The effect of dietary nitrate deprivation and subsequent supplementation on blood pressure in humans

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I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

Abstract:

Introduction: Cardiovascular disease (CVD) is the leading cause of mortality globally, accounting for approximately 17.5 million deaths annually. Hypertension is both the strongest predictor and most preventable risk factor of CVD; thus, the prevention of hypertension is central to reducing CVD-associated mortality. Dietary nitrate (NO_3^-) supplementation has emerged as a potential therapeutic strategy to manage hypertension and prevent CVD. Therefore, it is also possible that dietary NO_3^- deprivation may cause hypertension. However, research is required to investigate whether dietary NO_3^- deprivation causes a measurable increase in blood pressure in humans. Furthermore, rodent studies have demonstrated a 'super compensation' effect when NO_3^- supplementation is administered after a period of dietary NO_3^- deprivation. This phenomenon may have important applications as an anti-hypersensitive intervention. This thesis investigates the effect of dietary NO_3^- deprivation and subsequent supplementation on blood pressure in humans.

Methods: In a repeated measures, crossover design study, thirteen healthy subjects ingested 180 $mg.d^{-1}$ NO_3^- for 3 days, followed by a low NO_3^- diet (< 30 $mg.d^{-1}$ NO_3^-) or a standard NO_3^- diet (180 $mg.d^{-1}$ NO_3^-) for 7 days. Finally subjects ingested > 800 $mg.d^{-1}$ NO_3^- for 3 days. Subjects reported to the lab after each diet allocation for blood pressure measurements.

Results: No interaction effects were observed following 7 days dietary NO_3^- deprivation for systolic blood pressure (SBP), diastolic blood pressure (DBP) or mean arterial pressure (MAP) ($P > 0.05$). However, a significant main effect of time was observed (*P*

< 0.05; η^2_p= 0.30) in SBP with *post-hoc* t-test analysis demonstrating a significant decrease after 3 days NO_3^- supplementation compared to baseline (- 4 ± 3 mmHg; *P* = 0.001) in the standard condition. There were no significant differences in blood pressure variables following NO_3^- deprivation (*P* > 0.05). Additionally, NO_3^- supplementation following NO_3^- deprivation did not reduce blood pressure to a greater extent than NO_3^- supplementation following a standard NO_3^- diet.

Conclusion: 7 days dietary NO_3^- deprivation does not cause a significant increase in blood pressure within healthy humans. Moreover, 3 days NO_3^- supplementation reduced SBP following a standard diet. However, supplementation, when administered after a period of deprivation does not accentuate the blood pressure-lowering effects of dietary NO_3^-. These findings contribute to our understanding of the regulation of blood pressure by dietary NO_3^-. Specifically, the results suggest that the NOS-dependent pathway performs compensatory NO generation which offsets the decrease in NO generation from the NO_3^--NO_2^--NO pathway during a period of dietary NO_3^- deprivation.

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Symbols and abbreviations

[ ]	Concentration
ADI	Acceptable daily intake
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BP	Blood pressure
Ca^{2+}	Calcium
cGMP	Cyclic guanosine monophosphate
CHO	Carbohydrate
CVD	Cardiovascular disease
DASH	Dietary Approaches to Stop Hypertension
DBP	Diastolic blood pressure
eNOS	Endothelial nitric oxide synthase
HR	Heart Rate
iNOS	Inducible nitric oxide synthase
KNO_3	Potassium nitrate
L-NAME	L-arginine analogue nitro-L-arginine methyl ester
LND	Low nitrate diet

L-NMMA	NG-monomethyl-L-arginine
MAP	Mean arterial pressure
mmHg	Millimetre of Mercury
$NaNO_3$	Sodium nitrate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOCs	N-nitrosamine compounds
NOS	Nitric oxide synthase
NO_2^-	Nitrite
NO_3^-	Nitrate
O_2	Oxygen
PLA	Placebo
SBP	Systolic blood pressure
SD	Standard deviation
sGC	Soluble guanylate cyclase
SIT	Sprint interval training
WHO	World Health Organisation

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Introduction and Literature Review

Cardiovascular disease and hypertension

The World Health Organisation (WHO) has reported that cardiovascular disease (CVD) is a leading global cause of mortality. Indeed, the 2013 Global Burden of Disease study established that CVD accounts for 31.5 % (17.5 million) of all deaths (Naghavi et al., 2013). Trends project that the mortality rate associated with CVD will continue to rise and studies have ascertained that hypertension is the most important risk factor for CVD (Van Kleef and Spiering, 2017; Forouzanfar et al., 2017). Importantly, hypertension is also the most preventable risk factor for premature death (Smith et al., 2012; He and Whelton, 1999). Hypertension is diagnosed when diastolic blood pressure (DBP) is ≥ 90 mmHg or when systolic blood pressure (SBP) is ≥ 140 mmHg (Chobanian et al., 2003). In the UK, approximately 30 % of the population suffers from hypertension (Falaschetti et al., 2014) and in the USA, the figure is closer to one-third of the population (Yoon et al., 2015). It is estimated that the global cost of hypertension treatment over the next 10-year period will be $ 1 trillion (Gaziano et al., 2009).

Multiple treatments such as thiazide diuretics have been developed over recent decades either to lower or prevent high blood pressure and the result has been a decrease in the incidence of cardiovascular morbidity, mortality and strokes (Collins et al., 1990). However, the management of hypertension with pharmaceuticals can carry the risk of adverse side effects such as hyponatremia, hypokalemia and hyperkalemia (Sica, 2004). Furthermore, many anti-hypertensive pharmaceutical products are only effective in approximately half of cases (50.1 %; Egan et al., 2010), calling for

alternative, natural therapies to be explored in the management and prevention of hypertension.

The projection of CVD prevalence to increase may have catastrophic effects on global health services and economies. The Dietary Approaches to Stop Hypertension (DASH) was an initial trial in the United States designed to promote lifestyle changes and reduce sodium intake, in order to reduce the incidence of hypertension (Sacks et al., 1995). The study established that a diet combining high intakes of fruit and vegetables, alongside low-fat dairy products with reduced saturated and total fat significantly reduced both SBP and DBP (Sacks et al., 2001). Research in overweight individuals with hypertension identified reductions in both SBP (- 11.2 mmHg) and DBP (- 7.5 mmHg) after adhering to the DASH diet for 4 months, demonstrating the benefit of the DASH diet to reduce blood pressure in populations with hypertension (Blumenthal et al., 2010). The results have led the US Government to promote the DASH diet in national guidelines. In the UK, the Department of Health has already invested heavily in the 5-A-Day Campaign as advised by the WHO (1990). This campaign advocates the consumption of 400 g via five portions (80 g) of different fruits and vegetables per day (Christian et al., 2013). However, a heightened awareness of the dietary recommendations has not resulted in adults meeting the 5-a-day target, with adults in England averaging 4.1 portions of fruit and vegetables per day (Rooney et al., 2017; Bates et al., 2014). The failure of many adults to comply with these dietary recommendations emphasises the importance of understanding alternative methods that both treat and prevent key risk factors for CVD. One such strategy to complement the benefits of the DASH diet and 5-A-Day Campaign is dietary nitrate (NO_3^-)

supplementation. This thesis aims to improve our understanding of the role of dietary NO_3^- within human physiology, specifically the influence of dietary NO_3^- intake on blood pressure.

Nitric Oxide and its physiological role in blood pressure control

Nitric oxide (NO) is an inorganic free-radical, gaseous signalling molecule, which is critical to a plethora of physiological processes within the human body. These processes include vascular tone integral for blood flow and pressure regulation, neurotransmission (Moncada and Higgs, 1993), myocardial contractile function (Kelly, Balligand, and Smith, 1996), skeletal muscle glucose uptake (Merry, Lynch and McConell, 2010) and mitochondrial respiration (Cooper and Giulivi, 2007) and biogenesis (Nisoli et al., 2003). Given its short biological half-life which in vivo is approximately 0.1 s (Kelm and Schrader, 1990) and physiological importance, continuous NO generation is fundamental both at rest and during exercise**.**

Historically, NO was considered to be solely generated via the oxidation of the amino acid, L-arginine (NOS-dependent pathway), which is synthesised by NO synthase (NOS) enzymes and uses molecular oxygen (Moncada et al., 1989). Endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoforms activate the production of NO at numerous sites within the body (Stamler and Meissner, 2001). The generation of NO via the NOS dependent pathway entails a five-electron oxidation of L-arginine and reaction with molecular oxygen to catalyse the production of L-citrulline and NO (Moncada and Higgs, 1993). This complex metabolic reaction is catalysed via specific heme-enzymes: calcium-calmodulin, flavin adenine dinucleotide (FAD), flavin

mononucleotide (FMD) nicotinamide adenine dinucleotide phosphate (NADPH) and tetrahydrobiopterin (BH4) are all integral co-factors within this reaction (Verhaar et al., 2004). However, previous research has suggested poor L-arginine bioavailability following L-arginine supplementation resulting from arginase metabolising L-arginine in the liver (Schwedhelm et al., 2008; Morris, 2004; Castillo et al., 1993).

Interestingly, the concentration of intracellular L-arginine exceeds the Michaelis-Menten constant (K_m) by 15 - 30 fold (Böger, 2004) for maximal NO generation via eNOS. Therefore, NO_3^- rather than L-arginine supplementation, may be a more promising strategy for NO generation. However, despite the theoretical enzyme saturation, previous research within human populations has suggested that pure L-arginine supplementation may augment NO bioavailability (Bode-Böger et al., 2003); this is referred to as the 'L-arginine paradox' (Tskias et al., 2000).

Nitrate and Nitric Oxide production (NOS and NO_3^--NO_2^--NO pathway)

Dietary NO_3^- and nitrite (NO_2^-) were initially understood to be carcinogens due to their link with N-nitroso compounds (Buiatti et al., 1990), and thought to be inert oxidative by-products of NOS-derived NO synthesis. However, the existence of an alternative pathway of NO generation from NO_3^- and NO_2^-, the NO_3^--NO_2^--NO pathway (Lundberg et al., 2008), has since been established. In this pathway, NO can be physiologically recycled within the blood and tissues back to form NO_2^- and NO_3^-. The formation of NO from the NO_3^--NO_2^--NO pathway results, in part, from the acidic and enzymatic reduction of inorganic NO_2^-, following the reduction of NO_3^- to form NO_2^- in the oral cavity (Jansson et al., 2008; Duncan et al., 1995). It is understood that NO_3^- and NO_2^-

have longer half-lives than NO and new evidence demonstrates they can be stored in various tissues (Piknova et al., 2015). This finding provides a clear rationale for NO_3^- and NO_2^- to be considered an alternative storage reservoir for NO generation and a promising target for enhancing NO bioavailability.

The stepwise reduction of the NO_3^--NO_2^--NO pathways uses both NO_3^- and NO_2^- as a substrate, followed by the one-electron reduction to NO which can be catalysed by xanthine oxidase (Godber et al., 2000), aldehyde oxidase (Li et al., 2008), deoxyhemoglobin (Cosby et al., 2003), deoxymyoglobin (Shiva et al., 2007), cytochrome P-450 (Li et al., 2006), the mitochondrial electron transfer complexes (Tischner et al., 2004), sulfite oxidase (Wang et al., 2015), mitochondrial amidoxime-reducing component (Sparacino-Watkins et al., 2014) and NOS (Webb et al., 2008). Importantly, this NO_3^--NO_2^--NO pathway does not require oxygen or multiple cofactors, ensuring that NO generation can be maintained during periods of reduced NOS activity (Knowles and Moncada, 1994; Lundberg et al., 2008; Giraldez et al., 1997).

The generation of NO by the NO_3^--NO_2^- -NO pathway is permitted both endogenously, by the products of NOS reactions and/or via the reduction of ingested dietary NO_3^-. Following dietary NO_3^- ingestion, NO_3^- enters the upper gastrointestinal tract and enters the systemic circulation. Approximately 25 % of the NO_3^- is absorbed by the salivary glands and enters the entero-salivary system by the active transporter, sialin (Qin et al., 2012), prior to being concentrated at least 10 - 20 fold in saliva (Doel et al., 2005). The majority of the remaining NO_3^- is extracted by the kidneys for urinary excretion (Pannala et al., 2003). Within the oral cavity, commensal facultative anaerobic bacteria, namely *Niesseria* and *Veionella spp*, located on the dorsal surface of the tongue (Koch et al.,

2017; Hyde et al., 2014; Tannenbaum et al., 1974), reduce ~ 20 % of salivary NO_3^- to NO_2^-. These bacteria use NO_3^- as an alternative electron acceptor in the absence of oxygen to generate adenosine triphosphate (ATP) (Pittman and Kelly, 2005). A proportion of the swallowed NO_2^- is reduced further via the acidic gastric juice of the stomach, forming nitrous acid which further decomposes to NO amongst other nitrogen intermediates (McKnight et al., 1997). It is evident that some NO_2^- is absorbed into the systemic circulation as increased circulating plasma NO_2^- is measurable 1.5 - 2.5 hours post-ingestion (Kapil et al., 2010).

The NO_3^--NO_2^--NO pathway plays an important role in NO production during exercise. Hypoxia and acidosis are physiological states, typically induced via muscular contraction (Modin et al., 2001; Robergs et al., 2004) and potentiate the reduction of NO_2^- to NO (Lundberg and Govoni, 2004). These are also physiological conditions in which the activity of the traditional NOS-dependent pathway may be impaired and, as a result, the complementary NO_3^--NO_2^--NO pathway ensures NO may be generated across a range of oxygen tensions and pH (Jones, 2014; Richardson et al., 1995).

NO_3^- as a potential contaminant?

Initially, NO_3^- was regarded as a contaminant within the diet. Indeed, it was thought that NO_3^- and NO_2^- were carcinogenic due to their capacity to form N-Nitroso compounds (Swann, 1977). Once NO_3^- is reduced to NO_2^-, it can react with amines and amides in the acidity of the stomach which can cause the formation of endogenous N-nitrosamine compounds (NOCs) and lead to carcinogenesis resulting from DNA adductions (Bartsch and Montesano, 1984).

NO_3^- from dietary consumption can also be sourced from processed meats where NO_3^- salts are added as a preservative, and from drinking water, which can be influenced by nitrogen fertilizers used within agriculture washing into rivers and streams. In 2010, a study identified an increased risk of thyroid cancer with high NO_3^- levels in drinking water (> 5 $mg.L^{-1}$; Ward et al., 2010). Guidance from the WHO (2017) is to limit NO_3^- in water to 50 $mg.L^{-1}$ in public supplies of developed countries. In the USA, the maximum contaminant level is 10 $mg.L^{-1}$ due to an understanding that NO_3^- within drinking water is a leading factor contributing to infantile methemoglobinemia (Avery, 1999).

Additionally, research has established that red and processed meats are associated with cancer and suggested that the NO_3^- and NO_2^- within the meats could be responsible for the elevated risk (Ferrucci et al., 2010; Cross et al., 2010). The results of this earlier literature brought forth an acceptable daily intake (ADI) in the UK of 3.7 $mg.kg.d^{-1}$, the equivalent of 278 $mg.d^{-1}$ for a 75 kg adult. This figure represents a relative amount that can be consumed daily throughout a lifetime without appreciable health risk (WHO, 1987). The ADI has led to strict regulations of NO_3^- within the water, meat and agricultural industries at a huge economic cost.

However, studies that have suggested a link between NO_3^- and cancer have been considered epidemiologically weak (Gilchrist et al., 2010). The International Agency for Research on Cancer, (2010) determined “limited evidence” between the association of NO_3^- and NO_2^- intake on incidences of stomach cancer. Additionally, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) stated there was not currently enough evidence to support the view that NO_3^- is carcinogenic (Speijers and Brandt, 2003). More recently, research has demonstrated that NO_2^- ingested without the co-

ingestion of a carcinogenic nitrosamine precursor, is not linked to carcinogenesis (Bryan et al., 2012), suggesting NO_3^- and NO_2^- consumption in the form of green leafy vegetables is not associated with increased risk of cancer.

Early research identified several micronutrients, such as ascorbate, polyphenols, vitamin C and vitamin E found in NO_3^- rich foods such as green leafy vegetables can limit the formation of form N-Nitroso compounds (Tannenbaum et al., 1991; Wu et al., 1993; Bartsch et al., 1988; Zhu et al., 2014). Additionally, it is suggested the protection against cancer from NO_3^- generated NO can be due to the NO production and the generation of its by-products to have anti-tumour activity and activate white blood cells, a vital component of the immune system (Weitzberg and Lundberg, 2013; Nathan, 1997). Recent research has also established an inverse relationship between dietary NO_3^- intake and cancer risk (Xie et al., 2016). Up to 85 % of NO_3^- consumed within the diet is via green leafy vegetables (Hmelak Gorenjak and Cencič, 2013; WHO, 1995). The protective effects of dietary NO_3^- via vegetables against cancer is supported by research involving vegetarians. Studies within Europe have demonstrated that the NO_3^- intake of vegetarians is up to 3 fold greater than the remaining population (Mitek et al., 2013). A meta-analysis by Huang and colleagues (2012) assessing 124,706 participants from seven studies established that the overall cancer incidence in vegetarians was 18 % lower than the remaining. It is important to appreciate, however, that NO_3^- intake within vegetarians can fluctuate widely depending on vegetable preferences.

Therefore, the traditional interpretation of NO_3^- as a potentially carcinogenic contaminant is flawed. The more recent evidence regarding NO_3^- intake within vegetarians which has been estimated at 340.1 $mg.d^{-1}$ NO_3^- (Mitek et al., 2013),

surpassing the ADI, challenges the notion that NO_3^- is detrimental to human health and suggests that the current ADI is potentially unwarranted. Instead, NO_3^- has a beneficial effect on a wide array of physiological systems and, in the form of vegetables, can be protective against cancers. These findings indicate further research is required to assess dietary NO_3^- intake on the health of humans.

Estimates of dietary NO_3^- intake

Inorganic NO_3^- is a fundamental constituent of a range of vegetables, resulting from the extraction of NO_3^- from the earth to provide a source of nitrogen, which is a key mineral required for plant development (Prasad and Chetty, 2008; Lezhneva et al., 2014). Green leafy vegetables including spinach, lettuce and beetroot are particularly abundant in NO_3^-, typically containing > 2500 mg $NO_3^-.kg^{-1}$ of fresh weight produce (Santamaria, 2006). In addition, NO_2^- content within food and vegetables is lower than NO_3^-, with average values of 1 - 2 mg $NO_2^-.kg^{-1}$ fresh weight (Walker, 1996). However, the NO_3^- and NO_2^- content of such vegetables fluctuates greatly and depends on factors such as the time of year, the use of nitrogen-based fertilizers and the NO_3^- content of the earth where the vegetables are grown (Santamaria et al., 1999).

To fully understand whether NO_3^- is essential to human health and, thus, whether the current ADI represents an appropriate consumption limit that will not bring forth appreciable health risk, it is important to understand estimates of daily NO_3^- intake across different geographical locations. Furthermore, to understand whether the current ADI encapsulates the minimum requirement for health benefits, it is important to assess the impact on human health when NO_3^- is removed from the diet.

Interestingly, Japan has one of the longest life expectancies in the world, partly due to low incidences of CVD and cancer which has been attributed to their diet (Kurotani et al., 2016). The typical Japanese diet involves a wide variety of seafood and vegetables including kombu (seaweed) and mushrooms. Researchers estimated the traditional Japanese diet to include 18.8 $mg.kg.d^{-1}$ (Sobko et al., 2010) of NO_3^-. Additionally, the typically NO_3^--rich Mediterranean diet, including foods such as spinach and celery, has also been associated with reduced incidence of CVD, type 2 diabetes, and cancer (Shannon et al., 2018; Mentella et al., 2019). NO_2^- content in foods is generally low and estimates range from 0 – 20 $mg.d^{-1}$ (WHO, 2017; Pennington, 1998).

It is estimated that approximately 80 - 95 % of daily NO_3^- intake comes from vegetables in a western diet (Machha and Schechter, 2011). To estimate dietary NO_3^- intake, Temme et al. (2011) used two 24 h recalls for approximately 3000 subjects > 15 years old from the Belgian population. The study ascertained that the mean intake of NO_3^- was 1.38 $mg.kg.d^{-1}$ which represents 38 % of the ADI. Interestingly this is a reduction from 2.11 $mg.kg.d^{-1}$ calculated in the Belgian population in 1994 (Dejonckheere et al., 1994). However, Temme and colleagues note that the earlier study used household values rather than individuals and did not compensate for food wastage, so the true value was likely less. The study by Temme and colleagues also estimated that NO_3^- intake would be 76 $mg.d^{-1}$ if it were not to include non-alcoholic beverages, therefore suggesting that NO_3^- from water sources makes up approximately 20 % of total daily intake.

In the UK, the estimated total NO_3^- intake is approximately 70 $mg.d^{-1}$ (Ashworth and Bescos, 2017) and in the USA it was estimated to be 88 $mg.d^{-1}$. There are many potential

explanations for the lower NO_3^- intake observed within the western diet, one being a reduction in vegetables consumed and the NO_3^- content of the vegetables ingested. Bok Choy, a popular type of cabbage in Asia, can contain 102.3 - 309.8 $mg.kg^{-1}$ of NO_3^- whereas lettuce, a vegetable more prominent in the western diet, contains 12.3 - 267.8 $mg.kg^{-1}$ (Hord et al., 2009).

Nitric Oxide bioavailability

In humans, resting values of plasma NO_3^- and NO_2^- can vary considerably, which can be attributed, in part, to dietary intake, fitness levels and health status. Previous research has established that athletic populations have significantly elevated resting plasma NO_3^- and NO_2^- values in comparison to non-athletic groups, owing to exercise causing the upregulation of eNOS (Jungersten et al., 1997; Green et al., 2004), thus enhancing NO bioavailability. Conversely, disease states, such as atherosclerosis (Cannon, 1998) and type 2 diabetes mellitus (Woodman et al., 2006), are associated with diminished NO release resulting from a dysfunctional vascular endothelium.

Extensive research has established that NO_3^- supplementation in the form of NO_3^- salts such as potassium NO_3^- (KNO_3) and sodium NO_3^- ($NaNO_3$) or via NO_3^- rich vegetable products such as beetroot juice, elevates both plasma [NO_3^-] and [NO_2^-] (Kapil et al., 2010; Lansley et al., 2010; Bailey et al., 2009). Wylie and colleagues (2013) investigated the pharmacodynamics and dose-response relationship following acute beetroot supplementation (4.2, 8.4 and at least 16.8 mmol NO_3^-) on plasma [NO_3^-] and plasma [NO_2^-] in 10 healthy subjects. The study demonstrated a dose-dependent relationship between NO_3^- supplementation and plasma [NO_3^-] and [NO_2^-] up to 16.8 mmol NO_3^-. Following 4.2 and 8.4 mmol NO_3^- supplementation, plasma [NO_3^-] peaked at

130 and 282 μM respectively, 1 h after ingestion. Following 16.8 mmol NO_3^- supplementation, plasma [NO_3^-] peaked at 580 μM 2 h after ingestion. After 4.2 and 8.4 mmol NO_3^- supplementation, plasma [NO_2^-] peaked at 150 and 291 nM respectively, 2 h after ingestion. Following 16.8 mmol NO_3^- supplementation, plasma [NO_2^-] peaked at 425 nM 4 h after ingestion. The delayed time to attain peak plasma [NO_2^-] may be attributed to the requirement for a reduction of salivary NO_3^- to NO_2^- within the oral cavity prior to entering the systemic circulation. Interestingly, this research identified, for SBP, the peak reduction of 4.2 (5 ± 5 mmHg), 8.4 (10 ± 5 mmHg), and 16.8 mmol NO_3^- (9 ± 4 mmHg) occurred 4 h post supplementation. For DBP, the peak reduction of 8.4 mmol NO_3^- (3 ± 3 mmHg) occurred 4 h post supplementation and 2 h post supplementation of the 16.8 mmol NO_3^- (4 ± 4 mmHg), the 4.2 mmol NO_3^- dose did not significantly reduce DBP at any of the measured time points. These results suggest a dose-dependent reduction in blood pressure values up to 8.4 mmol NO_3^-, demonstrating the optimal dose of dietary NO_3^- supplementation in the form of beetroot juice to elicit anti-hypertensive effects and that greater NO_3^- doses do not provide an additional benefit. However, research conducted using KNO_3 supplementation in the form of capsules, identified a dose-dependent reduction in blood pressure values up to 24 mmol NO_3^-, suggesting the form of NO_3^- supplementation may influence the dose-dependent limit of NO_3^- blood pressure-lowering effect (Kapil et al., 2010).

Physiological effects of NO_3^- supplementation on blood pressure

The advantages to cardiovascular health resulting from a diet consisting of high levels of fruit and vegetables have long been known. A review by Ness and Powles (1997) into the cardioprotective effect of fruit and vegetable intake on CVD established that fruit and

vegetable intake reduced the incidence of coronary heart disease and strokes. An epidemiologic study by Bazzano and colleagues (2002), assessed 9608 subjects in the US that were CVD-free at the commencement of the study and measured their fruit and vegetable intake at baseline via a food-frequency questionnaire. Medical records and death certificates were used to assess mortality resulting from CVD. After approximately 20 years, 1145 deaths were related to CVD, 45.2 % of the total deaths during the study. This study concluded that subjects who consumed 3 or more daily portions of fruit and vegetables had 27 % fewer strokes. Stroke mortality was reduced by 42 % and CVD mortality was also 27 % lower in comparison to subjects that consumed < 1 portion of fruit and vegetables a day, suggesting a minimum intake of fruit and vegetables is required.

A recent prospective study investigated the influence of fruit, vegetables and legume intake on CVD and deaths over a 10-year period across 18 low, medium and high-income countries across Africa, Asia, Europe, North America and South America (Miller et al., 2017). 135,335 CVD-free subjects enrolled in the study, country-specific food-frequency questionnaires were used to record fruit and vegetable intake, and death certificates and medical records were used to record major health incidents. The study found that subjects consumed, on average, 3.91 portions of fruit, vegetables and legumes per day and established an inverse relationship between portion intake and incidence of CVD. In a clinical trial investigating dietary patterns on blood pressure (Appel et al., 1997), 459 subjects were randomly assigned to either a diet rich in fruits and vegetables, a control diet containing low quantities of vegetables, fruit and dairy products, or a “combination” diet rich in fruits, vegetables, and low-fat dairy products.

After eight weeks, the “combination” diet reduced SBP (6 mmHg) and DBP (3 mmHg) compared to the control. However, the fruit and vegetable diet also reduced both SBP by 2.8 mmHg and DBP by 1.1 mmHg compared to the control diet, demonstrating fruit and vegetable intake to be important in preventing CVD risk factors such as hypertension. Further research has investigated the effect of specific types of food protecting against coronary heart disease, the research indicated that green leafy vegetables provided the greatest protection against coronary heart disease (Joshipura et al., 2001). It has been suggested that the cardioprotective effect of green leafy vegetable intake is largely a function of their high NO_3^- content (Lundberg et al., 2006).

In a landmark study, Larsen et al. (2006) observed, subsequent to 3 days NO_3^- supplementation (0.1 mmol $NaNO_3^-.kg^{-1}.d^{-1}$) in 17 active subjects, a significant reduction in diastolic blood pressure (DBP) of 4 mmHg. Further research has since established that both SBP and DBP are reduced (10 ± 3 and 8 ± 2 mmHg) following the ingestion of a single bolus of 22.5 mmol NO_3^- administered via 500ml beetroot juice in 14 healthy subjects. This effect was evident just 1 h post NO_3^- supplementation (Webb et al., 2008). Subsequent research identified reductions in mean arterial pressure (MAP) by 2 mmHg at 1 h post supplementation of 4.2 mmol NO_3^- (Wylie et al., 2013), confirming an acute, single bolus of NO_3^- is sufficient to invoke marked reductions in blood pressure measures.

A study by Jonvik et al. (2016) investigated the effect of different forms of NO_3^- supplements on blood pressure, plasma $[NO_3^-]$ and $[NO_2^-]$. The study found that all supplements increased plasma $[NO_3^-]$ and $[NO_2^-]$ to a similar extent but with beetroot juice and $NaNO_3$ supplementation attaining the peak earliest. Compared to baseline,

SBP was reduced after the spinach, rocket salad and beetroot juice conditions and DBP was reduced in all conditions, suggesting that vegetables other than beetroot can reduce blood pressure to a similar degree. However, the research identified beetroot juice supplementation reduced blood pressure measures to a greater extent than $NaNO_3$ supplementation. It also identified that dietary NO_3^- in the form of beetroot juice can offer anti-hypertensive benefits without an increase in dietary salt consumption which can be detrimental to human health (He and MacGregor, 2009).

Additional research has investigated NO_3^- supplementation in the form of vegetable smoothies and increasing whole vegetable consumption on blood pressure variables. Specifically, NO_3^- rich vegetable smoothies reduced blood pressure, but the decrease was abolished when co-ingesting thiocyanate-rich vegetables (Dewhurst-Trigg et al., 2018). Additionally, interventions involving high NO_3^- vegetable diets have had mixed responses to lowering blood pressure in both healthy and clinical populations (Ashworth et al., 2015; Sundqvist et al., 2020).

A later exercise study (Thompson et al., 2018), compared the effect of a 4-week sprint interval training (SIT) intervention alongside NO_3^- supplementation in the form of either beetroot juice or KNO_3. The results corroborated previous findings that beetroot juice provides a greater blood pressure-lowering effect than an equimolar NO_3^- dose administered as a salt, but also identified that completing SIT with NO_3^- supplementation in the form of beetroot juice elicited greater exercise capacity adaptations than with KNO_3. These findings suggest that it is more effective to use beetroot juice as opposed to NO_3^- salts for NO_3^- supplementation. The presence of vitamin C and/or polyphenols (Gago et al., 2007), facilitate the formation of NO in the reduction of NO_3^- from the NO_3^--

NO_2^--NO pathway, which may explain the greater anti-hypertensive and exercise benefits of NO_3^- supplementation in the form of beetroot juice compared to NO_3^- salts.

Further research by McDonagh et al., (2018) investigated the effect of NO_3^- supplementation in various beetroot forms including, concentrated beetroot juice, non-concentrated beetroot juice and beetroot flapjack. Each form of supplement resulted in increases in plasma [NO_2^-], interestingly, beetroot flapjack and concentrated beetroot juice were most effective at reducing systemic blood pressure leading to the use of beetroot flapjack and concentrated beetroot juice as the form of NO_3^- supplementation in the present study.

Research conducted by Lansley et al., (2010) investigated the influence of NO_3^- supplementation in the form of beetroot juice on blood pressure. For six days, nine active males ingested 500ml of beetroot juice (~ 6.2 mmol NO_3^-) or the same volume of placebo (~ 0.003 mmol NO_3^-). Following 6 days NO_3^- supplementation, there was a significant reduction in systolic blood pressure (SBP), in comparison to the placebo (129 ± 9 vs. 124 ± 10 mmHg) although no changes in diastolic blood pressure (DBP) were observed. Importantly, this study was the first to use NO_3^- depleted beetroot juice (Beet It, James White Drinks Ltd, Ipswich) as the placebo, developed via passing the juice through an ion-exchange resin which selectively removed NO_3^-. The unchanged plasma NO_2^- concentration and SBP with the NO_3^- depleted beetroot juice in comparison to the beetroot juice, also demonstrated that NO_3^- is the active ingredient responsible for the associated changes, rather than other components of the beetroot such as vitamin C and betaine.

To determine the physiological effects of long-term NO_3^- supplementation, Vanhatalo and colleagues (2010) assessed the effects of NO_3^- rich beetroot juice over a 15-day period. Eight healthy subjects consumed 5.2 mmol $NO_3^-.d^{-1}$ for 15 days via 500 ml of beetroot juice. There was a reduction of both SBP (~ 4 mmHg) and DBP (~ 4 mmHg) following both acute and long-term supplementation periods, demonstrating that the observed reductions in blood pressure following acute supplementation could be maintained for > 2 weeks with daily NO_3^- supplementation. Interestingly, previous research suggests that multiple concomitant mechanisms underpin vasodilation resulting from dietary NO_3^- supplementation (Carlström et al., 2018). Earlier pharmacodynamic and dose-response research identified a delay in the reduction of blood pressure measures following NO_3^- supplementation, with the delay in the blood pressure-lowering effect of NO_3^- coinciding with the delay of peak plasma [NO_2^-] attainment (Kapil et al., 2010). Previous research has also demonstrated NO_2^- to be responsible for vasodilation in the human forearm (Cosby et al., 2003). However, to date, a specific NO_2^- receptor has not been identified in smooth muscle. Therefore, without a NO_2^- receptor, NO_2^- cannot be directly coupled mechanistically to a down-stream signalling event in smooth muscle resulting in vasodilation, suggesting other mechanisms may explain the concentration-response relationship between NO_2^- and vasodilation (Bailey et al., 2014). It has been postulated that for vasodilation to be triggered, NO_2^- may require entry within the tissue, whereby NO_2^- reductases reduce NO_2^- to NO prior to interaction with soluble guanylate cyclase (sGC), forming cyclic guanosine monophosphate (cGMP) via guanosine triphosphate and causing vasodilation (Ignarro et al., 1981). However, research has also demonstrated NO_2^-

transport within the tissue may not always be necessary for NO_2^--mediated vasodilation. Two proteins, XOR and deoxyhemoglobin have shown NO_2^- reduction activity can take place through red blood cells, with this reduction being, in part, responsible for NO_2^--mediated vasodilation (Ghosh et al., 2013; Webb et al., 2008).

Additionally, nitrosylation of target proteins such as protein kinase C (PKC) in the vasculature can play an important role in inhibiting vasoconstriction (Choi et al., 2011). Recent mechanistic studies have suggested that oral NO_2^- supplementation in the form of $NaNO_2^-$ can increase PKC nitrosylation which is an important mechanism preventing angiotensin II-induced vasoconstriction (Pinheiro et al., 2021). Therefore, it is possible that elevating systemic NO_2^- can have anti-hypertensive benefits via inhibiting vasoconstriction alongside NO_2^--mediated vasodilation.

An earlier study investigated the effect of NO_2^--derived NO on vasodilation in the aorta of rodents (Modin et al., 2001). The researchers observed vasodilation within the aorta following the generation of NO_2^--derived NO. Interestingly, the vasodilation was prevented when the cGMP inhibitor ODQ was administered. Additionally, Kapil and colleagues (2010) observed an increase in plasma [cGMP] corresponded with a reduction in blood pressure subsequent to KNO_3^- supplementation. These findings support the notion that NO_2^--derived NO can signal the sGC/cGMP pathway resulting in vasodilation. Interestingly, literature remains inconsistent regarding additional mechanisms responsible for vasodilation via the NO_3^--NO_2^--NO pathway. Research investigating the effect of beetroot supplementation on the nervous system identified reduced muscle sympathetic nerve activity following acute NO_3 supplementation (Notay et al., 2017). This suggests that the CVD benefits, such as reduced blood pressure

following NO_3 supplementation may result from reduced sympathetic outflow. However, additional research is necessary to further understand the relationship between muscle sympathetic nerve activity and blood pressure.

A study by Gao and colleagues (2015) demonstrated vasodilation to be induced within the renal microcirculation via NO_2^-. The authors observed varied vasodilatory responses to NO_2^- and associated this with the varying NO_2^- reductases in the vascular wall. In addition, the authors observed a blunted vasodilation response of NO_2^- following the addition of XO inhibitors, promoting the significance of XO being pivotal to the reduction of NO_2^- in renal microcirculation. Furthermore, the study identified reduced NADPH oxidase-dependent superoxide formation within rodents, which contributes to hypertension via greater arteriolar reactivity (Datla and Griendling, 2010). The authors suggested that reducing NADPH oxidase-dependent superoxide formation is likely a central mechanism explaining how inorganic NO_3^- and NO_2^- may lower blood pressure.

Research by Dejam and colleagues (2007) provided support for the role of deoxyhemoglobin in NO_2^--mediated vasodilation. The authors proposed other mechanisms that must be considered too. Ascorbate, which can react with protonated NO_2^- forming NO, has been identified to also lower superoxide concentrations, which has been identified to inactivate NO_3^-, mitigating the blood pressure lowering effects of NO_3^- (Carlström et al., 2010; Wilcox, 2005).

It is evident that multiple mechanisms play a role in vasodilation resulting from NO_3^- supplementation via the NO_3^--NO_2^--NO pathway. However, further research is required to better understand how the mechanisms overlap and whether alternative systems are responsible for NO_3^--mediated vasodilation. In addition, it is important to note that the

range of blood pressure change reported following dietary NO_3^- supplementation is broad. An early study by Larsen et al. (2006) observed reductions in only DBP (~ 3.7 mmHg) whereas Bailey and colleagues (2009) observed a reduction solely in SBP (~ 8 mmHg). Although, even a slight reduction in blood pressure variables can have clinically meaningful consequences (Chobanian et al., 2003).

Reductions in blood pressure following NO_3^- supplementation is not exclusive to healthy populations. In fact, evidence suggests normotensive participants appear less responsive to NO_3^- supplementation than those who are hypertensive. Of note, Kapil and colleagues (2015) assessed blood pressure in patients with hypertension. Subjects consumed 250 ml (6.4 mmol NO_3^-) of beetroot juice or a placebo (0.007 mmol NO_3^-) for 4 weeks. Following the NO_3^- supplementation, SBP measured in the clinic was reduced by 7 mmHg and DBP was reduced by 2 mmHg compared to baseline values; these reductions were not observed in the placebo group. These findings carry importance because they support NO_3^- supplementation as being effective for blood pressure control within populations already with hypertension.

However, research with type 2 diabetic subjects indicated no blood pressure-lowering effect following 2 weeks NO_3^- supplementation (7.2 $mmol.d^{-1}$), likely due to multiple biochemical perturbations such as dyslipidaemia, hyperglycaemia and increased oxidative stress, reducing NO activity which may mitigate the enhanced NO_3^- bioavailability arising from supplementation (Gilchrist et al., 2013; Yan et al., 1994; Kawamura et al., 1994). Additionally, patients with heart failure have reduced NO signalling (Cai and Harrison, 2000) and NO_3^- supplementation is being used as an additional source of NO (Lundberg et al., 2011). However, nine days of NO_3^-

supplementation (12.9 mmol NO_3^-) resulted in no changes in blood pressure measures compared to the placebo (Hirai et al., 2017). The authors suggested that the greater NO bioavailability resulting from the NO_3^- supplementation may be detrimental to eNOS activity (Zhen et al., 2008), negating the beneficial effects of NO_3^- supplementation and explaining the unchanged blood pressure measures observed. Further research into clinical populations such as patients with chronic obstructive pulmonary disease also demonstrated no significant differences in SBP or DBP following 2.5 days of NO_3^- supplementation (6.77 mmol NO_3^-) (Shepherd et al., 2015). These findings contribute to the suggestion that NO_3^- supplementation may not beneficially lower blood pressure in all disease populations.

While much research has investigated the physiological effects of NO_3^- supplementation, no research to date exists on the effect of deprivation of dietary NO_3^- on blood pressure. Understanding the response of blood pressure to dietary NO_3^- deprivation will further our understanding of NO_3^- on physiological systems.

The muscle NO_3^- reservoir

Previously, only internal organs such as the liver and heart were regarded as active sites of NO storage (Li et al., 2008; Totzeck et al., 2012). Although skeletal muscle is one of the largest organs within the human and mammalian body, it was not initially recognised as a contributor to the NO cycle other than as a target organ. However, extensive research has now established that NO is produced and stored in skeletal muscle tissue at rest, whilst production increases dramatically during muscular contractions (Jackson et al., 2007; Balon and Nadler, 1994; Patwell et al., 2004).

A landmark study by Piknova et al. (2015) demonstrated that NO_3^- concentrations are distributed non-homogenously within mammalian bodies. A steep concentration gradient was observed such that muscle NO_3^- values were 3 fold greater than that of the blood and 17 fold greater than the liver, demonstrating that skeletal muscle represents an endogenous reservoir of NO_3^- ions. Contrary to the observed distribution of NO_3^-, the study indicated that [NO_2^-] does not differ significantly between locations, although the highest value reported was in skeletal muscle tissue. The authors completed a follow-up study (Piknova et al., 2016), which ascertained that muscle NO_3^- levels decreased and muscle NO_2^- levels increased following exercise in Wistar rats, with XOR identified as the catalyst for this NO_2^- generation. Collectively, these results indicate that muscle is a major store of NO_3^- and can be used to produce NO_2^- and NO during exercise.

More recently, Wylie and colleagues (2019) investigated whether the purported muscle NO_3^- reservoir is evident in humans and to what extent it might be influenced by exercise and dietary NO_3^- supplementation. Thirteen subjects consumed a low NO_3^- diet (< 25 mg NO_3^-) the day before testing and muscle and blood samples were collected prior to and following, the consumption of either beetroot juice containing 12.8 mmol NO_3^- or placebo (0.04 mmol NO_3^-), a third muscle and blood sample were collected at the end of a high-intensity step exercise test. The results of this study aligned with earlier research using a rodent model: NO_3^- content at rest in human skeletal muscle tissue was ~ 4 fold greater than that of plasma. After NO_3^- supplementation, skeletal muscle tissue [NO_3^-] increased ~ 5 fold and plasma [NO_3^-] increased ~ 19 fold demonstrating that dietary NO_3^- contributes towards the human skeletal muscle NO_3^- reservoir. After severe-intensity exercise, muscle [NO_3^-] decreased by ~ 39 % and

plasma [NO_3^-] decreased by ~ 34 %. Interestingly, the concentrations did not alter after exercise in the placebo condition. Collectively, the findings suggest that skeletal muscle acts as a NO_3^- reservoir which can be increased via dietary NO_3^- supplementation and utilised as a source for NO generation. However, it remains to be established how the NO_3^- reservoir responds to a period of NO_3^- deprivation.

NO_3^- deprivation and the effect on blood pressure

The weight of evidence demonstrating the anti-hypertensive effects following NO_3^- supplementation and the recent understanding that muscle tissue acts as a reservoir to store NO_3^- following dietary NO_3^- supplementation demonstrates the benefits of dietary NO_3^- (Piknova et al., 2015; Wylie et al., 2013). It also provides a clear rationale for dietary NO_3^- supplementation as a therapeutic strategy to manage hypertension and prevent CVD. It is possible that the large prevalence of hypertension globally is associated with diets being deprived of NO_3^-, at least in Western societies. Thus, it is important to understand the physiological effects in humans when NO_3^- is removed from the diet.

In an earlier study, Jones et al. (2004) explored the effect of NOS inhibition via the systemic infusion of L-NAME on blood pressure. 7 active subjects were infused with either L-NAME (4 $mg.kg^{-1}$ in 50 ml saline) or a placebo (50 ml saline) over 1 h, which has been established to reduced NOS activity by 67 % (Frandsen et al., 2001) and MAP was recorded. There was a significant increase in MAP (~ 14 mmHg) after NOS inhibition. However, whether dietary NO_3^- deprivation might have similar effects on MAP is unknown.

A study by Kina-Tanada et al. (2017) is the only study to date to investigate the long-term effect of NO_3^- deficiency on the health of mice. 6-week old mice were fed either a standard chow with tap water or low NO_3^- chow with ultrapure water for 1.5 - 22 months. After as little as 3 months of a low NO_3^- diet (LND), mice had significantly reduced plasma NO_3^- and NO_2^- levels in concert with metabolic syndrome-like symptoms including increased cholesterol and greater epididymal white adipocyte size. Following 18 months LND, the metabolic syndrome-like symptoms were of greater severity, with the LND mice having significantly gained weight, insulin resistance and hypertension. At 22 months, approximately a third of LND mice (7 / 22) had died, as opposed to none of the standard diet mice. These findings indicate that long-term dietary NO_3^- deprivation causes metabolic syndrome, endothelial dysfunction and in some cases, cardiovascular death in mice.

In a later study, Gilliard and colleagues (2018) investigated the short-term effect of NO_3^- deprivation and subsequent NO_3^- loading on the endogenous NO_3^- reservoir in skeletal muscle. 15 Wistar rats were divided into groups of 3 (n = 5 per group) and administered a diet for 7 days. The control group rats were fed a standard chow, the high NO_3^- diet group consumed water infused with $1g.L^{-1}$ of $NaNO_3^-$ and the low NO_3^- diet group rats were fed a low NO_3^- chow. A separate group of rats consumed the low NO_3^- diet for 7 days after which they consumed the high NO_3^- diet for 3 and 7 days respectively, to establish the response of the muscle NO_3^- reservoir following a period of deprivation. Results showed that compared to control, NO_3^- supplemented rats increased $[NO_3^-]$ in muscle, blood and liver by 1.3 fold, 1.5 fold and 3 fold respectively. $[NO_2^-]$ increased in the muscle, blood and liver by 3.5 fold, 2.5 fold and 3.7 fold respectively. Following 7

days of NO_3^- deprivation there was a significant reduction in skeletal muscle, blood and liver [NO_3^-] compared to the values of the control by 0.4 fold, 0.7 fold and 0.8 fold respectively. [NO_2^-] also decreased to approximately 40 % of values on the standard diet. Interestingly, when NO_3^- was restored to the diet following deprivation, [NO_3^-] significantly increased within each measured organ. After 7 days, [NO_3^-] increased compared to the standard diet rats in the muscle, liver and blood by 3 fold, 2.6 fold and 2 fold respectively.

The results suggest that dietary NO_3^- deprivation results in the utilisation or redistribution of the endogenous muscle NO_3^- reservoir. Furthermore, the findings also indicate that dietary NO_3^- supplementation following a period of deprivation restores skeletal muscle [NO_3^-] highly efficiently. Indeed, following 3 days of high NO_3^- water, NO_3^- levels within all tissues were restored to levels that were equal to or greater than the control group. Interestingly, 7 days of the high NO_3^- diet increased [NO_3^-] within muscle and blood by 2.3 and 1.2 fold in comparison to the high NO_3^- group that was not previously deprived of NO_3^-, NO_2^- levels responded similarly.

These findings demonstrate a ‘super compensation’ response of NO_3^-. This response is akin to the muscle glycogen response that has been reported following post-exercise carbohydrate loading in athletes (Bergström, J. and Hultman, 1966) and used as a strategy to promote athletic performance.

Research aims and hypothesis

The primary aim of this thesis was to investigate the effect of dietary NO_3^- deprivation and subsequent resumption of NO_3^- intake on blood pressure in a healthy human

population. This thesis will aim to address the current gap in the literature surrounding the physiological response, namely, blood pressure, to dietary NO_3^- deprivation in humans. Further understanding of the physiological role of dietary NO_3^- will aid in optimising supplementation of NO_3^- as a potential prophylactic for hypertension. Subjects will be enrolled onto 2 x 13-day diets whereby all food and drink they ingest will be strictly regulated. Although this brings complexity, it allows greater confidence in the results and furthers our knowledge of NO_3^- and its role within the diet and health of humans.

Hypotheses

The following hypotheses will be tested:

1. 7 days dietary NO_3^- deprivation will result in a significant increase in SBP, DBP and MAP in healthy humans.
2. Dietary NO_3^- deprivation followed by NO_3^- supplementation will result in a significantly greater reduction in all blood pressure measures to a greater extent than NO_3^- supplementation following a standard NO_3^- diet.

Methodology

Ethical approval and informed consent

All experimental testing was sanctioned by the University of Exeter's Sport and Health Sciences Ethics Committee and adhered to the principles of the Declaration of Helsinki, apart from registration in a clinical trials database. After reading the Participant Information Sheet outlining the requirements, potential benefits and possible risks involved, further questions or concerns raised by potential participants were answered. After a minimum of 24 h following subjects receiving the study information, all participants provided written informed consent prior to commencing the experimental testing. Participants were informed that they could withdraw without explanation at any point while participating in the study.

Health and safety

All experimental testing conformed to the Sport and Health Sciences Health and Safety Code of Practice 2015-16. The researchers ensured all laboratories provided a safe and hygienic environment for all testing procedures. All equipment was cleaned and deemed sterile at the commencement of each visit.

Participants

Participants were recruited from the student population of the University of Exeter (mean ± standard deviation (SD): age; 21.9 ± 2.0 years, body mass; 77.7 ± 7.8 kg, height; 177 ± 7 cm, male; n = 12, female; n = 1). Participants were required to report to the laboratory > 2 hours post-prandial after ingesting the relevant NO_3^- supplement, so

that the measures aligned with expected peak plasma [NO_3^-] and [NO_2^-] (Wylie et al., 2013). Participants were also asked to arrive in a rested and hydrated state having not consumed caffeine in the previous 12 hours or alcohol in the previous 24 hours as this may influence NO storage (Rocha et al., 2015). Additionally, participants were required to not be using antibacterial mouthwash or a tongue scraper for 2 weeks prior to commencing and throughout the study because it has been established to markedly attenuate the rise in plasma NO_2^- (Govoni et al., 2008). Participants were also instructed to avoid the use of any dietary or ergogenic aids which may influence the supplementation or blood pressure measures. In addition, participants were also informed not to complete strenuous exercise in the 24 h preceding experimental visits and were requested to maintain habitual physical activity throughout the study. All experimental testing was conducted at the same time of day (± 2 hours) to reduce the influence of diurnal variation on measures.

Exclusion / inclusion criteria

Participants were eligible to enrol in the study provided they satisfied specific criteria for SBP (90 - 130 mmHg), DBP (60 - 90 mmHg) and body mass index (18.5 – 24.9 kg/m^2). Additionally, participants were required to be recreationally active but not highly trained, who were free from disease and did not have a history of smoking. Participants were excluded should they have an existing medical condition such as diabetes or take dietary or medical supplements, such as L-Arginine, which would influence the supplementation or performance during the study.

Experimental design

The study followed a randomised, repeated measures, cross-over design. Subjects reported to the laboratory on six occasions to complete the experimental testing (figure 1 A). The first visit of each condition occurred following a 3 day standard NO_3^- diet, the second laboratory visit occurred after either a 7 day low NO_3^- diet or a 7 day standard NO_3^- diet and the final laboratory visit of each condition occurred following a 3 day high NO_3^- diet; a 2 week washout was implemented between conditions. Upon arrival, subjects were asked about their adherence to the diet, body mass was recorded, and 5 resting blood pressure measures were recorded at each of the 2 time points, the mean value of the two time points were used for statistical analysis. All data collection was repeated on each visit.

Preceding the commencement of the study, prospective participants attended the laboratory for health screening in which they completed informed consent forms, and a physical activity readiness questionnaire (PARQ) before measurements were made of body mass (Seca Digital Column Scale SEC-170, Seca, Hamburg, Germany), height (Seca Stadiometer SEC-225, Seca, Hamburg, Germany) and blood pressure (Dinamap Pro 100V2, GE Medical Systems Information Technologies 2002, Tampa, Florida, USA) to ensure subjects conformed to the eligibility criteria. Following meeting the study's criteria and > 24 h after informed consent, subjects were familiarised with the experimental testing procedures.

Diet and NO_3^- supplementation

Dietary NO_3^- supplementation and deprivation conditions were created, in part, via the use of NO_3^- rich (0.67 mmol) and NO_3^- depleted (< 0.05 mmol) beetroot flapjack and

beetroot juice (6.4 mmol) donated by James White Drinks (Ipswich, UK). The NO_3^- depleted flapjack was implemented in the current study as a placebo to conceal the NO_3^- diet condition from participants. The NO_3^- depleted, placebo flapjack was created via NO_3^- rich juice flowing through an ion-exchange resin to extract NO_3^- ions while maintaining the original properties of the beetroot juice (Lansley et al., 2010).

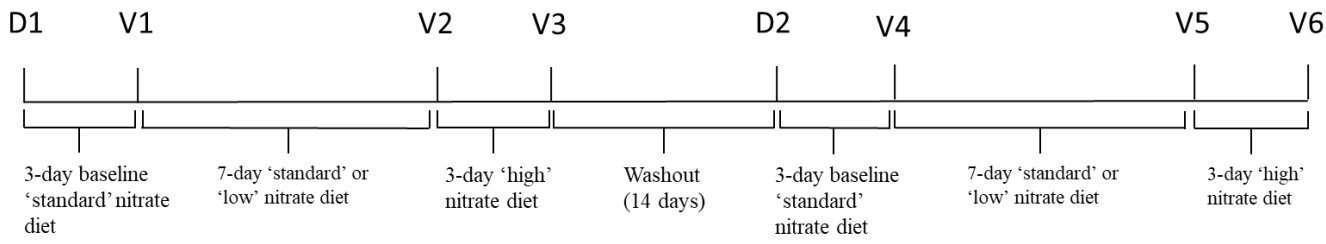
Subjects' diets were designed using a bespoke NO_3^- database created by Dr Nicholas McMahon, which collates the analysis of NO_3^- content of different food and drinks from previous research (McMahon et al., 2017), allowing the control of total NO_3^- content when creating the low (< 30 $mg.d^{-1}$), standard (~ 180 $mg.d^{-1}$) and high (> 800 $mg.d^{-1}$) NO_3^- diets. Using participants' height, weight, and physical activity levels alongside the bespoke NO_3^- database, individual diets were created which satisfied individual calorie expenditure and achieved recommended daily intake levels of macronutrients and micronutrients with the deliberate exception of NO_3^-. Subjects were also administered Buxton mineral water to consume throughout the study which has negligible NO_3^- content (< 0.1 $mg.L^{-1}$). Participants were administered bottled mineral water to prevent the consumption of tap water, for which the NO_3^- content is variable.

Table 1: Example of a prescribed diet to achieve < 30mg.d^{-1} NO_3^-

	Energy (Kcal)	Energy (Kj)	NO_3^- (mg)	NO_2^- (mg)	CHO (g)	PRO (g)	FAT (g)
Wheat biscuits with skimmed milk	330.8	1421.2	2.23	0.39	58.8	16.1	5.48
Apple	92.2	382.8	1.14	0.21	20.9	1.7	1.74
Breaded cod and chips	717.8	2967.6	6.87	0.26	94.8	23.3	26.3
Orange	94.7	409.6	3.33	0.051	20.5	2.6	0
Chocolate bar	237.8	997.6	0.70	0.14	37.1	2.3	8.7
Grilled chicken and chips	749.8	3069.6	6.23	1.31	83.8	61.9	16.7
Cheddar cheese and crackers	384.0	1584	1.14	0.18	18.9	17.7	25.8
Grapes	112.5	472.7	2.77	0.04	26.1	1.6	0
Buxton water	0	0	0.02	0	0	0	0
Total	2719.6	11305.1	24.43	2.58	360.9	127.2	84.7

Abbreviations; NO_3^- : Nitrate; NO_2^- : Nitrite; CHO : Carbohydrate; PRO: Protein; FAT: Fat.

A



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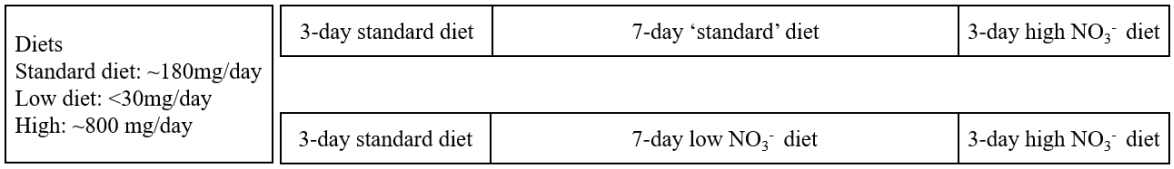

Figure 1 (A): Schematic diagram of the time-course of the study. **(B)** Supplement schematic illustrating the NO_3^- intake and length of each diet. Abbreviations; D: diet, V: visit.

Participants completed a 3 day diet with a standard (~ 180 $mg.d^{-1}$) NO_3^- intake, which was calculated from the European Estimate of Nitrate Intake (Hord et al., 2009). Subsequently, subjects completed a 7 day diet with either a low (< 30 $mg.d^{-1}$) NO_3^- intake, which following from previous research in mammalian studies, demonstrated that 7 days dietary NO_3^- deprivation elicited significant reductions in skeletal muscle, blood and liver [NO_3^-] compared to the values of the control (Gilliard et al., 2018). Or a 7 day standard (~ 180 $mg.d^{-1}$) NO_3^- diet, this was followed by a 3 day diet with a high (~ 800 $mg.d^{-1}$) NO_3^- intake.

Table 2: Daily NO_3^- supplementation for each NO_3^- condition

	Baseline Diet	7 day standard NO_3^- diet	7 day low NO_3^- diet	3 day NO_3^- diet
NO_3^- flapjack (3 x 8.5 g)	2 $mmol.d^{-1}$	2 $mmol.d^{-1}$	0	2 $mmol.d^{-1}$
NO_3^- depleted flapjack (3 x 8.5 g)	0	0	< 0.05 $mmol.d^{-1}$	0
Beetroot juice (2 x 70 ml)	0	0	0	12.8 $mmol.d^{-1}$

Total	2 mmol.d^{-1}	2 mmol.d^{-1}	< 0.05 mmol.d^{-1}	14.8 mmol.d^{-1}

Abbreviations; NO_3^- : Nitrate.

As illustrated in table 2, participants were instructed to ingest NO_3^- rich flapjack 3 x 8.5 g.d^{-1} (2 mmol.d^{-1}, one accompanying each meal) during the initial 3 day baseline diet and the 7 day standard NO_3^- diet. It is important to note, the NO_3^- content of the supplements were included in the calculations of total daily NO_3^- intake during each condition. During the 7 day low NO_3^- condition subjects ingested NO_3^--depleted flapjack (< 0.05 mmol.d^{-1}) 3 x 8.5 g.d^{-1}. Subsequently, during the 3 day high NO_3^- intake diet, participants were instructed to ingest 2 x 70 mL.d^{-1} (12.8 mmol.d^{-1} NO_3^-) beetroot juice supplements (Beet It, James White Drinks Ltd, Ipswich), 12 hours apart and 3 x 8.5 g.d^{-1} NO_3^- rich flapjack (2 mmol.d^{-1}). Additionally, participants were instructed to consume 2 x 70 mL beetroot juice 2 hours prior to attending the laboratory on day 14 of each condition, to ensure experimental testing corresponded with expected peak plasma [NO_3^-] concentrations (Wylie et al., 2013). It should be acknowledged, that this design cannot separate the NO_3^- storage effect from the acute systemic increase in NO_3^- and NO_2^-. Each supplementation condition was separated by a washout period of at least 14 days. Participants were informed prior to ingestion that the supplementation might cause the temporary and harmless side effect of beeturia (red urine) and red stools.

Measurement procedures

Blood pressure and heart rate

Heart rate (HR), SBP, DBP and MAP were assessed at the brachial artery via an automated sphygmomanometer at two time points each visit. The first measurement

was subsequent to a 10 minute period in a supine position in an isolated room during which body position, temperature and light exposure were controlled. The remaining time point was approximately 25 min after the first (figure 2), with the subjects' position and room temperature maintained throughout. Five measurements were recorded at each time point with 1 min rest in-between on the 6 experimental visits. Mean arterial pressure (MAP) was calculated manually using the equation 1/3 systolic pressure + 2/3 diastolic pressure. The first measurement at each time point was excluded from data analysis. The means of the last four measurements of SBP, DBP and MAP measures were calculated for each time point and the means of the time points were used for analysis.

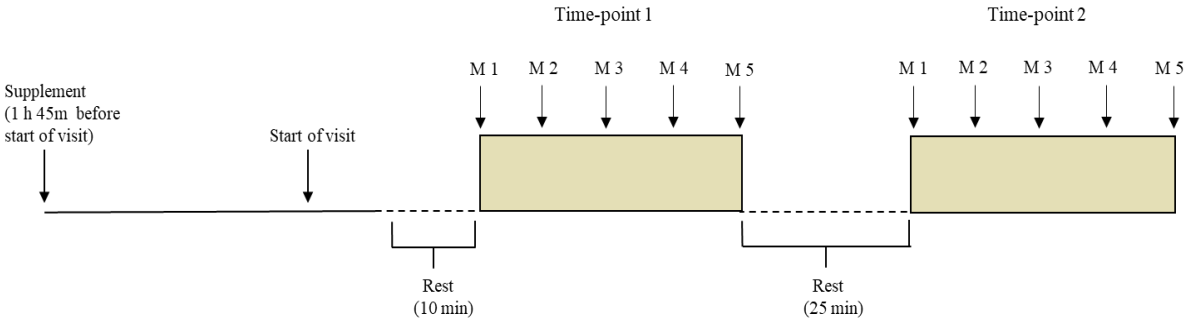

Figure 2: Schematic diagram of the measurement protocol of the study. Abbreviations; M: Blood pressure measure.

Dietary intake analysis

To estimate individual daily NO_3^- intake via their food and drink consumption, each subject was asked to complete a 3 day (2 weekdays and 1 weekend) food diary. Subjects were given a set of scales to record the weight of all food and drink consumed over this period. In the event that the weight couldn't be recorded, brand and packaging data were used. Subjects were also asked to record the time at which they consumed

the food and drink. The completed diaries were clarified with the subjects to check the data prior to analysis.

Nutrient intake analysis

Following completion of the food diaries, the diets were recorded and analysed using Nutritics Nutrition Analysis Software (version 4.267 Academic Edition, Nutritics, Dublin, Ireland). Protein, Fat and CHO intake was calculated. Diets were also analysed via input into the bespoke NO_3^- database, created to summarise previous literature which has conducted NO_3^- and NO_2^- analysis on different foods and allowed for subjects habitual NO_3^- intake to be calculated.

Statistical analyses

All data were analysed using the Statistical Package for the Social Sciences (SPSS), version 26. Prior to completing the research, a power calculation was conducted which determined, based on an effect size of d = 1.50 observed by Gilliard et al., (2018), a sample size of 8 was required for 95% statistical power at $P < 0.05$ level. 2 x 3 repeated-measures ANOVA was conducted using the combined means of the two time points to assess differences in SBP, DBP and MAP across 2 conditions (standard NO_3^- diet and low NO_3^- diet) at 3 time points: after the 3 day baseline diet (day 4), after the 7 day low or standard NO_3^- diet (day 11) and after the 3 day high NO_3^- diet (day 14). Paired sample t-tests were conducted to further analyse significant main and interaction effects. Values are expressed as mean ± SD unless otherwise indicated. The effect size was calculated as partial eta^2 (η^2_p), statistical significance was accepted at $P < 0.05$ level, a statistical trend was considered at $P < 0.10$ level.

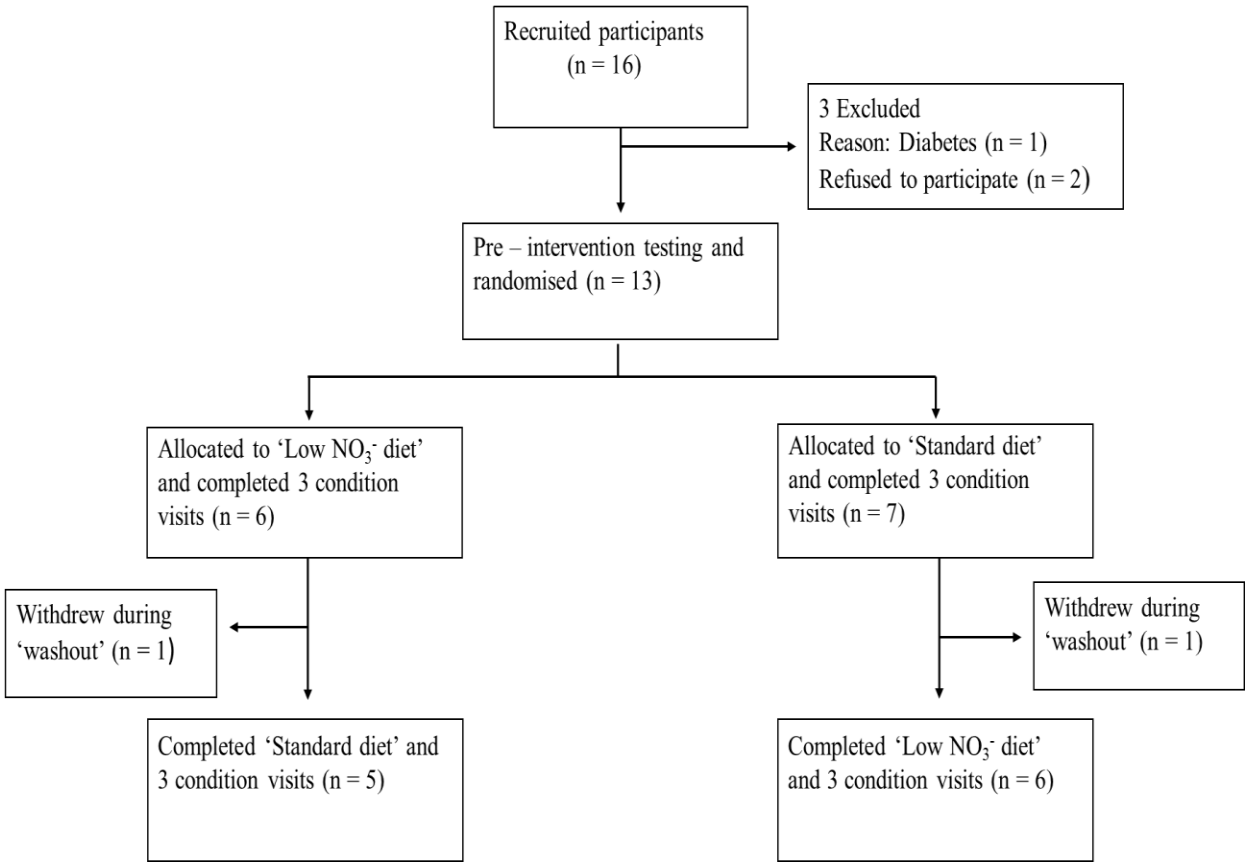

Figure 3: Flow diagram illustrating recruitment and compliance to the study

Results

Thirteen subjects completed the study (mean ± standard deviation (SD): age; 21.9 ± 2.0 years, body mass; 77.7 ± 7.8 kg, height; 177 ± 7 cm, male; n = 12, female; n = 1) and reported full compliance to the beetroot juice and flapjack supplementation. The beetroot supplementation was well tolerated by all subjects with no negative side effects. At baseline, prior to each diet, there was no statistical significance between SBP, DBP and MAP in either condition ($P > 0.05$)

Table 3: Baseline blood pressure values

Parameter	Standard NO_3^- Diet	Low NO_3^- Diet
SBP (mmHg)	114 ± 6	114 ± 9
DBP (mmHg)	64 ± 5	63 ± 5
MAP (mmHg)	81 ± 5	80 ± 5

Values are expressed as mean values ± SD. Abbreviations; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; MAP: Mean arterial pressure.

Dietary analysis

Each subjects' diet throughout the study was designed to meet their individual calorie intake and satisfy their macronutrient and micronutrient requirements. Throughout the study, excluding the beetroot flapjack and beetroot juice supplementation, the average NO_3^- intake, was 24.07 ± 1.96 $mg.d^{-1}$ with a range of 8.32 $mg.d^{-1}$ (19.02 – 27.34 $mg.d^{-1}$). The NO_3^- supplementation, in the form of beetroot flapjack and beetroot juice, elevated the daily NO_3^- intake of the diet from 24.07 $mg.d^{-1}$ to ~ 180 $mg.d^{-1}$ during the baseline and 7 day standard NO_3^- diet and to > 800 $mg.d^{-1}$ for the 3 day high NO_3^- diet. Analysis of the subjects' food diaries, via the bespoke NO_3^- database, to assess each subjects' 3

day habitual NO_3^- intake, demonstrated average habitual NO_3^- intake was 117.67 ± 13.36 $mg.d^{-1}$ and the average daily NO_2^- intake was 6.44 ± 0.26 $mg.d^{-1}$.

SBP

The effect of dietary NO_3^- deprivation and supplementation on SBP are displayed in figure 4. Analysis via repeated-measures ANOVA identified no interaction effect between the low NO_3^- and standard NO_3^- conditions across the experimental visits (P > 0.05). However, there was a significant main effect of time within the conditions (P = 0.03; η^2_p = 0.30).

Compared to baseline (114 ± 8 mmHg), there was no significant change in SBP (0 ± 5 mmHg; P > 0.05) after the 7 day low NO_3^- diet (114 ± 8 mmHg). Similarly, after the 3 day high NO_3^- diet (112 ± 10 mmHg), no significant change in SBP was observed compared to the 7 day low NO_3^- diet (- 2 ± 6 mmHg; P > 0.05) or between the 3 day high NO_3^- diet compared to baseline (- 2 ± 6 mmHg; P > 0.05).

Compared to baseline (114 ± 6 mmHg), there was no significant change in SBP after the 7 day standard NO_3^- diet (0 ± 4 mmHg; P > 0.05). However, compared to baseline, SBP was significantly reduced (- 4 ± 3 mmHg; P = 0.001) after the 3 day high NO_3^- diet (110 ± 7 mmHg) and there was a trend for the 3 day high NO_3^- diet to reduce SBP compared to the 7 day standard NO_3^- diet (- 3 ± 5 mmHg; P = 0.086).

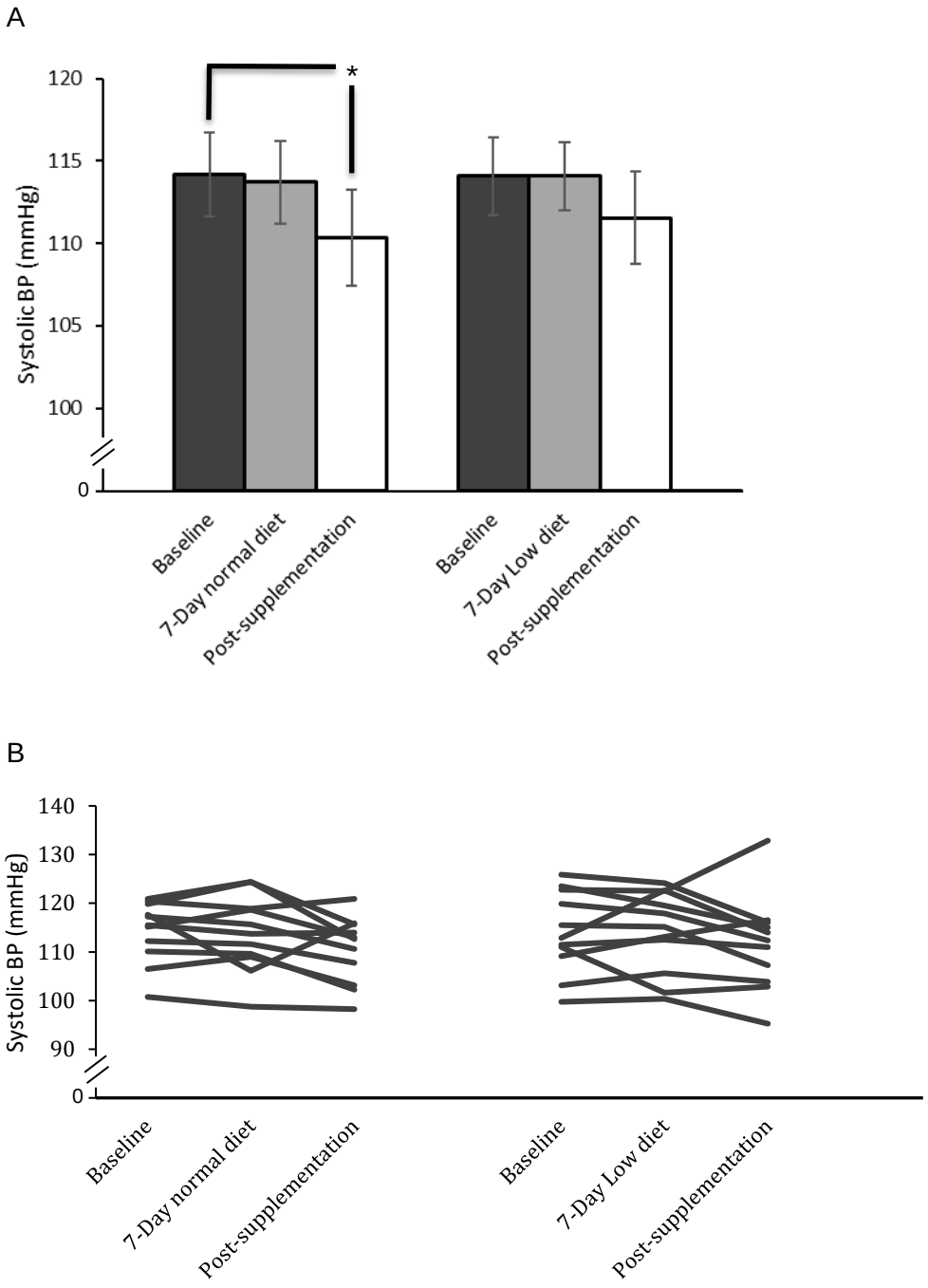

Figure 4: The effect of dietary NO_3^- deprivation and supplementation on systolic blood pressure. (A). The group mean response of systolic blood pressure at each time point across both conditions. Dark bars represent baseline values for each diet, grey bars represent the 7 day low NO_3^- or standard NO_3^- diet and white bars represent 3 days NO_3^- supplementation. Values presented as mean ± error bars. (B). The individual response of systolic blood pressure at each time point across both conditions. * represents a statistically significant reduction ($P < 0.05$). Abbreviations; BP: blood pressure.

DBP

The effect of dietary NO_3^- deprivation and subsequent supplementation on DBP are displayed in figure 5. Analysis via repeated-measures ANOVA revealed no significant interaction effect ($P > 0.05$) between NO_3^- intake conditions in DBP. ANOVA analysis did reveal a trend towards a significant main effect of time ($P = 0.081$; $\eta^2_p = 0.22$).

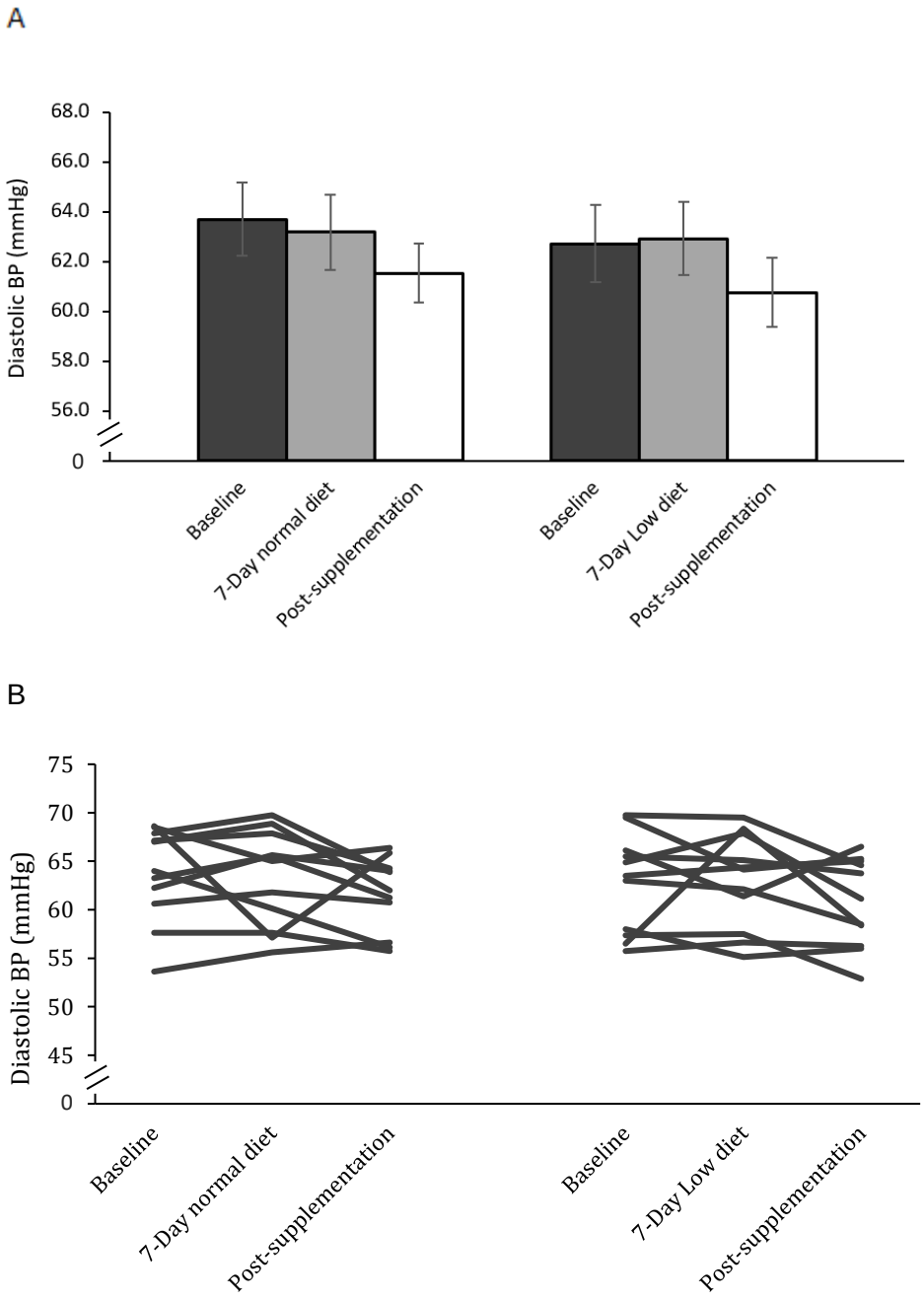

Figure 5: The effect of dietary NO_3^- deprivation and supplementation on diastolic blood pressure. (A). The group mean response of diastolic blood pressure at each time point across both conditions. Dark bars represent baseline values for each diet, grey bars represent the 7 day low NO_3^- or standard NO_3^- diet and white bars represent 3 days NO_3^- supplementation. Values presented as mean ± error bars. (B). The individual response of diastolic blood pressure at each time point across both conditions. Abbreviations; BP: blood pressure.

MAP

The effects of dietary NO_3^- deprivation and supplementation on MAP are displayed in figure 6. Repeated-measures ANOVA analysis identified no significant interaction effect ($P > 0.05$) between NO_3^- intake conditions and MAP. ANOVA analysis did reveal a trend towards a significant main effect of time ($P = 0.065$; $\eta^2_p = 0.24$).

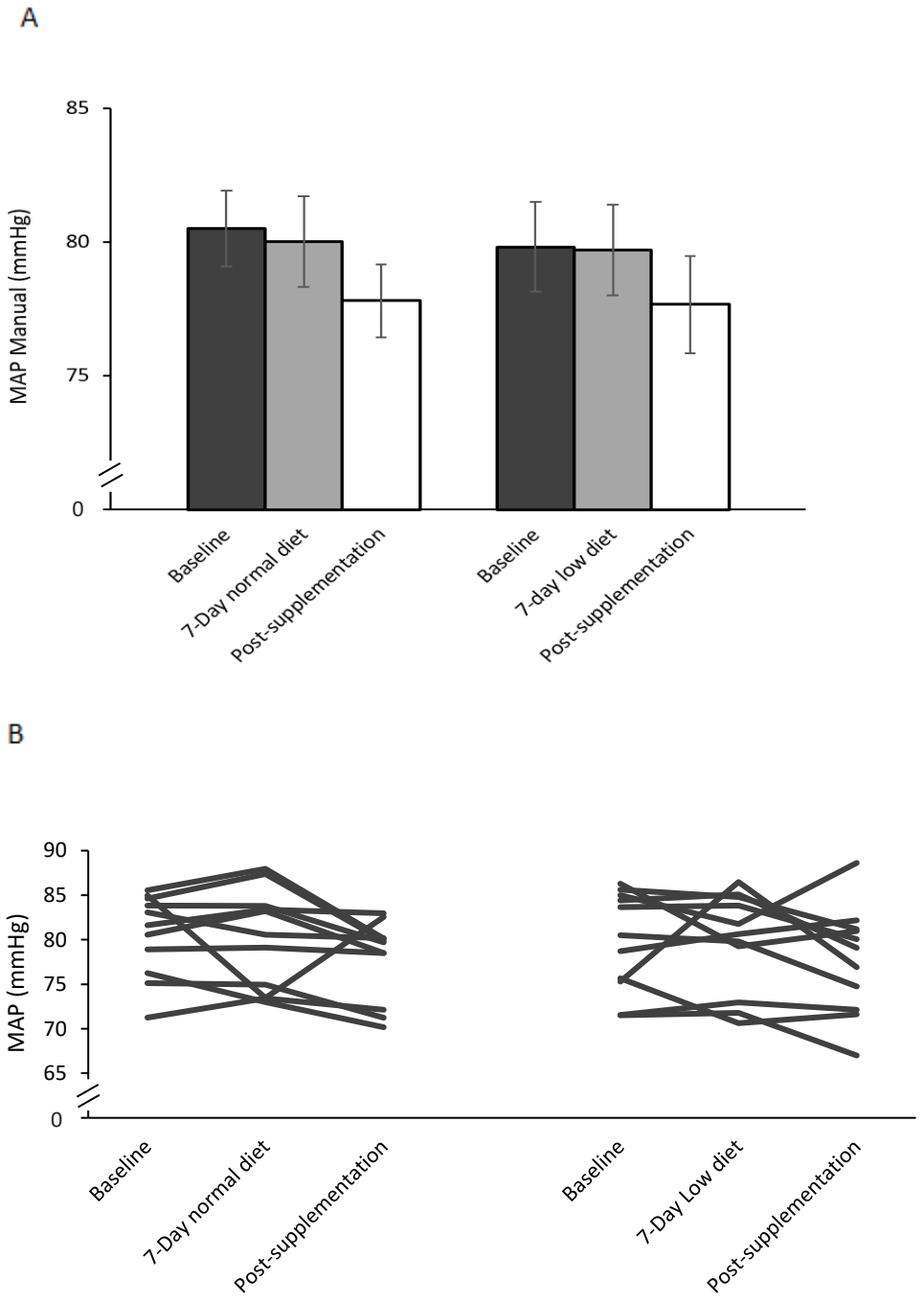

Figure 6: The effect of dietary NO_3^- deprivation and supplementation on mean arterial pressure (MAP). (A). The group mean response of MAP blood pressure at each time point across both conditions. Dark bars represent baseline values for each diet, grey bars represent the 7 day low NO_3^- or standard NO_3^- diet and white bars represent 3 days NO_3^- supplementation. (B). The individual response of MAP blood pressure at each time point across both conditions. Values presented as mean ± error bars. Abbreviations; MAP: mean arterial pressure.

Discussion

This was the first study to investigate the influence of dietary NO_3^- deprivation and subsequent supplementation on blood pressure in humans. In contrast to the experimental hypothesis, the main novel findings from the present research were that 7 days dietary NO_3^- deprivation does not cause a significant increase in SBP, DBP or MAP in healthy humans. Furthermore, 3 days NO_3^- supplementation following a low NO_3^- diet does not have a 'super compensatory' effect; it does not reduce SBP, DBP or MAP to a greater extent than NO_3^- supplementation following a standard NO_3^- diet. However, SBP was lowered compared to baseline after 3 days NO_3^- supplementation following a standard NO_3^- diet.

Habitual dietary analysis from subject food diaries revealed that the average daily NO_3^- intake differed by ~ 50 $mg.d^{-1}$ NO_3^- in comparison to previously recorded amounts in the UK diet (70 $mg.d^{-1}$; Ashworth and Bescos, 2017). A possible explanation for this disparity is that Ashworth and Bescos, (2017) used the mean NO_3^- content values across all vegetables, equating to 33.6 mg per 100 g, whereas the current study determined the NO_3^- content for each food and drink individually.

It could be suggested that a diet of 24.07 $mg.d^{-1}$ NO_3^- could not be considered deprived of NO_3^- when this is approximately 50% of the UK populations intake based on the report by Ashworth and Bescos (2017). However, NO_3^- is present in some capacity in most food and drinks, making further reductions difficult. Additionally, in the present study's population, the deprived NO_3^- diet was approximately 20 % of their habitual NO_3^- intake. Although, future research should consider subjects habitual NO_3^- intake prior to

enrolment and calculating an individualized approach to NO_3^- deprivation based on their habitual NO_3^- intake.

In contrast to the hypothesis, data from the current study suggests that dietary NO_3^- deprivation (24.07 $mg.d^{-1}$ for 7 days) results in no significant difference in SBP, DBP or MAP. This contrasts with earlier research by Jones et al. (2004), which reported that the infusion of L-NAME increased MAP by ~ 14 mmHg. Furthermore, earlier research using LNMMA, which can also inhibit NOS activity (Sander et al., 1999), resulted in a doubling of vascular resistance within the human forearm (Vallance et al., 1989). However, both aforementioned studies investigated the acute response of NOS inhibition via endogenous blockade. Considering the findings of the current study, it might be speculated that the two distinct processes of reducing NO generation, via inhibition of the NOS blockade via L-NAME infusion or reducing the NO^{-3}-NO^{-2}-NO pathway via dietary NO_3^- deprivation, produce contrasting effects on vascular physiology. Such that NOS inhibition via the infusion of L-NAME or LNMMA has a more potent adverse effect on blood pressure than dietary NO_3^- deprivation.

The mechanisms responsible for the unchanged blood pressure following a 7 day low NO_3^- diet were not measured in the current study. Importantly, for the findings of the current study, Carlström et al. (2015) investigated the inter-relationship between the NO_3^--NO_2^--NO pathway and the NOS-dependent pathway and found that prolonged dietary NO_3^- supplementation initiated a reversible reduction of eNOS activity in rats. The reduced NOS-dependent pathway contribution was identified by the reduction in the citrulline-to-arginine ratio in plasma. The reduction was contributed to by the down-

regulation of eNOS phosphorylation at Ser1177 and the up-regulation at Thr495 in the aorta.

These findings have important implications for the interpretation of results in the current study. This ‘cross-talk’, in which alterations in dietary NO_3^- intake, and subsequent up- or downregulation of the NO_3^--NO_2^--NO pathway can modify eNOS activity by effecting the NOS-dependent pathway, may be an important factor in the unchanged blood pressure response observed in the current study. Interestingly, Bryan et al. (2005) ascertained that blood pressure increases initially following doses of NO_2^- supplementation before declining. It should be acknowledged for interpreting the results of the current study, that the authors theorised that if NO is a pivotal molecule necessary for homeostasis, then the body will likely utilise negative feedback loops to assess and regulate endogenous NO production to control and maintain NO bioavailability. Thus, following dietary NO_3^- deprivation, the consequential decrease in NO_2^- concentrations from the NO_3^--NO_2^--NO pathway may signal the up-regulation of endogenous NO generation via increased eNOS activity from the NOS-dependent pathway. Such a change might offset the reduction in NO generation from the NO_3^--NO_2^--NO pathway and explain the unchanged blood pressure values observed in the current study. Future investigations might assess eNOS activity as well as plasma [NO_3^-] and [NO_2^-] following bouts of NO_3^- deprivation and supplementation.

Blood pressure control, therefore, seems to be carefully regulated and may partly explain why no significant decrease in blood pressure was observed after the 7 days NO_3^- depleted diet in the present study. It is important to acknowledge, however, that vascular control is not only NO-mediated. The endothelium releases other vasodilators

such as prostacyclin and EDHFs (Gomberg-Maitland and Olschewski, 2008; Durand and Gutterman, 2013), so it is important to understand how dietary NO_3^- deprivation impacts other mediators of vascular control. It should be noted, however, that young healthy individuals such as the present study's subjects, with normal endothelial function and coupled NOS may be more capable of compensatory upregulation in NOS activity following dietary NO_3^- deprivation. Whereas, the compensatory upregulation in NOS activity might not be possible in heart failure patients or individuals with endothelial dysfunction and uncoupled NOS (Bauersachs and Widder, 2008), although further research in heart failure populations response to dietary NO_3^- deprivation may explain this.

Previous research has determined that antibacterial mouthwash suppresses the activity of oral microflora responsible for the stepwise reduction of NO_3^- to NO_2^- (Govoni et al., 2008), ameliorating the benefits of NO_3^- supplementation. A study in rodents investigated the effect of NO_3^- supplementation and mouthwash on blood pressure over 10 days (Petersson et al., 2009). Results showed that NO_3^- supplementation reduced blood pressure in rats that were not given mouthwash, whereas the blood pressure-lowering effect was abolished in the rats given NO_3^- supplementation and mouthwash. However, the researchers observed no rise in blood pressure following the disruption of the NO_3^--NO_2^--NO pathway within the mouthwash condition. Although the substrate utilised for the NO_3^--NO_2^--NO pathway had been reduced below 'normal' levels, using a different method to that of the current study, the results have similarities, namely, there was no detrimental effect on blood pressure following the disruption of the NO_3^--NO_2^--NO pathway. The findings from the current study suggest that the NOS-dependent

pathway may compensate for the NO_3^--NO_2^--NO pathway when it is down-regulated. However, further research is necessary to better understand the 'cross-talk' between the two pathways during dietary NO_3^- deprivation and how these dynamics influence the development of hypertension in humans.

A key finding of the present study was that after 3 days of NO_3^- supplementation subsequent to a 7 day low NO_3^- diet, there were non-significant reductions in SBP, DBP and MAP. It should be noted that the lack of a blood pressure-lowering effect is not an unusual finding in normotensive, healthy young adults exposed to a period of NO_3^- supplementation. Some previous research has reported no significant difference in both SBP and DBP (Cermak et al., 2012) subsequent to 6 days NO_3^- supplementation. Haider and Folland (2014) also reported no significant reduction subsequent to 7 days NO_3^- supplementation (9.7 $mmol.d^{-1}$) in comparison to a control. In addition, the baseline values in the current study for SBP, in the low NO_3^- (114 mmHg) and standard NO_3^- (114 mmHg) diets are considered low. Previous research investigating blood pressure measures in different populations established that mean SBP across Europe was 124 mmHg, for participants aged between 35 and 39 years (Wolf-Maier et al., 2003). The lower baseline SBP values from a healthy population in the current study may have therefore limited the scope for reduction in blood pressure variables. This is supported by previous research that identified a negative correlation between peak decreases in SBP and DBP measures following NO_3^- supplementation and baseline blood pressure values (Kapil et al., 2010). Therefore, it is possible that the vascular effects of dietary NO_3^- deprivation and subsequent supplementation might be better detected in older or diseased populations.

It should be noted (see figures 4 B, 5 B and 6 B) that one subject had a large increase in each blood pressure measure following 3 days of NO_3^- supplementation after the low NO_3^- condition which may have influenced the overall findings due to the small sample size. This may explain why the reduction in SBP was lowered compared to baseline after the standard NO_3^- diet but not the low NO_3^- diet. However, the findings of the current study contrast with the hypothesis and suggest that there is no greater blood pressure-lowering effect of NO_3^- supplementation after dietary NO_3^- deprivation in comparison to NO_3^- supplementation following a standard NO_3^- diet.

These results indicate that those with a lower NO_3^- diet are not more likely to benefit from the anti-hypertensive benefits of NO_3^- supplementation. Research has demonstrated that sustained NO_3^- supplementation can beneficially alter commensal facultative anaerobic bacteria, such as *Niesseria* and *Veionella spp* which are integral for NO_3^- reduction (Burleigh et al., 2019). Further research by Vanhatalo and colleagues, (2018) established similar results, suggesting that increased dietary NO_3^- intake will beneficially alter NO_2^- producing oral microbiome allowing individuals to increase NO bioavailability and receive greater vascular benefits from NO_3^- supplementation. This has important implications for the results of the current study, suggesting subjects with a low NO_3^- diet will have a reduced oral microbiome capacity to reduce NO_3^- to NO_2^- than those ingesting greater dietary NO_3^-. This may explain why NO_3^- supplementation after a period of NO_3^- deprivation does not increase the benefit of NO_3^- supplementation following a standard NO_3^- diet.

Interestingly, studies in rodents have demonstrated that the replenishment of NO_3^- to the muscle NO_3^- reservoir is highly efficient. After 3 days NO_3^- supplementation following 7

days NO_3^- deprivation (Gilliard et al., 2018), the $[NO_3^-]$ in rodent muscle was restored to equal to that of the baseline values. A ‘super compensation’ effect in which muscle and blood $[NO_3^-]$ increased by 2.3 and 1.2 times of that measured in the NO_3^- supplemented group following a standard diet, was observed after 7 days NO_3^- supplementation following 7 days NO_3^- deprivation. Therefore, if the NO_3^- replenishment timeframe in humans is similar to that of rodents, it is possible that the duration of the 3 day high NO_3^- diet implemented in the present study was not long enough to elicit the physiological ‘super compensation’ adaptations anticipated from the NO_3^- supplementation following a period of NO_3^- deprivation.

A study by Wilkerson et al. (2012) involving 8 trained cyclists completing a 50 mile time trial after ingesting 500 ml of beetroot juice (6.2 mmol NO_3^-) or placebo (~ 0.0047 mmol NO_3^- depleted beetroot juice), identified that there was no significant improvement in exercise performance between supplements and the authors introduced the notion of ‘responders’ and ‘non-responders’ to NO_3^- supplementation. The authors argued that trained individuals have greater NOS activity and thus, the scale of physiological response to a standard NO_3^- dose will likely be blunted in comparison to sedentary individuals. Although the subjects in the current study were not highly trained, they were all highly active and healthy university students. McDonagh and colleagues (2019) noted factors such as health and fitness may affect the blood pressure response to NO_3^- supplementation, with normotensive and trained populations displaying lower reductions in blood pressure values than hypotensive and untrained populations. Therefore, the health and fitness status coupled with the aforementioned low baseline blood pressure

values of the participants may have reduced the scope for measurable benefits in the current study.

In the present study, the standard NO_3^- diet did not alter blood pressure values following the 7 day standard NO_3^- diet in comparison to baseline. However, there was a significant reduction in SBP after the 3 day NO_3^- supplementation in comparison to the baseline visit in the standard diet. In fact, SBP was lowered compared to baseline after the standard NO_3^- diet but not the low NO_3^- diet. A possible explanation for this increased antihypertensive effect following NO_3^- supplementation after the standard NO_3^- diet is the potentially increased activity of oral microflora responsible for the reduction of NO_3^- to NO_2^- following a period of standard dietary NO_3^- intake compared to a period of low dietary NO_3^- intake (Vanhatalo et al., 2018). This would allow a greater yield of NO_2^- and thus, NO following NO_3^- supplementation subsequent to a standard NO_3^- diet. However, future research should investigate the changes to oral microflora that facilitate the capacity to reduce NO_3^- to NO_2^- following a period of dietary NO_3^- deprivation to ascertain whether this is a possible explanation.

The results of the study also observed a trend towards a significant reduction in SBP after the 3 day NO_3^- supplementation compared to the 7 day standard diet.

The reduction in SBP following 3 days NO_3^- supplementation compared to the baseline measures in the control condition, supports the notion that NO_3^- supplementation in the form of beetroot juice can be utilised as a natural, cost-effective approach to treat hypertension and prevent CVD (Bonilla Ocampo et al., 2018). Although the reduction in SBP in the current study was small following NO_3^- supplementation, it has been suggested that a reduction in SBP by 5 mmHg can reduce the mortality associated with

CVD by 9 % (Chobanian et al., 2003). CVD is understood to account annually for 31.5 % of all deaths, at a cost of billions of dollars to global economies (Neghavi et al., 2013; Gaziano et al., 2009), therefore making the reduction of blood pressure measures of great health and economic benefit. As demonstrated by the reduction of SBP after NO_3^- supplementation in the current study, populations are able to benefit from the anti-hypertensive effect of dietary NO_3^- via the consumption of more green leafy vegetables, including products made from beetroot. With the demonstration that dietary NO_3^- supplementation can reduce blood pressure, future research should investigate how specific NO_3^-- rich vegetables could improve upon current government-supported dietary strategies such as the DASH diet and the 5-A-Day Campaign to maximise reductions of hypertension incidences.

It is noteworthy that one subject had a large reduction in each blood pressure measure after the 7 day standard diet which may have contributed to an underestimation of the effect of the subsequent 3 day high NO_3^- diet. Nevertheless, these findings agree with several previous studies (Vanhatalo et al., 2010; Webb et al., 2008; Ashworth et al., 2015; Siervo et al., 2013), that NO_3^- supplementation can reduce blood pressure measures in healthy young adults.

It should be acknowledged, the study's methodology implemented two blood pressure measures approximately 25 min apart and 2 h post-prandial following NO_3^- ingestion, this allowed a double-baseline measure to help identify anomalies. It could be noted that these measurements, therefore, assessed the acute effect of NO_3^- supplementation. However, during the baseline and standard NO_3^- diet, only a small quantity of NO_3^- was ingested via the NO_3^- rich flapjack (0.67 mmol) to maintain normal

dietary NO_3^- intake. Additionally, during the 7 day low NO_3^- diet, a minimal amount of NO_3^- was ingested via the NO_3^- depleted flapjack (< 0.05 mmol) therefore the acute effect of NO_3^- supplementation following these diets would have had a negligible influence on blood pressure measures. During the 3 day high NO_3^- diet subject consumed approximately > 800 mg NO_3^- via beetroot juice 2 h before testing. Within this condition, the study visits therefore coincided with the peak pharmacokinetic response following NO_3^- supplementation (Wylie et al., 2013) which was important to potentially demonstrate the 'super compensation' effect of NO_3^- supplementation following dietary NO_3^- deprivation.

An inherent limitation of the current study is the adherence to the strict diet employed in the experiment. Although the diet offered variety and researchers asked questions at the start of each visit to understand whether the subjects had deviated from the diet, subjects could have deviated and ingested additional (or less) dietary NO_3^- which would have influenced the findings without researchers knowing. Additionally, the daily diets did not control for phytochemical content within the foods, which have been demonstrated to contribute to blood pressure regulation (Biesinger et al., 2016).

A further consideration is the calculated dosage of NO_3^- administered throughout the study. An absolute, rather than relative, daily dose of NO_3^- was prescribed to the participants. The dietary analysis also revealed large inter- and intra-individual differences in habitual NO_3^- intake. In combination, these factors may have reduced the capacity for NO_3^- deprivation and supplementation to yield a detectable physiological response in the present study.

The study also did not measure biomarkers, the lack of these measures during the different dietary NO_3^- conditions has meant the study could not determine whether the NO_3^- deprivation diet did reduce circulating levels of plasma [NO_3^-] and [NO_2^-]. Understanding these measures at different time points throughout the conditions would have helped to interpret the results and specify whether 'cross talk' between the NOS-dependent pathway and the NO_3^--NO_2^--NO pathway explained the unchanged blood pressure values after 7 days NO_3^- deprivation. Future studies might consider measuring NO biomarkers to assess whether the low NO_3^- diets resulted in lower circulating levels of plasma [NO_3^-] and [NO_2^-] and also including baseline or habitual NO_3^- in determining the doses allocated to study volunteers.

Conclusion

The present study is the first in humans to investigate the effect of dietary NO_3^- deprivation on blood pressure and provides an important contribution to our understanding of dietary NO_3^- and its physiological role in blood pressure control within humans. The study established that following a 7 day low NO_3^- diet (24.07 $mg.d^{-1}$), there were no significant alterations in resting SBP, DBP or MAP. This suggests that short-term dietary NO_3^- deprivation may not contribute to hypertension.

Achieving a completely deprived dietary NO_3^- state is challenging due to the presence of NO_3^- in the majority of foods. However, with the deprived diet containing approximately 86 % less NO_3^- than the baseline diet, it is evident that the subjects were deprived of dietary NO_3^-. An important factor potentially explaining the lack of difference in blood pressure values between the low and standard NO_3^- diet in the current study is a 'cross-talk' relationship between the NO_3^--NO_2^--NO and NOS-dependent pathway, offsetting the reduced NO generation resulting from decreased dietary NO_3^- ingestion. Measuring plasma and muscle [NO_3^-] and [NO_2^-] in future studies would help interpret whether the 7 day low NO_3^- diet could reduce NO_3^- within the muscle NO_3^- reservoir and whether 'cross talk' between the NO_3^--NO_2^--NO and NOS-dependent pathways explained the unchanged blood pressure values after the 7 day low NO_3^- diet. Additionally, it is plausible that the 7 day low NO_3^- diet (24.07 $mg.d^{-1}$) may not have been sufficient to achieve a state of dietary NO_3^- deprivation within the subjects, resulting in changes to blood pressure values, or that subjects deviated from the diets and ingested additional, unaccounted NO_3^-. Therefore, measuring biomarkers in

subsequent dietary NO_3^- deprivation research would help ascertain whether the strict diet was adhered to and whether 24.07 $mg.d^{-1}$ NO_3^- was a sufficient amount of dietary NO_3^- to achieve a deprived NO_3^- state.

Additionally, the study also observed no greater effect of NO_3^- supplementation after a period of NO_3^- deprivation in comparison to a standard NO_3^- diet. In fact, it seemed to be less effective at lowering SBP compared to baseline than a standard NO_3^- diet. However, in support of earlier research, the study demonstrated that NO_3^- supplementation reduces resting blood pressure following a standard diet, indicating that NO_3^- supplementation might be considered an effective strategy to combat hypertension and prevent CVD. Further research is now required to better understand the physiological effect of dietary NO_3^- deprivation in humans. Specifically, future studies might consider assessing the effects of a more protracted NO_3^- deprivation, using subjects that consume the average habitual daily NO_3^- intake (Hord et al., 2009) and a dose that is relative to subjects' habitual daily NO_3^- intake alongside measuring NO biomarkers throughout each dietary NO_3^- intake condition.

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