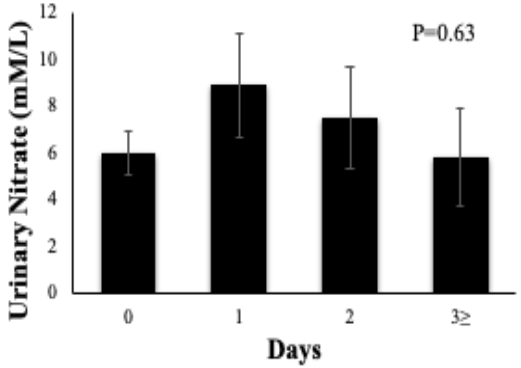
A1



A2

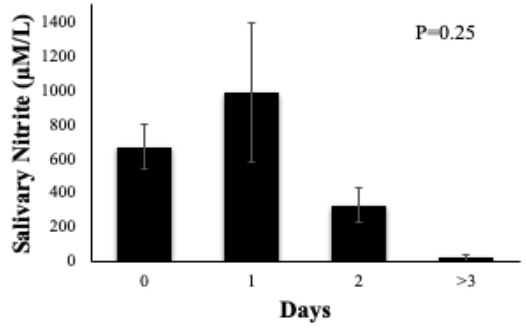


A3

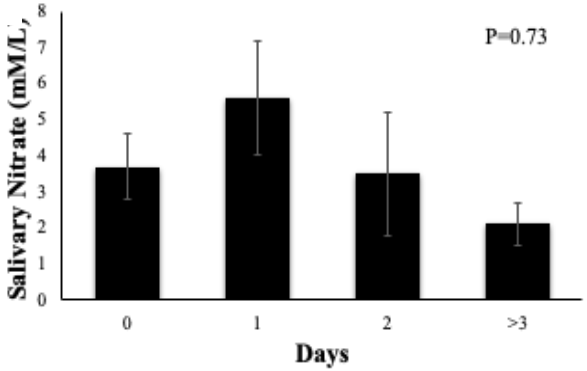


B) MN dose

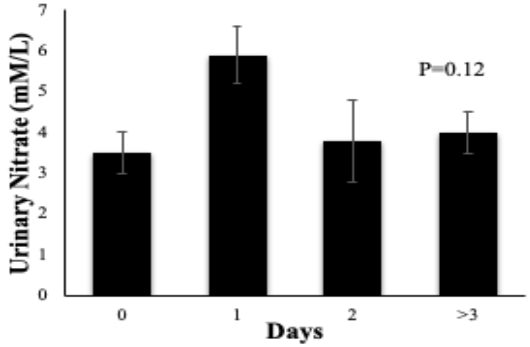
B1



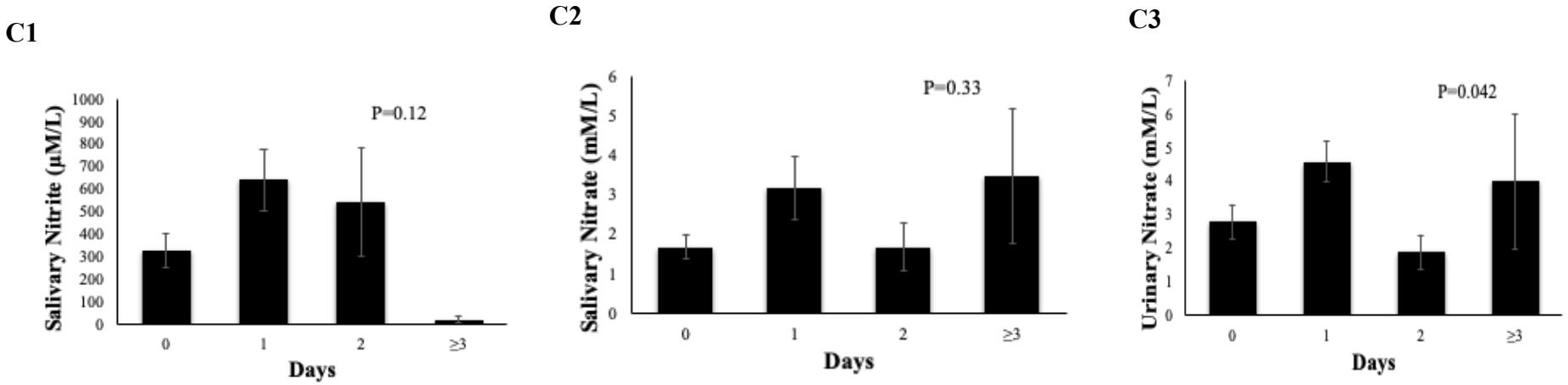
B2



B3



C) LN dose



D) PL

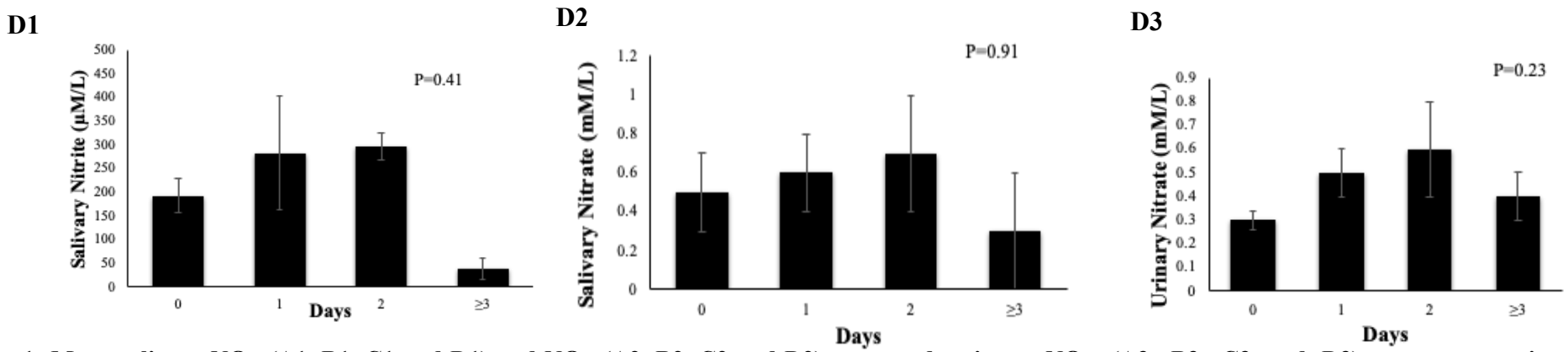


Figure1: Mean salivary NO_2^- (A1, B1, C1 and D1) and NO_3^- (A2, B2, C2 and D2) and urinary NO_3^- (A3, B3, C3 and D3) concentrations in samples received after a different number of days. One-way ANOVA was used to test differences in biomarker concentrations between different days. Data are expressed as mean ± SEM. (A) represent the figures related to HN (High NO_3^- ; two 70ml shots of BJ/day, morning and evening), (B)) represent the figures related to MN (Medium NO_3^- ; 70 ml of BJ/day), (C) represent the figures related to LN (Low NO_3^-; 70 ml of BJ every alternate days) and (D) represent the figures related to PL (placebo; 70 ml of NO_3^- depleted BJ).

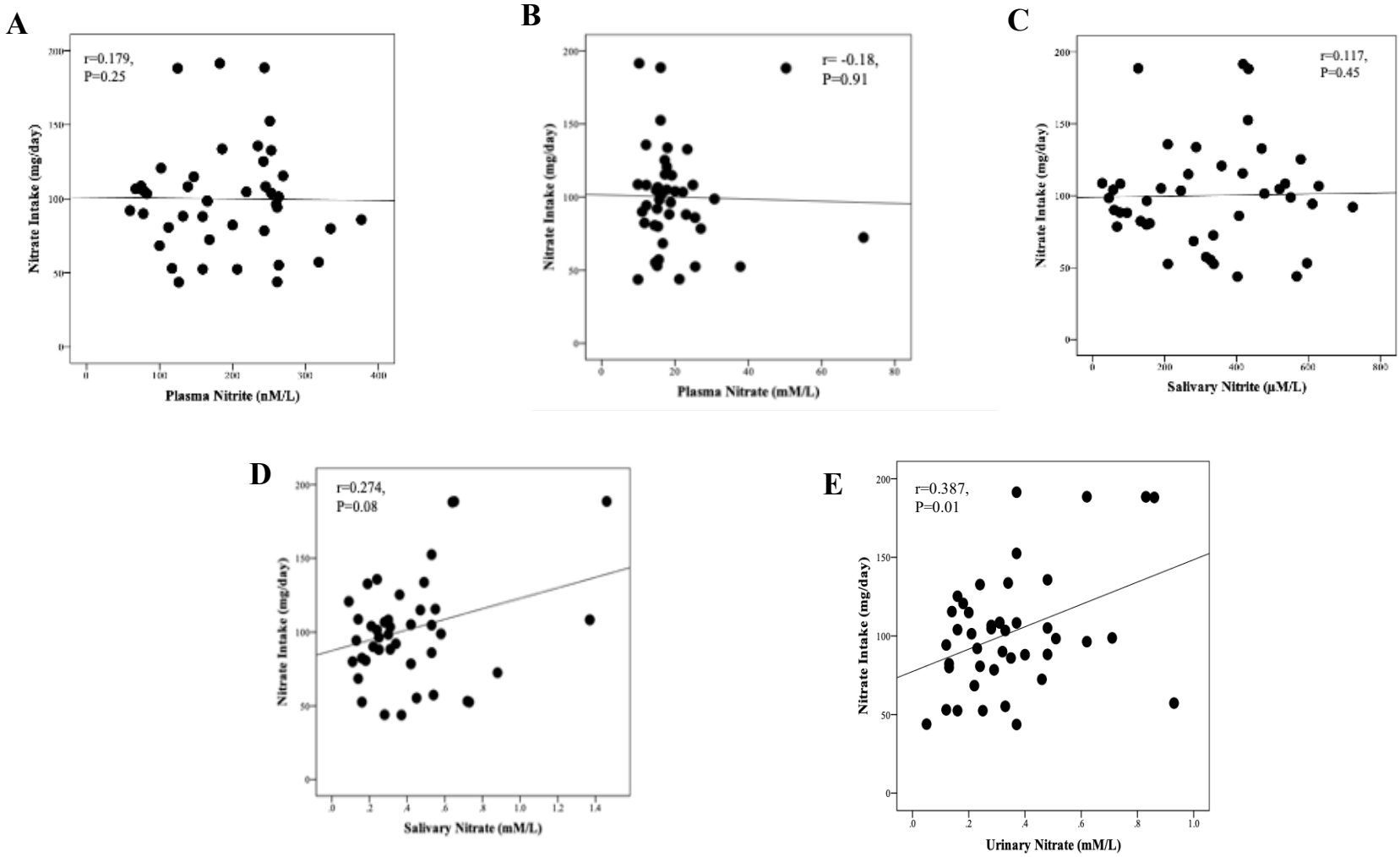


Figure 2: Scatterplot showing Pearson's correlation between the mean of NO_3^- intake and baseline plasma NO_2^- (A), plasma NO_3^- (B), salivary NO_2^- (C), salivary NO_3^- (D) and urinary NO_3^- concentration.

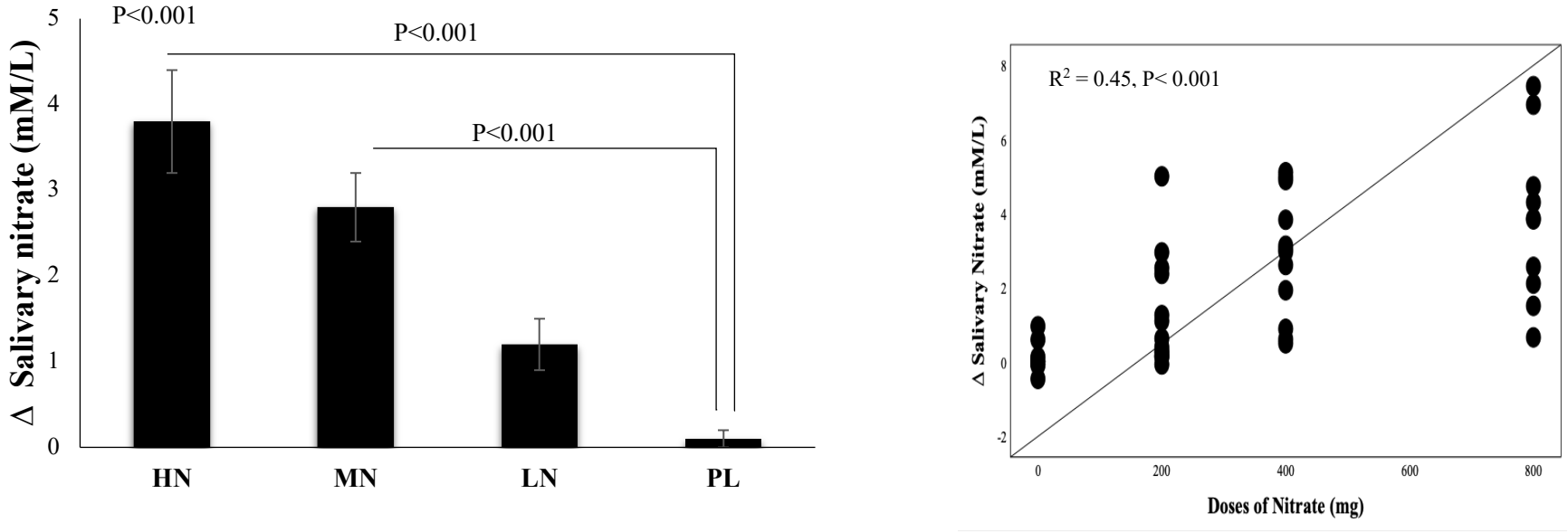


Figure 3: Mean of salivary NO_3^- concentrations after removing data from one participant.

Appendix 5.13: Feedback questionnaire with participants' answers

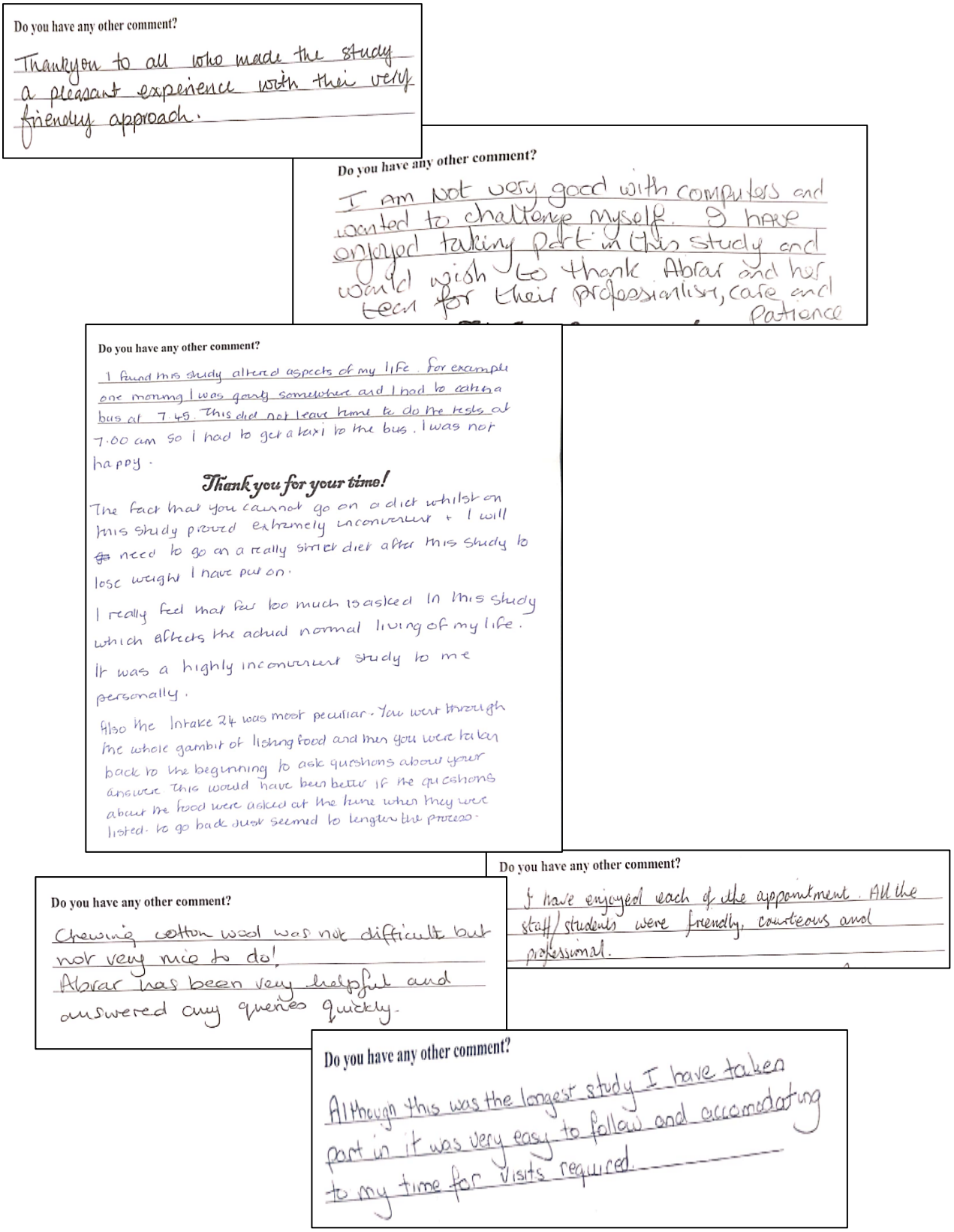
Questions related to nutritional supplementation	Total respondents (N=52)	Group1[1] (N=11)	Group2[2] (N=14)	Group3[3] (N=14)	Group4[4] (N=13)
Do you eat or drink beetroot regularly?					
• Yes	19	3	4	4	8
• No	33	9	9	9	6
If No, why?					
- No reasons reported	3	1	1	1	-
- Taste and smell	10	3	3	2	2
- Difficult to prepare	4	1	1	1	1
- Rarely/Limited availability	16	4	4	5	3
Would you recommend study product to a friend?					
• Yes	34	6	9	9	10
Why?					
- No reasons reported	8	2	4	-	1
- Taste	4	-	2	-	2
- Health benefits *	23	4	3	9	7
• No	18	6	4	4	4
Why?					
- No reasons reported	15	6	3	4	2
- Taste	3	-	1	-	2
Do you prefer having raw beetroot or still beetroot juice if you participating in another study?					
• Row	27	9	7	6	5
Why?					
- **No reasons reported**	1	1	-	-	-
- Better taste and tolerated	24	6	7	6	5
- Not processed and fresh	2	2	-	-	-
• Juice	20	2	5	6	7
Why?					
- No reasons reported	6	-	2	1	3
- Convenient	14	2	3	5	4
• No preference	5	1	1	1	2
Was it difficult to drink the dose of beetroot juice during the study?					
• Yes	14	3	4	4	3
Why?					
- Taste and smell	12	2	4	4	2
- Side effect	2	1	-	-	1
• No	38	9	9	9	10

Have you felt any difference as a result of the beetroot juice intervention?					
• Yes	14	4	3	4	3
Why?					
- Improved alertness and cognitive performance*	8	2	1	2	3
- Improved blood pressure*	3	-	1	1	1
- More energy	3	1	1	1	-
- Gastrointestinal side effect	1	1	-	-	-
• No	35	7	9	8	11
*one participant mentioned both					
(Placebo)					
If any, what was the most inconvenient aspect of taking the beetroot juice?					
- None	17	2	7	5	3
- Timing	11	2	3	1	5
- Remembering to take it out home	8	-	3	4	1
- Taste	10	2	4	1	3
- Saliva collection	1	1	-	-	-
- Stop donation	1	1	-	-	-
- Taking the juices on holiday	6	2	1	3	1
Protocol measurements					
Was the ONT protocol (collection of saliva samples) easy to follow?					
- Yes	47 (92)				
- No, reason: Too many samples (All)	4 (6)				
Did you have any difficulties recording your 24h recall diet and using the software (Intake24) every two weeks?					
- Yes, reasons: Time constraints in recording (7) Access and software compatibility (4) Format difficult to understand (3) Limited food choice (7)	21 (41)				
- No	30 (59)				
Was collecting saliva, urine and saliva strips every 4 weeks for 3 days acceptable for you?					
- Yes					
- No, reasons:	47 (92)				

Time consuming (All)	4 (6)
Did you feel any difficulty to post them?	
- Yes	2 (3)
- No	49 (96)
Did you find the general procedure of vascular assessment acceptable for you?	
- Yes	51 (100)
- No	0
Did you find the general procedure of cerebral blood flow assessment acceptable for you?	
- Yes	51 (100)
- No	0
Did you find the cognitive function assessment using COMPASS software acceptable for you?	
- Yes	49 (96)
- No	2 (3)
Would be acceptable for you if the tests to assess cognitive function last more than 30 minutes?	
- Yes	39 (78)
- No	11 (22)
Study design	
Was the number of visits appropriate?	
- Yes	50 (99)
- No	1 (1)
Others	
What were the main factors which motivated you to complete this study?	
- Commitment with study	1 (1)
- Health benefits	29 (57)
- Cognitive testing	28 (55)
- Interest in research	37 (73)
- Honorarium	1 (1)
- Improve dietary habits	

- Worry about dementia	1 (1)
- Health check up	1 (1)
	1 (1)

Appendix 5.14: Some examples of participants' comments about the study



Do you have any other comment?

Thankyou to all who made the study a pleasant experience with their very friendly approach.

Do you have any other comment?

I am not very good with computers and wanted to challenge myself. I have enjoyed taking part in this study and would wish to thank Abrar and her team for their professionlism, care and patience

Do you have any other comment?

I found this study altered aspects of my life. For example one morning I was going somewhere and I had to catch a bus at 7.45. This did not leave home to do the tests at 7.00 am so I had to get a taxi to the bus. I was not happy.

Thank you for your time!

The fact that you cannot go on a diet whilst on this study proved extremely inconvenient + I will need to go on a really strict diet after this study to lose weight I have put on.

I really feel that far too much is asked in this study which affects the actual normal living of my life.

It was a highly inconvenient study to me personally.

Also the Intake 24 was most peculiar. You went through the whole gambit of listing food and then you were taken back to the beginning to ask questions about your answers. This would have been better if the questions about the food were asked at the time when they were listed. To go back just seemed to lengthen the process.

Do you have any other comment?

I have enjoyed each of the appointment. All the staff/students were friendly, courteous and professional.

Do you have any other comment?

Chewing cotton wool was not difficult but not very nice to do!
Abrar has been very helpful and answered any queries quickly.

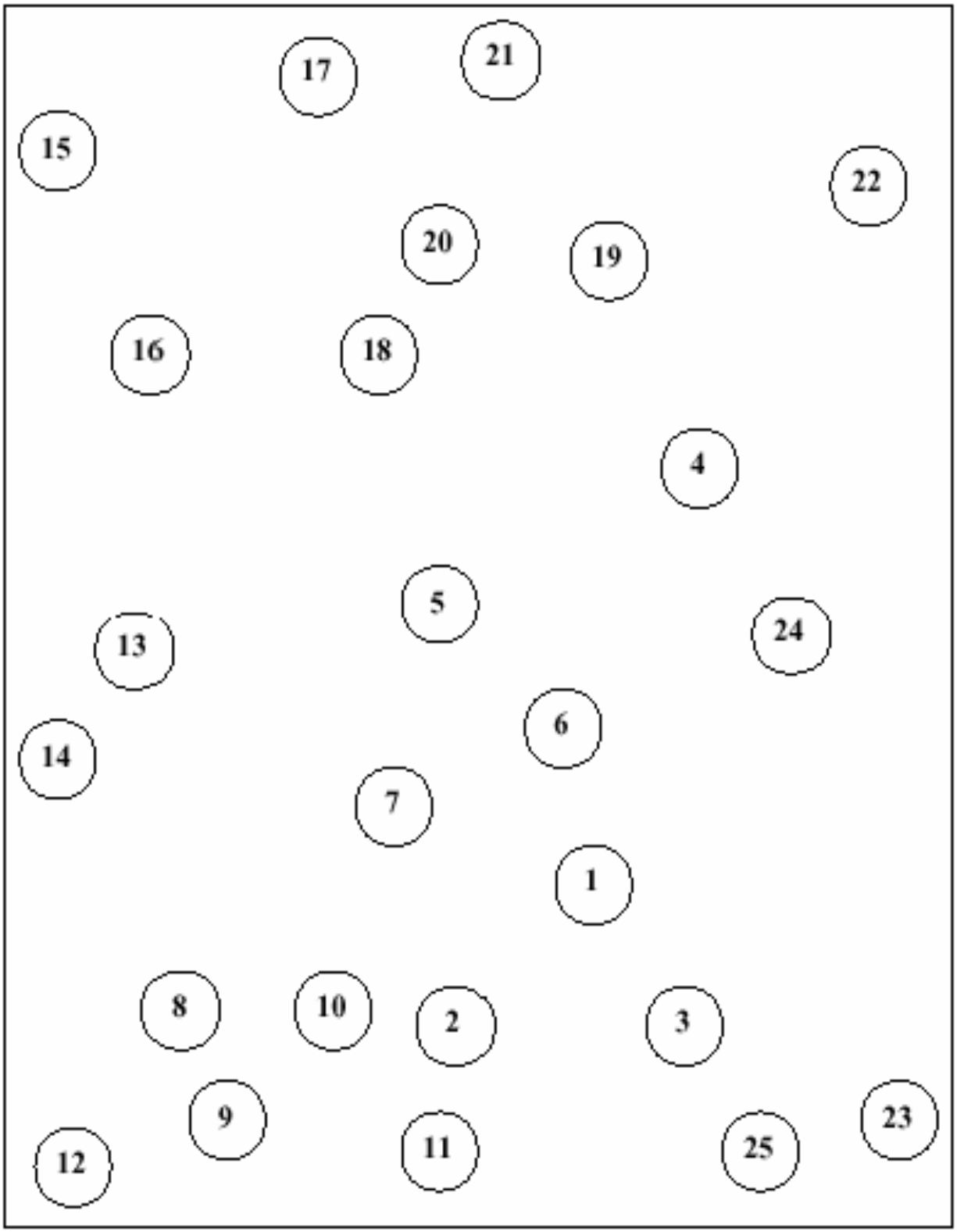
Do you have any other comment?

Although this was the longest study I have taken part in it was very easy to follow and accomodating to my time for visits required.

Appendix 6.1: Trail making Tasks forms

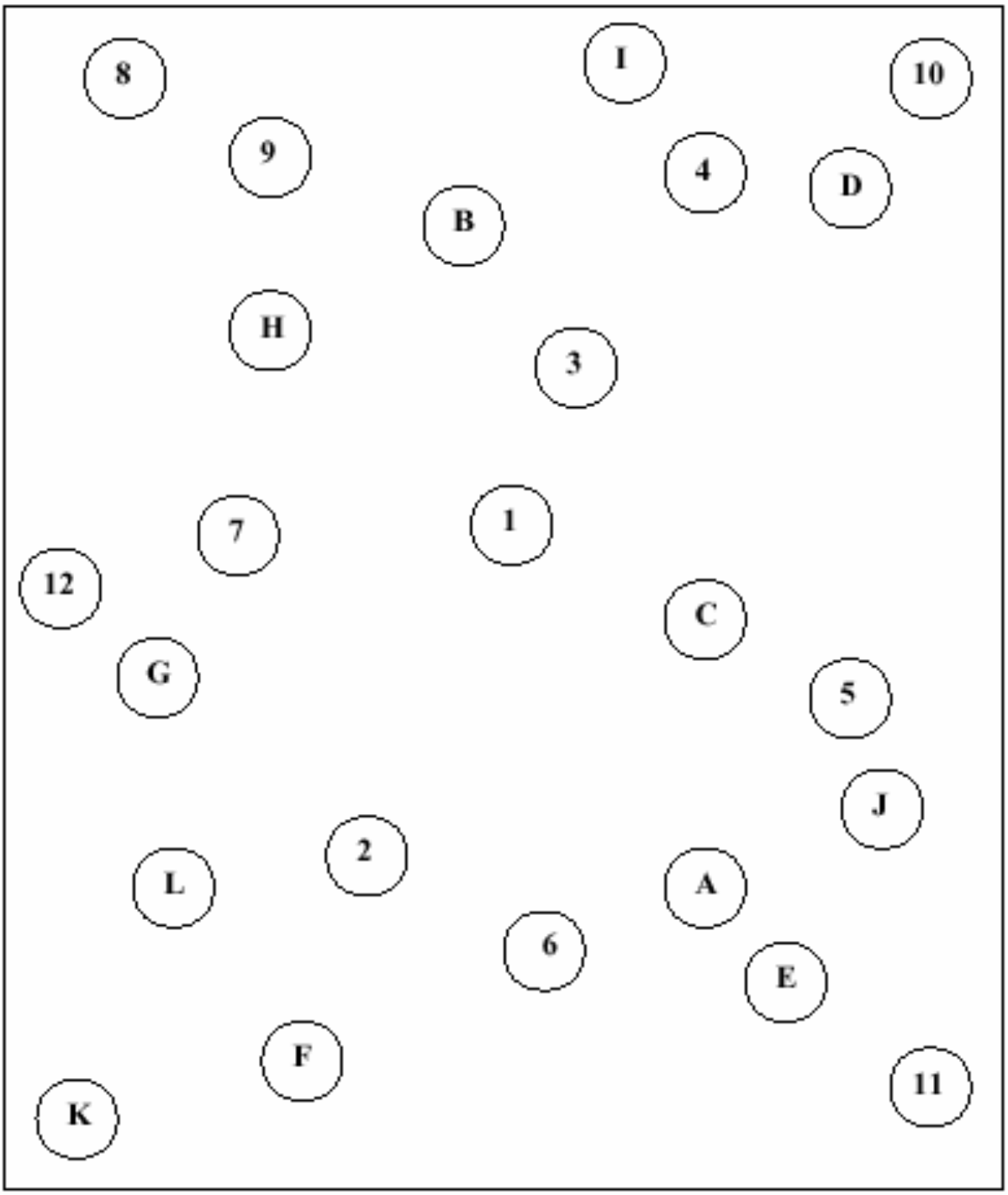
Trail Making Test -A

Code: **Date:**



Trail Making Test -B

Code: **Date:**



Appendix 6.2: Example of NIRS data analysis of one participant

1- Whole data recorded at baseline and 13-weeks of one participant (blue cells are the averages of each period)

		Baseline Data				13-week Data			
ID	Task	Ox%	[THC]	[HbO]	[Hb]	Ox%	[THC]	[HbO]	[Hb]
NCS2	average	59.259	37.293	22.222	15.071	64.709	39.106	25.328	13.778
NCS2	Data recorded during resting period (5 min)	59.112	37.155	22.07	15.084	64.626	38.635	24.985	13.65
NCS2		59.29	37.212	22.177	15.035	64.326	38.937	25.075	13.862
NCS2		59.359	37.297	22.249	15.049	64.367	38.967	25.105	13.862
NCS2		59.131	37.204	22.105	15.099	64.196	39.053	25.096	13.958
NCS2		58.998	37.137	22.01	15.128	64.444	39.034	25.176	13.858
NCS2		59.008	37.255	22.098	15.157	64.52	39.151	25.284	13.867
NCS2		59.248	37.283	22.195	15.088	64.312	39.09	25.166	13.923
NCS2		59.336	37.253	22.217	15.035	64.653	39.136	25.329	13.807
NCS2		59.55	37.363	22.379	14.984	64.771	39.203	25.424	13.78
NCS2		59.553	37.409	22.401	15.009	64.757	39.303	25.479	13.824
NCS2		59.452	37.347	22.322	15.025	64.424	39.182	25.266	13.916
NCS2		59.24	37.272	22.205	15.067	64.491	39.006	25.183	13.823
NCS2		59.384	37.241	22.238	15.003	65.232	39.191	25.589	13.602
NCS2		59.538	37.362	22.373	14.988	65.544	39.378	25.832	13.546
NCS2		59.538	37.364	22.375	14.99	65.082	39.208	25.542	13.665
NCS2		59.37	37.305	22.267	15.038	64.983	39.089	25.421	13.669
NCS2		59.237	37.311	22.232	15.079	65.133	39.324	25.641	13.683
NCS2		59.505	37.364	22.359	15.005	65.155	39.124	25.51	13.615
NCS2		59.21	37.321	22.226	15.094	65.176	39.165	25.548	13.618
NCS2		59.296	37.336	22.27	15.065	65.235	39.152	25.561	13.591
NCS2		59.065	37.244	22.13	15.114	65.224	39.3	25.653	13.647
NCS2		59.095	37.258	22.143	15.114	64.879	39.191	25.443	13.749
NCS2		59.25	37.271	22.207	15.064	65.186	39.194	25.57	13.623
NCS2		59.277	37.305	22.256	15.049	64.447	39.058	25.193	13.864
NCS2		59.113	37.278	22.168	15.11	64.37	38.965	25.104	13.862
NCS2		59.124	37.338	22.209	15.128	64.354	39.068	25.163	13.906
NCS2		59.24	37.29	22.218	15.071	63.911	38.802	24.823	13.979
NCS2		58.891	37.187	22.022	15.164	64.299	38.967	25.079	13.888
NCS2		59.156	37.398	22.253	15.145	64.6	39.103	25.28	13.822
NCS2		59.227	37.442	22.303	15.14	64.594	39.208	25.345	13.864
NCS2		Ox%	[THC]	[HbO]	[Hb]	Ox%	[THC]	[HbO]	[Hb]
NCS2	average	60.062	37.533	22.673	14.86	64.662	38.927	25.192	13.735
NCS2	Data recorded during serial subtraction 3 (3:20 min)	58.913	37.197	22.034	15.162	63.939	38.9	24.888	14.011
NCS2		59.146	37.398	22.25	15.148	63.8	38.907	24.84	14.066
NCS2		59.219	37.431	22.294	15.137	63.691	38.759	24.706	14.054
NCS2		59.351	37.404	22.32	15.084	64.179	38.968	25.03	13.938
NCS2		59.387	37.415	22.355	15.06	64.546	38.956	25.161	13.796
NCS2		59.224	37.378	22.277	15.1	64.617	38.949	25.19	13.76
NCS2		59.436	37.492	22.424	15.068	64.76	38.977	25.26	13.717
NCS2		59.716	37.612	22.591	15.02	64.96	39.059	25.397	13.661
NCS2		59.787	37.493	22.549	14.944	65.352	39.119	25.586	13.532
NCS2		59.906	37.57	22.646	14.924	65.182	39.002	25.445	13.556
NCS2		60.184	37.54	22.724	14.817	65.168	39.003	25.438	13.565
NCS2		60.393	37.556	22.806	14.751	65.13	38.938	25.378	13.56
NCS2		60.633	37.697	22.982	14.715	65.112	38.823	25.302	13.521
NCS2		60.796	37.677	23.036	14.641	64.958	38.957	25.331	13.626
NCS2		60.798	37.586	22.969	14.618	65.001	38.955	25.349	13.606
NCS2		60.765	37.631	22.989	14.642	64.548	38.859	25.107	13.752
NCS2		60.805	37.563	22.966	14.597	64.715	38.885	25.183	13.702
NCS2		60.923	37.688	23.097	14.593	64.445	38.807	25.029	13.777
NCS2		60.895	37.67	23.064	14.606	64.607	38.917	25.163	13.754
NCS2		60.938	37.653	23.072	14.581	64.547	38.809	25.07	13.738
NCS2		Ox%	[THC]	[HbO]	[Hb]	Ox%	[THC]	[HbO]	[Hb]
NCS2	average	61.304	37.749	23.259	14.491	63.991	38.575	24.704	13.871
NCS2	Data recorded during serial subtraction 7 (3:20 min)	60.864	37.631	23.032	14.599	64.294	38.746	24.931	13.813
NCS2		60.874	37.624	23.042	14.582	64.172	38.644	24.815	13.83
NCS2		61.09	37.7	23.16	14.541	63.73	38.53	24.579	13.952
NCS2		60.86	37.589	22.999	14.59	64.267	38.686	24.881	13.805
NCS2		61.1	37.675	23.146	14.528	64.101	38.609	24.769	13.841
NCS2		61.213	37.678	23.195	14.483	64.293	38.682	24.886	13.796
NCS2		61.182	37.676	23.17	14.505	64.511	38.755	25.016	13.738
NCS2		61.483	37.709	23.291	14.419	64.263	38.687	24.878	13.809
NCS2		61.302	37.696	23.219	14.477	63.911	38.49	24.618	13.871
NCS2		61.306	37.785	23.278	14.506	64.014	38.558	24.7	13.859
NCS2		61.433	37.753	23.296	14.457	63.877	38.498	24.614	13.884
NCS2		61.429	37.837	23.349	14.489	63.839	38.515	24.609	13.906
NCS2		61.307	37.753	23.256	14.497	63.586	38.354	24.409	13.944
NCS2		61.318	37.721	23.233	14.489	63.71	38.553	24.58	13.975
NCS2		61.438	37.767	23.31	14.458	64.081	38.614	24.76	13.853
NCS2		61.42	37.865	23.372	14.493	63.713	38.521	24.566	13.955
NCS2		61.598	37.891	23.441	14.45	63.557	38.453	24.46	13.993
NCS2		61.645	37.94	23.507	14.434	63.977	38.523	24.668	13.855
NCS2		61.623	37.896	23.47	14.426	64.135	38.622	24.785	13.837
NCS2		61.581	37.787	23.395	14.393	63.81	38.462	24.562	13.9
NCS2		Ox%	[THC]	[HbO]	[Hb]	Ox%	[THC]	[HbO]	[Hb]
NCS2	average	61.195	37.705	23.197	14.508	63.637	38.419	24.461	13.958
NCS2	Data recorded during Stroop (3 min)	61.808	37.773	23.469	14.304	64.435	38.706	24.96	13.747
NCS2		61.75	37.71	23.409	14.301	64.451	38.598	24.888	13.71
NCS2		61.503	37.741	23.327	14.413	64.226	38.545	24.771	13.774
NCS2		61.228	37.647	23.175	14.471	63.796	38.445	24.542	13.902
NCS2		61.023	37.627	23.09	14.538	63.628	38.381	24.433	13.949
NCS2		61.536	37.762	23.367	14.395	63.685	38.32	24.416	13.903
NCS2		61.475	37.81	23.368	14.441	63.874	38.428	24.559	13.868
NCS2		61.223	37.675	23.186	14.489	63.929	38.456	24.597	13.859
NCS2		61.11	37.701	23.177	14.524	63.643	38.418	24.463	13.954
NCS2		61.131	37.613	23.123	14.49	63.702	38.43	24.494	13.936
NCS2		61.162	37.737	23.212	14.525	63.326	38.355	24.297	14.057
NCS2		61.007	37.743	23.149	14.594	63.511	38.411	24.406	14.005
NCS2		61.014	37.674	23.104	14.569	62.935	38.269	24.094	14.175
NCS2		60.925	37.706	23.084	14.622	63.271	38.364	24.282	14.082
NCS2		60.763	37.623	22.978	14.644	63.01	38.203	24.084	14.12
NCS2		60.903	37.692	23.069	14.623	63.534	38.45	24.439	14.011
NCS2		60.881	37.687	23.064	14.622	63.29	38.394	24.312	14.081
NCS2		61.07	37.773	23.196	14.577	63.213	38.363	24.261	14.103
NCS2		Ox%	[THC]	[HbO]	[Hb]	Ox%	[THC]	[HbO]	[Hb]
NCS2	average	62.028	37.995	23.69	14.306	63.512	38.427	24.418	14.009
NCS2	Data recorded during peg & ball (2:30 min)	61.538	37.888	23.445	14.444	63.556	38.422	24.434	13.987
NCS2		61.253	37.843	23.305	14.537	63.534	38.509	24.48	14.03
NCS2		61.632	37.933	23.506	14.427	63.375	38.567	24.453	14.114
NCS2		61.727	37.913	23.525	14.388	63.143	38.432	24.281	14.151
NCS2		61.64	37.916	23.496	14.42	63.222	38.505	24.353	14.151
NCS2		62.035	37.999	23.693	14.306	63.131	38.338	24.219	14.119
NCS2		62.038	37.956	23.673	14.283	63.226	38.343	24.256	14.087
NCS2		62.467	38.078	23.9	14.178	63.504	38.457	24.437	14.02
NCS2		62.454	38.087	23.902	14.185	63.53	38.318	24.358	13.96
NCS2		62.585	38.102	23.966	14.136	63.956	38.46	24.61	13.851
NCS2		62.367	38.104	23.893	14.212	64.056	38.566	24.715	13.851
NCS2		62.007	37.997	23.687	14.31	63.661	38.356	24.428	13.928
NCS2		62.125	38.017	23.726	14.291	63.641	38.368	24.431	13.937
NCS2		62.278	38.054	23.815	14.24	63.54	38.361	24.383	13.979
NCS2		62.261	38.04	23.805	14.236	63.591	38.4	24.43	13.969

2- Change from resting period at baseline was calculated

		Baseline Data				13-week Data			
ID	Task	Ox%	[THC]	[HbO]	[Hb]	Ox%	[THC]	[HbO]	[Hb]
NCS2	average	59.259	37.293	22.222	15.071	64.709	39.106	25.328	13.778
NCS2	Data recorded during resting period (5 min)					64.626	38.635	24.985	13.65
NCS2		59.29	37.212	22.177	15.035	64.326	38.937	25.075	13.862
NCS2		59.359	37.297	22.249	15.049	64.367	38.967	25.105	13.862
NCS2		59.131	37.204	22.105	15.099	64.196	39.053	25.096	13.958
NCS2		58.998	37.137	22.01	15.128	64.444	39.034	25.176	13.858
NCS2		59.008	37.255	22.098	15.157	64.52	39.151	25.284	13.867
NCS2		59.248	37.283	22.195	15.088	64.312	39.09	25.166	13.923
NCS2		59.336	37.253	22.217	15.035	64.653	39.136	25.329	13.807
NCS2		59.55	37.363	22.379	14.984	64.771	39.203	25.424	13.78
NCS2		59.553	37.409	22.401	15.009	64.757	39.303	25.479	13.824
NCS2		59.452	37.347	22.322	15.025	64.424	39.182	25.266	13.916
NCS2		59.24	37.272	22.205	15.067	64.491	39.006	25.183	13.823
NCS2		59.384	37.241	22.238	15.003	65.232	39.191	25.589	13.602
NCS2		59.538	37.362	22.373	14.988	65.544	39.378	25.832	13.546
NCS2		59.538	37.364	22.375	14.99	65.082	39.208	25.542	13.665
NCS2		59.37	37.305	22.267	15.038	64.983	39.089	25.421	13.669
NCS2		59.237	37.311	22.232	15.079	65.133	39.324	25.641	13.683
NCS2		59.505	37.364	22.359	15.005	65.155	39.124	25.51	13.615
NCS2		59.21	37.321	22.226	15.094	65.176	39.165	25.548	13.618
NCS2		59.296	37.336	22.27	15.065	65.235	39.152	25.561	13.591
NCS2		59.065	37.244	22.13	15.114	65.224	39.3	25.653	13.647
NCS2		59.095	37.258	22.143	15.114	64.879	39.191	25.443	13.749
NCS2		59.25	37.271	22.207	15.064	65.186	39.194	25.57	13.623
NCS2		59.277	37.305	22.256	15.049	64.447	39.058	25.193	13.864
NCS2		59.113	37.278	22.168	15.11	64.37	38.965	25.104	13.862
NCS2		59.124	37.338	22.209	15.128	64.354	39.068	25.163	13.906
NCS2		59.24	37.29	22.218	15.071	63.911	38.802	24.823	13.979
NCS2		58.891	37.187	22.022	15.164	64.299	38.967	25.079	13.888
NCS2		59.156	37.398	22.253	15.145	64.6	39.103	25.28	13.822
NCS2									
NCS2		Ox%	[THC]	[HbO]	[Hb]	Ox%	[THC]	[HbO]	[Hb]
NCS2	average	60.062	37.533	22.673	14.86	64.662	38.927	25.192	13.735
NCS2	Data recorded during serial subtraction 3 (3:20 min)	58.913	37.197	22.034	15.162				
NCS2		59.146	37.398	22.25	15.148	63.8	38.907	24.84	14.066
NCS2		59.219	37.431	22.294	15.137	63.691	38.759	24.706	14.054
NCS2		59.351	37.404	22.32	15.084	64.179	38.968	25.03	13.938
NCS2		59.387	37.415	22.355	15.06	64.546	38.956	25.161	13.796
NCS2		59.224	37.378	22.277	15.1	64.617	38.949	25.19	13.76
NCS2		59.436	37.492	22.424	15.068	64.76	38.977	25.26	13.717
NCS2		59.716	37.612	22.591	15.02	64.96	39.059	25.397	13.661
NCS2		59.787	37.493	22.549	14.944	65.352	39.119	25.586	13.532
NCS2		59.906	37.57	22.646	14.924	65.182	39.002	25.445	13.556
NCS2		60.184	37.54	22.724	14.817	65.168	39.003	25.438	13.565
NCS2		60.393	37.556	22.806	14.751	65.13	38.938	25.378	13.56
NCS2		60.633	37.697	22.982	14.715	65.112	38.823	25.302	13.521
NCS2		60.796	37.677	23.036	14.641	64.958	38.957	25.331	13.626
NCS2		60.798	37.586	22.969	14.618	65.001	38.955	25.349	13.606
NCS2		60.765	37.631	22.989	14.642	64.548	38.859	25.107	13.752
NCS2		60.805	37.563	22.966	14.597	64.715	38.885	25.183	13.702
NCS2		60.923	37.688	23.097	14.593	64.445	38.807	25.029	13.777
NCS2		60.895	37.67	23.064	14.606	64.607	38.917	25.163	13.754
NCS2		60.938	37.653	23.072	14.581				
NCS2		Ox%	[THC]	[HbO]	[Hb]	Ox%	[THC]	[HbO]	[Hb]
NCS2	average	61.304	37.749	23.259	14.491	63.991	38.575	24.704	13.871
NCS2	Data recorded during serial subtraction 7 (3:20 min)	60.864	37.631	23.032	14.599				
NCS2		60.874	37.624	23.042	14.582	64.172	38.644	24.815	13.83
NCS2		61.09	37.7	23.16	14.541	63.73	38.53	24.579	13.952
NCS2		60.86	37.589	22.999	14.59	64.267	38.686	24.881	13.805
NCS2		61.1	37.675	23.146	14.528	64.101	38.609	24.769	13.841
NCS2		61.213	37.678	23.195	14.483	64.293	38.682	24.886	13.796
NCS2		61.182	37.676	23.17	14.505	64.511	38.755	25.016	13.738
NCS2		61.483	37.709	23.291	14.419	64.263	38.687	24.878	13.809
NCS2		61.302	37.696	23.219	14.477	63.911	38.49	24.618	13.871
NCS2		61.306	37.785	23.278	14.506	64.014	38.558	24.7	13.859
NCS2		61.433	37.753	23.296	14.457	63.877	38.498	24.614	13.884
NCS2		61.429	37.837	23.349	14.489	63.839	38.515	24.609	13.906
NCS2		61.307	37.753	23.256	14.497	63.586	38.354	24.409	13.944
NCS2		61.318	37.721	23.233	14.489	63.71	38.553	24.58	13.975
NCS2		61.438	37.767	23.31	14.458	64.081	38.614	24.76	13.853
NCS2		61.42	37.865	23.372	14.493	63.713	38.521	24.566	13.955
NCS2		61.598	37.891	23.441	14.45	63.557	38.453	24.46	13.993
NCS2		61.645	37.94	23.507	14.434	63.977	38.523	24.668	13.855
NCS2		61.623	37.896	23.47	14.426	64.135	38.622	24.785	13.837
NCS2		61.581	37.787	23.395	14.393				
NCS2		Ox%	[THC]	[HbO]	[Hb]	Ox%	[THC]	[HbO]	[Hb]
NCS2	average	61.195	37.705	23.197	14.508	63.637	38.419	24.461	13.958
NCS2	Data recorded during Stroop (3 min)	61.808	37.773	23.469	14.304	64.435	38.706	24.96	13.747
NCS2		61.75	37.71	23.409	14.301	64.451	38.598	24.888	13.71
NCS2		61.503	37.741	23.327	14.413	64.226	38.545	24.771	13.774
NCS2		61.228	37.647	23.175	14.471	63.796	38.445	24.542	13.902
NCS2		61.023	37.627	23.09	14.538	63.628	38.381	24.433	13.949
NCS2		61.536	37.762	23.367	14.395	63.685	38.32	24.416	13.903
NCS2		61.475	37.81	23.368	14.441	63.874	38.428	24.559	13.868
NCS2		61.223	37.675	23.186	14.489	63.929	38.456	24.597	13.859
NCS2		61.11	37.701	23.177	14.524	63.643	38.418	24.463	13.954
NCS2		61.131	37.613	23.123	14.49	63.702	38.43	24.494	13.936
NCS2		61.162	37.737	23.212	14.525	63.326	38.355	24.297	14.057
NCS2		61.007	37.743	23.149	14.594	63.511	38.411	24.406	14.005
NCS2		61.014	37.674	23.104	14.569	62.935	38.269	24.094	14.175
NCS2		60.925	37.706	23.084	14.622	63.271	38.364	24.282	14.082
NCS2		60.763	37.623	22.978	14.644	63.01	38.203	24.084	14.12
NCS2		60.903	37.692	23.069	14.623	63.534	38.45	24.439	14.011
NCS2		60.881	37.687	23.064	14.622	63.29	38.394	24.312	14.081
NCS2		61.07	37.773	23.196	14.577	63.213	38.363	24.261	14.103
NCS2		Ox%	[THC]	[HbO]	[Hb]	Ox%	[THC]	[HbO]	[Hb]
NCS2	average	62.028	37.995	23.69	14.306	63.512	38.427	24.418	14.009
NCS2	Data recorded during peg & ball (2:30 min)	61.538	37.888	23.445	14.444	63.556	38.422	24.434	13.987
NCS2		61.253	37.843	23.305	14.537	63.534	38.509	24.48	14.03
NCS2		61.632	37.933	23.506	14.427	63.375	38.567	24.453	14.114
NCS2		61.727	37.913	23.525	14.388	63.143	38.432	24.281	14.151
NCS2		61.64	37.916	23.496	14.42	63.222	38.505	24.353	14.151
NCS2		62.035	37.999	23.693	14.306	63.131	38.338	24.219	14.119
NCS2		62.038	37.956	23.673	14.283	63.226	38.343	24.256	14.087
NCS2		62.467	38.078	23.9	14.178	63.504	38.457	24.437	14.02
NCS2		62.454	38.087	23.902	14.185	63.53	38.318	24.358	13.96
NCS2		62.585	38.102	23.966	14.136	63.956	38.46	24.61	13.851
NCS2		62.367	38.104	23.893	14.212	64.056	38.566	24.715	13.851
NCS2		62.007	37.997	23.687	14.31	63.661	38.356	24.428	13.928
NCS2		62.125	38.017	23.726	14.291	63.641	38.368	24.431	13.937
NCS2		62.278	38.054	23.815	14.24	63.54	38.361	24.383	13.979
NCS2		62.261	38.04	23.805	14.236	63.591	38.4	24.43	13.969

3- Data after calculated the change (blue cells)

ID	Task	Ox%	[THC]	[HbO]	[Hb]
NCS52	average	8.3	13.4	22.8	2.1
NCs52	Resting period	6.7	11.5	19.0	2.5
NCS52		9.7	14.6	25.9	1.6
NCS52		7.4	12.3	20.8	2.4
NCS52		9.0	13.1	23.3	1.4
NCS52		6.9	13.1	21.1	3.5
NCS52		8.1	13.0	22.2	2.1
NCS52		5.7	11.3	17.7	3.6
NCS52		5.8	12.7	19.3	4.7
NCS52		6.8	12.8	20.5	3.6
NCS52		8.3	12.6	21.8	1.7
NCS52		5.4	11.3	17.4	3.7
NCS52		8.3	14.6	24.2	3.2
NCS52		8.3	13.9	23.5	2.6
NCS52		9.3	14.0	24.7	1.4
NCS52		9.4	13.8	24.5	1.0
NCS52		9.2	14.8	25.2	2.2
NCS52		10.2	15.1	26.9	1.1
NCS52		12.1	16.2	30.4	-0.3
NCS52		11.9	16.1	30.0	-0.2
NCS52		7.3	14.7	23.1	4.0
NCS52		8.7	15.0	25.0	2.4
NCS52		9.5	14.0	24.7	1.2
NCS52		6.9	12.6	20.3	3.1
NCS52		4.8	11.4	16.8	4.4
NCS52		8.2	12.9	22.1	1.7
NCS52		7.5	12.4	20.9	2.1
NCS52		7.1	12.5	20.6	2.5
NCS52		10.2	13.6	25.2	0.1
NCS52		10.3	13.4	25.1	-0.3
NCS52		8.9	12.0	22.1	-0.1
NCS52		Ox%	[THC]	[HbO]	[Hb]
NCS52	average	7.7	10.4	18.9	-0.8
NCS52	Serial subtraction 3	5.4	10.4	16.4	2.8
NCS52		7.9	11.0	19.6	0.2
NCS52		4.9	9.2	14.5	2.3
NCS52		6.7	10.5	17.9	1.0
NCS52		7.7	10.7	19.3	-0.4
NCS52		7.7	10.5	19.0	-0.7
NCS52		7.9	11.4	20.1	-0.1
NCS52		8.1	10.9	19.7	-0.9
NCS52		8.6	11.1	20.6	-1.4
NCS52		8.5	11.1	20.5	-1.5
NCS52		8.9	10.3	20.2	-2.5
NCS52		8.5	11.2	20.7	-1.3
NCS52		8.2	11.0	20.1	-1.3
NCS52		6.9	10.1	17.7	-0.3
NCS52		7.9	9.8	18.4	-1.8
NCS52		8.4	9.6	18.7	-2.5
NCS52		7.8	9.3	17.7	-2.2
NCS52		8.2	10.0	19.0	-2.1
NCS52		7.6	9.7	18.0	-1.4
NCS52		7.9	10.1	18.8	-1.5
NCS52		Ox%	[THC]	[HbO]	[Hb]
NCS52	average	9.4	10.3	20.5	-3.9
NCS52	Serial subtraction 7	7.0	11.4	19.3	0.4
NCS52		5.0	8.8	14.2	0.9
NCS52		8.3	10.5	19.5	-2.5
NCS52		7.4	9.5	17.5	-2.3
NCS52		8.6	9.9	19.3	-3.8
NCS52		11.1	11.1	23.3	-5.9
NCS52		10.2	10.9	22.1	-5.2
NCS52		12.0	11.8	25.0	-6.6
NCS52		10.7	11.8	23.5	-4.9
NCS52		10.8	11.3	23.1	-5.2
NCS52		10.2	10.1	21.3	-5.3
NCS52		10.2	10.0	21.0	-5.2
NCS52		9.1	9.6	19.4	-4.0
NCS52		11.4	11.0	23.4	-5.7
NCS52		9.1	10.3	20.1	-3.4
NCS52		9.5	10.1	20.3	-4.0
NCS52		9.7	9.9	20.3	-4.4
NCS52		9.0	9.2	18.7	-3.8
NCS52		8.6	9.3	18.3	-3.2
NCS52		9.9	10.1	20.7	-4.2
NCS52		Ox%	[THC]	[HbO]	[Hb]
NCS52	average	9.7	8.9	19.1	-4.8
NCS52	Stroop	11.2	10.6	22.6	-5.3
NCS52		9.4	10.5	20.6	-3.4
NCS52		11.0	11.3	23.2	-4.7
NCS52		11.1	11.6	23.5	-4.4
NCS52		11.8	11.1	23.7	-5.3
NCS52		9.7	8.4	18.5	-5.3
NCS52		9.2	7.9	17.3	-4.9
NCS52		10.3	8.7	19.3	-5.5
NCS52		8.9	8.2	17.5	-4.4
NCS52		9.0	8.4	17.7	-4.4
NCS52		9.5	8.8	18.7	-4.9
NCS52		10.2	8.9	19.7	-5.5
NCS52		9.7	8.3	18.4	-5.4
NCS52		9.4	7.6	17.3	-5.2
NCS52		8.9	7.3	16.4	-5.0
NCS52		8.8	7.4	16.4	-4.9
NCS52		8.5	7.6	16.3	-4.4
NCS52		8.5	7.8	16.6	-4.3
NCS52		Ox%	[THC]	[HbO]	[Hb]
NCS52	average	12.6	9.3	22.7	-7.2
NCS52	Peg & ball	10.4	7.8	18.6	-5.9
NCS52		10.4	8.6	19.5	-5.7
NCS52		10.8	8.0	19.4	-6.3
NCS52		13.4	9.7	24.0	-7.9
NCS52		12.7	9.4	23.0	-7.3
NCS52		12.3	9.1	22.3	-7.0
NCS52		12.0	8.7	21.5	-7.1
NCS52		13.6	9.3	23.9	-8.3
NCS52		12.8	9.5	23.3	-7.4
NCS52		13.0	9.6	23.5	-7.2
NCS52		13.6	9.7	24.3	-7.8
NCS52		12.3	9.7	22.9	-6.4
NCS52		13.6	9.8	24.3	-7.5
NCS52		14.1	10.1	25.2	-8.1
NCS52		14.3	10.2	25.6	-8.3

4- The average of each period was used for statistical analysis

ID	Task	Ox%	[THC]	[HbO]	[Hb]
NCS52	Resting	8.3	13.4	22.8	2.1
NCS52	Serial 3	7.7	10.4	18.9	-0.8
NCS52	Serial 7	9.4	10.3	20.5	-3.9
NCS52	Stroop	9.7	8.9	19.1	-4.8
NCS52	Peg & ball	12.6	9.3	22.7	-7.2

Appendix 6.3: Results of linear mixed model analysis for cognition

Measure	HN (1)		MN (2)		LN (3)		PL (4)		P.value	Δ 1vs 4 (P.value)	Δ 2 vs 4 (P.value)	Δ 3 vs 4 (P.value)
	Baseline (N=16)	13-weeks (N=10)	Baseline (N=17)	13-weeks (N=13)	Baseline (N=14)	13-weeks (N=14)	Baseline (N=15)	13-weeks (N=13)				
IWR correct (number)	5.4±0.4	5.4±0.5	6.5±0.4	6.6±0.4	5.6±0.5	5.9±0.5	6.8±0.5	6.0±0.5	T: 0.64 G: 0.16 T*G: 0.42	0.76±0.7 (0.28)	0.89±0.7 (0.18)	1.0±0.6 (0.12)
IWR error (number)	0.2±0.2	0.1±0.2	0.4±0.2	0.6±0.2	0.6±0.2	0.5±0.2	0.3±0.2	0.2±0.2	T: 0.93 G: 0.23 T*G: 0.72	0.04±0.3 (0.88)	0.3±0.3 (0.31)	0.02±0.3 (0.94)
NWM (% accuracy)	96.2±2.0	95.9±2.4	92.2±1.9	93.1±2.2	96.4±2.2	95.2±2.3	92.2±2.1	93.2±2.3	T: 0.80 G: 0.47 T*G: 0.53	-1.4±2.0 (0.49)	-0.2±1.9 (0.90)	-2.5±1.9 (0.19)
NWM-RT (msec)	1129.3±85.8	1142.6±70.6	1210.4±83.2	1061.3±62.9	1098.8±91.7	1167.5±62.4	1137.7±88.6	1040.6±63.7	T: 0.32 G: 0.94 T*G: 0.23	109.4±120.4 (0.36)	-52.1±115.4 (0.65)	165.8±117.4 (0.16)
CRT (% accuracy)	98.6±0.5	98.0±0.6	98.4±0.5	98.8±0.5	98.7±0.5	98.7±0.5	98.9±0.5	98.3±0.5	T: 0.52 G: 0.93 T*G: 0.44	0.01±0.8 (0.99)	1.1±0.8 (0.16)	0.6±0.8 (0.43)
CRT RT (msec)	601.7±28.3	577±32.3	628.0±27.5	586.6±29.8	645.1±30.3	649.6±30.9	594.5±29.3	588.3±30.8	T: 0.13 G: 0.46 T*G: 0.44	-17.6±32.0 (0.58)	-35.1±30.0 (0.25)	10.7±29.6 (0.72)
Stroop %accuracy*	99.3±0.6	99.7±1.3	98.9±0.6	97.7±1.1	99.9±0.7	99.7±1.1	99.5±0.6	97.7±1.1	T: 0.20 G: 0.45 T*G: 0.55	2.15±1.7 (0.20)	0.6±1.6 (0.69)	1.6±1.5 (0.29)
Stroop RT (msec)*	1312.5±93.1	1260.3±113.2	1448.9±85.2	1435.6±99.9	1523.5±93.1	1479.9±102.8	1270.9±88.1	1299.9±101.4	T: 0.62 G: 0.22 T*G: 0.89	-81.4±117.6 (0.49)	-42.4±110.9 (0.70)	-72.7±107.6 (0.50)
DV (%accuracy)	95.7±2.7	93.3±4.9	87.7±2.6	81.6±4.3	86.4±2.9	84.3±4.2	85.2±2.8	84.2±4.3	T: 0.20 G: 0.07 T*G: 0.85	-1.4±6.6 (0.83)	-5.2±6.1 (0.40)	-1.1±6.1 (0.86)

DV RT (msec)	482.9±10.8	481.4±23.6	497.6±10.5	455.4±20.9	511.9±11.9	508.3±20.6	502.2±11.2	501.5±21.1	T: 0.24 G: 0.27 T*G: 0.39	-0.8±29.9 (0.97)	-41.5±27.9 (0.14)	-2.9±27.6 (0.92)
DV false alarms (number)	2.3±0.8	2.9±1.1	5.1±0.8	4.5±0.9	5.4±0.9	5.4±0.9	5.5±0.9	5.0±0.9	T: 0.79 G:0.06 T*G:0.83	1.0±1.3 (0.43)	-0.1±1.2 (0.94)	0.5±1.2 (0.71)
Corsi blocks span	5.4±0.4	5.6±0.4	4.9±0.3	4.9±0.4	5.2±0.4	5.5±0.4	5.2±0.4	4.9±0.4	T: 0.67 G: 0.55 T*G:0.51	0.5±0.5 (0.25)	0.4±0.4 (0.31)	0.6±0.4 (0.16)
P&B thinking time	4552.9±471.1	4043.3±418.3	5110.6±457.0	4076.4±384.6	4745.9±503.7	4001.6±397.7	4704.7±486.6	4212.8±397.0	T:0.000 G: 0.95 T*G:0.64	-17.8±499.4 (0.97)	-542.4±474 (0.26)	-252.5±476.3 (0.59)
P&B working time (msec)	12302±1002	11301±938	14688±972	12638±839	14480±1071	12446±834	12647±1035	11748±850	T:0.004 G: 0.27 T*G: 0.74	-101.8±1432 (0.94)	-1160±1364 (0.39)	-1135±1377 (0.41)
P&B errors	3.4±1.2	4.3±1.1	3.0±1.2	2.5±0.9	4.1±1.3	3.4±0.9	5.9±1.3	3.1±1.0	T: 0.21 G: 0.63 T*G: 0.22	3.6±1.8 (0.04)	2.3±1.7 (0.16)	2.2±1.7 (0.20)
DWR correct (number)	3.8±0.5	4.1±0.6	5.3±0.4	4.2±0.5	3.5±0.5	4.4±0.5	5.0±0.5	4.9±0.5	T: 0.97 G:0.26 T*G: 0.03	0.3±0.7 (0.70)	-1.0±0.7 (0.13)	0.9±0.6 (0.16)
DWR error (number)	0.6±0.2	1.0±0.3	0.8±0.2	0.8±0.3	0.6±0.2	0.6±0.3	0.6±0.2	0.7±0.3	T:0.41 G:0.83 T*G: 0.71	0.3±0.4 (0.47)	-0.1±0.4 (0.70)	-0.1±0.4 (0.78)
WR (%accuracy)	76.9±2.2	75.4±2.7	78.6±2.2	80.1±2.4	77.9±2.4	76.9±2.3	82.4±2.3	81.9±2.4	T:0.80 G: 0.11 T*G: 0.91	-0.9±4.5 (0.83)	2.0±4.3 (0.64)	0.4±4.3 (0.93)
WR RT (msec)	1300±78.7	1281.2±94.2	1113±76.3	1135.9±87.3	1212±84.2	1202.9±91.1	1271±81	1349.8±90.5	T: 0.53 G:0.33 T*G: 0.66	-97.3±88.3 (0.3)	-54.5±82.5 (0.51)	-87.4±81.2 (0.28)

Data are presented as Estimated marginal means ± SEM. Data were analysed using linear mixed model.
*Due to non-compliance with the task instructions, data of some participants was removed: High NO_3^- (Baseline n=13, 13-weeks n=9), Moderate NO_3^- (Baseline n=15, 13-weeks n=11), Low NO_3^- (Baseline n=13, 13-weeks n=13), Placebo (Baseline n=14, 13-weeks n=11).
CRT; Choice reaction time, DV; Digit vigilance, DWR; Delayed word recall, HN; High NO_3^- dose, IWR; Immediate word recall, LN; Low NO_3^- dose, MN; Medium NO_3^- dose, NWM; Numeric working memory, PL; Placebo, P&B; Peg and ball, RT; Reaction time. WR; Word recognition.

Appendix 6.4: Results of linear mixed model analysis for blood pressure

Measure	HN (1)		MN (2)		LN (3)		PL (4)		P.value	Δ 1vs 4 (P.value)	Δ 2 vs 4 (P.value)	Δ 3 vs 4 (P.value)
	Baseline (N=16)	13-weeks (N=10)	Baseline (N=17)	13-weeks (N=13)	Baseline (N=14)	13-weeks (N=14)	Baseline (N=15)	13-weeks (N=13)				
SBP	130.8±3.7	124.8±4.0	136.1±3.6	126.7±3.6	139.4±3.9	131.2±3.7	134.1±3.8	136.4±3.7	T: 0.002 G:0.36 T*G: 0.06	-8.9±4.3 (0.09)	-11.7±4.5 (0.01)	-10.5±4.5 (0.02)
DBP	75.8±2.4	73.0±2.8	77.4±2.4	75.7±2.5	77.6±2.6	72.7±2.6	76.9±2.5	78.8±2.5	T:0.14 G: 0.72 T*G: 0.26	-4.6±3.6 (0.21)	-3.6±3.4 (0.03)	-6.8±3.4 (0.05)

Data are expressed as estimated marginal means ± SEM. Data were analysed using Linear mixed model, significant at < 0.05. T; Time, G; Group of intervention, T*G; Interaction between time and group of intervention. SBP; Systolic blood pressure, DBP; Diastolic blood pressure, HN; High NO_3^- dose, LN; Low NO_3^- dose, MN; Medium NO_3^- dose, PL; Placebo. Δ: Changes in each nitrate group are compared to placebo.

Appendix 6.5: Chapter 6 supplementary figures

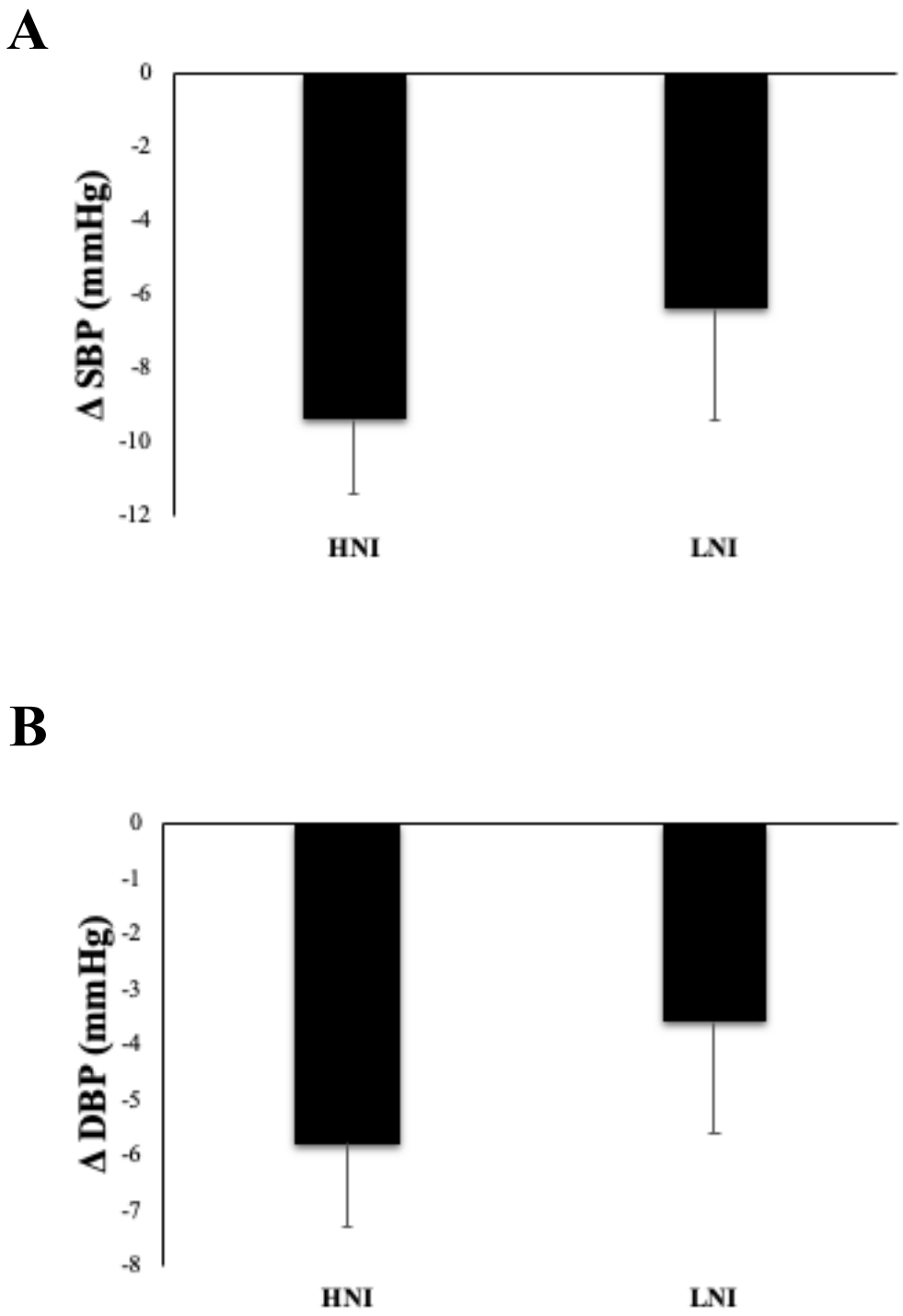


Figure 1: Mean changes from baseline of SBP (A) and DBP (B). NO_3^- intake data from all participants in all NO_3^- groups were pooled and dichotomised at the median. Analyses were conducted by one-way ANOVA. Data are expressed as mean ± SEM, n = 32.