

The therapeutic effect of dietary nitrate supplementation in healthy adults,
individuals with type 2 diabetes mellitus and chronic obstructive pulmonary
disease.

Submitted by Anthony Ian Shepherd to the University of Exeter
as a thesis for the degree of
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Abstract

Introduction and aim

Increases in the bioavailability of nitric oxide have been shown to reduce the oxygen (O₂) cost of exercise, improve exercise performance, alter gastric blood flow and mediate glucose uptake in healthy individuals. Aim; does dietary nitrate reduce the O₂ cost of exercise, improve walking performance in individuals with type 2 diabetes mellitus (T2DM) and chronic obstructive pulmonary disease (COPD) or alter hepatic diffusion and positively affect glucose homeostasis in healthy adults?

Methods

Experimental chapters utilised a double-blind, placebo-controlled, randomised, experimental design. Breath by breath pulmonary gas analysis was utilised to assess the O₂ cost of exercise in 48 individuals with T2DM and 13 with COPD. Walking performance was assessed via the six minute walk test (6MWT) in cohorts 1 and 2. Magnetic resonance imaging was used to assess portal vein flux, velocity and the apparent diffusion coefficient, in order to assess hepatic microvascular diffusion (apparent diffusion coefficient (ADC)). Blood pressure (BP) was measured in all trials.

Results

Relative to placebo, beetroot juice resulted in a significant increase in plasma nitrate and nitrite. There were no differences between placebo vs. beetroot juice for the O₂ cost of walking (T2DM: placebo; 946 ± 221 vs. beetroot juice; 939 ± 223 ml·min⁻¹; *P*=0.59) or cycling (COPD: placebo; 933 ± 323 vs. beetroot juice; 939 ± 302 ml·min⁻¹; *P*=0.88), distance covered in the 6MWT (T2DM: placebo;

550 ± 83 vs. beetroot juice; 554 ± 90m; $P=0.17$ or COPD: placebo; 456 ± 86 vs. beetroot juice; 449 ± 79 m; $P=0.37$) or BP (T2DM: systolic: placebo; 134 ± 10 vs. beetroot juice; 132 ± 12 mmHg, $P=0.17$; diastolic: placebo; 77 ± 7: vs. beetroot juice; 76 ± 11 mmHg, $P=0.27$. COPD: systolic: placebo; 123 ± 14 vs. beetroot juice; 123 ± 14 mmHg; $P=0.91$; diastolic: placebo; 77 ± 9 vs. beetroot juice; 79 ± 9 mmHg; $P=0.27$). No differences were seen between placebo and beetroot juice for ADC (young adults: $F_{(3, 45)} = 0.25$, $P = 0.74$; older adults; $F_{(3, 42)} = 1.3$, $P = 0.28$), portal vein flux (young adults: $F_{(3, 45)} = 0.339$, $P = 0.79$; older adults; $F_{(3, 42)} = 1.65$, $P = 0.19$) however, there was an interaction effect in the young adults: ($F_{(3, 45)} = 2.9$, $P = 0.04$) but not in the older adults; $F_{(3, 42)} = 1.8$, $P = 0.16$) between visits for portal vein velocity.

Nitrate supplementation did not reduce plasma glucose concentrations (young adults: $F_{(3, 45)} = 0.96$, $P = 0.42$; older adults; $F_{(3, 42)} = 0.04$, $P = 0.99$). Nitrate supplementation did not reduce systolic blood pressure (young adults: $F_{(3, 45)} = 0.20$, $P = 0.89$; older adults; $F_{(3, 42)} = 1.7$, $P = 0.18$) or diastolic blood pressure (young adults: $F_{(3, 45)} = 0.25$, $P = 0.86$; older adults; $F_{(3, 42)} = 0.45$, $P = 0.72$).

Conclusion

Dietary nitrate supplementation does not alter the O_2 cost of exercise, improve walking performance or reduce BP in individuals with T2DM or COPD. Nitrate supplementation does not alter hepatic diffusion, glucose homeostasis or BP.

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In experimental chapter 1; the high-performance liquid chromatography analysis of the antioxidant content of the beetroot juice was performed by Ewa Rembialkowska group in Warsaw, Poland.

Abbreviations

Nitric oxide; NO \cdot

Nitric oxide synthase; NOS

Oxygen; O $_2$

Neuronal nitric oxide synthase; nNOS

Inducible nitric oxide synthase; iNOS

Endothelial nitric oxide synthase; eNOS

Nicotinamide adenine dinucleotide phosphate hydrogen; NADPH

Flavin adenine dinucleotide; FAD

Flavin mononucleotide; FMN

Calmodulin; CaM

Tetrahydrobiopterin; BH $_4$

Nitrate; NO $_3^-$

Nitrite; NO $_2^-$

Type 2 diabetes mellitus; T2DM

Cyclic guanosine monophosphate; cGMP

Blood pressure; BP

Oxygen uptake; $\dot{V}O_2$

Peak oxygen uptake; $\dot{V}O_{2peak}$

Work rate; WR

Gross efficiency; GE

Delta efficiency; DE

Systolic blood pressure; SBP

Diastolic blood pressure; DBP

Mean arterial blood pressure; MAP

Ischemic reperfusion; IR

Flow mediated dilation; FMD

Moderate intensity; MI

Rating of perceived exertion; RPE

Severe intensity; SI

Maximal oxygen uptake $\dot{V}O_2$
Low intensity; LI
High intensity; HI
Phosphocreatine; PCr
Inorganic phosphate; Pi
Adenosine diphosphate; ADP
Adenosine tri phosphate; ATP
Gas exchange threshold; GET
Peripheral artery disease; PAD
Arterial oxygen saturation; S_aO_2
Limit of tolerance; T_{lim}
Phosphorylation efficiency; P/O ratio
Power output; PO
Time trial; TT
Time to exhaustion; TTE
Low nitrate food; LNF
High nitrate food HNF
Partial pressure of oxygen; PO_2
Dimethoxybenzaldehyde; DMBA
Near infrared spectrometry; NIRS
Soluble guanylate cyclase; sGC
Respiratory quotient; RQ
Maximal voluntary contraction; MVC
Red blood cells; RBC
Chronic obstructive pulmonary disease; COPD
Glycated haemoglobin; HbA_{1c}
Asymmetric dimethylarginine; ADMA
Glucagon like peptide 1; GLP-1
Glucose dependant insulintropic peptide; GIP
Glucagon like peptide 1 receptor; GLP-1R

Glucagon like peptide 2; GLP-2
Guanosine triphosphate; GTP
GTP cyclohydrolase; GTPCH1
Peroxynitrite; OONO_2^-
Dihydropterin; BH_2
Dihydrofolate reductase; DHFR
Asymmetric dimethylarginine; ADMA
Symmetrical dimethylarginine; SDMA
Monomethyl-L-arginine; L-NMMA
Protein-arginine methyltransferases; PMRTs
Superoxide dismutase; SOD
Angiotensin-converting enzyme inhibitor; ACEi
Angiotensin receptor blockers; ARBs
Cardiovascular disease; CVD
Diabetes related nephropathy; DRN
Forced expiratory volume in the first second; FEV_1
Quality of life; QoL
Reactive oxygen species; ROS
Mean pulmonary artery pressure; mPAP
Six minute walk test; 6MWT
Cyclic guanosine 3' 5' monophosphate; cGMP
Pulmonary artery hypertension; PAH
Phosphodiesterase type 5; PDE-5
Sodium hydroxide; NaOH
Zinc sulphate; ZnSO_4
Vanadium chloride; VCl
Spike recovery; SR
Sodium Iodide; NaI
Amplitude; A_p
Time delay; TD_p

Time constant; τ_p

Mean response time; MRT

Region of interest; ROI

Signal intensity in low flow sensitivity image; S_0

Signal intensity in flow sensitive image; S_1

Magnetic field gradient used in low flow sensitivity image= 250 s/mm^2 ; b_0

Magnetic field gradient used in flow sensitive image= 750 s/mm^2 ; b_1

Standard deviation; SD

Interquartile range; IQR

Gas exchange threshold; GET

Publications and awards arising from work contained in this thesis

Journal articles:

1. Anthony I Shepherd, Daryl P Wilkerson, Lee Dobson, James Kelly, Paul G Winyard, Andrew M Jones, Nigel Benjamin, Angela C Shore, Mark Gilchrist (2015). The effect of dietary nitrate supplementation on the oxygen cost of cycling, walking performance and resting blood pressure in individuals with chronic obstructive pulmonary disease: A double blind placebo controlled, randomised control trial. *Nitric Oxide*. [doi:10.1016/j.niox.2015.01.002](https://doi.org/10.1016/j.niox.2015.01.002).
2. Anthony I Shepherd, Mark Gilchrist, Paul G Winyard, Andrew M Jones, Ewelina Hallmann, Renata Kazimierczak, Ewa Rembialkowska, Nigel Benjamin, Angela C Shore, Daryl P Wilkerson (2015). Effects of dietary nitrate supplementation on the oxygen cost of exercise and walking performance in individuals with type 2 diabetes: a randomised, double blind, placebo-controlled cross-over trial. *Free Radical Biology & Medicine*. [doi:10.1016/j.freeradbiomed.2015.05.014](https://doi.org/10.1016/j.freeradbiomed.2015.05.014)

Abstracts;

1. Anthony I Shepherd, Mark Gilchrist, Paul G Winyard, Andrew M Jones, Nigel Benjamin, Angela C Shore, Daryl P Wilkerson. (2014). The effect of dietary nitrate supplementation on the ability of individuals with Type 2 diabetes to perform walking exercise: A randomised control trial. Diabetes UK Professional Conference. Liverpool.
2. Anthony I Shepherd, Mark Gilchrist, Paul G Winyard, Andrew M Jones, Nigel Benjamin, Angela C Shore, Daryl P Wilkerson. (2014). Effects of dietary nitrate supplementation on blood pressure, the O₂ cost of

exercise, and walking performance in individuals with Type 2 diabetes: A randomised control trial. The British Association of Sport and Exercise Sciences. Student Conference. Portsmouth.

Award: Best postgraduate oral presentation.

3. Anthony I Shepherd, Mark Gilchrist, Paul G Winyard, Andrew M Jones, Nigel Benjamin, Angela C Shore, Daryl P Wilkerson. (2014). Effects of dietary nitrate supplementation on blood pressure, the O₂ cost of exercise, and walking performance in individuals with Type 2 diabetes. The European College of Sport Science. Amsterdam.

Shortlisted for the young investigator award.

Chapter 1: Introduction

Nitric oxide ($\text{NO}\cdot$) was first found to be an endothelium derived relaxation factor in 1987 by Ignarro et al. (1987). The research questions surrounding its functions and biochemical pathways have expanded vastly since the identification of a stepwise reduction on nitrate (NO_3^-) to nitrite (NO_2^-) via facultative anaerobic bacteria and subsequently a conversion to $\text{NO}\cdot$ within the stomach (Lundberg et al., 1994, Benjamin et al., 1994).

More recently, trials investigating the impact of supplementation of inorganic NO_3^- have shown some remarkable findings, predominantly in young healthy individuals. These include: improvements in mitochondrial efficiency (Larsen et al., 2011); reduction in the metabolic rate (Larsen et al., 2014), increases in blood flow (matching to skeletal muscle requirements and delivery to the brain) (Presley et al., 2011); reductions in systolic and diastolic blood pressure (Webb et al., 2008a, Vanhatalo et al., 2010, Vanhatalo et al., 2011, Lansley et al., 2010, Lansley et al., 2011a, Engan et al., 2012, Wylie et al., 2013b, Thompson et al., 2014, Muggeridge et al., 2014, Keen et al., 2014, Levitt et al., 2015, Treichler et al., 2015, Kocoloski and Crecelius, 2015, Bond Jr et al., 2013, Bond et al., 2014) and increase glucose uptake (Merry et al., 2010). In this introduction the biochemical pathways of $\text{NO}\cdot$ synthesis will be reviewed along with exploration of what is known surrounding NO_3^- supplementation and the potential clinical applications of these beneficial effects.

1.1: Inorganic nitrate (NO₃⁻), nitrite (NO₂⁻) & NO·

1.1.1: NO· synthesis in man:

NO· is a gaseous signalling molecule involved in many physiological processes such as vasodilation (Ignarro et al., 1987), neuronal signalling (Garthwaite, 2008) calcium handling (Hart and Dulhunty, 2000) and protection against bacterial pathogens (Chakravorty and Hensel, 2003). There are two major pathways in which NO· is produced in humans, firstly; the nitric oxide synthase (NOS) pathway, which is oxygen (O₂) dependent and creates endogenously derived NO·. There are three NOS isoforms which are bi-domain in structure including; (i) neuronal NOS (nNOS), (ii) inducible NOS (iNOS) and (iii) endothelial NOS (eNOS), all of which produce NO· in different locations of the body (see figure 1). The families of NOS's catalyse a five stage oxidation of L-arginine to produce the biologically active molecule NO· and also L-citrulline (Stamler and Meissner, 2001). This reaction relies on a constant substrate availability of six key compounds in order to maintain NO· production via the NOS pathway (Crabtree et al., 2009), these are: nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), haem, calmodulin (CaM) and tetrahydrobiopterin (BH₄) (Alderton et al., 2001). If any of these substrates are not present the production of NO· via NOS enzymes is diminished. For eNOS activation to occur, the release of its inhibition is required by two CaM attachments which transfer electrons from the reductase domain of the NOS to the oxygenase domain (Andrew and Mayer, 1999).

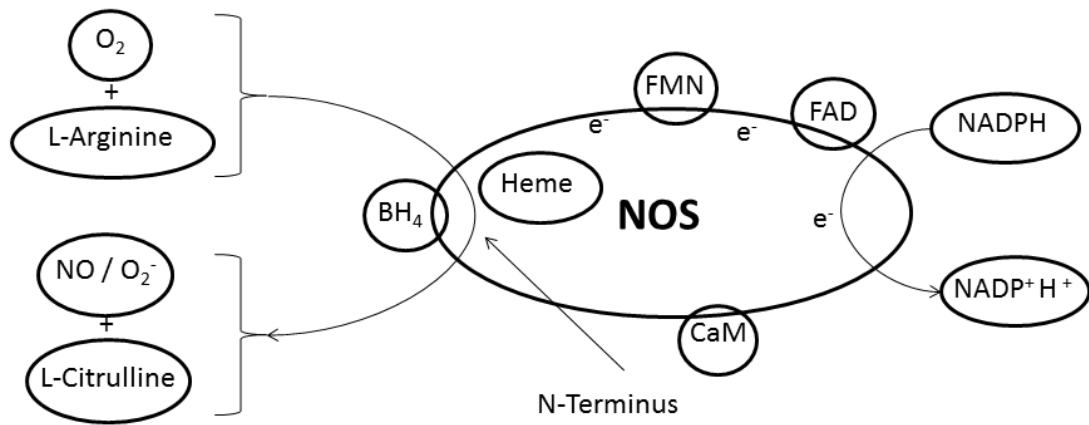


Figure 1: NO[•] production via NOS.

This graphical representation shows the movement of electrons through the NOS enzyme with its key co-factors. The net outcome produces NO[•].

The second is the entero-salivary pathway (NO₃⁻, NO₂⁻ & NO[•] conversion); this pathway can act as a pool to provide additional NO[•]. This pathway functions with the body absorbing inorganic NO₃⁻ from food and from NO₃⁻ produced endogenously within the body. 60-80% of a western dietary intake of NO₃⁻ is derived from vegetables (Ysart et al., 1999), with beetroot, leafy greens and cruciferous vegetables containing the highest concentrations of dietary NO₃⁻. The conditions in which plants is grown can have an impact on where the NO₃⁻ is stored within the plant (Gilchrist et al., 2010). If the plant is grown in conditions with small amounts of light it will store the NO₃⁻ as it cannot be converted to amino acids during photosynthesis. Beetroot store copious amounts of NO₃⁻ in their roots as its main anion (Gilchrist et al., 2010).

NO₃⁻ consumption through normal habitual dietary means has been described as part of a healthy balanced diet (Bryan and Hord, 2010). The average consumption of NO₃⁻ per day is ~1-2 mmols in a typical western diet (ECETOC., 1988), of which the majority of the available NO₃⁻ is quickly absorbed in the upper gastrointestinal tract (Florin et al., 1990, van Velzen et al., 2008). Approximately 60% of the NO₃⁻ from our diets is excreted in our urine (Lundberg and Govoni, 2004) within 48 hours (Wagner et al., 1983). Of the available NO₃⁻

either produced endogenously or taken in from the diet, 25% passes into the entero-salivary circulation where it is concentrated by a magnitude of 10 fold in salivary glands (Lundberg and Weitzberg, 2010).

The entero-salivary pathway is reliant on a number of factors, principally the symbiotic relationship that humans share with facultative anaerobic bacteria that live on the surface of the tongue (Duncan et al., 1995). The salivary glands secrete high concentrations of NO_3^- in our oral cavity. NO_3^- acts as an alternative electron acceptor (Duncan et al., 1995) where bacteria on the tongue reduces the NO_3^- to NO_2^- . The NO_2^- is swallowed and some of the NO_2^- is further reduced to $\text{NO}\cdot$ in the acid environment of the stomach (Benjamin et al., 1994). Not all of the NO_2^- is reduced to $\text{NO}\cdot$ in the stomach, some is absorbed into the circulating plasma where it can act as a storage pool for later reduction to $\text{NO}\cdot$ (Lundberg and Govoni, 2004).

This NO_2^- to $\text{NO}\cdot$ reduction can also be catalysed by deoxyhaemoglobin, deoxymyoglobin, xanthine oxide, NOS, aldehyde oxidase, cytochrome P-450 and the mitochondrial electron transfer complexes (Cosby et al., 2003, Shiva et al., 2007, Zhang et al., 1998, Vanin et al., 2007, Li et al., 2008b, Kozlov et al., 2003, 1999). Other specific conditions can also expedite the conversion of NO_2^- to $\text{NO}\cdot$ such as hypoxia (Castello et al., 2006) and acidic environments (Modin et al., 2001), which can occur during exercise (Bailey et al., 2009). This may be important for clinical populations, such as those with T2DM, who find day to day activities difficult, and have a reduced NOS derived $\text{NO}\cdot$ (Wu and Meininger, 2009). Lauer et al. (2008) used plasma NO_2^- concentration change as a marker of $\text{NO}\cdot$ production. Lauer et al. (2008) suggest that a reduced concentration of NO_2^- is associated with a reduced exercise tolerance.

1.1.2: The beneficial effects of NO_3^- , NO_2^- and $\text{NO}\cdot$:

Consuming a diet that is high in fruits and vegetables has been shown to lower blood pressure (Appel et al., 1997, Rouse et al., 1983). These diets have the ability to lower the risk of morbidity and mortality from cardiovascular disease (Bazzano et al., 2001, Joshipura et al., 1999, Joshipura et al., 2001). Due to large variation in the diet between individuals, it is difficult to elucidate which components decrease the risk of mortality and have cardio protective effects. Individuals who consume greater quantities of vegetables, such as those following the DASH diet (Appel et al., 1997), may take in up to 20 mmol of NO_3^- per day (Hord et al., 2009). Individuals who consume the greatest amounts of green leafy and cruciferous vegetables, which typically have high NO_3^- contents, have the largest protective effect from cardiovascular disease (Joshipura et al., 1999, Joshipura et al., 2001). More recently it has been shown that diets rich in green leafy vegetables may also protect against the risk of developing type 2 diabetes (T2DM) (Carter et al., 2010) and COPD (Hirayama et al., 2009, Watson et al., 2002).

1.1.3: Exercise efficiency & performance:

A basic premise of exercise physiology was that an individual's O_2 requirement for an exercise bout could be accurately predicted if the work load was known (Jones and Poole, 2005).

The O_2 cost of moderate intensity exercise is unaffected by having a disease such as T2DM, ageing, having a sedentary lifestyle and poor health status (Jones and Poole, 2005). Exercise training (in the short term) will not modulate the O_2 cost of moderate intensity exercise (see Jones and Poole, 2005 for

review). It is, therefore, surprising that dietary supplementation with inorganic NO_3^- can elicit a reduction in the O_2 cost of exercise.

A 3-5% reduction in the O_2 cost of exercise has been shown following NO_3^- supplementation with beetroot juice (Bailey et al., 2009, Larsen et al., 2007b) and pharmacological sodium NO_3^- (Larsen et al., 2010). Subsequent to these studies, a reduction in the O_2 cost of exercise following submaximal exercise and/or improvements in exercise performance following supplementation of beetroot juice or sodium NO_3^- in recreationally active individuals have been shown in multiple studies (Bailey et al., 2009, Lansley et al., 2010, Larsen et al., 2011, Larsen et al., 2007b, Larsen et al., 2010, Vanhatalo et al., 2010, Bescos et al., 2011, Cermak et al., 2012b, Wylie et al., 2013a, Wylie et al., 2013b, Breese et al., 2013). It should be noted that some studies have not reported such positive effects (Christensen et al., 2013, Murphy et al., 2014, Martin et al., 2014, Fulford et al., 2013) (for more examples see review table 1 and 13 a,b and c).

The question that remains in the clinical context is: can this reduction in the O_2 cost of exercise be seen in populations that may benefit from improved exercise efficiency and elicit improvements in quality of life and activities of daily living? NO_3^- supplementation in clinical populations is a novel concept and there is limited research published. At the commencement of this body of work only one study has been conducted with NO_3^- supplementation in a clinical population. It demonstrated an increase in walking time, time to onset of claudication pain and improved muscle oxygenation in individuals with peripheral arterial disease (Kenjale et al., 2011). However, this study is very small ($n=8$) and was not

double blinded. Thus the robustness of the conclusions should be interpreted with care.

A recent review (Bailey et al., 2012) suggests that as athletes have elevated plasma NO_3^- and NO_2^- (Jungersten et al., 1997, Cuzzolin et al., 2002) concentrations compared to non-athletic controls, well-trained athletes may not gain the same benefits as recreationally active individuals following NO_3^- supplementation. This perhaps indicates the existence of a ceiling limit, whereby further increases in the bioavailability of NO_2^- may not have an additive effect on exercise performance. Other evidence exists to suggest that it is not a ceiling limit per se but it is more likely to have diminishing effects as the aerobic capacity of the individual increases. A recent study demonstrated that a cohort of well-trained cyclists over a 50 mile time trial did not cover the distance in a shorter time (Wilkerson et al., 2012). Interestingly, there was a significant correlation between a rise in plasma NO_2^- concentration and time trial performance. Elite athletes tend to have a higher plasma NO_2^- concentration (Wilkerson et al., 2012). This may diminish the ability of athletes to gain ergogenic effects from NO_3^- supplementation. If this hypothesis is correct then individuals with reduced plasma NO_3^- and NO_2^- concentration such as individuals with T2DM (Vanhoutte, 2009) may stand to get the greatest benefits from NO_3^- supplementation. Although Vanhoutte presents data suggesting individuals with T2DM have a reduced plasma NO_3^- and NO_2^- concentration the method used to assess this was the greiss method and is less sensitive at lower concentrations.

$\text{NO}\cdot$ increases following exercise have been positively correlated to increases in cardiovascular health (Jungersten et al., 1997). There is an association

between the increase of endogenously produced NO_2^- and an improvement in exercise tolerance (Dreißigacker et al., 2010) whilst an increase in plasma NO_2^- concentration following beetroot juice has been correlated with improvements in time trial performance (Wilkerson et al., 2012). If an individual's tolerance to exercise improves then they have the potential to perform the same amount of exercise whilst feeling less physiological stress or do more work while feeling the same physiological stress as incurred previously at a lower level of exercise.

Enabling people with chronic disease to exercise more, via reducing early onset fatigue, may lead to significant health and quality of life benefits. Exercise performance could improve in individuals with reduced plasma NO_3^- and NO_2^- by doing regular exercise via increases in endogenously produced $\text{NO}\cdot$. Whilst this is unlikely to have a large effect it could occur due to increases in vascular production of $\text{NO}\cdot$ due to shear stress (Cooke et al., 1991, Miller and Burnett, 1992). As the individual exercises the subsequent increase in cardiac output will create shear stress producing more $\text{NO}\cdot$ (van Citters and Franklin, 1969). Given that individuals with reduced $\text{NO}\cdot$ bioavailability such as people with T2DM, and they have a lower $\dot{V}\text{O}_2$ max (Regensteiner et al., 1995b, Regensteiner et al., 2009), NO_3^- supplementation could enable individuals with disease to begin exercising at a lower $\dot{V}\text{O}_2$, thus exercise feels relatively easier and have the potential to yield more endogenously derived $\text{NO}\cdot$. Another potential means of improving exercise tolerance would be to improve efficiency in a non-haemodynamic process such as an improved mitochondrial efficiency or calcium handling.

1.1.4: NO· mechanisms for the reductions in the O₂ cost of exercise:

Multiple potential mechanisms for the reduction in O₂ cost of exercise following supplementation with inorganic NO₃⁻ have been hypothesised. These include a reduced O₂ cost of mitochondrial ATP re-synthesis (Clerc et al., 2007, Basu et al., 2008) and or a reduction in the ATP turnover (Bailey et al., 2010b). A vasodilatory effect has also been postulated, allowing for a better coupling of blood flow to metabolic demand and finally an increase in the anaerobic energy production (Larsen et al., 2007b, Bailey et al., 2009). These mechanisms will be discussed below.

The reduction in the O₂ cost of exercise has been shown in numerous studies with athletes, recreationally active individuals and groups of individuals with disease (See table 1 and 13). The reduction in the O₂ cost of mitochondrial ATP synthesis may be brought about via two different mechanisms (Larsen et al., 2011). The first is that NO₂⁻ could act as an alternative electron acceptor via the inhibition of cytochrome-c (Basu et al., 2008). Cytochrome-c is the last enzyme in the electron transport chain. NO· could become a surrogate for O₂ at cytochrome-c's binding site, replacing the need for O₂ during ATP production (Brown and Cooper, 1994). This, *in vitro* study appears to show a near linear relationship between increasing NO· concentration (via light releasing nitroprusside) and decreasing O₂ consumption of brain synaptosomes. At 60nM NO· concentration and 30µM O₂ concentration half of the cytochrome-c binding sites were inhibited (Brown and Cooper, 1994). This is of great importance given the difficulties of O₂ delivery in many clinical populations.

The second proposed mechanism involves an increase in the phosphate - O₂ (P/O) ratio. The term P/O ratio was coined by Hinkle (2005). The P/O ratio is

used as a measure of mitochondrial oxidative phosphorylation efficiency. The P/O ratio is the term given for the number of ATP molecules that can be produced for an molecule of O₂. If the ratio is relatively higher, then more ATP can be produced for the same amount of O₂. The reduction in the O₂ cost is thought to have been brought about via a reduced slippage of protons across the mitochondrial proton pumps essentially reducing energy waste and increasing oxidative phosphorylation. This is thought to occur due to one of two reasons; firstly there is an increase in the efficiency of the electron transport chain and secondly a decrease in the proton gradient consuming process (Clerc et al., 2007). P-magnetic resonance spectrometry has been used to access the turnover rate of ATP. Bailey et al., measured phosphocreatine, ADP and inorganic phosphate in addition to pH. Bailey et al., showed that with NO₃⁻ supplementation you can significantly reduce the estimated turnover rate of ATP (essentially reduce the O₂ cost of exercise) (Bailey et al., 2010b).

NO· is a potent vasodilator. The potential for NO· to vasodilate hypoxic and anoxic areas to improve oxygenation is a theory often referred to in NO· related blood pressure lowering effects (Webb et al., 2008b, Gautier et al., 2006). NO· production via eNOS is important for maintaining vascular tone. If the microvasculature has a greater flux of oxygenated blood, it is possible that a greater matching of O₂ delivery to O₂ demand within the working muscles may occur. However, this may be impaired in individuals with O₂ delivery deficiencies such as individuals with T2DM and chronic obstructive pulmonary disease (COPD). Moreover, if there is a greater matching of O₂ to the working muscles then uptake and utilisation may increase. Therefore, O₂ delivery is an unlikely source of reduction in steady state O₂ consumption but may mediate increased performance.

Given the reduced O_2 cost of exercise another potential mechanism for this improvement in efficiency could be an increase, proportionally, by the anaerobic contribution. This is unlikely based on two studies showing no increase in blood lactate levels measured via capillary blood sampling (Larsen et al., 2007b, Bailey et al., 2009) post NO_3^- supplementation. Bailey et al., (2012) showed via p-magnetic resonance spectrometry that the pH was unchanged during moderate and severe exercise, further suggesting that the reduction in the O_2 cost of exercise is not due to an increase in anaerobic contribution to ATP production.

With regards to the reduction in the O_2 cost of exercise, the evidence suggests that $NO\cdot$ acts as a surrogate for O_2 in the electron transport chain (Basu et al., 2008, Brown and Cooper, 1994) in conjunction with an increase in the P/O ratio via mitochondrial protein changes (Larsen et al., 2011, Clerc et al., 2007). Larsen's 2011 protocol utilised muscle biopsies harvested from healthy young individuals. The primary skeletal muscle cell cultures were conducted in ambient conditions. This would place the cells in an artificially elevated O_2 rich environment which would cause oxidative stress. In order to assess the O_2 consumption relative to the muscle the O_2 concentration could be lowered to ~3% which would match the pO_2 at the muscle. This makes the findings within this study questionable as the utilisation of $NO\cdot$ in different redox balances is likely to be vastly different. The limitations of these data supporting a reduction of proton slippage at the proton pumps and improvements of efficiency via the P/O must be borne in mind when seeking to understand the mechanism behind the reduction in O_2 cost of exercise. However, the data shows the potential for improvements in efficiency and more research is warranted in this area.

1.1.5: NO·, cGMP and sGC in vascular function:

NO· is a small size molecule that carries no charge. Due to this, it can diffuse easily across membranes without the need for a transport system, removing a rate limiting step (Lancaster, 1996, Lancaster, 1994, Denicola et al., 1996). NO· is relatively 'unreactive' and moves freely from the cell in which it was created to a target cell prior to being utilised (Denninger and Marletta, 1999). Within plasma the half-life of NO· is thought to be very short ~ 3 seconds (Yoshizumi et al., 1993, Lundberg and Govoni, 2004); NO₂⁻ is ~ 110 seconds (Kelm, 1999) and NO₃⁻ is ~ 5 hours (McKnight et al., 1997).

The bioavailability of NO· is paramount for normal cellular function and in particular, smooth muscle relaxation and signalling transduction. Many actions of NO· mediated vascular function are produced via cyclic guanosine monophosphate (cGMP) (Alzawahra et al., 2008). cGMP is created by the reduction of guanosine triphosphate (GTP) which is catalysed by guanylate cyclase (GC) (Denninger and Marletta, 1999). GC can be soluble (sGC) or attached to a receptor on the cell membrane. NO· increases GC activity (Schultz et al., 1977, Arnold et al., 1977) and subsequently cGMP concentrations (Denninger and Marletta, 1999). The elevations in cGMP lead to the phosphorylation of protein kinase C activity which phosphorylates proteins and decreases intracellular levels of Ca²⁺ via compartmentalisation or inhibits its release (Lohmann et al., 1997). This reduction leads to relaxation of vascular smooth muscle which ultimately lowers blood pressure (Lohmann et al., 1997).

1.1.6: Entero-salivary pathway and its role in blood pressure regulation:

The importance of reduction of NO₃⁻ to NO₂⁻ in the entero-salivary pathway was initially tested by having participants refrain from swallowing their own saliva

(Webb et al., 2008a) or by spitting post NO_3^- supplementation (Lundberg and Govoni, 2004). These studies suggest that by preventing the swallowing of NO_2^- , the beneficial effects of NO_3^- supplementation are removed due to interruption of the pathway. Recently, it has been shown by limiting the amount of microflora in the oral cavity (via the use of antibacterial mouthwash) the circulating plasma NO_2^- concentration is reduced without any decrease in the plasma NO_3^- concentration (Kapil et al., 2012; Petersson et al., 2009) again providing evidence that oral microflora are a key step in the entero-salivary pathway (Spiegelhalter et al., 1976, Lundberg et al., 2004, Duncan et al., 1995). Consequently, the use of antibacterial mouthwash has also been shown to increase the blood pressure of the participants involved (Kapil et al., 2012, Petersson et al., 2009) (shown in human and animal models), therefore, suggesting that antibacterial mouthwash may attenuate the $\text{NO}\cdot$ stimulated blood pressure reducing effects. Individuals with elevated blood pressure could potentially have raised systolic and diastolic pressures due to a depletion in $\text{NO}\cdot$ (either due to increased scavenging, reduced NOS production, poor diet and or other failures in the entero-salivary pathway), as opposed to earlier hypotheses that a high blood pressure causes a reduced $\text{NO}\cdot$ level (Rockey and Chung, 1998).

The role of NO_3^- and its subsequent conversion to $\text{NO}\cdot$ has many beneficial effects. Previously, Lundberg et al., (2006) hypothesised that NO_3^- rich vegetables may play a key role in blood pressure reduction. Subsequently, there have been numerous reports of a reduction in blood pressure, either with sodium NO_3^- (Larsen et al., 2006) or beetroot juice (Bailey et al., 2010b, Lansley et al., 2010).

At the start of this project the only study to examine a clinical population with NO_3^- supplementation was in individuals with peripheral artery disease which found a statistically significant reduction in diastolic blood pressure (BP) (7 mmHg) but no change in systolic BP (Kenjale et al., 2011). For more details on blood pressure changes in NO_3^- supplementation studies see table 1 and 13.

Much of the early literature (and a number of more recent studies) used a placebo that is not a 'true' placebo, i.e. blackcurrant juice or prune/orange juice. Whilst the increase in exercise time or reductions in the O_2 cost of exercise in these studies are of interest, there are significant limitations. By utilising fruit juice they are likely to have a substantially different antioxidant content which could alter $\text{NO}\cdot$ bioavailability (Bondonno et al., 2012). This could therefore explain some of the variability in these studies. Moreover, related to the lack of a 'true' placebo where the participant did not know whether active or placebo juice was being taken, the widely reported beneficial effects of beetroot juice [in the lay press] on exercise performance/tolerance may have given rise to a "placebo" effect in those informed volunteers taking beetroot juice. For example the Daily Mail online published an article stating beetroot juice 'helps lower blood pressure by 7%'. On the 29th of June 2010 the Daily Mail stated that beetroot juice 'could save your life' (DailyMail, 2013).

Beetroot is known to contain a number of antioxidants within the juice and it was an important stage in NO_3^- research to determine if antioxidants could reduce the O_2 cost of exercise. Bailey (2011) found that supplementing with N-acetylcysteine (an antioxidant) had no changes in the O_2 cost of sub maximal exercise. There is, however, some evidence to suggest that antioxidants and polyphenols can reduce NO_2^- to $\text{NO}\cdot$ (Gago et al., 2007, Carlsson et al., 2001)

albeit in the stomach and urine. Beetroot juice has a variety of antioxidants (see experimental chapter 1 for antioxidant content of beetroot juice). Lansley et al. (2010) removed the NO_3^- content from the beetroot juice to act as a placebo. This was performed via an ion exchange resin. They subsequently supplemented their participants with the active and placebo juices. These results provided evidence to suggest that in young healthy individuals it is NO_3^- that is the active ingredient as there was no reduction in the O_2 cost of exercise with the NO_3^- depleted juice.

Table 1. A summary of dietary NO₃⁻ supplementation and its effects on plasma NO₃⁻/ NO₂⁻ concentrations, BP and exercise (2007 - 2012).

Author	Participants & Protocol	NO ₃ ⁻ supplement	Plasma NO ₃ ⁻ & NO ₂ ⁻ concentration (BR vs. PL respectively)	BP changes	Exercise efficiency or performance and/or clinical relevance	Primary outcome successful?
Larsen et al. (2007b)	9 healthy well-trained males. Min 10 day washout. 5 mins cycling at 45, 60, 70, 80, 85 and 100% of $\dot{V}O_2$ max	3 days supplementation of NaNO ₃ ⁻ (0.1 mmol Kg ⁻¹ day ⁻¹)	NO ₃ ⁻ = 182 ± 55 and 27 ± 6.9 μM NO ₂ ⁻ = 226 ± 87 and 124 ± 28 nM.	↓SBP ↓DBP	↓ $\dot{V}O_2$ over 4 lowest WR ↑ average GE over 4 lowest WR ↑ average DE over 4 lowest WR	Yes
Webb et al. (2008a)	14 healthy subjects (10 in phase two), min 7 day washout period. 3BP measurements taken every 15mins for 1 hour.	500ml BR juice (NO ₃ ⁻ = 45 mmol/L) or water	16 fold ↑ in plasma NO ₃ ⁻ 2 fold ↑ in plasma NO ₂ ⁻ (Values not given)	2.5h peak ↓SBP 3h peak ↓DBP and MAP	IR of the forearm slowed the FMD and protected against IR injury (phase two).	Yes
Bailey et al. (2009)	8 healthy males. With a min of a 10 day washout period. 3BP measurements taken at the 6 th supplementation point. 4 MI and 2 SI bouts on days 4, 5 & 6.	500ml/day of BR juice (NO ₃ ⁻ = 5.5mmol/day) or black current cordial for 6 days	NO ₂ ⁻ = 273 ± 44 and 140 ± 50 nM	↓ SBP	↓ $\dot{V}O_2$ amplitude in MI ↓ $\dot{V}O_2$ slow component in SI ↑ time to exhaustion in SI ↓ deoxyhaemoglobin ↑ haemoglobin ↑ total haemoglobin	Yes

Bailey et al. (2010b)	7 healthy makes. With a min of a 10 day washout period. 3BP measurements taken at the 6 th supplementation point. Metabolic milieu measured via P-MRS.	500ml/day of BR juice ($\text{NO}_3^- = 5.1 \text{ mmol/day}$) or black current cordial for 6 days	$\text{NO}_2^- = 643 \pm 110$ and $206 \pm 93 \text{ nM}$	<p>↓SBP ↓DBP ↓MAP</p>	<p>↓ $\dot{V}\text{O}_2$ amplitude in LI ↓ $\dot{V}\text{O}_2$ slow component in HI ↑ time to exhaustion in SI ↓ muscle ATP turnover rate ↓ muscle ADP accumulation ↓ muscle Pi accumulation ↓ muscle PCr depletion</p>	Yes
Vanhatalo et al. (2010)	8 healthy physically active subjects. With a min of a 10 day washout period. 4BP measurements were taken on each visit. Protocol consisted of a ramp and MI exercise.	500ml/day of BR juice ($\text{NO}_3^- = 5.2 \text{ mmol}$) or juice cordial for an acute dose (2.5h) and chronic exposure (5 and 15 days)	$\text{NO}_2^- = 454 \pm 81 \text{ nM}$ at baseline and increased by 39% (post 2.5h), 25% (after 5 days) and 46% after 15days.	<p>↓SBP ↓MAP</p> <p>For all conditions</p>	<p>For acute and chronic supplementations. ↓ $\dot{V}\text{O}_2$ amplitude in MI</p> <p>After 15 days only: ↑$\dot{V}\text{O}_2\text{max}$ ↑peak work rate during the ramp test ↑GET & WR</p>	Yes

Larsen et al. (2010)	Nine (7 male) healthy subjects. With a min of a 7 day washout period. 2 BP measurements were taken on the final visit.	Acute (1h prior to exercise) & Chronic exposure; 2 days NaNO_3^- supplementation ($0.1 \text{ mmol Kg}^{-1} \text{ day}^{-1}$) or placebo which was equally dosed NaCl.	$\text{NO}_2^- = 142 \pm 35$ and $61 \pm 11 \text{ nM}$ $\text{NO}_3^- = 230 \pm 31$ and $17.3 \pm 3 \mu\text{M}$	No change in resting SBP & DBP. \downarrow DBP post ex.	Acute ingestion; $\dot{V}\text{O}_2$ during LI cycle exercise Chronic ingestion; \uparrow cGMP $\uparrow \dot{V}\text{O}_2\text{max}$	Yes
Vanhatalo et al. (2011)	9 healthy (7 male) physically active participants. BP was measured prior to exercise commencement after breathing normoxic or hypoxic air for 15 minutes.	750ml/day of BR juice ($\text{NO}_3^- = 9.3 \text{ mmol}$) or PL ($\text{NO}_3^- = 0.006 \text{ mmol}$). Juice was consumed 24, 12 and 2.5h prior to exercise.	$\text{NO}_2^- = 194 \pm 51$ and $129 \pm 23 \text{ nM}$	\downarrow SBP \downarrow DBP \downarrow MAP	\uparrow PCr recovery \downarrow S_{aO_2} in BR and PL compared to control. \downarrow T_{lim} PL compared to control \leftrightarrow T_{lim} BR to control	No
Larsen et al. (2011)	Mitochondria harvested from healthy volunteers post supplementation.	3 days NaNO_3^- supplementation ($0.1 \text{ mmol Kg}^{-1} \text{ day}^{-1}$)	$\text{NO}_2^- = 163 \pm 29$ and $35 \pm 7 \text{ nM}$ $\text{NO}_3^- = 169 \pm 18$ and $27 \pm 2.6 \mu\text{M}$	N/A	\uparrow Mitochondrial P/O ratio by 19%, suggesting a higher max ATP turnover which increased by 23% $\downarrow \dot{V}\text{O}_2$ in LI exercise	Yes

Lansley et al. (2011b)	9 healthy active males. With a min of a 10 day washout period. 4 BP measurements were taken on each visit to the lab (10 occasions)	500ml/day of BR juice ($\text{NO}_3^- = 6.2\text{mmol/day}$) or PL($\text{NO}_3^- = 0.003\text{mmol/day}$) for 6 days	$\text{NO}_2^- = 373 \pm 211$ and 183 ± 119 nM	↓SBP	↓ $\dot{V}\text{O}_2$ whilst walking ↓ $\dot{V}\text{O}_2$ amplitude in MI ↓ end exercise $\dot{V}\text{O}_2$ in SI ↑ time to exhaustion in SI	Yes
Lansley et al. (2011a)	9 healthy male club level cyclists. With a min of a 48h washout period. Exercise involved a 4 & 16.1km TT.	500ml/day of BR juice ($\text{NO}_3^- = 6.2\text{mmol}$) or PL($\text{NO}_3^- = 0.005\text{mmol/day}$) 2.5h prior to exercise	$\text{NO}_2^- = 575 \pm 199$ and 241 ± 125 nM	↓SBP	↑ TT performance (time=↓) ↑PO in TT	Yes
Kenjale et al. (2011)	8 (4 males) PAD patients. With a 7 – 14 day washout period. 1 BP measurement was taken prior to the intervention and 2h post juice. ABI and endothelium function were assessed.	500ml/day of BR juice ($\text{NO}_3^- = 18,181$ $\mu\text{mol/l}$) or PL (orange juice) 3h prior to exercise.	$\text{NO}_2^- = 943 \pm 826$ and 152 ± 72 nM	↓DBP	↓ time to onset of claudication ↑ walking distance ↓ deoxyhaemoglobin ↑ haemoglobin ↑ total haemoglobin	Yes
Bescós et al. (2011)	11 healthy participants. With a 7 day washout period.	NaNO_3^- supplementation (10 $\text{mg}\cdot\text{kg}^{-1}$) or equivalent dosed PL (NaCl).	$\text{NO}_2^- = 2.3$ $\mu\text{M} \pm 157$ nM and 2 $\mu\text{M} \pm 206$ nM $\text{NO}_3^- = 250 \pm 80$ and 29 ± 8 μM	N/A	↓ $\dot{V}\text{O}_2$ peak without affected TTE or max power.	Yes

Cermak et al. (2012a)	12 active male cyclists. With a 14 day washout period. Exercise involved a 60min sub max cycling followed by a 10-km TT.	500ml/day of BR juice ($\text{NO}_3^- = 8\text{mmol/day}$) or PL (NO_3^- depleted juice) for 6 days.	Abstract only, figures not presented.	N/A	\downarrow TT time \uparrow Power output \downarrow MI $\dot{V}\text{O}_2$	Yes
Cermak et al. (2012b)	20 well-trained males. Exercise included a ~1h TT.	140ml BR 2.5h prior to exercise ($\text{NO}_3^- = 8.7\text{mmol}$).	$\text{NO}_2^- = 532 \pm 32$ and $271 \pm 13\text{ nM}$	N/A	\leftrightarrow TT time \leftrightarrow Power output	No

\uparrow = statistical significant increase; \leftrightarrow = no statistical reduction; \downarrow = statistical significant reduction; $\dot{V}\text{O}_2$ = oxygen uptake; $\dot{V}\text{O}_{2\text{peak}}$ = peak oxygen uptake; WR = work rate; GE = gross efficiency; DE = delta efficiency; SBP = systolic blood pressure, DBP = diastolic blood pressure; MAP = mean arterial blood pressure; IR = ischemic reperfusion, FMD = flow-mediated dilation; MI = moderate intensity; RPE = rating of perceived exertion ; SI = severe intensity; $\dot{V}\text{O}_{2\text{max}}$ = maximal oxygen uptake; LI = low intensity; HI = high intensity; PCr = phosphocreatine; Pi = inorganic phosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; GET = gas exchange threshold; PAD = peripheral arterial disease; cGMP = cyclic guanosine monophosphate; S_{aO_2} = arterial O_2 saturation; Tlim = limit of tolerance; P/O ratio = phosphorylation efficiency; PO = power output; TT = time trial; TTE = time to exhaustion; LNF = low nitrate food; HNF = high nitrate food; PO_2 = partial pressure of oxygen; DMBA = dimethoxybenzaldehyde (a powerful organ-specific laboratory carcinogen); NIRS = near infrared spectrometry and sGC = Soluble guanylate cyclase; RQ = respiratory quotient; MVC = maximal voluntary contraction. Where NO_3^- & NO_2^- values are given it is from the final supplementation period if multiple doses given.

From 2008 until the start of this body of work, 20 papers were published in the field of NO_3^- supplementation (regardless of the primary outcome). Variability of NO_3^- dose between studies must be considered when comparing results from different trials. Webb et al., (2008a) administered ~ 22.5 mmol of NO_3^- in a single 500ml bolus. Many studies have supplementation regimens between ~ 4.5 and 6.5 mmol (see table 1 above for published values). These differences are also seen in plasma NO_3^- concentration values, which can range from 17.3 to 27 μM ($23.8 \pm 5.6 \mu\text{M}$) after placebo juice supplementation compared with the active juice ranging from 169 to 230 μM ($194 \pm 32 \mu\text{M}$). Plasma NO_2^- concentrations have greater variability between studies with concentrations following placebo juice supplementation from 29nM to 2 μM ($326 \pm 524 \text{ nM}$) and concentrations following active juice supplementation ranging from 142nM to 2.3 μM ($543 \pm 575 \text{ nM}$). A minority of published papers have reported plasma NO_2^- concentration in the μM range with a minimal increase post supplementation (Bescos et al., 2011). The majority of the inorganic NO_3^- supplementation studies have used chemiluminescence to determine NO_3^- and NO_2^- concentrations. Unsupplemented or placebo plasma NO_2^- concentrations are predominantly in the nM range. The measurement of NO_2^- poses significant challenges with substantial environmental contamination and plasma values that are often approaching the limits of detection of most assays. Furthermore there are a range of assay techniques available, with varying sensitivities. When this study is removed (Bescos et al., 2011) from the NO_2^- and NO_3^- data there is still a ~ 10 fold difference between the largest and the smallest baseline plasma NO_2^- values which some researchers would argue is due to training status. It is unlikely that the variation in reported plasma NO_2^- values is entirely due to genuine biological variation and dietary intake. Dietary intake accounts for $\sim 1 - 2$ mmol of NO_3^- per day

(ECETOC., 1988) and would therefore not elicit these variations with much less than this being derived from the L-arginine pathway. Assay variation remains a likely contributor to these large variations. A consensus within and between groups would help to establish norms within populations.

Variation in length of supplementation of NO_3^- ranges from 2.5 hours to 15 days with variation in washout periods for acute and chronic supplementation ranging from 2.5 days to 28 days. For supplementation periods that do not have time to elicit protein expression within the mitochondria (i.e. < 3 days) (Larsen et al., 2011) the washout period is less important. Conversely, longer washout periods are needed for longer supplementation periods. Research examining the length of RNA expression changes following supplementation withdrawal would elucidate how long it would take to lose these mitochondrial adaptations.

1.1.7: Endothelial dysfunction and red blood cells:

Endothelial function and the blood pressure lowering effects of $\text{NO}\cdot$ have been closely linked in previous research studies. It has been hypothesised that eNOS could act as another NO_2^- reductase in areas where it may be needed, such as hypoxia (Webb et al., 2008b) and anoxia (Gautier et al., 2006). Plasma NO_2^- concentrations have been shown to mirror endothelial dysfunction in humans (Kleinbongard et al., 2006), with dysfunction in endothelial function and reduced bioavailability of $\text{NO}\cdot$ being closely linked to cardiovascular disease (Forte et al., 1997, Vanhoutte, 2009). This potentially could be the reason for poor control of vascular tone (Forte et al., 1997).

Endothelial dysfunction is a systemic disease of the vascular endothelium which is brought about via a disparity between vasodilation and vasoconstricting substances.

This endothelial dysfunction is associated with T2DM and has been strongly linked to a reduced bioavailability of NO \cdot (Calver et al., 1992, Cohen, 1993, Cockcroft et al., 2000). This is likely to be underpinned by multiple factors; firstly, an impairment of NOS (Ito et al., 2002, Huynh and Tayek, 2002) to create NO \cdot and secondly, increased inactivation of NO \cdot by oxidants or reactive O $_2$ species (Vallejo et al., 2000, Angulo et al., 1996). A third possibility includes scavenging of NO \cdot in the vascular lumen which can be expedited by four key proteins such as, oxyhaemoglobin (to form methemoglobin and NO $_3^-$), deoxyhaemoglobin (to create nitrosyl haemoglobin), O $_2$ and thiols (creating S-nitrosohaemoglobin) (Kharitonov et al., 1995, Gow et al., 1999, Gow and Stamler, 1998, Lancaster, 1996).

Nitrosyl-haemoglobin levels are higher in individuals with type 1 diabetes compared to healthy controls. It would appear that higher concentrations of glycated haemoglobin will reduce the bioavailability of NO \cdot (Milsom et al., 2002). S-nitrosohaemoglobin related dilation is inversely related to oxygenation status (James et al., 2004). However, the deoxygenation of haemoglobin has been proposed as a mechanism of NO \cdot delivery (Jia et al., 1996). NO \cdot delivered via red blood cells (RBC) has been suggested to play a role in vasodilation in healthy participants under hypoxic conditions and contributes to a role in healthy physiology (McMahon and Stamler, 1999). If these metabolites do act as a NO \cdot storage pool then they could be key for NO \cdot mediated vasodilation in conditions such as acidosis or hypoxia (James et al., 2004) which are particularly prevalent conditions in skeletal muscle of individuals with T2DM and the respiratory muscles of individuals with chronic obstructive pulmonary disease (COPD). However, more NO \cdot is bound to glycated haemoglobin (HbA $1c$) which appears to reduce the vasodilatory functions of red blood cells (James et al., 2004) and thus individuals with T2DM will retain more NO \cdot .

than non-diabetic controls. This RBC derived NO[•] delivery appears to be expedited under pathological conditions such as ischemia reperfusion injury (Duranski et al., 2005), pulmonary hypertension (Hsu et al., 2007), atherosclerosis (Kim-Shapiro et al., 2006) and cerebral vasospasm (Pluta et al., 2005).

Blood vessel relaxation can be induced by insulin and is mediated at least in part via nitric oxide (Scherrer et al., 1994). Vascular function is impaired in T2DM (Steinberg et al., 1996). However, the perturbations in nitric oxide production and metabolism in T2DM and consequent modulation of vascular health are currently not clear. Some investigators suggest basal nitric oxide production is reduced in individuals with T2DM compared with healthy controls (Woodman et al., 2006, Scherrer and Sartori, 2000, Tessari et al., 2010). This could be a result of the increased expression of the intracellular inhibitor of endothelial nitric oxide synthase (García-Cardeña et al., 1997), caveolin-1 in the adipose tissue of individuals with T2DM (Catalán et al., 2008), elevated levels of NOS inhibitors such as asymmetric dimethylarginine (ADMA) (Abbasi et al., 2001) or BH₄ oxidation. Other groups report elevated levels of oxidised products of nitric oxide (Maejima et al., 2001, Chien et al., 2005, Ghasemi et al., 2010) with associations with specific genetic polymorphisms of eNOS noted (Monti et al., 2003). Together these reports would suggest T2DM is accompanied by either impaired production of NO[•], rapid scavenging of nitric oxide before it can cause downstream changes or a failure of activation of secondary messengers such as cGMP which are known to mediate NO[•]'s vascular effects (Young and Leighton, 1998).

1.1.8: NO· hepatic glucose uptake and T2DM:

Following a meal, glucose along with other carbohydrates and dietary constituents gets absorbed from the gastrointestinal tract. These nutrients are taken directly to the liver by the portal blood vessels allowing the liver to control the levels of nutrients like glucose in the blood. Glucose is the predominant energy source for mammalian cells and these cells require a steady supply of glucose to maintain normal function. Blood glucose regulation is maintained by numerous actions including: the rate of glucose consumption, carbohydrate uptake from the intestine, skeletal muscle glucose uptake and utilisation, and the rate of uptake/release via the kidney (Nordlie and Arion, 1964) and via the generation of endogenous glucose when required. Under normal physiological conditions glucose does not accumulate in the plasma. The surplus glucose gets metabolised, stored as glycogen (glycogenesis) predominantly in the liver) or converted to fat. Following ingestion of food, portal vein diameter widens increasing blood flow to the liver (Pazahr et al., 2014). This process may expedite glucose disposal.

Incretins, such as glucagon like peptide (GLP-1) and Glucose dependent insulinotropic peptide (GIP), are a group of gastrointestinal hormones that are released into the blood stream from cells in the small intestine in response to ingestion of food (Holst, 2007). They are predominantly involved in the regulation of blood glucose levels via their ability to have an insulinotropic (i.e. they promote insulin secretion) effect essentially augmenting the glucose-induced insulin secretion (known as the incretin effect). Other functions of incretins include regulation of gut motility, gall bladder constriction, nutrient absorption and secretion of gastric acids and pancreatic enzymes (Drucker, 2006). Glucagon-like peptide receptors (GLP-1Rs) have been found in many cardiovascular tissues including vascular and

coronary endothelium smooth muscle, cardiomyocytes and endocardium (Wei and Mojssov, 1995, Bullock et al., 1996). A diverse range of beneficial cardiovascular effects have been shown by multiple groups which include: regulation of blood pressure and heart rate, vascular tone and myocardial contractility (Barragán et al., 1996, Vila Petroff et al., 2001, Yamamoto et al., 2002, Green et al., 2008). Importantly these beneficial effects have been shown in healthy adults and also individuals with T2DM (Nikolaidis et al., 2004a, Nystrom et al., 2004, Özyazgan et al., 2005, Sokos et al., 2007).

L-cells present in the intestines secrete the biologically active GLP-1 (7-37), GLP-1 (7-36 amide) and GLP-2 (Baggio and Drucker, 2007) into the microcirculation to which the cells are closely associated. GLP-1 is known to slow gastric emptying (Meier et al., 2006) which reduces feelings of hunger (Zander et al., 2002) and in combination with receptors within the brain, GLP-1 creates a feeling of satiety (Goke et al., 1995) which have been shown to improve weight loss in individuals with T2DM. Other beneficial effects have been shown such as increased myocardial and endothelial function (note, in cardiac disease patients) (Basu et al., 2007, Nikolaidis et al., 2004a, Nikolaidis et al., 2004b).

A consensus within the literature has emerged that individuals with T2DM have an impaired incretin response compared to healthy controls (Nauck et al., 2011). This appears to be due to both reduced GLP-1 secretion and a resistance to GIP. The impaired incretin response increases as T2DM progresses (Nauck et al., 2011). GLP-1 has been shown to elicit myocardial glucose uptake (Bhashyam et al., 2010) and increases skeletal muscle microvascular blood flow, glucose uptake, nitric oxide production and insulin clearance mediated via nitric oxide-dependant mechanisms

(Chai et al., 2012). Specifically, GLP-1 analogues in animal models (dog) have also been shown to up-regulate the production of the vasodilator NO \cdot (Post et al., 2010) whilst GIP has been shown to stimulate NO \cdot production from eNOS in the portal vein but not in the hepatic artery (Ding et al., 2004). The portal vein is of paramount importance for glucose disposal; it collates tributaries from the stomach, proximal small intestines and the spleen and directs nutrient rich blood towards the hepatic vasculature / parenchyma for processing.

NO \cdot mediated vasodilation facilitates insulin stimulated glucose uptake into skeletal muscle (measured via an L-NAME NO \cdot blockade) (Roy et al., 1998). In endothelial cells insulin promotes nitric oxide synthesis (via eNOS) leading to activation of GLUT4 translocation and increased glucose uptake into the cell (Jiang et al., 2014). Therefore, increasing NO \cdot concentration may increase glucose uptake into cells via both activation of GLUT's translocation and/or via vasodilation. As described above dietary NO $_3^-$ is converted to NO $_2^-$ which is rapidly absorbed in the stomach and proximal small intestine. NO $_2^-$, absorbed by the stomach and the proximal small intestine will be delivered via the portal vein along with other micro and macronutrients to the stomach. Therefore the highest plasma concentrations of NO $_2^-$ in the body will occur in the portal circulation. Nitrite may play a vital role in glucose homeostasis following a meal either by direct signalling as a vasodilator or following conversion to NO \cdot (Petersson et al., 2007). The portal vein is partially deoxygenated. Deoxyhaemoglobin is a NO $_2^-$ reductase (Cosby et al., 2003) and thus there may be an increase in the bioavailability of NO \cdot . As the elevated plasma NO $_2^-$ concentrations reach the liver, many more NO $_2^-$ reductases are present such as neuroglobin, cytoglobin, xanthine oxidase and aldehyde oxidase which may increase the bioavailability of NO \cdot (Martin et al., 2004, Li et al., 2008b).

Over recent years experimental evidence has shown that NO \cdot may be key for glucose uptake (particularly in animal models) with skeletal muscle and in the intestines. In 2002 Kingwell et al, used a NOS inhibitor to assess the role of glucose uptake with skeletal muscle of an individuals with T2DM. They present a 13% drop in glucose uptake (Kingwell et al., 2002). One year later Guan et al., infused GLP-2 into neonatal piglets with an without a NOS inhibitor and found a marked reduction in glucose uptake with L-NAME (Guan et al., 2003). L-NMMA has also be shown to reduce glucose uptake in skeletal muscle in a mouse model (Merry et al., 2010). Another animal model used eNOS deficient mice, supplemented half the mice with nitrate rich water for 10 weeks. Carlstrom et al., demonstrate reduction in HbA1c, baseline plasma glucose concentrations and post prandial glucose concentrations and reduction in weight and circulating triglycerides (Carlström et al., 2010). Together these animal models suggest that NO \cdot may be a mediator of glucose uptake. Potentially via an increase in GLUT-4 translocation (Bedard et al., 1997) or increased perfusion with the parenchyma. One study has also shown that nitrate supplementation in healthy young individuals increases glucose uptake compared to placebo during exercise (Wylie et al., 2013b).

An intervention that can increase the bioavailability of NO \cdot within the liver (a major site for glucose disposal) may help expedite reductions in postprandial plasma glucose concentrations.

1.1.9: eNOS coupling

Uncoupling is a term used to describe the physiological process where eNOS no longer produces NO \cdot but creates O $_2^-$ (Li and Forstermann, 2013). Coupled and uncoupled NOS are commonly present in the same cell (Li and Forstermann, 2013).

As the coupled NOS produces $\text{NO}\cdot$ and the uncoupled NOS produces O_2^- , the potential for an increase in peroxynitrite concentration and subsequently a change in redox balance increases (Zou et al., 2004).

eNOS is mainly found in the endothelium but also in the cardiomyocytes (Wei et al., 1996), airway endothelium (Giaid and Saleh, 1995), and the tubular cells of the kidneys (Tracey et al., 1994). The eNOS N-terminus (i.e. the start of a polypeptide) contains the oxygenase aspect of the NOS, BH_4 , heme and also the L-arginine binding sites.

L-arginine and BH_4 stabilise the eNOS protein when in its dimeric form (non-covalent bonded macromolecule of two similar sub-units (Reif et al., 1999, Venema et al., 1997)). Zinc thiolate is key in maintaining the BH_4 binding site and maintains its structure in a dimeric form (Raman et al., 1998). Therefore maintaining BH_4 and zinc thiolate is key to prevent uncoupling and an increase in oxidative stress. Guanosine triphosphate (GTP) is converted to BH_4 via GTP cyclohydrolase (GTPCH1). Under elevated conditions of oxidative stress BH_4 is converted to dihydropterin (BH_2) which ultimately leads to a perpetual increase in NOS uncoupling. O_2^- and $\text{NO}\cdot$ combine to produce peroxynitrite (OONO^-), which is a powerful free radical. This is a reversible reaction which can be catalysed by dihydrofolate reductase (DHFR) (Crabtree et al., 2011). Individuals with T2DM are in a pro-oxidant state, which is likely to lead to uncoupled NOS and in particular eNOS (Siasos et al., 2007) which will generate O_2^- . $\text{NO}\cdot$ can react with O_2^- which will create OONO_2^- . This will reduce the bioavailability of $\text{NO}\cdot$ (Beckman and Koppenol, 1996).

Methylation of L-arginine naturally occurs in the cytoplasm of any normal functioning cell. Methylation of L-arginine can create one of three distinct derivatives of this

amino acid (i), Asymmetric dimethylarginine (ADMA), (ii) symmetrical dimethylarginine (SDMA) and (iii) monomethyl-L-arginine (L-NMMA). Two distinct enzymes are required to produce ADMA and SDMA: they are type 1 (forms ADMA) and type 2 (forms SDMA) protein-arginine methyltransferases (PMRTs). ADMA can inhibit all three NOS isoforms and has a greater affinity for eNOS (Tousoulis et al., 2007). ADMA is elevated in individuals with T2DM (Abbasi et al., 2001, Lu et al., 2011, Schulze et al., 2006), and hypertension (Surdacki et al., 1999). Elevated ADMA has been shown to predict cardiovascular disease (Ravani et al., 2005, Lajer et al., 2008, Lu et al., 2011, Schnabel et al., 2005). Moreover, plasma ADMA concentrations have been shown to independently predict not only non-fatal myocardial infarctions but also death from cardiovascular causes (Schnabel et al., 2005, Meinitzer et al., 2007) in patients with coronary artery disease. Similar findings have been shown in haemodialysis patients (Zoccali et al., 2001) and angina (Lu et al., 2003a). Hyperglycaemia impairs dimethylargininase in the endothelium and smooth muscles which results in raised ADMA levels in individuals with T2DM (Lin et al., 2002). ADMA causes atherosclerosis lesions and renal damage which impairs NO \cdot output in eNOS deficient mice (Suda et al., 2004). These deleterious effects are caused by upregulation of angiotensin-converting enzyme and elevated levels of O $_2^-$ (Suda et al., 2004).

1.1.10: Preventing eNOS uncoupling via pharmaceutical methods:

Uncoupling of eNOS decreases O $_2$ dependent NO \cdot production and elevates oxidative stress (Li and Forstermann, 2013). Strategies that increase NO \cdot bioavailability either through pharmacological methods or through dietary sources could alter the redox balance to prevent NO \cdot and BH $_4$ oxidation. This may lead to reduced superoxide production from uncoupled NOS (Förstermann and Sessa,

2012). Below is a summary of supplements/drugs that can help prevent the uncoupling of eNOS and therefore elevate NO· bioavailability.

BH₄

BH₄ supplementation has been shown to vasodilate blood vessels after arterial-infusion in patients with coronary heart disease (Cunnington et al., 2012). Its effectiveness as an anti-hypertensive is limited by its rapid oxidation. When BH₄ is administered as an oral treatment it has no effect on endothelial function (Cunnington et al., 2012). There is some evidence that BH₄ elevates iNOS activity (McNeill and Channon, 2012). Sepiapterin is a molecule that is converted to BH₄ via Sepiapterin-reductase and DHFR. These reductases have been shown to be down-regulated in hypertension (Youn et al., 2012). Therefore, given the rapid oxidation of BH₄ as an oral agent (Cunnington et al., 2012), its use as a supplement is unlikely to be beneficial. Maintaining redox balance however, will prevent the uncoupling of NOS (Zou et al., 2004).

Antioxidants

There are data to suggest that beetroot juice will not only increase the bioavailability of NO· by the entero-salivary pathway involving inorganic NO₃⁻ but also via its antioxidant content (see experimental chapter 1 for table of antioxidant content) (Kanner et al., 2001). Antioxidant supplementation *per se* has the potential to alleviate oxidative stress via reductions in cell damage (de Boer et al., 2005), anti-inflammatory effects (Kelley et al., 2006) an inhibition of uric acid production (Jacob et al., 2003) and potentially prevent NOS uncoupling.

Statins

Statins are a group of drugs that are utilised to lower plasma cholesterol levels via the inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase. Statins enhance the availability of superoxide dismutase (SOD) and GPx isoforms (Carrepeiro et al., 2011, Landmesser et al., 2005). Statins also have an anti-atherosclerotic effect which is may in part be due to their impact on NO· bioavailability (Antonopoulos et al., 2012). *In vivo*, statins have been shown to increase the expression of BH₄ and also GTPCH1 in humans (Antoniades et al., 2011). This increase in GTPCH1 is likely to be responsible for the increase in BH₄ and ultimately reducing eNOS uncoupling (Hattori et al., 2003, Antoniades et al., 2011).

Organic nitrates

Organic NO₃⁻ act as NO· donors (such as isosorbide mononitrate). Pentaerithrityl tetranitrate has been shown to prevent eNOS uncoupling (again via elevated BH₄ concentrations (Schuhmacher et al., 2011)), induce SOD isoforms (Oppermann et al., 2009), HO-1, reduce NADPH oxidase and xanthine oxidase activity.

ARB, ACEi and Beta Blockers

Angiotensin-converting enzyme inhibitor (ACEi) and angiotensin receptor blockers (ARBs) impact upon the renin-angiotensin-aldosterone system. ACEi have been shown to increase NO· production via reductions in NAPDH oxidase, O₂⁻ and OONO⁻ whilst elevating BH₄ concentrations in an the wantanabe heritable hyperlipidemic rabbits (Imanishi et al., 2008). ARBs have antioxidant properties (Warnholtz et al., 1999) and long term exposure leads to re-coupling of eNOS (Warnholtz et al., 1999). This appears to be via the prevention of BH₄ oxidation (Wenzel et al., 2008). GTPCH1 mediates BH₄ synthesis (Wenzel et al., 2008, Satoh et al., 2008) and by up-regulation of dihydrofolate reductase mediates reduction of BH₂ to BH₄ (Oak and

Cai, 2007). Nebivol, a beta blocker, not only stimulates eNOS to produce NO \cdot (Münzel and Gori, 2009) but has the ability to reverse its uncoupling (Mollnau et al., 2003) by inhibiting NADPH oxidase.

eNOS transcriptional enhancers (AVE3085)

AVE3085 is an eNOS transcription factor which is involved in the regulation of eNOS protein expression (Wohlfart et al., 2008). AVE3085 has been shown to elevate vascular BH $_4$ concentrations and thus reduce eNOS uncoupling (Wohlfart et al., 2008). AVE3085 also appears to have antioxidant effects and reduces the expression of NADPH oxidase (Westermann et al., 2009) which may contribute to elevated NO \cdot bioavailability.

1.1.11 Ageing and NO \cdot

Two key co-factors in the production of NO \cdot are reduced in concentration as we age. These are BH $_4$ and L-arginine (Delp et al., 2008). This may mean that as we age we get a greater increase in uncoupled NOS, creating more superoxide and subsequently peroxynitrite (Kang et al., 2009). Moreover, plasma nitrite concentrations have also been shown to be reduced in older adults (Sindler et al., 2011). Given the putative benefits of NO \cdot in healthy young adults, using a dietary source of nitrate may increase NO bioavailability in healthy older adults and enable them to benefit from the positive association that has been shown between NO \cdot and vascular health (Ignarro et al., 1999).

1.1.12: Toxicology:

Methemoglobinemia occurs when the heme group in a haemoglobin is oxidised to its ferric state (Fe $^{3+}$) and is therefore incapable of transporting O $_2$ (Avery, 1999). As far back as 1868 the combination of NO $_3^-$ and blood has been shown to produce

methemoglobinemia (Eusterman and Keith, 1929, Gamgee, 1868, Reichert, 1880). This was later corroborated in animal models as large doses of NO_3^- caused the formation of methemoglobinemia (Binz and Geringer, 1901). In 1945 concerns about nitrates' potential toxicity emerged again as fears over methemoglobinemia or blue baby syndrome arose. Blue baby syndrome was first reported by Comly (1945) with the observation that the well-water had large contamination of NO_3^- . Occurrences of methemoglobinemia were shown in (Walton) 1951 to be rare in water that contained $< 44\text{mg/l}$ of NO_3^- . Given this knowledge, the level at which the US (44 mg/l) and the European union (50 mg/l) set the NO_3^- levels in drinking water was addressed. The notion that NO_3^- by itself can cause methemoglobinemia has been strongly argued against by Avery (1999). Avery proposed a more likely scenario that NO_3^- in the well water contributed to methemoglobinemia. Bacterial contamination within the well was converting NO_3^- to NO_2^- . Furthermore, given the beneficial effects of NO_3^- that are described later in this chapter, the removal of NO_3^- from water supplies could be detrimental to human health by reducing the daily intake of NO_3^- .

N-nitrosamines were shown to cause hepatic tumours in rats (Magee and Barnes, 1956). In 1976 two independent groups from Germany and the USA introduced the concept that NO_3^- could be transformed into N-nitrosamines under acidic environments (Tannenbaum et al., 1976, Spiegelhalder et al., 1976). Given that NO_3^- and NO_2^- have been used as a preservative in food for centuries and that NO_3^- is abundant in many foods which epidemiological evidence has suggested to have cardio protective effects such as vegetables (Joshi-pura et al., 1999, Joshi-pura et al., 2001), the publication by (Newberne, 1979) stating that NO_2^- caused lymphomas in rats was surprising. The food and agriculture organisation and the world health organisation expert committee on food additives jointly released a statement which

stated “Overall, the epidemiological studies showed no consistently increased risk for cancer with increasing consumption of NO_3^- . These data, combined with the results of the epidemiological studies considered by the Committee at its forty-fourth meeting, do not provide evidence that NO_3^- is carcinogenic to humans” (Speijers and Brandt, 2003).

1.2: Introduction to Type 2 Diabetes Mellitus:

T2DM is a chronic disease and progressive in nature. The aetiology of the disease is chronic hyperglycaemia caused via insulin resistance and diminishing pancreatic beta cell insulin production (Stumvoll et al., 2007). These characteristics cause micro and macrovascular complications (such as cardiovascular disease) which ultimately result in elevated morbidity and mortality (Jaffe et al., 1984, Sprafka et al., 1991). T2DM is emerging as an epidemic in both the developed world as well as the developing world. Life expectancy has risen sharply over recent years (Oeppen and Vaupel, 2002) whilst obesity is rising due to a sedentary lifestyle and excessive intake of calories. This review will aim to explore the demographics of T2DM, insulin resistance and the development of T2DM, measurement of T2DM, genetics, response to exercise, macrovascular, microvascular disease and metabolic complications and NO.

1.2.1: T2DM demographics and costs:

T2DM accounts for 90 - 95% of all cases of diabetes worldwide (Harris, 1998, Hariri et al., 2006). Physical inactivity and obesity increases the prevalence of this metabolic disease (Wild et al., 2004). As we age (on a population level) we do less and less activity (Sallis, 2000) which may explain some of the increased risk with aging. As the global population expands the number of people with T2DM is increasing (Wild et al., 2004). The chances of developing diabetes increases from 0.3 - 0.5 % per year for 50-60 year olds to 0.5 - 1% for 60-70 year olds and greater than 1% a year for over 70 year olds (Berger et al., 1999). Wild et al., (2004) projected that by 2030 this figure of 171 million will have doubled to over 340 million worldwide. However, this may be conservative given these calculations were based on obesity levels remaining stable and the observation that obesity levels are in fact

increasing. More recent estimates of 439 million by 2030 have been suggested after extrapolation from the 2010 figure of 285 million individuals (Shaw et al., 2010). In the UK 3.1 million people (diagnosed and undiagnosed) have T2DM, which equates to 7.4% of the population (PHO, 2010). With increasing numbers of individuals developing T2DM the associated costs increase (Caro et al., 2002). T2DM is responsible for 8% of the NHS annual budget equating to £8.8 billion per annum. The total cost of direct and indirect care comes to a total of £20.5 billion (Hex et al., 2012).

1.2.2: Insulin resistance, beta cell dysfunction, and type 2 diabetes mellitus:

Reduction in sensitivity to the hormone insulin causes three major abnormalities to occur in the body: firstly, a reduced insulin mediated uptake of glucose by skeletal muscle, secondly, an increase in free fatty acid metabolism via adipose tissues and finally over production/secretion of glucose by the liver (Kabir et al., 2005). As insulin resistance progresses hyperglycaemia will begin to manifest itself in the fasted state (Lebovitz, 1999). Insulin resistance is a precursor to the development of T2DM in which the cells within the bodies fail to respond to the hormone correctly. The body produces adequate concentrations of insulin, however, changes in the cell surface receptors, (Kolterman et al., 1980), mitochondrial dysfunction (Holloszy, 2009), endoplasmic reticulum stress (Cnop et al., 2012), fat in skeletal muscle and glucose modification (Hart et al., 2011) (via the hexosamine biosynthesis pathway) prevent the correct response (i.e. the uptake of glucose into the cell). As peripheral insulin resistance progresses in skeletal muscle, liver, and fat tissues circulating plasma glucose concentrations will rise. Rising plasma glucose concentrations stimulate the pancreatic β -cells to secrete more insulin (hyperinsulinemia) in an attempt to

maintain metabolic homeostasis. This will continue until total β -cells' death (Stumvoll et al., 2005) (See figure 2 below).

The aetiology of T2DM and the subsequent insulin resistance and beta cell dysfunction is multifactorial. Of primary importance are the following: relative excessive calorie intake (Bloomgarden, 2000), aging (Harris et al., 1998), and a lack of exercise (Morgan et al., 2002, Shaw et al., 2010). This means that insulin resistant individuals are in a chronically elevated energy balance (Stumvoll et al., 2005, Eriksson, 2007, Kahn et al., 2006). If an individual has a persistent positive energy balance (i.e. over and above the requirements for daily living) adipocytes will uptake triglycerides until they become saturated, creating adiposopathy (Bays, 2011). This mainly occurs in the visceral adipocytes. If adipocytes are at maximum capacity the ability to reduce circulating fats is diminished (Tushuizen et al., 2007). Hyperglycaemic mediated insulin production has been shown to be stunted by exposure of the β -cells to fatty acid deposits, which are present in higher concentrations due to reduced clearance (Lee et al., 1994, Tushuizen et al., 2007). Furthermore, persistent chronically elevated fatty acid and triglyceride levels lead to abnormal amounts of these fats being stored in skeletal muscle (Boden, 1997, Boden and Chen, 1995) and the liver (Kabir et al., 2005). Storage of fats in skeletal muscle and the liver expedites the insulin resistance as skeletal muscle is the predominate source of glucose uptake (Berg et al., 2001, Kelley et al., 1993).

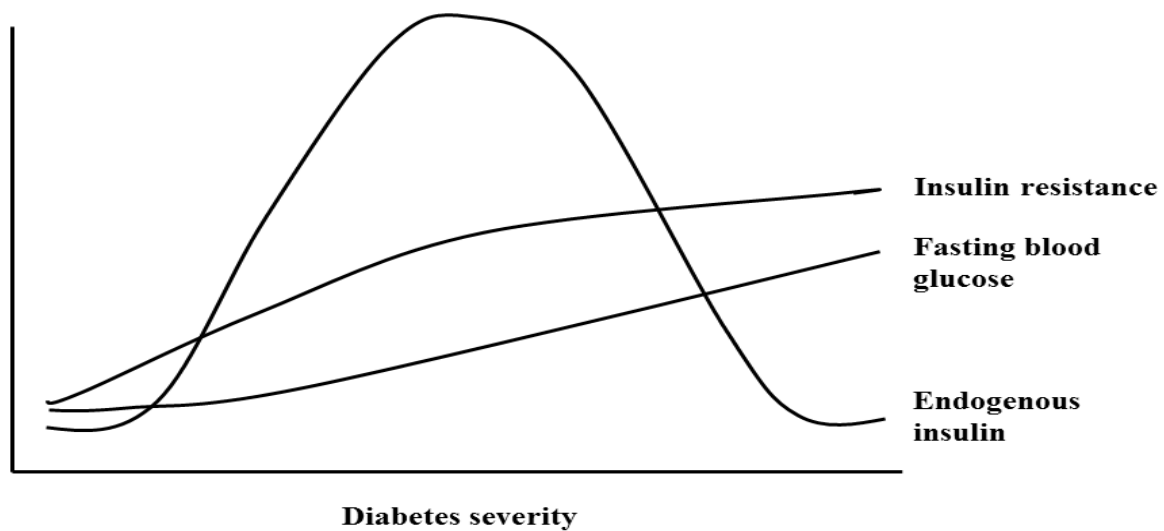


Figure 2: Change in insulin resistance, insulin production and fasting blood glucose as diabetes severity increases.

1.2.3: Measurement of insulin resistance and T2DM

HbA_{1c} is haemoglobin that has been glycosylated. HbA_{1c} is created via an Amadori reaction which causes an irreversible binding of glucose molecules to the β -N-terminal valine residues on the globin chains. During the reaction the haemoglobin is rearranged to 1-deoxy-1-N-valyl-fructose (Bookchin and Gallop, 1968, Dixon, 1972, Bunn et al., 1975). Although exposure of haemoglobin to glucose during the 120 day life time is stable in individuals with controlled diabetes, the HbA_{1c} concentration is higher in older erythrocytes (Huisman and Dozy, 1962, Fitzgibbons et al., 1976). When circulating glucose concentrations rise the proportion of HbA_{1c} rises as well, therefore the higher the plasma glucose the higher the HbA_{1c} (Rohlfing et al., 2002). HbA_{1c} is used as a measure of glycaemic control over a prolonged period as it is stable over time, typically accurate over a 3 month period due to the replacement of new red blood cells. The WHO have published a report on the use of HbA_{1c} as a diagnostic tool for diabetes (WHO, 2011).

1.2.4: Genetics

T2DM appears to have a heritable component with heritability of T2DM being suggested to be greater than 50% in studies of monozygotic twins (Herder and Roden, 2011, Poulsen et al., 2009), and first degree relatives of individuals with T2DM. (Hemminki et al., 2010, Poulsen et al., 2009, Tattersall and Fajans, 1975, Saxena et al., 2007). Genetic mapping over recent years has shown more than 40 loci that are thought to be partly responsible for the development of T2DM (Herder and Roden, 2011, Voight et al., 2010, Dupuis et al., 2010, Zeggini et al., 2008, Saxena et al., 2010). Most of these loci are associated with the dysfunction of pancreatic β -cells with the most common named 'TCF7L2' (Dupuis et al., 2010). The identified loci can only explain 10% of the heritability in T2DM (Herder and Roden, 2011). The remaining cause of heritability is unknown. However, Nolan and colleagues (2011) suggest that variants in allele frequency maybe involved. Genetics also have a role to play in the development of microvascular disease, there appears to be an abnormality in the loci within chromosomes 7 and 20 which increases susceptibility to nephropathy (Imperatore et al., 1998).

Ethnicity and environment can affect the risk of developing T2DM and it can also affect the likelihood of complications. Fasting plasma glucose levels were measured in Pima Indians (Pettitt et al., 1980, McCance et al., 1994), Egyptians (Engelgau et al., 1997), sections of the US population (Alberti and Zimmet, 1998) and Caucasians (Jarrett et al., 1984) independently. Measurements of the associated risk diabetes related complications and fasting plasma glucose concentrations were correlated with the mg/dL ranging from 108-140. African American and Mexican Americans have been shown to be at greater risk of diabetes related retinopathy compared to Caucasian Americans. In African American individuals this could be explained by a

larger % of the population having high blood pressure and a higher HbA1c. However, in Mexican Americans this is unexplained (Harris et al., 1998).

1.2.5: T2DM and response to exercise:

Fatigue:

During exercise T2DM can lead to increased discomfort and early fatigue (Regensteiner et al., 1995a, Regensteiner et al., 1998) which may impair people with T2DM's ability to achieve the recommended amounts of exercise that are a key part of the management of T2DM (NICE, 2008). This premature fatigue has been attributed to abnormalities occurring at multiple points during the transport of O₂ from the lungs to the muscles where it is utilised during exercise (Scognamiglio et al., 1998). Possible explanations for this impairment include compromised cardiac function which can manifest with reduced stroke volume (Gusso et al., 2008), reduced cardiac innervation (Scognamiglio et al., 1998) reduced left ventricular diastolic function (Poirier et al., 2000) and impaired cardiac autonomic function (Gerritsen et al., 2001). Other explanations of exercise impairment include diminished skeletal muscle O₂ diffusion and a reduced mitochondrial O₂ utilisation (Regensteiner et al., 1995a, Regensteiner et al., 1998, Baldi et al., 2002). These effects are likely underpinned by reductions in mitochondrial density (Mårin et al., 1994, Mathieu-Costello et al., 2005, Ritov et al., 2005) and amplified levels of mitochondrial dysfunction (Padilla et al., 2007). This dysfunction is characterised by an impaired bioenergetic capacity (specifically diminished NADH and citrate synthase (Kelley et al., 2002) and a reduced ability to uptake glucose (Mathieu-Costello et al., 2003).

Maximal O₂ uptake:

Individuals with T2DM have an elevated resting O₂ consumption coupled with a profound reduction in maximal O₂ uptake (Baldi et al., 2002, Regensteiner et al., 1998, Regensteiner et al., 1995a, Wilkerson et al., 2011, O'Connor et al., 2015). Maximal O₂ uptake is an important predictor of all-cause mortality (Myers et al., 2002, Galant et al., 2012). Therefore, individuals with T2DM are likely to be closer to their maximum exercise capacity than individuals without diabetes for any given intensity of exercise (Regensteiner et al., 1998). The consequence of being closer to the maximal exercise capacity is that a greater physiological stress and thus greater discomfort will be experienced by the individual (Regensteiner et al., 1998), resulting in a reduction in exercise tolerance. If two individuals exercise at the same workload, the one with higher O₂ consumption will typically find the exercise more difficult. This may translate with individuals with T2DM having greater perceived exertion at the same workload. Difficulties completing submaximal exercise tasks may manifest as a barrier to attain the recommended amounts of exercise recommended by the WHO (Huebschmann et al., 2009). The reduction in maximal O₂ uptake is ultimately caused by an irregularity in O₂ delivery (Regensteiner et al., 1995a, Regensteiner et al., 1998, Baldi et al., 2002, Wilkerson et al., 2011).

O₂ delivery:

Many of the abnormalities that are related to premature fatigue in individuals with T2DM are likely caused via O₂ delivery perturbations. These include O₂ transportation from the lungs to the muscle during exercise (Scognamiglio et al., 1998), along with reduced stroke volume (Gusso et al., 2008), cardiac innervation (Scognamiglio et al., 1998), cardiac autonomic function (Gerritsen et al., 2001) and left ventricular diastolic function (Poirier et al., 2000). O₂ delivery may also be limited

by a reduced total and fractional O₂ extraction into the mitochondria which is caused via reduction in mitochondria density, size and function (Regensteiner et al., 1995a, Regensteiner et al., 1998, Baldi et al., 2002, Padilla et al., 2007, Shore et al., 1994, MÅrin et al., 1994, Mathieu-Costello et al., 2005). Individuals with T2DM also have elevated plasma endothelin-1 (endothelin-1 are a group of peptides that modulate vascular tone) (Schneider et al., 2002), reduced capillary density (MÅrin et al., 1994, Mathieu-Costello et al., 2003), impaired haemodynamics (Padilla et al., 2007, Shore et al., 1994) and mitochondrial function (Kelley et al., 2002) in the skeletal muscle.

$\dot{V}O_2$ kinetics and exercise tolerance:

The rate at which $\dot{V}O_2$ projects towards the steady state is a major determinant in the ability of an individual to tolerate exercise. The quicker an individual can attain a steady state following the onset of exercise, the greater the contribution of oxidative compared to non-oxidative metabolism (Jones and Poole, 2005). By increasing the speed of the O₂ uptake response, individuals can reduce the intracellular changes in metabolic milieu that have been linked to the fatigue process. By using a larger proportion of oxidative metabolism, the speed at which lactic acid concentrations build up are slowed. Lactic acid disassociates into lactate and H⁺, the H⁺ causes acidosis and thus is an important determinant of fatigue (Bruton et al., 1998, Pate et al., 1995, Westerblad et al., 1997, Wiseman et al., 1996). Similarly, in anaerobic respiration, phosphocreatine is broken down into creatine and inorganic phosphate (Pi). Pi concentrations appear to have a larger contribution to fatigue than reduced pH levels (at least at physiological temperatures) (Dahlstedt et al., 2001, Dahlstedt et al., 2000, Dahlstedt and Westerblad, 2001, Fryer et al., 1995, Kabbara and Allen, 1999, Kabbara and Allen, 2001). Fatigue may however be expedited in low pH levels by inhibition of key enzymes involved in glycolysis and glycogenolysis. This may

slow the adenosine diphosphate to adenosine triphosphate turnover rate (Westerblad, Allen, & Lännergren, 2002).

The $\dot{V}O_2$ kinetics of individuals with T2DM have been shown to be slowed (Bauer et al., 2007, Regensteiner et al., 1998, Regensteiner et al., 1995a, Brandenburg et al., 1999). Conversely, Wilkerson et al. (2011) showed no difference in the $\dot{V}O_2$ kinetics of older individuals with T2DM compared to healthy age matched controls. More recently, the putative slowed $\dot{V}O_2$ kinetics shown by some studies in older individuals with T2DM has been revisited by O'Connor et al. (2015) to assess whether the lack of effect shown in some studies in the kinetics of older individuals with T2DM was due to disease duration or age. Disease duration was matched between a younger group and an older group of individuals with T2DM. Their findings indicate that middle aged men have impaired $\dot{V}O_2$ kinetics (i.e. a longer time constant) compared to healthy matched controls. However, older (65 years old) individuals with T2DM were similar to their healthy matched controls, replicating Wilkerson et al's findings in 2011. This suggests that aging is the predominant cause for slowed $\dot{V}O_2$ kinetics.

Medication and exercise performance:

T2DM is a disease that affects multiple systems. Many of the medications that are used to treat one aspect of their condition can also have many other beneficial effects. Drug treatment used to reduce hyperglycaemia such as sulfonylureas, insulin and metformin have all been shown to help reduce microvascular complications (Turner et al., 1998, UKPDS, 1998, Ohkubo et al., 1995, Patel et al., 2008) and improve exercise performance. For instance insulin when used to treat patients with T2DM and heart failure as a comorbidity have been shown to improve ventilatory efficiency and $\dot{V}O_2$ (Guazzi et al., 2003). Moreover, ramipril has been

shown to increase treadmill walking time compared with a placebo in individuals with peripheral artery disease (Ahimastos et al., 2013).

1.2.6: Micro & macrovascular Complications:

Between 1950 and 1995 the absolute risk of myocardial infarction, strokes and intermittent claudication has fallen by ~50% in individuals with diabetes (Fox et al., 2004). However, individuals who have T2DM still have a two to four fold increased chance of developing coronary heart disease compared to individuals without T2DM (Stamler et al., 1993, Hanefeld et al., 1991, Wei et al., 1998, Wilson et al., 1991). Although the overall risk of CVD has fallen in individuals with T2DM over recent decades, more of the risk is attributable to T2DM (Fox et al., 2007) (the Framingham heart study). Having diabetes and CVD increases the risk of mortality compared to non-diabetic controls following a myocardial infarct by 50% (Sprafka et al., 1991). Jaffe et al., also showed an increased mortality in individuals with T2DM compared with controls. However when both groups were stratified for congestive heart failure the survival rates were similar (Jaffe et al., 1984).

Cholesterol is one of the major components involved in the pathogenesis of CVD (Lu et al., 2003b). Superficially, individuals with T2DM appear to have a similar lipid profile to age matched controls, for instance they have total cholesterol and LDL-cholesterol that are very similar (Laakso et al., 1985). Individuals with T2DM have a lower HDL-cholesterol and higher triglycerides (Wilson et al., 1985, Taskinen, 2003) which in turn are known risk factors for the development of CVD (Fontbonne et al., 1989, Assmann and Schulte, 1991).

Poor glycaemic control is detrimental to microvascular function and has been strongly associated with elevated microvascular dysfunction (Stratton et al., 2000).

The risk for microvascular disease is thought to be determined by the level of glycaemia, with extremely high glucose concentrations creating the greatest risk (Krolewski et al., 1995). Every percentage point decrease in HbA_{1c} (and thus improving control) results in a 35% reduction in microvascular complications (UKPDS, 1998). This group also suggested that by improving glycaemic control via diet and metformin as opposed to insulin therapy, microvascular complications can be reduced by 32%. Some of these findings have since been replicated in a Japanese cohort (Shichiri et al., 2000) when comparing 3 or more insulin injections compared with 1-2 injections. When more injections were used a greater level of glycaemic control was delivered which correlated with a reduced risk of microvascular complications. Not all of the experimental and epidemiological evidence supports this notion. In 2009 evidence was published to suggest that intensive glycaemic control in individuals with T2DM had no effect in controlling for microvascular complications or even major cardiovascular events (ADVANCE trial 2009) (Duckworth et al., 2009). Another study in 2008 showed no effect where aggressive treatment of HbA_{1c} with intensive therapy (increased medications with monthly visits to assess glycaemic control) compared with conventional therapy (as prescribed by their clinician) actually increases mortality (ACCORD (Hertzel C. Gerstein et al., 2008)).

1.2.7: NO[•] and related microvascular diseases:

The availability of NO[•] is important in the pathophysiology of diabetes related vascular disease, with reduced availability leading to reduced endothelial relaxation (Creager et al., 2003). This impairment ultimately contributes to diabetes related microvascular angiopathy. This microvascular disease is a hallmark of diabetes related retinopathy and nephropathy (Santilli et al., 2004). Polymorphisms of eNOS

have been shown to increase the severity of retinopathy and nephropathy in individuals with T2DM (Awata et al., 2004). By contrast a recent study has shown that an eNOS genotype is not associated with the development of diabetes related retinopathy (de Syllos et al., 2006, Neugebauer et al., 2000). However, eNOS polymorphisms are associated with nephropathy progression (Neugebauer et al., 2000). de Syllos and Awata et al's cohorts have ~50 participants with similar methodologies. The differences in these trials may be explained by differences in the cohorts. de Syllos et al's cohort had diabetic retinopathy for ~4 years more than Awata cohort. Awata et al's cohort also appear to have an abnormally low BMI for individuals with T2DM, at ~ 24 kg/m².

1.2.8: Retinopathy:

Awata et al., suggest that the down-regulation of basal eNOS expression resulted in the macular oedema (caused via protein deposits and fluid build-up under the macula) which leads to a degradation of the blood-retina barrier. Larger cohorts are required to definitively explore the effects of eNOS genetic variation on retinal status. If the retardation of eNOS is limiting NO· availability through the traditional NOS pathway, this may reduce its vasodilatory effect in the microvasculature. Habitual supplementation of NO₃⁻ could alleviate the damage by providing a pool of NO₂⁻ for later conversion to NO· in the microvasculature of the retina along with other micro vessels. Retinopathy is the changes in the microvasculature of the retina. Initially the changes are characterised by increased vascular permeability which can progress to proliferative diabetic retinopathy (PDR) and eventually blindness. PDR is characterised by growth of new blood vessels on the retina and vitreous with an increase in blood flow (Patel et al., 1992). Macular oedema caused by retinal thickening is a common development with this disease (Fong et al., 2004). Within the

first two decades of diagnosis > 60% of individuals with T2DM have retinopathy (Fong et al., 2004). The greatest predictor for diabetes related retinopathy and progression is the duration of diabetes. As the years of disease increase so does the incidence of retinopathy, after 3 years there is a 8% prevalence which increased to 25% after 5 years and 60% at 10 years (Klein et al., 1984).

1.2.9: Nephropathy:

Diabetes related nephropathy (DRN) is a progressive disease that affects the kidneys. It is caused by angiopathy of the capillaries within and surrounding the glomerulus (Christensen, 1971). DRN affects approximately 40% of all individuals with T2DM (Parving et al., 2001) with 5-10% of individuals with T2DM newly diagnosed with microalbuminuria each year (Ravid et al., 1993, Nelson et al., 1996, Gæde et al., 1999, HOPE, 2000). Approximately 40% of all end stage renal failure occurs due to diabetes related nephropathy (USRDS, 2012). Patients that have microalbuminuria have a 10-20 fold increased risk of developing nephropathy (Parving, 1996). Glomerular filtration rates may be elevated at diagnosis with T2DM due to hyperfiltration with or without microalbuminuria. Once macroalbuminuria develops the glomerular filtration rate progressively declines (Nelson et al., 1996). Ethnicity has a large impact upon nephropathy rates. However, age doesn't appear to have an impact. African American individuals have the highest risk followed by Asian and Hispanic with Caucasian individuals having the lowest risk factor (USRDS, 2012) with costs rising sharply to treat nephropathy with estimates ranging from \$68,000 to \$88,000 (USRDS, 2012, Foley and Collins, 2007).

1.2.10: Neuropathy:

Neuropathy is a microvascular complication thought to be brought about by damage to blood vessels that supply blood to nerves. Approximately 10% of individuals with

T2DM have neuropathy (Said, 2007). The prevalence of neuropathy is believed to increase with poor glycaemic control and the individual's duration of T2DM (Pirart, 1978, TDCCTRG, 1993, Martin et al., 2006). Neuropathy is usually a distal symmetric disease accounting for approximately 80% of all cases (Palumbo et al., 1978, Freitas et al., 1992). Progression of polyneuropathy can also be triggered by amyloid polyneuropathies (Said, 1981). Competitive inhibition of the breakdown of amyloid- β in individuals with T2DM, by insulin degrading enzyme, occurs predominantly due to hyperinsulinemia. In individuals with an impaired insulin response (Qiu and Folstein, 2006), this process may lead to increases in amyloid related polyneuropathies that are diagnosed as diabetic neuropathy in individuals with T2DM.

1.2.11: Conclusion:

T2DM metabolic disease with a rising prevalence that affects hundreds of millions of people worldwide. Attenuating sedentary time in individuals with T2DM appears to improve markers of metabolic health (Falconer et al., 2015). Therefore, strategies aimed at making exercise easier (thus removing barriers to exercise and fatigue) or reducing hyperglycaemia are needed to help combat the economic burden and improve quality of life in individuals with T2DM.

1.3: Introduction to chronic obstructive pulmonary disease

1.3.1: Background:

Chronic obstructive pulmonary disease (COPD) is an umbrella term given to group of diseases; emphysema, chronic bronchitis and chronic obstructive airways disease (Sin and Vestbo, 2009). Chronic obstructive pulmonary disease (COPD) is characterised by constricted airflow that is treatable but not entirely reversible, with a gradual decline in function (Kirkham and Rahman, 2006). Individuals with COPD typically report: exertional breathlessness, a chronic cough, regular sputum production, bronchitis and wheezing (GOLD., 2011). Multiple factors lead to relative hypoxemia in individuals with COPD such as reduced cardiac function (Jardin et al., 1984), muscle deconditioning (Maltais et al., 2014), irregular pulmonary blood flow distribution leading to incomplete gas exchange (Marshall et al., 1994) and cor pulmonale. These manifestations often lead to a significant reduction in quality of life (DiBonaventura et al., 2012) and feelings of breathlessness and fatigue (Nici et al., 2006). This review will consist of a brief introduction, demographics and economic burden, causes of the disease, COPD and exercise, pulmonary hypertension, dynamic hyperinflation and treatments.

1.3.2: Demographics and economic burden:

It is projected that by 2020 COPD will be the third most common cause of death worldwide (GOLD., 2011), due to an ageing population which have more exposure to COPD risk factors (Mathers and Loncar, 2006). There are approximately 3 million people living in the UK with COPD. However, 2 million of these remain undiagnosed (Healthcare-Commission, 2006). COPD accounts for 30,000 deaths a year in the UK with ~90% of these deaths occurring in those over 65 year olds (DoH, 2009). COPD accounts for 23% of all respiratory deaths in the UK (Society, 2006). There is a

substantial economic burden to society as a result of COPD. The Chief Medical Officer for England and the United Kingdom's governmental Chief Medical Advisor reported that COPD accounts for ~£800 million in direct health care costs (DoH, 2005), with other reports estimating the cost to be as high as £930 million (Society, 2006). The healthcare commission also states that more than 50% of these costs are related to direct provision of care, and as such it is the single most costly provision of care within hospitals (Healthcare-Commission, 2006). The total economic cost of COPD is much larger given that it accounts for the loss of 24 million working days in the UK each year (Healthcare-Commission, 2006).

1.3.3: Chronic bronchitis & emphysema:

Chronic bronchitis is a disease defined by bronchial hypersecretion and a cough which is present for a minimum of 3 months at a time in at least two consecutive years (MRC, 1965). However, not all patients with chronic bronchitis will develop airflow limitations (Vestbo and Lange, 2002). Chronic bronchitis is associated with a chronic secretion of mucus combined with a progressive decline in lung function. Chronic bronchitis increases incidences of hospitalisation and mortality (Vestbo et al., 1996). Chronic bronchitis is predominantly caused by smoking. Evidence following on from the clean air act of 1956 which improved urban air quality dramatically has shown an associated reduction in the incidence of COPD and its associated diseases.

As the disease progresses the alveoli are broken down which leads to large voids within the lungs, drastically reducing surface area for gas exchange (Snider, 1985). The pathology of emphysema involves the infiltration of alveoli by neutrophils, macrophages and lymphocytes (Taraseviciene-Stewart and Voelkel, 2008). Emphysema is also known to create a loss of elastic recoil within the lungs which is

caused by activated macrophages which release MMP-12 and neutrophil elastase which breaks down the elastin matrix between the alveoli (Mercer et al., 2004). Other cells such as epithelial and endothelial cells can also produce and secrete proteases (Mercer et al., 2004, Taraseviciene-Stewart et al., 2005). Subsequent scarring to lung tissue and a reduction in elastic recoil causes hyperinflation which causes major limitation to gas exchange (O'Donnell and Laveneziana, 2007). As the physiological drive to expire CO₂ rises and demand for O₂ increases, ventilation will increase (Fishman et al., 1955). The air enters the lung as the rib cage expands and pulls the scarred connective tissues. When the individual breathes out the air becomes trapped as the proximal and distal airways collapse. When the air becomes trapped the ability to breathe off CO₂ decreases which in turn will increase ventilation and create a vicious cycle of air trapping (Fishman et al., 1955).

1.3.4: COPD assessment:

Severity or grade of COPD is accessed via the measurement of the forced expiratory volume in the first second (FEV₁ (GOLD., 2011)). The speed at which FEV₁ declines is a good indicator of survival (Anthonisen et al., 1986). The authors also showed that those individuals with a lower FEV₁ at the beginning of the trial had the smallest reduction in FEV₁ relative to baseline. Moreover, with individuals with relatively preserved FEV₁ their speed of reduction correlated well with their response to a bronchodilator (Anthonisen et al., 1986). This landmark study also demonstrated an average reduction in FEV₁ of 44 ml/year over three years (n = 985). The severity of dyspnoea correlated significantly with Quality of Life (QoL) as measured via a health related questionnaire (SF-36) but not significantly with FEV₁ (Mahler and Mackowiak, 1995). This suggests that as FEV₁ does not correlate well with the QoL of an individual (Mahler and Mackowiak, 1995), a qualitative approach should be

considered when assessing QoL in individuals with COPD. This may be due to COPD being a multisystem disease.

1.3.5: Smoking and oxidative stress:

The recent report (GOLD., 2011) looking at the effects and causes of COPD has emphasised the impact of 'noxious particles and gases' and recently defined COPD as "a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases" (GOLD., 2011). COPD has been linked to elevations in oxidative stress within the lungs (Roy et al., 2007, Langen et al., 2003). Experimental research provides evidence that oxidants play a critical role in the pathogenesis of COPD. The principal contributory factor to the increase in oxidants is widely accepted to be cigarette smoke (Ciencewicki et al., 2008). Of all COPD related mortality, 85% is attributed to smoking (Healthcare-Commission, 2006). Cigarette smoke and smoke in general is known to have high concentrations of reactive oxygen species (ROS) (Chen et al., 2015). These ROS are thought to upregulate a transcription factor, NF- κ B, known to be involved in inflammation (Li et al., 2009). Increases in ROS will therefore increase the inflammatory response and lead to alveoli breakdown. However, cigarette smoke is not the only cause of COPD. COPD can be caused via exposure to occupational air pollution for example, from burning wood or other biofuels (Salvi and Barnes, 2009). This is important because there is a delicate balance within the body between ROS and antioxidants termed as a redox balance. When this balance is shifted past the antioxidant capacity, ROS cause damage to proteins, lipids and DNA (Ciencewicki et al., 2008). ROS are also endogenously produced in epithelial and inflammatory cells

in response to cellular damage/injury and metabolism of O₂ (Ciencewicki et al., 2008, Thannickal and Fanburg, 2000).

1.3.6: Smoking and exercise:

Reduced exercise tolerance is often found in individuals with COPD. The reduction in exercise tolerance is not a linear relationship dependent upon the number of cigarettes smoked (Wüst et al., 2008). This suggests that it is either an acute effect or, that after a certain number of cigarettes, the amount of intolerance to exercise that an individual experiences will reach a plateau (Wüst et al., 2008). This reduction in tolerance to exercise could simply be due to a sedentary lifestyle which is often linked to smoking (Larsson et al., 1998). Approximately 80% of individuals diagnosed with COPD are current or were former smokers. The only known way to slow the decline in pulmonary function is smoking cessation. Aggressive smoking interventions can significantly slow the decline in FEV₁ (Anthonisen et al., 1994). The majority of the reduction in FEV₁ decline was shown within the first year and the individuals who stopped smoking for the longest periods (within the year) experienced the greatest pulmonary function protective effects. Other medications which are usually utilised for asthmatic relief are used to ease the symptoms of the disease such as mucolytic agents, corticosteroids or bronchodilators (GOLD., 2011).

1.3.7: Impact of pulmonary hypertension, and dynamic hyperinflation on exercise:

The formation of abnormal pulmonary blood vessels in individuals with COPD creates hypoxia via reduced gas exchange (O'Donnell and Laveneziana, 2007). Subsequent to these deleterious effects a diminished O₂ saturation occurs which contributes to pulmonary hypertension via pulmonary vasoconstriction (Hopkins & McLoughlin, 2002). Pulmonary hypertension is defined by a resting mean pulmonary artery pressure (mPAP) of 20-25 mmHg. The mPAP can be used as a prognostic

tool. For mPAP > 25, the 5 year survival rate is 36% compared to < 25 mmHg is 62% (Oswald-Mammosser et al., 1995). The mPAP has been treated effectively via O₂ therapy (Zieliński et al., 1998) and NO· inhalation (in rats) (Kouyoumdjian et al., 1994). Over time pulmonary hypertension leads to increasing vascular damage and further decreasing lung function (Hopkins and McLoughlin, 2002). Pulmonary hypertension is strongly associated with COPD, especially in the advanced stages of the disease (Barberà and Blanco, 2009, Barberà et al., 2003). Pulmonary hypertension is also a key predictor of mortality in COPD (Burrows et al., 1972), and can have significant implications for morbidity (Chaouat et al., 2005). There are conflicting views as to the prevalence of pulmonary hypertension depending on which of these values are used. The occurrence of pulmonary hypertension can range from 20% to 91% of the COPD cohort (Oswald-Mammosser et al., 1991, Weitzenblum et al., 1984, Scharf et al., 2002, Thabut et al., 2005).

Tibetans appear to be able to utilise NO· to control hypoxia (due to altitude) via an elevated blood flow without the negative effects of pulmonary hypertension or vascular resistance (Erzurum et al., 2007). Non-indigenous people who go to high altitude have higher mPAP which impedes NO· bioavailability (Bailey et al., 2010a). Other research groups have suggested that high endogenous NO· levels are correlated with less severe injury within the lungs and may have a protective effect for pulmonary and systemic endothelium and may act as a protective function for acute lung injury (McClintock et al., 2007).

The significance of pulmonary hypertension to individuals with COPD can be stark. Pulmonary hypertension is usually stable when patients are assessed at rest. However, pulmonary hypertension is exacerbated during exercise (Thabut et al., 2005). Blood pressure rises during exercise which can increase the pressure of

blood moving through the lungs. The severity of pulmonary hypertension has a direct effect on exercise performance in the 6 minute walk test (6MWT) (Cuttica et al., 2010, Sims et al., 2009). Individuals with COPD, with or without pulmonary hypertension, may have a significant overlap within measures of lung function such as: S_pO_2 , peak O_2 uptake, ventilatory efficiency, maximal ventilation and spirometry levels has been previously demonstrated (Holverda et al., 2008). Reduction in S_pO_2 at rest which exacerbate when exercising have a clear correlation with pulmonary hypertension (Holverda et al., 2008).

When individuals with COPD exercise they can have a further hyperinflation known as dynamic hyperinflation. End-expiratory lung volume increases due to a superimposed increase above the hyperinflation at rest (Marin et al., 2001, O'Donnell et al., 1998, Gallagher, 1994).

1.3.8: COPD, exercise and medicines:

COPD is not only a pulmonary disease, but also a systemic disease. Impairments in FEV_1 do not account for all of the limitations associated with COPD (Roy et al., 2009). Diminished functional capacity is brought about via a higher resting O_2 requirement and lower peak $\dot{V}O_2$ (Gosker et al., 2003). The elevated energy requirement at rest represents a much higher fraction of their maximal exercise capacity (Gosker et al., 2003) and thus patients experience greater physiological stress during exercise. Other reasons for a reduced exercise tolerance are muscular atrophy, fibre type plasticity (changes from slow to fast) (Wüst and Degens, 2007), changes in pulmonary blood flow distribution (Marshall et al., 1994) and cardiac de-conditioning (Maltais et al., 2014).

Some improvements in exercise capacity have been shown following structured exercise programs (Griffiths et al., 2000, Cockcroft et al., 1981). To date, three processes have been shown to aid in this improvement: physical reconditioning (Casaburi et al., 1991), improved ventilatory efficiency (Casaburi et al., 1997) and desensitisation to dyspnoea (Cooper, 2009). Some researchers have also suggested that improvements in ventilatory muscle function could also aid these improvements (Cooper, 2009).

Individuals with COPD can have numerous exercise limiting cardiovascular defects. These consist of:-

(i) An impaired left ventricular function as a direct result of hypoxemia. Hypoxemia creates a reduced pulmonary blood flow (Cooper, 1995) which exacerbates the pulmonary hypertension causing a negative feedback loop.

(ii) There is an increasing amount of experimental evidence that suggests that deconditioning due to a sedentary lifestyle in individuals with COPD brings about markers of fatigue such as elevated blood lactate levels (Cooper, 1995).

(iii) A shift from aerobic to anaerobic metabolism due to a reduced delivery of O₂ to the muscle causes lactate production (Cooper, 1995). This process stimulates increased ventilation which in turn further reduces the time available for exhalations, creating a vicious cycle of air trapping (Cooper, 2001).

Reductions in exercise tolerance are also brought about via feelings of dyspnoea and early onset fatigue. Cooper et al., (2009) showed that a general physical training program elicited an improved feeling of dyspnoea as determined via a visual analogue scale following a 6MWT, whereas inspiratory muscle training had no effect.

However, participants with COPD can become 'acclimatised' to exercise after being deconditioned for a prolonged period which leads to an alleviation of the fear dyspnoea (Agle et al., 1973).

1.3.9: Nitric oxide (NO·) and its effects as a bronchodilator:

NO· is an endothelium derived relaxant factor in the vasculature. NO· and the ROS that develop from this molecule play a critical role in COPD pathophysiology (Bove and van der Vliet, 2006). NO· acts as a bronchodilator (Dupuy et al., 1992) via mediation of the actions of nitrovasodilator drugs such as glyceryl trinitrate and nitroprusside. These vasodilators relax smooth muscle in the airways which subsequently increases production of soluble guanylyl cyclase (sGC) and cyclic guanosine 3' 5' monophosphate (cGMP) (Dupuy et al., 1992, Gruetter et al., 1989). In animal models, inhaled NO· diffuses across the alveolar membrane into the vascular smooth muscle due to its highly lipophilic properties (Shaw and Vosper, 1977) and induces vasodilation, which reduces hypoxia induced pulmonary vasoconstriction (Frostell et al., 1991, Fratacci et al., 1991). However, when NO· comes into contact with haemoglobin a rapid oxidation (Gruetter et al., 1981) occurs which essentially makes the NO· inert due to its oxidation to NO₂⁻, diminishing its potent vasodilatory effects in the systemic circulation. COPD can cause an increase in expired NO· (Clini et al., 2001) compared to healthy controls.

1.3.10: COPD therapies and treatments:

There are numerous therapies used in the treatment of individuals with COPD and its associated co-morbidities, some of which are briefly reviewed below.

Smoking cessation

Smoking cessation is the only known intervention that has been shown to slow the progression of the disease (Barnes, 2003) and therefore is the most important intervention (Pauwels, 2000). An aggressive smoking cessation program can significantly reduce the progressive decline of FEV₁ shown in smokers (Anthonisen et al., 1994), in addition to improving long term prognosis in individuals with mild-moderate COPD (Scanlon et al., 2000).

Broncodilators

COPD is characterised by airway obstruction caused via airway narrowing in combination with cholinergic vagal bronchoconstriction (GOLD., 2011). The ability to use a drug in order to vasodilate the airways is key for day to day activities of individuals with COPD but also to reduce symptoms during exacerbations. There are currently 3 classes of broncodilators in clinical use: anticholinergics, β_2 -sympathomimetic agonists and methylxanthines. All these classes of drugs when administered work via smooth vessel relaxation. The mechanism of action however, is different. Anticholinergics cause antagonism of acetylcholine within the airway smooth muscle (Barnes, 1995) and β_2 -sympathomimetic agonists stimulate β_2 -receptors inducing elevations in cAMP (Lulich et al., 1988), methylxanthines work by inhibiting phosphodiesterase which breaks down cAMP.

Simvastatin & Beta-blockers

Simvastatin, a drug usually used to lower cholesterol, has been shown to reduce pulmonary arterial hypertension (PAH (Sun and Ku, 2008, Girgis et al., 2003)) in animal models (male adult Sprague-Dawley rats) perhaps due to their anti-inflammatory, antioxidant and antithrombotic effects (Wang et al., 2008a). However,

Wilkins et al. (2010) provide evidence that simvastatin does also enhance exercise tolerance, as measured via the 6MWT (n = 42 in a parallel group design).

Beta-blockers are often prescribed for COPD patients with cardiovascular comorbidity. Approximately 27% of individuals with COPD have coronary heart disease (Karoli and Rebrov, 2005). Beta-blockers have been shown to decrease mortality in individuals with severe COPD (Chen et al., 2001) and improve outcomes following a myocardial infarction (Quint et al., 2013). Their use in some patients is limited by bronchoconstriction (Albouaini et al., 2007).

Sildenafil

Sildenafil is used to treat/reduce pulmonary hypertension and secondly it is used to treat erectile dysfunction. Both of these effects are mediated by an inhibition of phosphodiesterase type 5 (PDE-5) enzyme which lowers the activity of PDE-5 via competitive inhibition of cGMP (Corbin, 2004). Therefore, cGMP concentrations rise. Intracellular cGMP levels are key as they play a critical role in the activation of protein kinases, protein phosphorylation and subsequent smooth muscle cell (SMC) relaxation via calcium modulation (Lucas et al., 2000, Wang et al., 2008b). There are a total of 11 known PDE isoenzymes which are all thought to act on specific tissue targets and substrates (Ramani and Park, 2010). Critically, PDE-5 has been a focus for PAH therapeutics due to its high expression with lung tissues, vascular beds and systemic circulation (Francis and Corbin, 2005). Sildenafil inhibits PDE-5 which extends the half-life of cGMP (the intracellular mediator for NO[•] vasodilatory pathways) and enhances SMC relaxation creating an increase in pulmonary vasodilation (Steiner et al., 2005).

Nitric Oxide gas

NO \cdot inhalation has been shown to have both positive and negative effects in individuals with COPD. In individuals with COPD and CVD, it can reduce peripheral vascular resistance (Kieler-Jensen et al., 1994) and it has been shown to increase the exercise capacity of heart failure patients with pulmonary hypertension (Koelling et al., 1998). However, there is evidence to suggest that in some individuals the NO \cdot gas can lead to increased venous return to the lungs which can cause elevated pulmonary capillary pressure and exacerbate pulmonary hypertension in heart failure patients (Ichinose et al., 2004). Furthermore, to gain the greatest beneficial effects, the NO \cdot needs to be administered with a small amount of O $_2$ (Yoshida et al., 1997). This has been shown to enable a greater PaO $_2$ in comparison to breathing O $_2$ alone or when mixed with air (Barberà et al., 1996). O $_2$ and NO \cdot inhalation has subsequently been shown to work over a 3 month period with decreases in pulmonary arterial pressure and pulmonary vascular resistance with increases in cardiac output (Vonbank et al., 2003).

1.4: Aims, objective and hypothesis

Dietary NO_3^- supplementation appears to offer a low cost therapeutic supplement that may alter the O_2 cost of exercise, improve exercise performance, lower blood pressure (in individuals with T2DM and COPD) alter hepatic diffusion and improve glucose homeostasis in healthy young and older adults.

The global aim of this thesis is to assess the therapeutic effects of inorganic dietary NO_3^- supplementation in individuals with type 2 diabetes (T2DM), chronic obstructive pulmonary disease (COPD) and healthy adults.

Specifically, in individuals with T2DM and COPD, will dietary NO_3^- :

1. Lower the O_2 cost of exercise?
2. Improve walking performance?

Additionally, in healthy young and older adults, can an acute NO_3^- rich meal alter:

1. Hepatic diffusion?
2. Affect glucose homeostasis?

Secondary objectives;

1. To assess the effects of dietary NO_3^- on blood pressure in all the above cohorts.

Hypothesis

1. Dietary nitrate supplementation will reduce the oxygen cost of exercise in individuals with T2DM and COPD.
2. Dietary nitrate supplementation will improve walking performance in individuals with T2DM and COPD.
3. Dietary nitrate supplementation will reduce blood pressure in individuals with T2DM and COPD.
4. Dietary nitrate supplementation will increase portal vein velocity, flux and microvascular diffusion in healthy young and older adults.
5. Dietary nitrate supplementation will reduce postprandial plasma glucose concentrations in healthy young and older adults.
6. Dietary nitrate supplementation will reduce blood pressure in healthy young and older adults.

2.0: Methods

2.1: Chemiluminescence

2.1.1: Blood collection and sampling

Blood sampling and processing were identical for all three experimental chapters. Free flowing blood samples for NO_3^- and NO_2^- analysis were drawn into room temperature lithium heparin tubes (Sarstedt S-Monovette, Nümbrecht, Germany). Samples were immediately centrifuged at 3600 rpm for 10 minutes at 4°C. The plasma was then aliquoted into (barcoded eppendorf's for experimental chapter 1) eppendorf's and flash frozen in liquid nitrogen prior to storing in -80°C freezers. Plasma samples were stored with minimal headspace between the sample and lid to minimise $\text{NO}\cdot$ / NO_2^- contamination. Eppendorf's were triple washed and left to dry overnight to remove NO_2^- contamination. Determination of plasma NO_3^- and NO_2^- concentrations were performed on a nitric oxide analyser via ozone chemiluminescence (Sievers NOA 280; Analytix Ltd, Durham, UK).

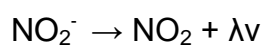
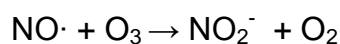
2.12: Deproteinisation

Samples were thawed in batches at room temperature on the laboratory bench. Samples were only used for a single freeze thaw cycle. Samples were completely defrosted prior to analysis (~1 hour) and deproteinised as soon as thawing had occurred. 200 μl of sample was added to the triple washed eppendorf's. 400 μl of 1M sodium hydroxide (NaOH) was added to the eppendorf, vortexed for 1 minute and incubated for 10 minutes (at room temperature). 400 μl of zinc sulphate (ZnSO_4 (10% w/v)) was added and vortexed once more and left to stand for a further 10 minutes. Samples were centrifuged for 5 minutes and decanted into triple washed eppendorfs.

Fresh reagents used in the deproteinisation process were created each day to prevent the accumulation of NO· from the ambient air (Pelletier et al., 2006).

2.1.3: NO₃⁻ analysis

For both NO₃⁻ and NO₂⁻ assays, following their respective reduction of NO_x content to NO·, the NO· reacted with ozone and created nitrogen dioxide, which on creation emits light (via the release of a photon). This luminescence is detected by a thermoelectrically cooled, red-sensitive photomultiplier tube. This tube is held within the chemiluminescence nitric oxide analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK) (see figure 3).



$\lambda\nu$ = photon

O₃ = Ozone

The analysis of NO₃⁻ was performed with an injection of 100µl of deproteinised plasma and performed in duplicate. To analyse plasma NO₃⁻ concentration it was reduced in a stepwise order to NO· via a 5ml solution of vanadium (III) chloride (VCl) in 1M hydrochloric acid (0.8% w/v) at 95°C. To quantify NO· concentration within the plasma samples it was bubbled through the purge vessel (where the VCl reduced NO₃⁻ to NO·), condenser and NaOH trap with an inert gas (nitrogen). The NaOH trap was required in order to neutralise any corrosive gases that had bubbled through with the NO· and nitrogen prior to reacting the analyser.

The effectiveness of the reagents diminished over the course of the assay. For NO_3^- analysis the VCI remained stable for ~12 injections. The reagents reduced NO_3^- at a slower speed. This manifested as an area under the curve that was stable but the peak had lowered. In order to account for this a biological standard was used at the start and end of each assay and the known concentration checked. The assay was accepted if the biological standard was within 10% of the previously determined inter assay mean. Reagents were changed in a timely manner, usually for each assay.

Equation 1: *Plasma NO_3^- reduction*

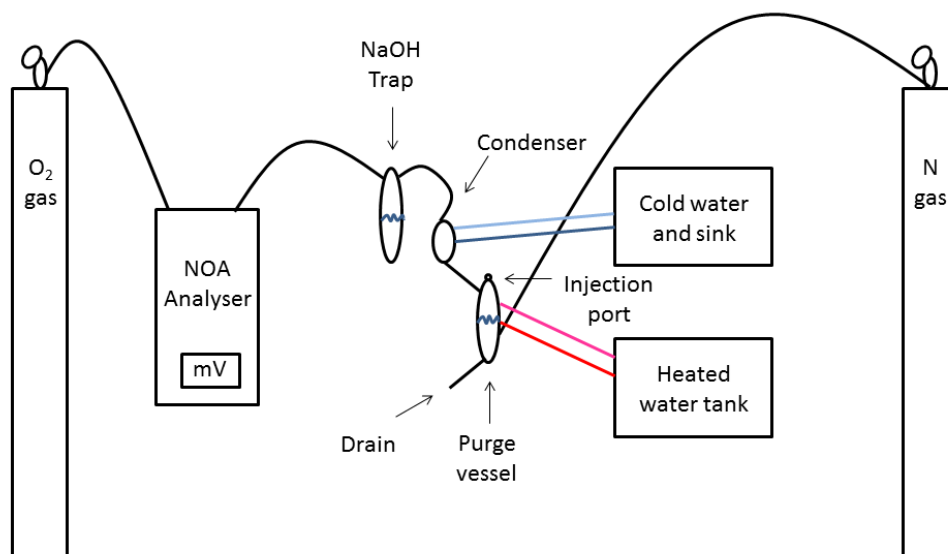
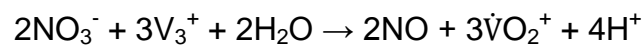


Figure 3. NOA analyser and associated equipment for nitrate reduction.

Depicts the NOA analyser and associated equipment for nitrate reduction to NO . 100 μl of deproteinised plasma was injected in the injection port. The VCI reduced the nitrate and nitrite to NO , where nitrogen bubbles the NO through the purge vessel, condenser, NaOH trap and into the analyser.

2.1.4: NO_3^- assay validation

To ensure that NO_3^- was being accurately measured NO_3^- spiked samples were used. A plasma standard was created by following the blood collection procedures above and subsequently pooling 300 ml of plasma from 3 individuals in a NO_3^- and NO_2^- free sterile beaker prior to aliquoting. Post deproteinisation spiked recovery was used to quantify the concentration of the NO_3^- and NO_2^- concentration in the plasma samples. Two separate, known concentrations of (100 μl) standards (typically 1 μM and 5 μM) were added to separately to different eppendorf's with 100 μl of biological standard prior to deproteinisation. These were vortexed for ~1 minute and deproteinised. 4x 200 μl of plasma was also deproteinised and analysed to compared with the spike recovery concentration and repeated on numerous days. An average of the 4x 200 μl of plasma was taken for the equation.

$$(\text{Average} + \text{standard concentration} / 2) / (\text{concentration of the spiked plasma} \times 100)$$

Equation 2: A working example (spike recovery):

$$((44.7 \mu\text{M} + 20 \mu\text{M}) / 2 = 32.35\mu\text{M}) / (34.55 \mu\text{M} \times 100 = 93.6\%)$$

Initial attempts to perform a spike recovery revealed that the method was either not being reliably executed or that there was contamination. The initial spike recoveries ranged between 140 – 210% of predicted concentration. Standard operating procedure was being strictly followed and therefore it was more likely to be explained by contamination. To get a reliable spike recovery (SR) for the plasma NO_3^- concentration distinct methodological changes were required. Numerous

amendments to the protocol were required in order to establish where the poor repeatability was emanating from. Firstly, the vortex was used for a longer period to ensure the plasma was mixed well with the ZnSO_4 and NaOH . This was done for ~60 seconds and the mixing was confirmed by eye. A visible swirl of the mixture can be seen moving down the Eppendorf. When it reached the bottom of the Eppendorf it was mixed. This maximised the removal of as much protein in the precipitate.

Another key aspect was to use VCI as the purge vessel was warming up. Previously, the vessel was washed with deionised water. The high temperature and the VCI cleaned the vessel of all detergent and debris. Prior to the analysis fresh reagent was added to the reaction chamber. This step allowed for a repeatable standard curve. When these errors were corrected the spike recovery was much more reliable. The final five plasma samples averaged $45.68 \pm 2.91 \mu\text{M}$, the spike recovery with $10\mu\text{M}$ sodium NO_3^- concentration averaged $95.45 \pm 4.1 \mu\text{M} \%$ and the $20\mu\text{M}$ sodium NO_3^- concentration averaged $97.23 \pm 4.6 \mu\text{M} \%$.

Table 2. Nitrate plasma standards and spike recovery reproducibility.

This table depicts the raw values, means, SD and CV for plasma standards, and two different spike recovery concentrations (10 and 20 μM).

Nitrate	Plasma standard μM	SR10	SR20
	48.4	97.3	93
	47.7	95.7	100
	41.9	99.8	102.3
	44.7	89	93.6
	46.7	93.5	N/A
	50.1	N/A	N/A
Mean	46.58	95.06	97.23
SD	2.91	4.09	4.63
CV %	6.252	4.307	4.767

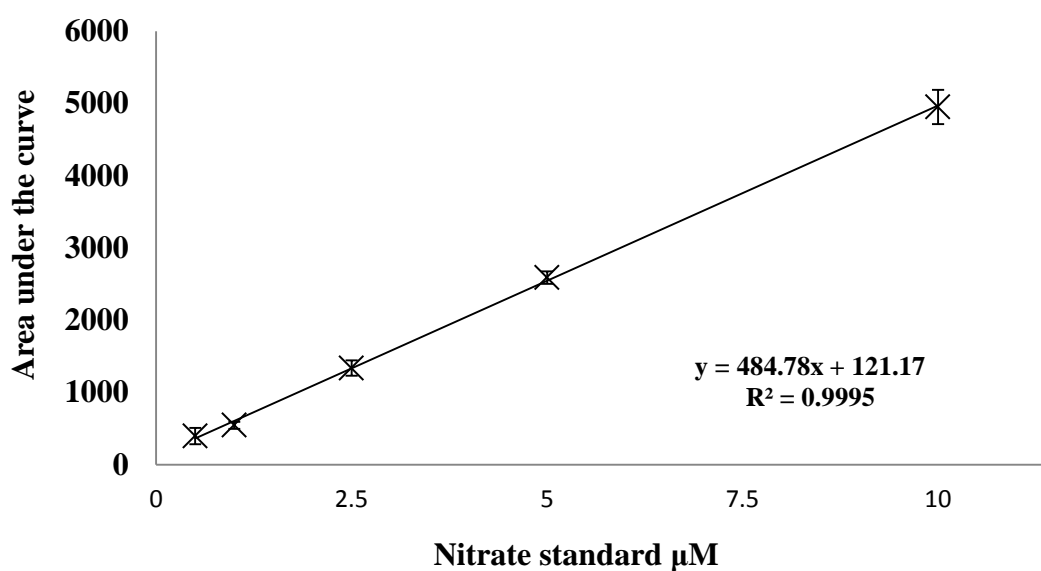


Figure 4. NO_3^- , representative standard curves.

This figure depicts the average \pm SD of 10 representative NO_3^- standard curves

2.1.5: NO_2^- analysis

To analyse plasma NO_2^- concentration it was reduced to $\text{NO}\cdot$ via 5ml glacial acetic acid and 1ml sodium iodide (NaI) (0.5% w/v) solution at 30°C. To quantify $\text{NO}\cdot$ (post reagent reduction) within the plasma samples it was carried through the reduction agents with nitrogen. Note the NaOH trap was not required with this reaction. The analysis of NO_2^- was performed with an injection of 100 μl of deproteinised plasma and performed in duplicate. For NO_2^- , the acetic acid and NaI was able to reduce the NO_2^- to $\text{NO}\cdot$ for longer (~20 injections) with consistent peaks.

Equation 3: *Plasma NO_2^- reduction*

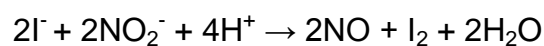


Figure 5. NOA analyser.

Depicts the NOA analyser and equipment used to reduce the NO_2^- to $\text{NO}\cdot$.

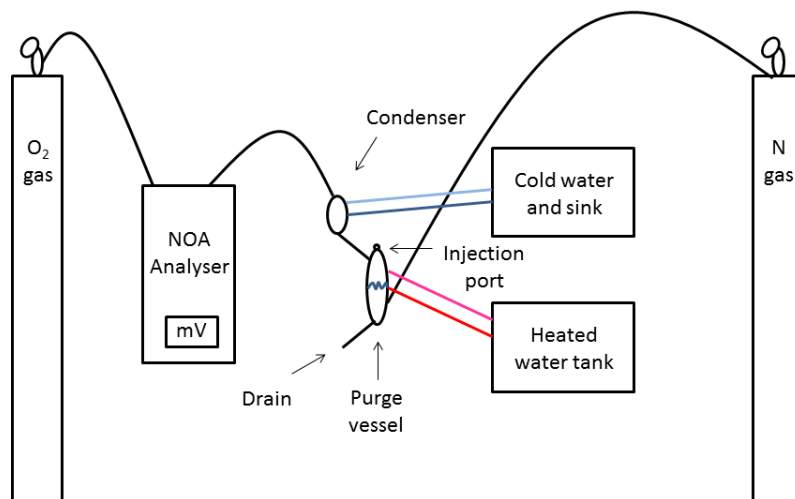


Figure 6. NOA analyser and associated equipment.

Shows the NOA analyser and associated equipment for nitrite reduction to NO^\cdot . $100\mu\text{l}$ of deproteinised plasma was injected in the injection port. The NaI reduced the nitrite to NO^\cdot , where nitrogen bubbles the NO^\cdot through the purge vessel, condenser and into the analyser.

To quantify the concentrations of NO_3^- and NO_2^- standard curves were created daily using sodium NO_3^- at known concentrations of 500nM to $10\mu\text{M}$ and sodium NO_2^- from 10nM to 250nM and the luminescence was plotted via signal area in mV. At least 5 points were used to create each curve.

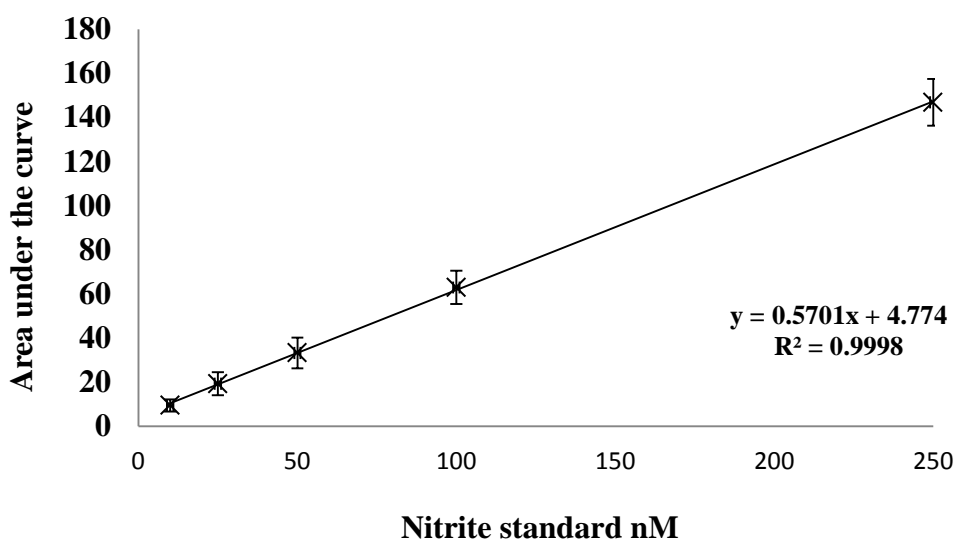


Figure 7. Nitrite, representative standard curves.

This figure depicts the average \pm SD of 10 representative NO_2^- standard curves.

Table 3. Nitrite plasma standards and spike recovery reproducibility.

Table depicts the raw values, means, SD and CV for plasma standards, and two different spike recovery concentrations (100 and 250 nM).

Nitrite	Plasma standard nM	SR100	SR250
	508	118	93
	502.5	127	102
	518.5	119	94
	571	117	100
	547	132	Missing value
	493	111	118
Mean	523.33	120.67	101.40
SD	28.90	6.58	4.43
CV %	5.522	5.453	4.364

2.2: O₂ uptake measurement and analysis

For experimental chapter 3 and 4, breath-by-breath pulmonary gas exchange and ventilation were measured during walking (MedGraphics, CardiO₂ Cardiopulmonary Diagnostic Systems, St. Paul, MN, USA) and cycling exercise (VmaxTM Encore, Yorba, Linda, CA) respectively. Before each session the O₂ and CO₂ analysers were calibrated using known concentration gases. The volume transducer was calibrated using a 3-litre syringe (Hans Rudolph, Kansas City, MO, USA).

Participant's breath-by-breath $\dot{V}O_2$ data were initially checked for 'erroneous' breaths. These breaths can be caused by coughing and swallowing and were

removed (Lamarra et al., 1987). Breaths over 4 standard deviations from the local mean were removed. For each individual breath-by-breath data were time aligned and interpolated for each transition to give second by second values (chapter 3 had 3 transitions and chapter 4 had two). A nonlinear least squares algorithm was then used to fit the ensemble-averaged data, as described in the following equation.

Equation 4: Nonlinear least square algorithm:

$$\dot{V}O_2(t) = \dot{V}O_{2 \text{ baseline}} + A_p(1 - e^{-(t - TD_p)/\tau_p})$$

where $\dot{V}O_2(t)$ represents the absolute $\dot{V}O_2$ at a given time t ; $\dot{V}O_{2 \text{ baseline}}$ represents the mean $\dot{V}O_2$ in the baseline period (when participants were stood still on the treadmill or when cycling at 0 watts); A_p , TD_p , and τ_p represent the amplitude, time delay, and time constant, respectively, describing the increase in $\dot{V}O_2$ above baseline when exercise had commenced. The end-exercise $\dot{V}O_2$ was defined as the mean $\dot{V}O_2$ measured over the final 30 seconds of exercise. The mean response time (MRT) was calculated by fitting a single exponential curve to the data from the onset to the end of exercise.

Equation 5 Nonlinear least square algorithm (without TD):

$$\dot{V}O_2(t) = \dot{V}O_{2 \text{ baseline}} + A_p(1 - e^{-t/\tau_p})$$

The O_2 deficit was calculated as the product of the $\dot{V}O_2$ response amplitude (i.e. the baseline to the point that a steady state was attained) and the MRT. The coefficient of variation for day to day variability of O_2 uptake for treadmill walking using the MedGraphics equipment can be seen in table 4.

Table 4. $\dot{V}O_2$ repeatability data.

This table shows day to day $\dot{V}O_2$ repeatability. Data from 1 individual after interpolation and modelled as described above for 3 transitions, with the mean, SD and coefficient of variation calculated over 3 separate days.

	Time constant	MRT	O_2 deficit	Baseline	Amplitude	End $\dot{V}O_2$
Day 1	42.35	47.62	0.86	296.15	1488.17	1744.62
Day 2	42.18	48.40	0.84	306.88	1467.04	1737.20
Day 3	41.82	48.92	0.78	327.62	1357.33	1641.72
Mean	42.12	48.31	0.83	310.21	1437.51	1707.85
SD	0.22	0.54	0.03	13.06	57.35	46.86
CV %	0.52	1.11	3.99	4.21	3.99	2.74

For the experimental overview, exercise modality and transitions, please see individual experimental chapters.

2.3: The apparent diffusion co-efficient of the liver, portal vein flux and velocity

For experimental chapter 3 a 1.5 T Philips (Amsterdam, The Netherlands) magnetic resonance imaging (MRI) scanner was used in order to examine modifications in velocity and flow to the liver (portal vein) and estimate capillary diffusion in the posterior right lobe of the liver following NO_3^- or placebo supplementation.

The human body is comprised predominantly of water. An MRI scanner creates a homogenous magnetic field which allows protons within hydrogen nuclei to occupy one of two distinct energy states. If radiofrequency radiation is then transmitted at the appropriate frequency it is possible to cause transitions of protons between these

two energy levels. The presence of protons in the excited energy state can be detected via the use of appropriate detector devices and it is this mechanism that gives rise to the means of generating MRI images.

2.3.1: Portal vein (flow & velocity);

Initially, structural scans were obtained in different planes to identify the location and orientation of the portal vein. Subsequently, an 8mm thick imaging slice was selected that lay perpendicular to the long axis of the vein. Landmarks (such as the inferior vena cava) were used to ensure replication of slice positioning between each repeat measurement and for visits 1 and 2. The circumference of the portal vein was checked between all scans from V1 and V2. To determine flow and velocity a cardiac triggered velocity sensitive phase encoding imaging sequence (Gatehouse et al., 2005) was employed which obtained image data at 20 time points throughout the cardiac cycle. Analysis was subsequently undertaken using a package supplied as part of the general philips scanner software. For each separate measurement the circumference of the vessel was manually drawn and recorded for each of the 20 time points to establish a defined region of interest (ROI). Within this ROI, flow and velocity were automatically calculated to give profiles throughout the cardiac cycle.

2.3.2: Apparent diffusion coefficient (ADC);

When examined as a group of voxels, capillary flow represents a pseudo-diffusion process, such that fluid flow is taking place in a 3D isotropic fashion. To examine this, a magnetic resonance (MR) sequence sensitive to flow, via the application of magnetic field gradients in three orthogonal directions, was employed. From this a parameter known as the apparent diffusion coefficient (ADC), dependent upon capillary flow averaged over all directions, could be determined. To calculate ADC within the posterior left lobe of the liver, a ROI (typical dimensions = 2500mm³) was

manually drawn using the scanner software and the signal intensity within determined. ADC was subsequently calculated based upon the ratio of signal intensity from the two images generated from the MR sequence employed, one of which was sensitive to flow, whereas the other had a low sensitivity to flow, where:

Equation 6: ADC:

$$ADC = 1/(b_1 - b_0) \ln(S_0/S_1)$$

S_0 signal intensity in low flow sensitivity image

S_1 signal intensity in flow sensitive image

b_0 magnetic field gradient used in low flow sensitivity image = 250 s/mm²

b_1 magnetic field gradient used in flow sensitive image = 750 s/mm²

2.3.3: ADC and portal vein repeatability

To establish the repeatability of the portal vein velocity (see table 5 below) and ADC measurement (see table 6 below) 6 different, fasted individuals were assessed over two separate mornings. A CV was created between each of the ADC indices and then averaged.

Table 5. MRI reproducibility.

This table describes the CV for the portal vein flux and velocity for 6 fasted individuals on two separate mornings.

	Portal vein flux	Portal vein velocity	ADC
Mean	16.64	13.61	1.15
SD	2.28	2.18	0.12
CV %	13.36	16.366	9.77

Table 6. Description of multiple ROI and its effect on reproducibility.

This table describes the mean, SD and CV for the ADC for 6 fasted individuals on two separate mornings. Different numbers of regions of interests (ROI) were used to find the optimum number to increase repeatability.

	ADC	SD	CV
6 ROI	1.09	0.33	30.32
3 ROI	1.10	0.23	21.34
2 ROI	1.11	0.21	15.29
1 ROI	1.15	0.12	9.77

For study analysis the ADC and the portal vein assessment (velocity and ADC), the baseline scans of each day are compared with 1, 2 and 3 hours post supplementation. See experimental chapter 3 for a description for detailed description of experimental procedures.

2.4: Standardised breakfast

For chapter 5, (experimental chapter 3 / DiMPLe) the participants came to the MRI research centre at St Luke's Campus in a fasted state. Participants were asked to fast from 10pm the night before however water was admissible. Approximately 1 hour after arrival participants were provided with breakfast. Each participant was supplemented (breakfast) with 76g of carbohydrates which is comparable to the glucose dose given in an OGTT (75g(Bartoli et al., 2011b)). The carbohydrate supplement was in the form of 2 beetroot juice sports shots (32g), 2 slices of brown bread (25g), butter (~0g) and jam (19g).

An OGTT test begins with an overnight fast and an acclimatisation period with the patient seated throughout the testing period. A baseline plasma glucose

measurement is then taken. Two hours after the carbohydrate bolus another plasma glucose measurement is taken. In 1997 the American Diabetes Association (Gavin et al., 1997) lowered the fasting plasma glucose measurement cut criteria for impaired fasting glycaemia from 140 mg/dl (7.7) to 126 (6.99mmol/L). In 1979 the national diabetes data group set a distinct cut of value for impaired glucose tolerance which was given following the 2 hour post plasma glucose concentrations for impaired glucose tolerance as 140 (7.7) - 199 mg/dl (11mmol/L) (NDDG, 1979). In the present study (experimental chapter 3), blood glucose concentrations were measured every hour following the standard breakfast for 3 subsequent hours.

2.5: General data handling and methods

Prior to data collection for any of the experimental chapters Microsoft Access (Redmond, WA, USA) databases were set up with all appropriate variables encoded. Data was double entered by an independent researcher. When both data sets were entered and the study complete a macro was run between the files to check for anomalies. All discrepancies were checked within the clinical records folder and amended into one database. Patient identifiable data were entered into a separate database and saved on a non-networked password encrypted hard drive in the diabetes and vascular research centre.

Variables were transposed into SPSS software version 21.0 (Chicago, IL, USA). All data were tested for normality prior to determination of statistical processing (i.e. parametric or non-parametric). Statistical difference was accepted when $P < 0.05$. For specific randomisation blinding and statistical tests please see experimental chapters.

3.0: Experimental chapters

3.1: Experimental chapter 1(Dietary NO₃⁻ supplementation in T2DM)

Title: Effects of dietary nitrate supplementation on the oxygen cost of exercise and walking performance in individuals with type 2 diabetes: a randomised, double blind, placebo-controlled cross-over trial.

3.1.1: Authors: Anthony I Shepherd^{1,2}, Mark Gilchrist², Paul G Winyard², Andrew M Jones¹, Ewelina Hallmann³, Renata Kazimierczak³, Ewa Rembialkowska³, Nigel Benjamin², Angela C Shore², Daryl P Wilkerson¹

3.1.2: Affiliations: College of Life and Environmental Sciences, Sport and Health Sciences, University of Exeter, Devon, UK, ² University of Exeter Medical School and NIHR Exeter Clinical Research Facility, Royal Devon and Exeter Hospital, Exeter, Devon, UK, ³ Warsaw University of Life Sciences, Warsaw, Poland.

3.1.3: Abstract

Background: Dietary NO₃⁻ supplementation has been shown to reduce the O₂ cost of exercise and enhance exercise tolerance in healthy individuals. This study assessed whether similar effects could be observed in individuals with type 2 diabetes (T2DM).

Methods: In a randomised, double blind, placebo-controlled cross-over study, 48 participants with T2DM supplemented their diet for four days with either NO₃⁻ rich beetroot juice (70 ml/day, 6.43 mmol NO₃⁻/day) or NO₃⁻ depleted beetroot juice as placebo (70 ml/day, 0.07 mmol NO₃⁻/day). After each intervention period, resting plasma NO₃⁻ and NO₂⁻ concentrations were measured subsequent to participants completing moderate-paced walking. Pulmonary gas exchange was measured to

assess the O₂ cost of walking. Following a rest period, participants performed the six-minute walk test (6MWT).

Results: Relative to placebo, beetroot juice resulted in a significant increase in plasma NO₃⁻ (placebo; 57 ± 66 vs. beetroot juice; 319 ± 110 μM; *P* <0.001) and plasma nitrite concentration (placebo; 680 ± 256 vs. beetroot juice; 1065 ± 607 nM; *P* <0.001). There were no differences between placebo juice vs. beetroot juice for the O₂ cost of walking (placebo; 946 ± 221 vs. beetroot juice; 939 ± 223 ml·min⁻¹, respectively; *P* =0.59), and distance covered in the 6MWT (placebo; 550 ± 83 vs. beetroot juice; 554 ± 90m, respectively; *P* =0.17).

Conclusion: Nitrate supplementation did not affect the O₂ cost of moderate-paced walking or improve performance in the 6MWT. These findings indicate that dietary NO₃⁻ supplementation does not modulate the response to exercise in individuals with T2DM.

3.1.4: Introduction

Individuals with type 2 diabetes mellitus (T2DM) have profound reductions in their tolerance to exercise compared to healthy individuals (Regensteiner et al., 1995b). The discomfort experienced by individuals with T2DM during exercise may impact upon their ability or willingness to attain their recommended level of exercise, which is a key aspect of disease management (NICE, 2008). Exercise intolerance in this population has been attributed to abnormalities at multiple points during the transport of O₂ from the lungs to its site of utilisation in the muscles (Scognamiglio et al., 1998, Regensteiner et al., 1995a, Regensteiner et al., 1998). There is evidence that individuals with T2DM have reductions in the biosynthesis and bioavailability of the biological messenger nitric oxide (NO·) (Xu et al., 2007, Meininger et al., 2000). NO·

is known to play an important role in muscle contractility, skeletal muscle glucose uptake, calcium handling (Stamler and Meissner, 2001), vascular function, blood flow regulation (Dejam et al., 2007), and mitochondrial respiration and biogenesis (Cooper and Giulivi, 2007).

There are two known pathways by which $\text{NO}\cdot$ is synthesised, the L-arginine pathway and the entero-salivary pathway. The L-arginine pathway involves the synthesis of $\text{NO}\cdot$ from L-arginine by the nitric oxide synthase (NOS) family of enzymes. The entero-salivary pathway involves a stepwise conversion of NO_3^- to NO_2^- , and subsequently, NO_2^- to $\text{NO}\cdot$. Briefly, NO_3^- from the diet (or from $\text{NO}\cdot$ / NO_2^- oxidation) is absorbed into the circulation where it is concentrated in the salivary glands. NO_3^- is then reduced to NO_2^- via facultative anaerobic bacteria on the surface of the tongue (Duncan et al., 1995) and subsequently swallowed. Some of the swallowed NO_2^- is reduced to $\text{NO}\cdot$ in the acidic environment of the stomach, with important local effects on gastric function and protection against enteric pathogens (Gilchrist et al., 2010) and the remainder enters the circulation. Circulating NO_2^- acts as a reservoir for $\text{NO}\cdot$, with its reduction to $\text{NO}\cdot$ potentiated in acidic or hypoxic areas, such as contracting skeletal muscle (Bryan, 2006). It has been suggested that the entero-salivary pathway is a complementary system for $\text{NO}\cdot$ synthesis (Lundberg et al., 2008).

There is extensive evidence in healthy individuals that NO_3^- supplementation, via either sodium NO_3^- or NO_3^- rich beetroot juice, increases plasma NO_2^- concentration (Bailey et al., 2009, Lundberg and Govoni, 2004), lowers blood pressure (BP) (Bailey et al., 2010b, Larsen et al., 2006), and reduces the O_2 cost of exercise (Bailey et al., 2009, Larsen et al., 2007a, Wylie et al., 2013a). The reduction in the O_2 cost of exercise has been proposed to be related to a reduction in the ATP cost of muscle

force production (Bailey et al., 2010b), an improvement in mitochondrial efficiency (Larsen et al., 2011), or a combination of both (Bailey et al., 2012).

It has been reported that the bioavailability of NO \cdot is reduced in individuals with T2DM due to increased scavenging and/or reduced synthesis. Reactive oxygen species (ROS) (Crabtree et al., 2011) and hyperglycaemia (Ding et al., 2004) may result in lower L-arginine derived NO \cdot . Further, there is evidence that NO \cdot production is suppressed by NOS inhibitors such as asymmetric dimethylarginine (ADMA) (Abbasi et al., 2001) and caveolin-1 (Catalán et al., 2008), which are elevated in T2DM. Conversely, others have reported increased plasma NO $_3^-$ and NO $_2^-$ concentrations in individuals with T2DM (Ghasemi et al., 2010, Chien et al., 2005), which may indicate a quenching of NO \cdot activity or upregulation of NO \cdot synthesis to counteract resistance. It is possible that dietary NO $_3^-$ supplementation in this population could ameliorate the impact of T2DM on NOS dependent NO \cdot synthesis by increasing the amount of NO \cdot produced via the entero-salivary pathway (Lundberg et al., 2008). We recently reported no effect of dietary NO $_3^-$ supplementation on the 24h ambulatory BP in individuals with T2DM (Gilchrist et al., 2013), but the impact on the O $_2$ cost of exercise, and exercise tolerance in individuals with T2DM remains unknown.

The aim of this study was, therefore, to examine the effects of dietary NO $_3^-$ supplementation (NO $_3^-$ rich beetroot juice) on the O $_2$ cost of exercise and walking performance, and to confirm our previous finding regarding resting BP, in individuals with T2DM. It was hypothesised that, compared to NO $_3^-$ depleted beetroot juice, NO $_3^-$ rich beetroot juice would reduce the amount of O $_2$ required per unit of time to perform moderate-paced walking, and increase the distance covered in the 6-minute walk test.

3.1.5: Methods

Patients

Forty eight patients (35 males) with T2DM (see Table 7 for characteristics) volunteered to participate in this randomised, double blind, placebo-controlled cross-over study. Participants were recruited from the NIHR Exeter Clinical Research Facility, Exeter 10,000 cohort, a database of individuals who have consented to be contacted for research. Ethical approval was granted by the Cornwall and Plymouth NRES Committee 12/SW/0118 and the study was registered as a clinical trial on the ClinicalTrials.gov website, ID # NCT02206074. All participants provided written informed consent. Patients were recruited if they had been diagnosed with T2DM (as defined by the WHO) at least five years prior to enrolment in the study and were 35–75 years of age. Patients were excluded from the study if they had significant renal impairment (eGFR <30), uncontrolled hypertension, a BMI <25 or >35 (kg/m²), a history of myocardial infarction or cerebro-vascular event, were taking regular organic NO₃⁻ or nicorandil, or were smokers (or who had stopped smoking within the previous three months). Patients taking phosphodiesterase inhibitors were asked to refrain from doing so for the duration of the study. On experimental days, participants were asked to arrive at the laboratory in a rested and fully hydrated state, at least 3 hours postprandial, and having avoided caffeine and alcohol for 6 and 24 hours, respectively. Participants were also asked to avoid exercise for 24 hours prior to each testing session. Participants were asked to record their diet for 24 hours prior to each experimental visit. Following the crossover the participant were asked to replicate their previous diet and this was verbally confirmed at the second visit.

Table 7. Characteristics of patients included in the final analysis. Data are mean \pm SD, or as a % of the cohort on a medication.

Age (years)	63.35 \pm 7.27
Diabetes Duration (years)	10.31 \pm 5.26
HbA1c (mmol/mol)	60.83 \pm 13.37
Body Mass Index (kg/m ²)	30.17 \pm 2.93
Baseline systolic BP (mmHg)	142.13 \pm 12.84
Baseline diastolic BP (mmHg)	80.85 \pm 7.36
No. of 30+ minutes of exercise per week	3.83 \pm 3.16
Portions of fruit and vegetables per day	3.83 \pm 2.00
Metformin %	83.33
Insulin %	25.00
ARB & ACEi %	47.92
Statins %	83.33

Participants attended the NIHR Exeter Clinical Research Facility where we completed informed consent, a medical history, anthropometric measures, and a resting ECG. A familiarisation session was performed for the treadmill exercise, allowing the determination of the walking speed for each participant that would be used in the experimental visits. The target speed was a pace equivalent to the participants' usual walking speed that could comfortably be maintained for 6 minutes.

Experimental Overview

Following the pre-experimental visit, participants were assigned in a double-blind, randomised, crossover design to consume 70 ml/day of NO₃⁻ rich beetroot juice (beetroot juice; containing 6.43 mmol of NO₃⁻; Beet it, James White Drinks Ltd., Ipswich) or NO₃⁻ depleted beetroot juice (placebo; NO₃⁻ depleted beetroot juice

containing 0.07 mmol of NO_3^- ; Beet it, James White Drinks Ltd., Ipswich) for four days. The dose of 6.43 mmol of NO_3^- equated to $0.072 \text{ mmol.kg}^{-1}$ per day for 3.5 days. Similar dosing regimens (0.068 (Bailey et al., 2010b); 0.064 (Muggeridge et al., 2013b); 0.086 (Berry et al., 2014) mmol.kg^{-1}) to ours have been shown to be effective at this relative dose. Production of the placebo juice has been detailed previously (Lansley et al., 2010), with the final product being indistinguishable in taste, colour, texture, appearance and odour to the NO_3^- rich beetroot juice (Gilchrist et al., 2014). The final 70 ml of juice was consumed ~3 hours before the commencement of exercise on the morning of testing. Participants were required to abstain from using antibacterial mouthwash and chewing gum throughout the study, as this has been shown to reduce the concentration of oral bacteria responsible for the reduction of NO_3^- to NO_2^- (Govoni et al., 2008).

During the two experimental visits, resting BP was measured and venous blood samples were drawn into lithium-heparin tubes (Sarstedt S-Monovette, Nümbrecht, Germany) and prepared for NO_3^- and NO_2^- analysis as previously described (Gilchrist et al., 2013). Subsequently, participants performed 3 bouts of walking on a motorised treadmill (NordicTrack T14.0, Chaska, MN, USA) at the target speed that was determined in the pre-experimental visit. Each bout was preceded by a resting baseline period of three minutes, with the three bouts separated by 15 minutes of passive recovery. Following a further 15 minutes of seated rest, participants performed a 6 minute walk test (6MWT) to assess functional capacity. This test was conducted indoors on a straight, flat course between 2 cones which were 32.2 meters apart, with patients instructed to cover as much distance as possible in the allotted time. Standardised verbal encouragement was given throughout. Following

their first experimental visit, participants began a washout period (10-14 days) before entering the opposing arm of the study.

Measurements

Prior to any exercise testing during the experimental visits, resting BP of the brachial artery was measured using an automated sphygmomanometer (Omron M6, Kyoto, Japan). Five measurements were taken in total, with the mean of the final three measurements being recorded.

For the determination of plasma NO_2^- concentration, venous blood samples (~4 ml) were drawn into lithium-heparin tubes (Sarstedt S-Monovette, Nümbrecht, Germany), centrifuged, aliquoted and immediately frozen in liquid nitrogen and stored at -80°C . Prior to analysis all samples were deproteinized using a variant of the protocol used by Higuchi and Motomizu (1999). Fresh reagents were created each day and checked for contamination. No contamination was detectable for water, sodium hydroxide, zinc sulphate or sodium iodide. Determination of plasma NO_3^- and NO_2^- concentrations was performed on a nitric oxide analyser via ozone chemiluminescence (Sievers NOA 280; Analytix Ltd, Durham, UK) using the protocol described by Bateman et al. (2002).

Pulmonary gas exchange was measured breath by breath during all treadmill walking exercise (MedGraphics Cardio₂ Cardiopulmonary Diagnostic Systems, St. Paul, MN, USA). The volume transducer was calibrated before each test with a 3-liter calibration syringe (Hans Rudolph, Kansas City, MO, USA) and the O_2 and CO_2 analysers were calibrated using gases of known concentration. During treadmill walking exercise, heart rate (HR) was measured every 5 seconds via telemetry (Polar R5400sd, Kempele, Finland).

Data Analysis

The breath by breath $\dot{V}O_2$ uptake ($\dot{V}O_2$) data were initially inspected for errant breaths (e.g. associated with coughing and swallowing), with values lying more than four SDs from the local mean being removed. The breath-by-breath data were linearly interpolated to provide second-by-second values and, for each individual, the three bouts were time-aligned to the start of exercise and ensemble-averaged. A nonlinear least-square algorithm was used to fit the data, as described in the following equation:

$$\dot{V}O_2 (t) = \dot{V}O_{2 \text{ baseline}} + A_p (1 - e^{-(t - TD_p)/\tau_p})$$

where $\dot{V}O_2 (t)$ represents the absolute $\dot{V}O_2$ at a given time t ; $\dot{V}O_{2 \text{ baseline}}$ represents the mean $\dot{V}O_2$ in the baseline period (when participants were stood still on the treadmill); A_p , TD_p , and τ_p represent the amplitude, time delay, and time constant, respectively, describing the increase in $\dot{V}O_2$ above baseline when exercise had commenced. The end-exercise $\dot{V}O_2$ was defined as the mean $\dot{V}O_2$ measured over the final 30 seconds of exercise. The mean response time (MRT) was calculated by fitting a single exponential curve to the data with no time delay from the onset to the end of exercise. The O_2 deficit was calculated as the product of the $\dot{V}O_2$ response amplitude (i.e. the baseline to the point that a steady state was attained) and the MRT. For a schematic representation of the $\dot{V}O_2$ kinetics parameters, see Figure 1.

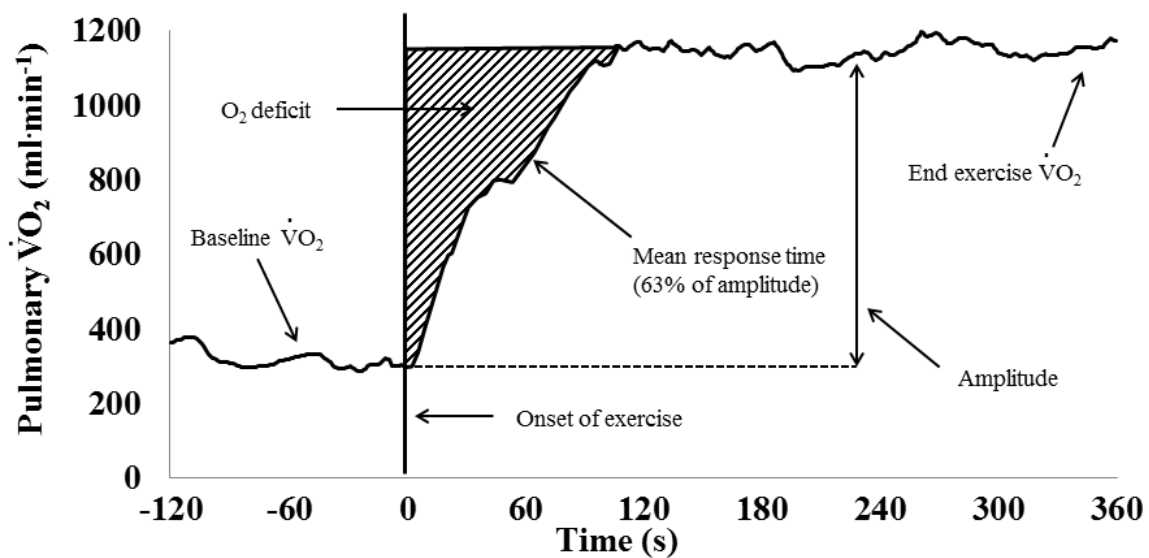


Figure 1. Schematic showing the parameters of the O_2 uptake kinetics on a representative participant's data.

Juice analysis

It has previously been suggested that the antioxidant content of beetroot juice may have beneficial effects (Lansley et al., 2011a). In order to establish whether the concentrations of known antioxidants in beetroot juice (polyphenols and betacyanins) were different in the active and placebo juice, both were analysed using the high-performance liquid chromatography technique according to our previously described methods (Kazimierczak et al., 2014).

Sample size and randomisation

An *a priori* sample size calculation was performed by a statistician from the University of Exeter Medical School. A pilot study ($n=6$) in individuals who have T2DM revealed a mean difference between beetroot juice and placebo juice of $45 \text{ ml}\cdot\text{min}^{-1}$ for end exercise $\dot{V}O_2$ (1SD) during cycling exercise. For 90% power and an α -level set at $P = 0.05$ (two tailed), to detect a 0.5 SD difference 44 patients were

required. In order to account for dropout, we anticipated that 48 patients would begin the study. Each participant was randomised by a research nurse, who also supplied them with the appropriate juice.

Data and statistical analysis

All data were tested for normality and are presented as means \pm standard deviation (*SD*) unless otherwise stated. Differences in plasma NO_3^- and NO_2^- concentrations, BP, $\dot{V}\text{O}_2$, and distance covered in the 6-minute walk test between the conditions were analysed using two-tailed, paired-samples *t*-tests. Where normality of data was not met, differences between the aforementioned data were tested using a non-parametric test (Wilcoxon rank-sum test). When the sample was split for patients taking different classes of medications, independent samples *t*-tests were performed. Pearson product–moment correlation coefficients were used to assess the relationships between variables. Statistical analyses were performed on SPSS software version 21.0 (Chicago, IL, USA), with statistical difference accepted when $P < 0.05$.

3.1.6: Results

48 subjects completed the study. For detailed information regarding participant recruitment and withdrawal, see Figure 8. Participants' self-reported adherence to beetroot juice and avoidance of mouth wash was 100% for both arms of the study. All participants reported similar physical activity and dietary patterns for both supplementation periods. The ingestion of both juices was well tolerated with no adverse side effects. As in previous studies using beetroot juice supplementation, participants reported beeturia (red urine) and red stools (Bailey et al., 2009).

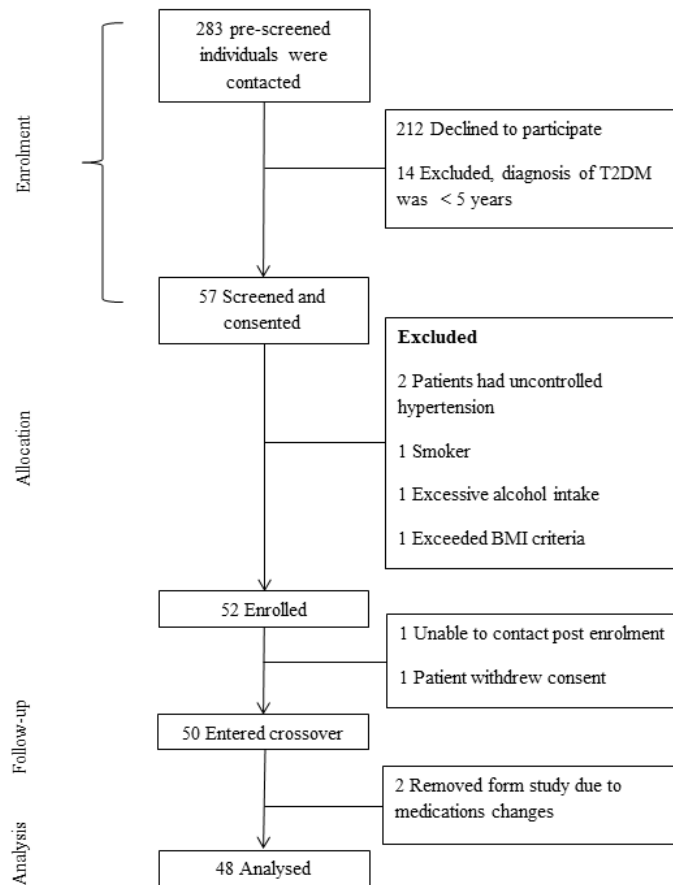


Figure 8. Flow diagram of trial

Plasma NO₃⁻ and NO₂⁻ concentrations: Relative to placebo, beetroot juice resulted in a significant increase in plasma NO₃⁻ concentration (placebo: 57 ± 66 vs. beetroot juice 319 ± 90 μM, $P < 0.001$, 95% CI -220, 302; Figure 9A). Plasma NO₂⁻ concentration also increased for beetroot juice in comparison to placebo (placebo: 680 ± 256 vs. beetroot juice 1065 ± 607 nM, $P < 0.001$, 95% CI -220, 548; Figure 9B). There were no differences in baseline (placebo) plasma NO₂⁻ when subjects were split into groups based on drug classes; ACEi and ARB (n= 25; difference vs. those not on these drugs -68 ± 75 nM, $P = 0.37$, 95% CI -218, 83), metformin (n = 40; difference -18 ± 105 nM, $P = 0.87$, 95% CI -231, 196), insulin (n= 12; difference 3 ± 86 nM, $P = 0.97$, 95% CI -171, 177) statins (n = 40; difference 164 ± 97 nM, $P =$

0.09, 95% CI -31, 360) and sulphonylurea (n = 15; difference 35 ± 134 nM, $P = 0.79$, 95% CI -237, 306). Other classifications of drugs were prescribed, however the sample size was too small to make inferences; gliptins (n = 2), exenatide (n = 2), sulfazalazine (n = 1), alpha blocker (n = 3), beta blocker (n = 1), calcium channel blocker (n = 6), loop diuretic (n = 1), thiazide (n = 2) $P > 0.05$).

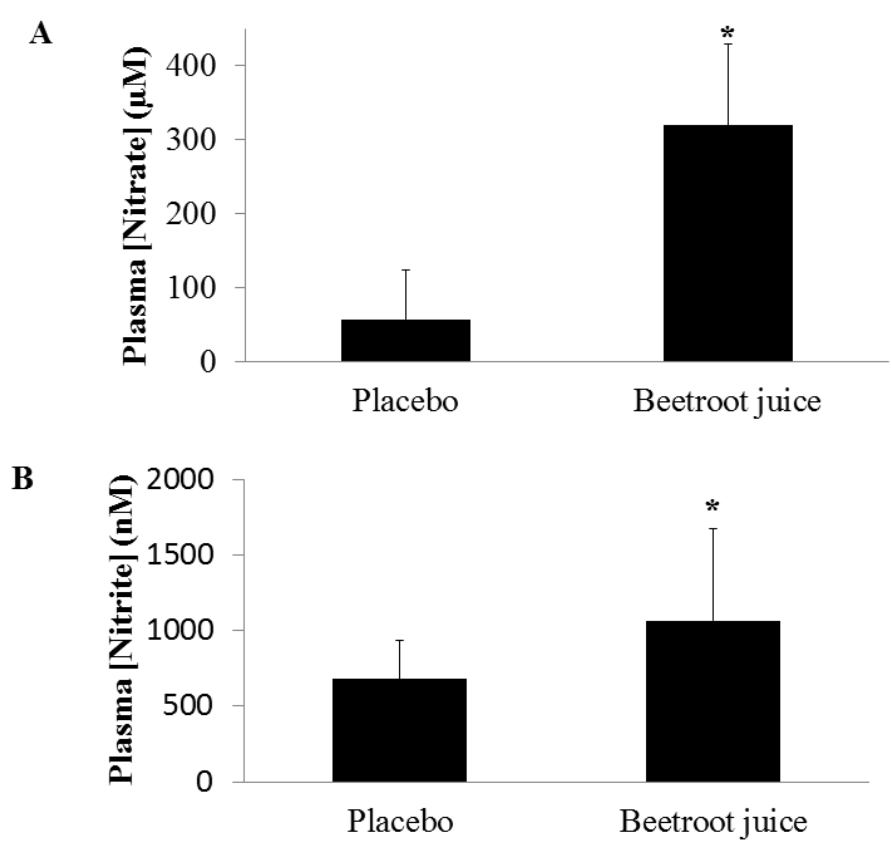


Figure 9. Plasma NO_3^- and NO_2^- concentration in individuals with T2DM.

A depicts plasma NO_3^- and B shows plasma NO_2^- group mean concentrations analysed via chemiluminescence. Values are means \pm SD. *significantly different from the placebo $P < 0.001$.

Treadmill walking, $\dot{V}O_2$ kinetics, and heart rate: Relative to placebo, beetroot juice supplementation had no effect on baseline (placebo: 282 ± 50 vs. beetroot juice: 281 ± 46 ml.min⁻¹; $P = 0.82$, 95% CI -12.9, 10.21) or end-exercise $\dot{V}O_2$ (placebo: 939 ± 223 vs. beetroot juice: 946 ± 221 ml.min⁻¹; $P = 0.60$, 95% CI -20, 34.3; Figure 10). The time constant and MRT of $\dot{V}O_2$ response, and the O_2 deficit were also not different between conditions (see Table 8). End-exercise heart rate was not different between conditions (placebo: 96 ± 12 vs. beetroot juice: 94 ± 11 BPM, $P = 0.36$, 95% CI -4.7, 1.7). No significant correlation was observed between the change in plasma NO_2^- concentration and end-exercise $\dot{V}O_2$ ($r = 0.04$, $P = 0.77$).

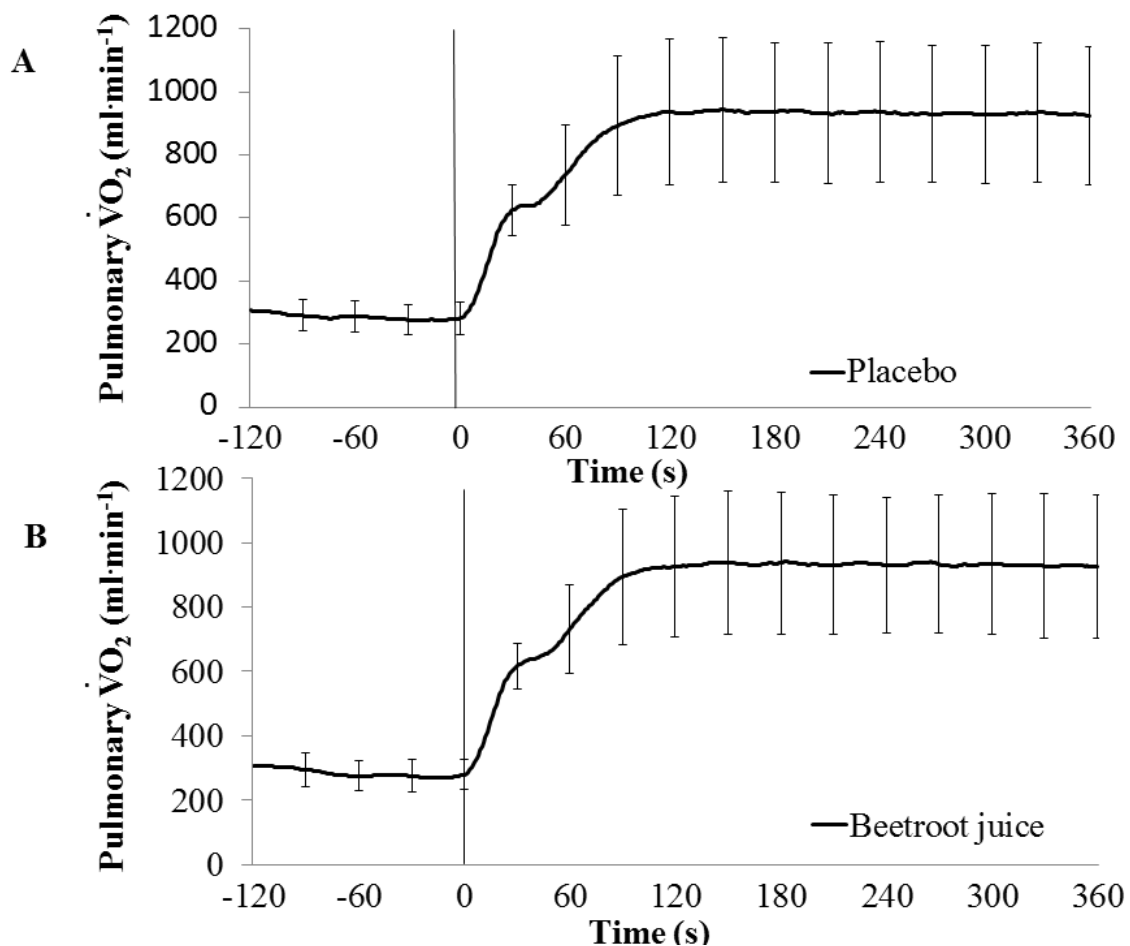


Figure 10. Pulmonary O_2 uptake response to beetroot juice in individuals with T2DM.

The group mean pulmonary O_2 uptake response for placebo (A) and NO_3^- rich beetroot (B). The vertical line represents the initiation of walking exercise from the standing baseline. Group mean responses are shown with error bars every 30s.

Table 8. O₂ uptake kinetics during walking exercise with placebo and beetroot juice supplementation.

	Placebo	Beetroot juice
Baseline (ml·min ⁻¹)	282 ± 50	281 ± 46
Primary amplitude (ml·min ⁻¹)	656 ± 205	665 ± 199
End-exercise (ml·min ⁻¹)	938 ± 223	946 ± 221
O ₂ deficit (L)	0.48 ± 0.17	0.50 ± 0.19
Mean response time (s)	45 ± 10	45 ± 10

Six minute walk test: No difference was found between placebo and beetroot juice for distance covered during the six minute walk test (placebo: 554 ± 90 vs. beetroot juice: 550 ± 83 m, $P = 0.17$, 95% CI -11, 2.04). No difference was observed for distance covered during the six minute walk test when the group was split for ACEi and ARB compared with individuals not on this classification of drug (change -7.6 ± 44.6 m, $P = 0.25$, 95% CI -20.6, 5.4).

Blood pressure: Comparisons between placebo and beetroot juice revealed no statistically significant effect on systolic BP (placebo: 134 ± 10 vs. beetroot juice: 132 ± 12 mmHg, $P = 0.17$, 95% CI -4.09, 1.17) or diastolic BP (placebo: 77 ± 7 vs. beetroot juice: 76 ± 11 mmHg, $P = 0.27$, 95% CI -4.04, 0.74; Figure 11 A and B).

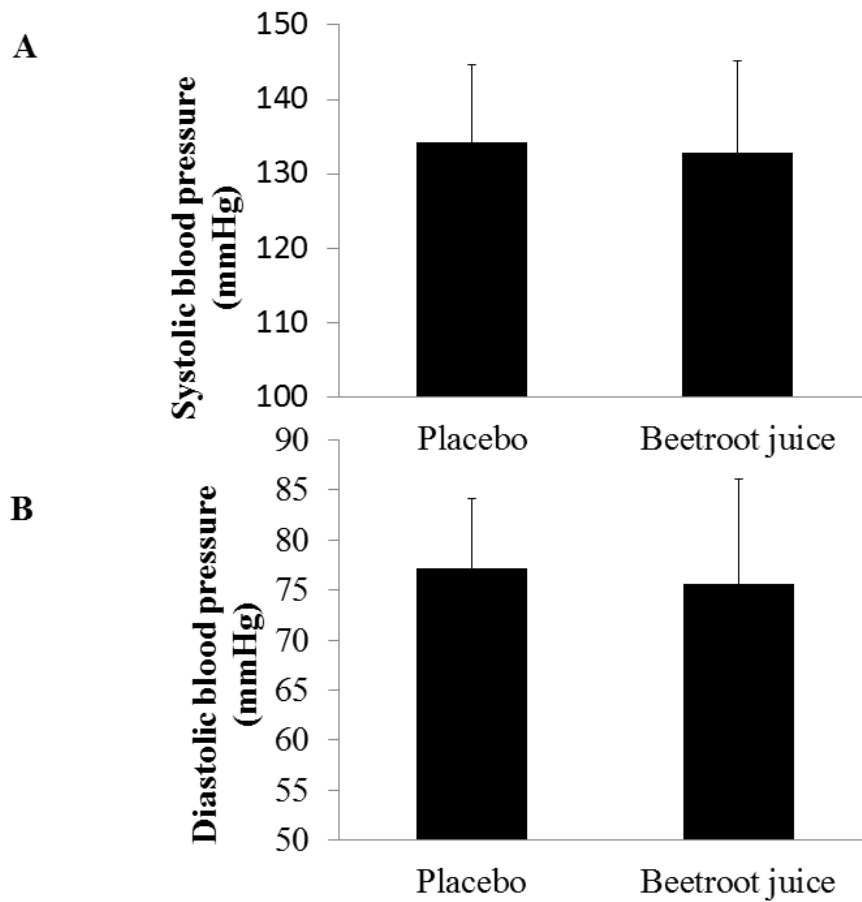


Figure 11. Systolic and diastolic blood pressure response to beetroot juice in individuals with T2DM.

Effects of NO_3^- on blood pressure, A: Groups means for systolic blood pressure and B depicts the group mean for diastolic blood pressure. Values are means \pm SD.

Antioxidants in beetroot juice: Antioxidant concentrations in placebo and active beetroot juice are displayed in Table 9. Betacyanins and polyphenol compounds in beetroot juice.

The content of total betacyanins and polyphenol compounds in two different beetroot juices. Table A, depicts all identified compounds and figure B shows the compound groups. Mean value \pm standard deviation (in mg/100 ml of fresh juice).

A

Identified compound	Beetroot juice	Placebo
Betanin-3-O-glucoside	452.71 \pm 19.56	519.31 \pm 11.43
Betanidine	6.03 \pm 0.40	3.61 \pm 0.19
Gallic acid	1.80 \pm 0.12	1.43 \pm 0.14
Chlorogenic acid	3.03 \pm 0.55	2.10 \pm 0.22
Caffeic acid	0.90 \pm 0.20	0.58 \pm 0.11
Ferulic acid	0.56 \pm 0.00	0.53 \pm 0.01
Rutinoside-3-O-queracetin	1.97 \pm 0.08	1.81 \pm 0.04
Glucoside-3-O-queracetin	0.89 \pm 0.04	0.83 \pm 0.02
Myricetin	0.44 \pm 0.01	0.47 \pm 0.02
Luteolin	0.23 \pm 0.01	0.16 \pm 0.01
Quercetin	0.25 \pm 0.01	0.19 \pm 0.00
Kaempferol	0.22 \pm 0.01	0.17 \pm 0.00

B

Group of compounds	Beetroot juice	Placebo
Total betacyanins	458.73 \pm 19.73	522.92 \pm 11.43
Total polyphenols	10.29 \pm 0.72	8.30 \pm 0.34
Total phenolic acids	6.30 \pm 0.67	4.65 \pm 0.34
Total flavonoids	3.99 \pm 0.10	3.65 \pm 0.05

3.1.7 :Discussion

This is the first study to investigate the effects of dietary NO_3^- supplementation on the exercise responses of individuals with T2DM. The principal findings of this investigation were that short-term dietary NO_3^- supplementation did not reduce the O_2 cost of walking, or increase the distance covered in the six minute walk test in this population. We also confirmed our previous finding that NO_3^- supplementation had no effect on resting blood pressure in individuals with T2DM. These findings may be considered surprising given the compelling effects of dietary NO_3^- supplementation reported in other populations. Possible explanations for the lack of effects in the present study relate to an elevated baseline plasma NO_2^- concentration in the control condition, and/or reductions in the bioavailability of $\text{NO}\cdot$ in individuals with T2DM.

Nitrate supplementation and plasma NO_3^- and plasma NO_2^- concentration.

Plasma NO_3^- and NO_2^- concentrations were significantly elevated following NO_3^- supplementation, which is consistent with previous studies examining young (Bailey et al., 2009, Lundberg and Govoni, 2004) and older healthy participants (Kelly et al., 2013a), and individuals with peripheral arterial disease (Kenjale et al., 2011). The post placebo plasma NO_2^- concentration was approximately two to six-times higher in the present study than those reported in the aforementioned studies. The elevated plasma NO_2^- concentration in individuals with T2DM in the placebo condition may be associated with the habitual up-regulation of iNOS which is endemic in this population (Krause et al., 2012). It is possible that the elevated baseline (placebo) plasma NO_2^- concentration is indicative of disease pathology and mitigates the attainment of the benefits of dietary NO_3^- supplementation that have been reported in other populations.

The plasma NO_2^- concentration was 680 ± 256 nM in the placebo arm in the present study. In the only other study which could be directly comparable, the plasma NO_2^- concentration was 232 nmol/L (200, 265), median (IQR) in the placebo arm (Gilchrist et al., 2013). The control plasma from Gilchrist et al. (2013) was re-measured during the current study to establish agreement between the NO_2^- values measured in the two studies. It is therefore unlikely that analytical error is the reason for our elevated 'baseline' plasma NO_2^- concentrations. There are notable differences between the two studies with respect to the timing of beetroot juice doses and plasma sampling. In the present work, studies were conducted at midday, with subjects having had breakfast including the beetroot juice and their usual morning hypoglycaemic and antihypertensive medications 3 hours previously. In our previous study in subjects with T2DM (Gilchrist et al., 2013), plasma sampling occurred after an overnight fast, with subjects having omitted their usual morning hypoglycaemic and antihypertensive medications, 16 hours after their last beetroot juice.

Multiple agents within these broad classes of medication have been shown to up-regulate eNOS activity (Davis et al., 2006, Ceconi et al., 2007, Andrade et al., 2013). When the current cohort was split for drug classifications, no differences in baseline plasma NO_2^- were detectable. It is therefore unlikely that drug classification affected plasma NO_2^- concentrations, though the study was not powered to detect any such difference. Furthermore, there are data to suggest circadian variation in eNOS activity, with the lowest levels in the morning, rising through the day before falling again at night (Elherik et al., 2002). This could further influence the difference between plasma NO_2^- concentrations in the two groups. With a growing number of studies in patient groups, understanding the effect of concomitant medication on plasma NO_2^- and NO_3^- concentrations is becoming increasingly important.

Accordingly, sufficiently powered research is required to examine the pharmacokinetics and dynamics of medications and NO_x concentrations in order to elucidate the possibility of NO₃⁻ related therapeutic effects on exercise performance.

Nitrate supplementation and pulmonary O₂ uptake

There was no difference between active and placebo juice in the $\dot{V}O_2$ responses to low intensity walking exercise in the present study. A reduction in the baseline (Lansley et al., 2010) and steady state (Bailey et al., 2009, Vanhatalo et al., 2010, Lansley et al., 2010) O₂ cost of low intensity exercise has previously been reported in young healthy individuals following similar NO₃⁻ dosage regimens to that which was implemented in the present study. No change in the O₂ cost of exercise was reported in healthy older individuals following NO₃⁻ supplementation (Kelly et al., 2013a). However, these authors reported a significant speeding of the $\dot{V}O_2$ kinetics, something that was not observed in the present study. It is therefore unlikely that aging *per se* explains the lack of effect on the $\dot{V}O_2$ kinetics in the present study. Other studies have reported no significant difference in the $\dot{V}O_2$ response to exercise subsequent to NO₃⁻ supplementation in healthy, well-trained athletes (Wilkerson et al., 2012, Bescos et al., 2011). This lack of effect has also been reported in individuals with COPD (Shepherd et al., 2015, Berry et al., 2014, Kerley et al., 2015). These studies all had relatively small sample sizes (n = 8 (Wilkerson et al., 2012), 11 (Bescos et al., 2011, Kerley et al., 2015), 13 (Shepherd et al., 2015), and 15 (Berry et al., 2014)).

One possible explanation for the lack of effect on the O₂ cost of exercise may relate to the elevated plasma NO₂⁻ concentration in the placebo condition (baseline) of this study which might reduce the scope for the further increases in plasma NO₂⁻

concentration that have been realised via NO_3^- supplementation in other populations. Thus the lack of effect of dietary NO_3^- supplementation on the O_2 cost of exercise noted in the present study may indicate the existence of an upper limit for the baseline plasma NO_2^- concentration, beyond which the scope for positive effects from further increasing plasma NO_2^- concentration is reduced. In support of this suggestion, a significant negative correlation has been reported between the changes in plasma NO_2^- concentration with beetroot juice vs. placebo juice and the change in performance during cycling exercise (i.e. participants whose plasma NO_2^- increased more following NO_3^- supplementation experienced a greater improvement in exercise performance, and *vice versa*) (Wilkerson et al., 2012). However, in the current study there was no correlation between either placebo plasma NO_2^- concentration, or change in plasma NO_2^- concentration with NO_3^- rich vs. placebo juice, and the O_2 cost of exercise.

An alternative suggestion for the lack of effect of NO_3^- supplementation on the O_2 cost of exercise may be related to the reduction in the bioavailability of $\text{NO}\cdot$ which has been reported in individuals with T2DM (Calver et al., 1992, Cockcroft et al., 2000, Cohen, 1993). This reduced bioavailability has been linked with the concentration of plasma ADMA (an analogue of L-arginine), which is elevated in individuals with T2DM (Devangelio et al., 2007). ADMA is known to inhibit all three NOS isoforms, particularly eNOS (Siasos et al., 2007), leading to eNOS uncoupling and the generation of superoxide radicals (Tousoulis et al., 2007), ultimately resulting in increased oxidative stress. When $\text{NO}\cdot$ and superoxide react, peroxynitrite is generated. This is known to result in a quenching of $\text{NO}\cdot$ activity and thus a reduction in its bioavailability, which may serve to diminish any positive impact of NO_3^- supplementation (Beckman and Koppenol, 1996, Coppey et al., 2001).

Furthermore, sustained exposure to oxidative and nitrative stress could result in damage to mitochondrial membranes (Kowaltowski and Vercesi, 1999) and thus reduce the P/O ratio (i.e more O₂ would be required to produce a given amount of ATP). It is possible that damaged mitochondria associated with T2DM (Kowaltowski and Vercesi, 1999) means that this population are less likely to benefit from the improved mitochondrial function subsequent to NO₃⁻ supplementation, which may underpin, in part, the reduced O₂ cost of exercise in healthy individuals (Cooper and Giulivi, 2007).

A further possible explanation for the lack of effect of NO₃⁻ supplementation on the exercise response in individuals with T2DM may relate to pathological consumption of NO₂⁻ during exercise. Post-exercise plasma NO₂⁻ concentration has been shown to fall markedly in individuals with cardiovascular risk factors (Rassaf et al., 2010) compared with the typical rise in plasma NO₂⁻ reported in healthy individuals (Cuzzolin et al., 2000). This may suggest that either the production of NO· cannot keep up with metabolic demand, or the NO· produced is scavenged more rapidly in individuals with cardiovascular risk factors compared with healthy individuals. It is plausible that a similar effect is seen in individuals with T2DM, with a marked net consumption of plasma NO₂⁻ during exercise. It should be noted, however, that this would not explain the lack of effect of NO₃⁻ supplementation on BP in our study as these measurements were conducted at rest.

It should be noted that although we have postulated a pro-oxidant state as an explanation for the lack of effect of NO₃⁻ supplementation in individuals with T2DM, beneficial effects of NO₃⁻ have been seen in other patient groups who are likely to be in a pro-oxidant state. Zamani et al. (2015) reported an improvement in exercise capacity (but no reduction in the O₂ cost of exercise) following beetroot juice

supplementation in individuals with heart failure. Similar findings have been reported in individuals with peripheral artery disease (Kenjale et al., 2011) and chronic obstructive pulmonary disease (Shepherd et al. (2015), Berry et al., 2014, Kerley et al., 2015). It should be noted, however, that to date no study has shown a reduction in the O_2 cost of exercise in a patient group, despite reporting improvements in exercise tolerance. It is feasible that the improvement in exercise tolerance in the aforementioned studies is associated with elevated muscle blood flow consequent to an elevated plasma NO_2^- concentration (Cosby et al., 2003), although this requires further investigation to confirm.

Nitrate supplementation and functional Capacity.

There was no difference between the active and placebo juice conditions for the distance covered in the 6MWT. This is consistent with the only other study to have investigated the impact of NO_3^- supplementation on exercise performance using the 6MWT (in healthy older individuals) (Kelly et al., 2013a). Since any improvement in walking performance could reasonably be assumed to be underpinned by alterations in the $\dot{V}O_2$ response to exercise, and considering that this was not modulated by NO_3^- supplementation, it is perhaps not surprising that functional capacity was also not different between conditions. It is likely that the explanation for the lack of effect of NO_3^- supplementation on walking performance is synonymous with the potential explanations for the lack of effect on the O_2 cost of exercise (see previous section).

Walking performance has been shown to be enhanced in individuals who are taking ACEi (Ahimastos et al., 2013). Our cohort included 48% of individuals on ACEi and/or ARBs, thus any potential improvements in walking performance subsequent to NO_3^- supplementation may have been masked. When we separated the group into

those prescribed ACEi or ARBs compared to the remaining individuals, no difference was seen between groups for walking performance. The groups were split 48% (n = 23) with prescribed ACEi or ARBs and 52% (n = 25) not taking ACEi/ARB. One possible explanation for the finding is that the 6MWT was the last of the experimental procedures to be conducted and followed 18 minutes of treadmill walking. The prior exercise (and time since last NO_3^- supplementation) may have meant that some of the additional NO_2^- which was available following NO_3^- supplementation had been utilised, especially given our cohort's elevated cardiovascular risk (Rassaf et al., 2010).

Nitrate supplementation and resting blood pressure.

In agreement with our previous study examining the effect of dietary NO_3^- supplementation on blood pressure in individuals with T2DM (Gilchrist et al., 2013), we found no difference in BP between conditions. NO_3^- doses similar to that which was administered in the present study (6.43 mmol per day) have elicited reductions systolic BP in healthy young (Bailey et al., 2009, Bailey et al., 2010b, Lansley et al., 2010) and old (Kelly et al., 2013a) individuals and reduced diastolic BP in healthy young individuals (Bailey et al., 2009) and those with peripheral arterial disease (Kenjale et al., 2011).

There are a number of possible explanations for the lack of effect of NO_3^- supplementation on the resting BP of individuals with T2DM. A reduced NO-responsiveness has been linked to vascular stiffening in older individuals (Lyons et al., 1997). However, aging *per se* is unlikely to explain the lack of effect in the present study as BP was significantly reduced subsequent to NO_3^- supplementation in healthy older adults (on no medication) (Kelly et al., 2013a). Secondly, the

elevated oxidative stress prevalent in individuals with T2DM would be expected to result in an increase in the scavenging of NO·, potentially diminishing any hypotensive effects from NO₃⁻ supplementation.

Antioxidants in beetroot juice

It has been demonstrated that the beneficial effects of beetroot juice are largely explained by its NO₃⁻ content (Lansley et al., 2010). However, it has also been suggested that an antioxidant effect may also be occurring, such as is observed under in vitro experiments with polyphenols (Kanner et al., 2001). Though there are small differences in antioxidants between the two juices used (placebo and active), the magnitude of this difference is unlikely to be physiologically relevant. Typical total daily polyphenol intake across multiple populations has been estimated to be in the region of 1g daily (Bohn, 2014); the effective dose from either juice in the current study therefore represents less than 1% of average total intake. Betacyanins were present in much higher quantities. The bioavailability of these compounds is however uncertain, but it appears to be very low with typical estimates from <1% to 4% of an oral dose, with some individuals having far greater absorption (Watts et al., 1993, Tesoriere et al., 2004). Furthermore, the removal of betanin from plasma is rapid, with a $t_{1/2}$ of 0.94±0.07 hours. These factors suggest the betacyanin content of beetroot juice is unlikely to have a clinically meaningful effect.

Strengths and limitations.

To date this is the largest trial, implementing robust methods, to examine the effect of inorganic NO₃⁻ or pharmacological NO₃⁻ supplementation on the O₂ cost of exercise. The *a priori* sample size calculation was designed to enable the study to detect an 8% reduction in the O₂ cost of low intensity exercise with a 0.05 alpha level

and an 80% power. From the 95% CI it is likely that the actual difference for the O₂ cost of low intensity exercise following NO₃⁻ supplementation in individuals with T2DM was between -2.1% and 3.6%. Therefore the minimum detectable reduction in the O₂ cost of exercise in our study is 2.1%. This is very similar to the day to day variability of $\dot{V}O_2$ measurement, thus it is unlikely that a larger sample size would elucidate a clinically significant difference in the O₂ cost of walking exercise in this patient group. A potential limitation of the present study is that the 6MWT was completed up to 4 hours subsequent to the consumption of the final beverage, and after 18 minutes of walking when the increments in NO₂⁻ from supplementation may have been utilised already. As plasma NO₂⁻ concentration was not determined prior to the 6MWT, we cannot be certain that this remained elevated following NO₃⁻ supplementation compared to the placebo condition. However, this does mean that the study provides knowledge of the level of improvement (or lack of it) that a patient with type 2 diabetes could expect in the early afternoon of normal daily living.

3.1.8: Conclusion

In contrast to much of the literature in young healthy individuals and despite a statistically significant and physiologically meaningful increase in plasma NO₂⁻ concentration, four days of beetroot juice supplementation with 6.43 mmol of NO₃⁻ did not reduce the O₂ cost of walking, improve functional capacity as determined by the 6MWT, or reduce resting BP in individuals with T2DM. The lack of effects of dietary NO₃⁻ supplementation in individuals with T2DM may be explained by increased oxidative stress and its impact on the bioavailability of NO[•], or an elevated 'baseline' plasma NO₂⁻ concentration which reduces the scope for the beneficial effects reported in other populations.

3.2: *Experimental chapter 2 (Dietary nitrate supplementation in COPD)*

Title: The effect of dietary nitrate supplementation on the oxygen cost of cycling, walking performance and resting blood pressure in individuals with chronic obstructive pulmonary disease: A double blind placebo controlled, randomised control trial.

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3.2.3: *Abstract*

Background

Chronic obstructive pulmonary disease (COPD) results in exercise intolerance. Dietary NO₃⁻ supplementation has been shown to lower blood pressure (BP), reduce the O₂ cost of exercise, and enhance exercise tolerance in healthy volunteers. This study assessed the effects of dietary NO₃⁻ on the O₂ cost of cycling, walking performance and BP in individuals with mild-moderate COPD.

Methods

Thirteen patients with mild-moderate COPD were recruited. Participants consumed 70 ml of either NO₃⁻ rich (6.77 mmol NO₃⁻; beetroot juice) or NO₃⁻ depleted beetroot juice (0.002 mmol NO₃⁻; placebo) twice a day for 2.5 days, with the final supplement ~3 hours before testing. BP was measured before completing two bouts of moderate-intensity cycling, where pulmonary gas exchange was measured throughout. The six-minute walk test (6MWT) was completed 30 minutes subsequent to the second cycling bout.

Results

Plasma NO_3^- concentration was significantly elevated following beetroot juice vs. placebo (placebo; 48 ± 86 vs. beetroot juice; 215 ± 84 μM , $P=0.002$). No significant differences were observed between placebo vs. beetroot juice for O_2 cost of exercise (933 ± 323 vs. 939 ± 302 $\text{ml} \cdot \text{min}^{-1}$; $P=0.88$), distance covered in the 6MWT (456 ± 86 vs. 449 ± 79 m; $P=0.37$), systolic BP (123 ± 14 vs. 123 ± 14 mmHg; $P=0.91$), or diastolic BP (77 ± 9 vs. 79 ± 9 mmHg; $P=0.27$).

Conclusion

Despite a large rise in plasma NO_3^- concentration, two days of NO_3^- supplementation did not reduce the O_2 cost of moderate intensity cycling, increase distance covered in the 6MWT, or lower BP.

3.2.4: Introduction

Exercise in individuals with COPD is limited by multiple factors which can result in hypoxemia. These include loss of normal lung architecture, impaired cardiac function (Jardin et al., 1984), abnormal pulmonary blood flow distribution (Marshall et al., 1994) and peripheral muscle de-conditioning (Maltais et al., 2014). O_2 uptake in the lungs and delivery of O_2 to working muscle is impaired by increases in pulmonary blood flow which increase shunting through blood vessels resulting in incomplete gas exchange (Cooper, 2009) and cor pulmonale later in the disease course. These abnormalities result in feelings of breathlessness and fatigue (Nici et al., 2006), with individuals often finding that activities of daily living are physically challenging.

The beneficial effects of a diet rich in vegetables upon cardiovascular health (Gilchrist et al., 2010), risk of morbidity and mortality (Joshi et al., 2001), and

COPD development (Hirayama et al., 2009, Watson et al., 2002) have been well described. These positive effects have, in part, been attributed to inorganic NO_3^- (NO_3^-) which is found in particularly high quantities in leafy green vegetables and some root vegetables such as beetroot (Bryan and Hord, 2010). Nitrate supplementation in the form of sodium NO_3^- or NO_3^- rich beetroot juice has been shown to have remarkable effects in healthy young individuals and athletes, including reductions in the O_2 cost of exercise (Bailey et al., 2009), enhanced exercise tolerance/performance and reduced blood pressure (BP) (Bailey et al., 2009, Bailey et al., 2010b). Some of these effects have subsequently been observed in individuals with peripheral artery disease following dietary NO_3^- supplementation (Kenjale et al., 2011). These findings have been attributed to an increase in the bioavailability of nitric oxide ($\text{NO}\cdot$).

$\text{NO}\cdot$ is a signalling molecule with multiple functions including regulation of vascular tone, mitochondrial respiration and skeletal muscle function (Cosby et al., 2003, Merry et al., 2010, Larsen et al., 2011). These factors are important in the physiological response to exercise. $\text{NO}\cdot$ is produced in two distinct ways in man. The best known is the classical L-arginine nitric oxide synthase (NOS) pathway which is O_2 dependent (Alderton et al., 2001). The second is the entero-salivary pathway and is O_2 independent. Briefly, NO_3^- from the diet is rapidly and extensively absorbed in the stomach and proximal small intestine with bioavailability approaching 100% (Florin et al., 1990). Nitrate is then concentrated in the salivary glands, with concentrations 10 fold greater in saliva than in plasma. NO_3^- secreted in saliva is reduced to NO_2^- by facultative anaerobic bacteria on the dorsum of the tongue (Duncan et al., 1995). On swallowing, the acidic environment of the stomach results in $\text{NO}\cdot$ formation with important local effects on gastric function and host defence

(Gilchrist et al., 2010, Benjamin et al., 1994). Some NO_2^- is absorbed into the circulation where it acts as a storage pool for subsequent $\text{NO}\cdot$ production (Cosby et al., 2003). The conversion of NO_2^- to $\text{NO}\cdot$ is expedited in conditions of acidosis (Modin et al., 2001) or hypoxemia (Cosby et al., 2003) which often occur in the exercising muscle of individuals with COPD (Fiaccadori et al., 1987).

In many individuals with COPD, functional capacity is reduced to a level where activities of daily living may impose a challenge due to an energy requirement representing a high fraction of their maximal O_2 uptake. While a number of cardiovascular and physiological benefits have been shown as a result of dietary NO_3^- supplementation in healthy populations, little is known about possible effects in clinical populations. We aimed to determine whether dietary NO_3^- supplementation has a beneficial impact upon the O_2 cost of sub-maximal cycling exercise, walking performance and BP in individuals with COPD.

Purpose

The aim of this study was to assess the effects of 2.5 days of dietary NO_3^- supplementation on the O_2 cost of sub-maximal cycling, walking performance, and resting BP in individuals with mild-moderate COPD.

3.2.5: Methods

Patients

Fourteen individuals with mild-moderate COPD (see table 10 for patient characteristics) gave written informed consent to participate in this double-blind, placebo-controlled, cross-over design study between April 2013 and January 2014. The study was registered as a clinical trial at ClinicalTrials.gov (NCT01712386). The

Exeter NRES Committee gave ethical approval (12//SW//0327). Patients were recruited if lung function was between 30-80% of predicted FEV₁ values, aged 40-75 years old and able to give informed consent. Participants were excluded if they had chronic kidney disease (estimated glomerular filtration rate <30 ml/min/1.73 m²), uncontrolled hypertension (systolic BP > 160 mmHg or diastolic >100 mmHg), were smokers (smoked within past 3 months), consumed regular organic NO₃⁻ or nicorandil. Patients taking phosphodiesterase inhibitors were asked to refrain from doing so for the duration of the study.

Table 9. Characteristics of patients included in the final analyses.

Data are mean ± SD or as % of the cohort on a medication.

Age (years)	64.7 ± 7.7
FEV ₁ (%)	57 ± 9
FVC (l)	3.6 ± 0.9
FEV ₁ /FVC	41 ± 16
Body Mass Index (kg/m ²)	29 ± 8
Baseline SBP (mmHg)	132 ± 15
Baseline DBP (mmHg)	85 ± 10
No. of 30+ minutes of exercise per week	4.7 ± 3.6
ARB /ACEi (%)	23
Calcium channel blocker (%)	15
Short-acting β ₂ -agonists (%)	77
Long-acting β ₂ -agonists (%)	77

Pre-experimental tests

Participants arrived at the Heart and Lung unit at Torbay hospital where informed consent, medical history, anthropometric measures, BP, lung function and an ECG were performed. Participants completed a ramp incremental cycle ergometer test (10 W·min⁻¹) to determine their gas exchange threshold (GET). Breath-by-breath pulmonary gas exchange was measured throughout and the GET was determined using the V-slope method as described previously (Beaver et al., 1986).

Experimental Overview

Participants consumed 70 ml of NO₃⁻ rich beetroot juice (beetroot juice; 6.77 mmol NO₃⁻; Beet it, James White Drinks Ltd., Ashbocking, UK) or NO₃⁻ depleted beetroot juice as a placebo (placebo; 0.002 mmol NO₃⁻; Beet it, James White Drinks Ltd., Ashbocking, UK), with one beverage in the morning and one in the evening for two days preceding testing. On study days, participants consumed a final 70 ml beetroot juice drink ~3 hours prior to exercising. Participants self-reported concordance with the supplementation regime which was confirmed by measurement of plasma NO₃⁻ concentration. After exercise testing the participants began a washout period (7 days) before entering the opposing arm of the study. The placebo was indistinguishable from the NO₃⁻ rich juice in taste, colour, texture, appearance and odour as described previously (Gilchrist et al., 2014).

Participants arrived at the laboratory in a fully hydrated state, having avoided consumption of caffeine, alcohol, cruciferous vegetables, leafy greens, beetroot, and completion of strenuous exercise 24 hours prior to testing. Participants were asked to record their food intake for 24 hours prior to testing and to replicate this after the crossover and this was verbally confirmed on the second exercise visit. Participants

avoided antibacterial mouthwash for 7 days prior to testing. Participants arrived 45 minutes before the initiation of exercise following ingestion of the randomised juice with their morning meal. Brachial artery BP was taken, after a 10 minute resting period whilst supine, with an automated sphygmomanometer (Omron M6, Kyoto, Japan). Five measurements were performed and the mean of the last three was recorded. Venous blood was drawn and processed for plasma NO_3^- concentration as per our previously described chemiluminescence technique (Gilchrist et al., 2013). Participants completed two bouts of cycling at 80% of their GET on a cycle ergometer (Ergoselect 100, Bitz, Germany) with 30 minutes recovery between bouts. Following 30 minutes rest, participants performed a six-minute walk test to assess functional capacity. Participants walked around a clear rectangular corridor (14 x 12m) for a total of 52m per lap, covering as much distance as possible. Standardised verbal encouragement was given throughout.

Measurements

Pulmonary gas exchange and ventilation were measured during the cycling exercise (Vmax^{TM} Encore, Yorba, Linda, CA). Before each session the analysers were calibrated using gases of known concentration. The volume transducer was calibrated using a 3-litre syringe (Hans Rudolph, Kansas City, MO, USA).

Outcome measures

Primary outcome measure: does 2.5 days of NO_3^- supplementation reduce the O_2 cost of moderate intensity cycling? Secondary outcome measure (i): does 2.5 days of NO_3^- supplementation improve functional capacity as measured via the six-minute walk test? Secondary outcome measure (ii): does 2.5 days of NO_3^- supplementation reduce resting BP?

Sample size and randomisation

An *a priori* sample size calculation was performed. Previous literature in healthy young volunteers has shown a mean change between beetroot juice and control of 69 ml for end exercise pulmonary O₂ uptake ($\dot{V}O_2$) (1SD) and 121 ml for $\dot{V}O_2$ amplitude (2SD) (Bailey et al., 2009). For 90% power and an α -level set at P=0.05 (two tailed), to detect a 1 SD difference 13 patients were required. The reproducibility of these measures in patients with COPD are similar to healthy controls (Covey et al., 1999). An unrestricted computer generated sequence was used by a research nurse to assign each participant a randomisation number and supply them with the requisite juice.

Data and statistical analysis

Participant's breath by breath $\dot{V}O_2$ data were initially checked for erroneous breaths (caused by coughing and swallowing). Breaths > 4 SDs away from the local mean were removed prior to interpolation. Breath by breath data for each cycling bout were time aligned and interpolated to provide second by second values. A nonlinear least squares algorithm was then used to fit the ensemble-averaged data. The overall $\dot{V}O_2$ kinetics were described using the mean response time (MRT), which was calculated by fitting a single exponential curve to the data with no time delay from the onset to the end of exercise. The O₂ deficit was calculated as the product of the $\dot{V}O_2$ response amplitude (i.e. the baseline to the point that a steady state was attained) and the MRT. For a schematic representation of the kinetics parameters, please see figure 12.

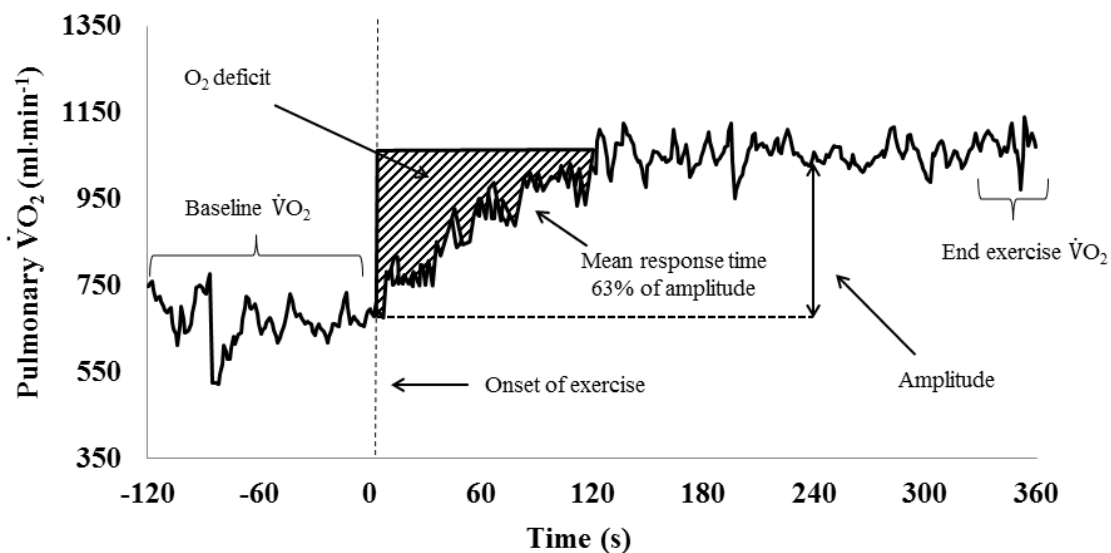


Figure 12. Parameters of O₂ uptake kinetics.

All data were tested for normality. Statistical differences were assessed using paired t-tests for normally distributed data and Wilcoxon rank-sum test for non-normally distributed data. All data are presented as means \pm standard deviation (*SD*). Statistical analysis was performed on SPSS software version 21.0 (Chicago, IL, USA). Statistical difference was accepted when $P < 0.05$.

3.2.6: Results

14 individuals with COPD provided written informed consent. Following screening, one individual was withdrawn due to $FEV_1 < 30\%$. 13 participants were randomised to start in either the beetroot juice or placebo condition of the study. All participants reported 100% adherence to the supplementation regime. Participants reported similar dietary patterns and physical activity during both study arms. Dietary NO_3^- supplementation was well tolerated with no adverse events apart from red stools and beeturia, as in previous studies (Bailey et al., 2009).

Plasma NO₃⁻ concentration: Relative to placebo, beetroot juice significantly increased plasma NO₃⁻ concentration (placebo; 48 ± 85 vs. beetroot juice; 215 ± 84 μM, *P* = 0.002, 95% CI 75, 260; Figure 13).

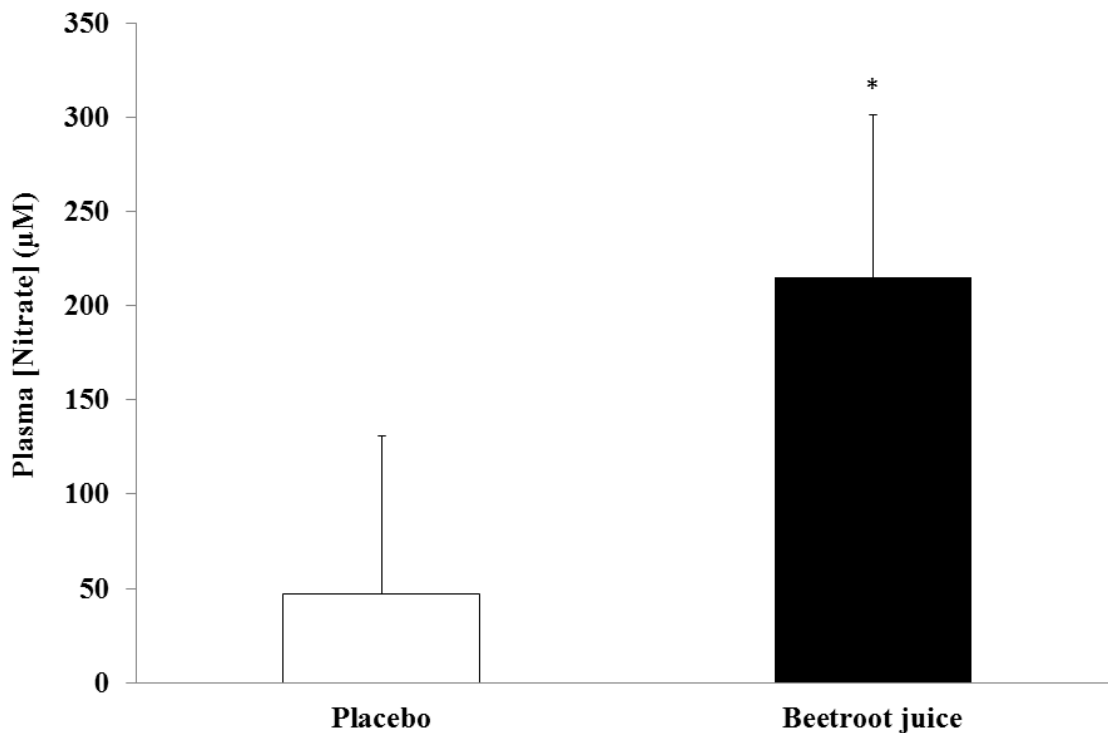


Figure 13. Plasma NO₃⁻ concentration in individuals with COPD.

Plasma NO₃⁻ concentration following placebo and beetroot juice supplementation. The open bar represents placebo and the closed bar, beetroot juice. *Significantly different from placebo *P* < 0.01.

Effects on the O₂ cost of cycling exercise: The group mean pulmonary $\dot{V}O_2$ response to exercise for both placebo and beetroot juice conditions can be seen in figure 14, with the $\dot{V}O_2$ kinetics resulting from the model fits displayed in Table 11. Relative to placebo, beetroot juice supplementation had no effect on baseline $\dot{V}O_2$ (placebo; 634 ± 233 vs. beetroot juice; 622 ± 253 ml·min⁻¹, *P* = 0.56, 95% CI -57, 32) or end exercise $\dot{V}O_2$ (placebo; 933 ± 323 vs. beetroot juice; 939 ± 302 ml·min⁻¹, *P* = 0.88,

95% CI -68, 78). There were no differences between conditions for the MRT ($P = 0.90$, CI -25, 28) or the O_2 deficit ($P = 0.71$, CI -.2, .3).

Table 10. Pulmonary gas exchange during moderate intensity cycling following placebo and beetroot juice supplementation.

	Placebo	Beetroot juice
Baseline $\dot{V}O_2$ (ml/min)	634 ± 233	622 ± 253
$\dot{V}O_2$ Amplitude (ml/min)	292 ± 80	308 ± 85
End-exercise $\dot{V}O_2$ (ml/min)	933 ± 323	939 ± 302
O_2 deficit (L)	0.39 ± 0.19	0.43 ± 0.29
MRT (s)	80 ± 19	81 ± 35

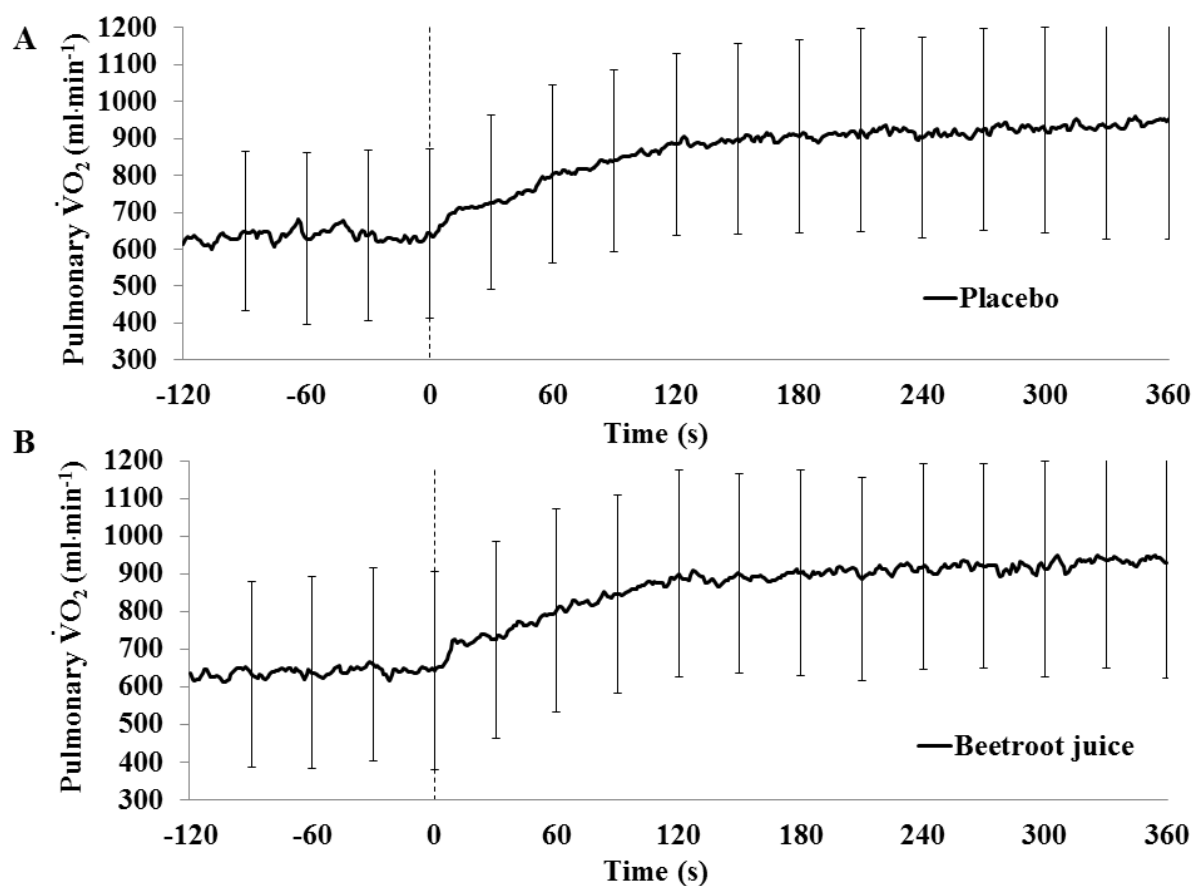


Figure 14. Pulmonary O_2 uptake response to beetroot juice in individuals with COPD.

The pulmonary O_2 uptake response during the transition from unloaded cycling to cycling at 80% of the GET for 6 minutes following placebo (A) and beetroot juice supplementation (B). The vertical line denotes the transition from baseline to moderate intensity cycling.

Effects on functional capacity: There was no difference between conditions for distance covered during the six-minute walk test (placebo; 456 ± 86 vs. beetroot juice; 449 ± 79 m, $P = 0.17$, 95% CI -22, 9).

Effects on resting blood pressure: Compared to the placebo juice, beetroot juice did not significantly reduce systolic BP (placebo; 123 ± 14 vs. beetroot juice; 123 ± 14 mmHg, $P = 0.91$, 95% CI -5, 4) or diastolic BP (placebo; 78 ± 9 vs. beetroot juice; 79 ± 9 mmHg, $P = 0.25$, 95% CI -2, 5; Figure 15).

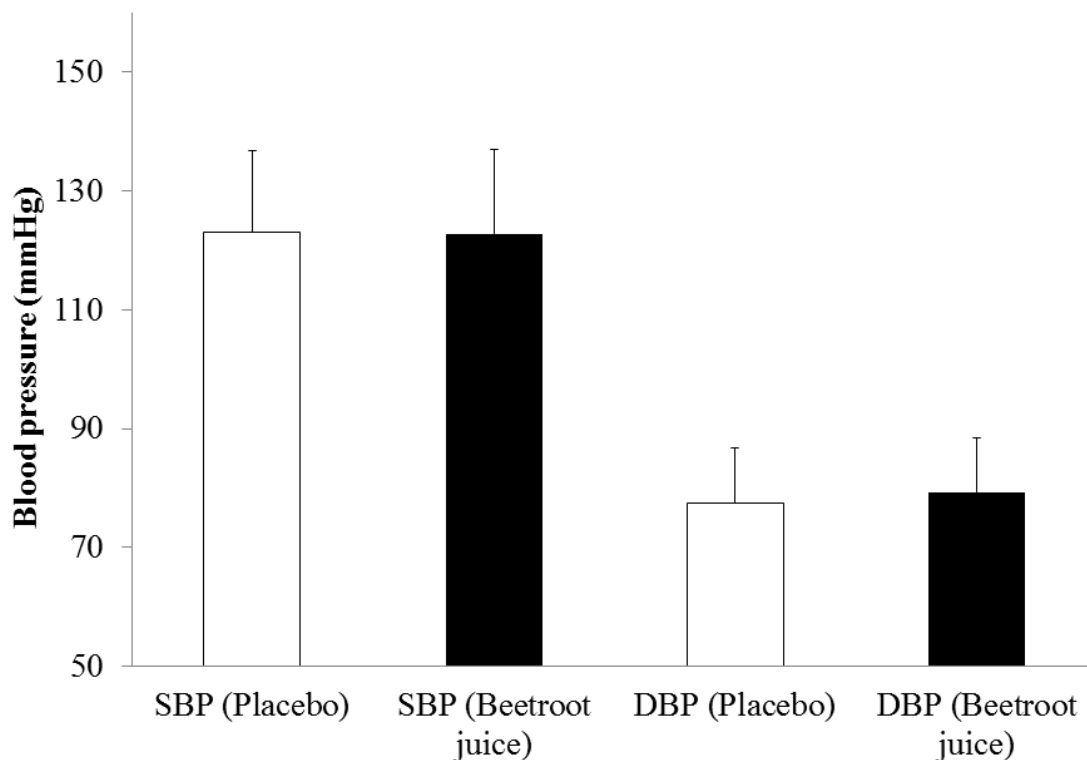


Figure 15. Systolic (SBP) and diastolic (DBP) blood pressure following placebo and beetroot juice supplementation.

The open bar represents placebo and the closed box, beetroot juice.

3.2.7: Discussion

Beetroot juice supplementation, (NO_3^- ; 6.77 mmol) twice daily, for 2.5 days did not reduce the O_2 cost of cycle ergometer exercise, improve functional capacity or reduce resting BP in individuals with COPD. There was no difference between conditions for these variables despite a statistically significant and physiologically meaningful rise in plasma NO_3^- concentration following NO_3^- supplementation.

Possible explanations for the lack of effect in this study include NO_3^- dosage, efficacy of NO_3^- reduction to NO_2^- , oxidative stress, and the age of the participants.

Nitrate supplementation and effects on plasma NO_3^- concentration.

Plasma NO_3^- concentration was $48\mu\text{M}$ post placebo and $215\mu\text{M}$ following NO_3^- rich beetroot juice, which is consistent with much of the literature in healthy young individuals (Larsen et al., 2010, Bescos et al., 2011) and individuals with type 2 diabetes (Gilchrist et al., 2014). Similar changes in plasma NO_3^- concentrations have been shown to elicit reductions in the O_2 cost of exercise, improved exercise tolerance/performance and reductions in BP (Larsen et al., 2007a, Muggeridge et al., 2013b, Wylie et al., 2013a). Due to logistical constraints, plasma NO_2^- concentration was not assessed in this study. In all previous studies involving dietary NO_3^- supplementation where plasma NO_2^- concentration has been determined, a rise in plasma NO_3^- concentration similar to the magnitude observed in the present study has been accompanied by a physiologically meaningful and statistically significant rise in plasma NO_2^- concentration (Muggeridge et al., 2013b, Larsen et al., 2007a, Larsen et al., 2011). However, we cannot exclude the possibility that there is an impaired capacity for reduction of NO_3^- to NO_2^- in individuals with COPD. Such an impairment could potentially be related to differences between individuals with COPD and healthy individuals in oral microflora due to oral steroids and repeated exposure to courses of antibiotics (Mobbs et al., 1999).

Nitrate supplementation and effects on the O_2 cost of cycling exercise.

We found no reduction in the O_2 cost of cycling exercise at baseline or end-exercise in individuals with COPD following NO_3^- rich beetroot juice supplementation compared to placebo. Nitrate supplementation in healthy young individuals has

previously resulted in reductions in the O₂ cost of exercise (Bailey et al., 2009). However, we recently reported that the O₂ cost of exercise was not altered by dietary NO₃⁻ supplementation in a group of healthy older adults (Kelly et al., 2013a). The current study is the first to examine the effects of NO₃⁻ supplementation on the O₂ cost of exercise in any clinical population. The supplementation regime used in this study, consisting of 6.77 mmol twice a day for 2.5 days, has previously been shown to increase plasma NO₂⁻ concentrations (Bailey et al., 2009, Vanhatalo et al., 2010, Lansley et al., 2010) and elicit reductions in the O₂ cost of exercise (Muggeridge et al., 2013b). It is therefore unlikely that the dosage and the timing of NO₃⁻ supplementation explain why no effect on the O₂ cost of exercise was observed.

One possible explanation for the reduction in O₂ cost following dietary NO₃⁻ supplementation in other populations is an increase in the P/O ratio (i.e. less O₂ being consumed to produce a given amount of ATP). Larsen et al (Larsen et al., 2011) reported an increase in the P/O ratio of harvested mitochondria following three days NO₃⁻ supplementation. However, we did not observe a reduction in the O₂ cost of exercise, which may be related to the impact of oxidative stress, which is reported to damage mitochondrial membranes (Kowaltowski and Vercesi, 1999), potentially resulting in a reduction in the P/O ratio. COPD is associated with increased oxidative stress, with reactive oxygen species (ROS) being produced within the inflammatory cells and epithelial cells of the airways in conjunction with increased systemic generation of ROS (Ciencewicz et al., 2008). Oxidative stress leads to uncoupling of the NO· synthase enzymes (Li and Forstermann, 2013), thus reducing NO· bioavailability and creating a negative feedback loop of diminishing NO· production and elevated NO· scavenging. This may be a substantial barrier to NO· based therapeutics in COPD.

Nitrate supplementation and effects on functional capacity.

No statistical difference in distance covered for the six-minute walk test was observed between conditions. Considering that the O_2 cost of exercise and rate of adaptation of $\dot{V}O_2$ were not altered following NO_3^- supplementation, it is perhaps not surprising that functional capacity was also not different between conditions. It is likely that these lack of effects share a common explanation, which may be related to the impact of oxidative stress on the bioavailability of $NO\cdot$ (Li and Forstermann, 2013) (see previous section).

The only other studies that have examined the impact of dietary NO_3^- supplementation on walking performance have reported both positive and neutral effects. Kenjale et al. (2011) reported an increased walking time to exhaustion (17%) in a cohort of peripheral artery disease patients. However, dietary NO_3^- supplementation had no effect on the distance covered in a six-minute walk test in healthy older individuals (Kelly et al., 2013a). Since plasma NO_3^- (and NO_2^- in Kenjale et al. 2011 and Kelly et al. 2013) concentrations were similar for the present study and two previous studies, the differences in walking performance post NO_3^- supplementation are likely related to methodological differences. Kenjale et al. (2011) assessed walking performance via an incremental test to exhaustion on a treadmill, whereas in the present study and that of Kelly et al. (2013a) walking performance was assessed via completion of a (submaximal) six-minute walk test. It is likely that the higher exercise intensity encountered by the participants in Kenjale et al. (2011) resulted in the development of a hypoxic and acidic cellular environment that is known to be conducive for the reduction of NO_3^- to NO_2^- (Modin et al., 2001). Such an environment would have been less likely to occur during the lower exercise intensity in the present and Kelly et al.'s (2013a) study. Finally, Kenjale et al. (2011)

did not use a placebo that was indistinguishable from their active juice, thus a 'placebo effect' cannot be ruled out.

Nitrate supplementation and effect on resting BP.

There was no difference in systolic or diastolic resting BP following NO_3^- rich beetroot juice compared to placebo. This may be related to a factor specific to COPD such as the elevated oxidative stress (Ciencewicki et al., 2008) in this population would be expected to increase the scavenging of $\text{NO}\cdot$, thus reducing its effectiveness. Alternatively there are multiple other factors which may modify the BP effect. Studies investigating the effects of dietary NO_3^- supplementation in older subjects with and without pathology have reported inconsistent BP effects. Gilchrist et al (2013) examined the impact of dietary NO_3^- supplementation in individuals with type 2 diabetes, and found no statistical difference in mean 24h ambulatory BP. In subjects with peripheral artery disease Kenjale et al. (2011) reported a statistically significant reduction in diastolic BP (7 mmHg) but no change in systolic BP. It is possible that ageing *per se* may attenuate $\text{NO}\cdot$ mediated BP reduction, however in Kelly et al. (2013a) study of healthy older adults dietary NO_3^- supplementation resulted in reductions in systolic and diastolic BP of 5 and 3 mmHg, respectively. In contrast, more recently, a larger study by Bondonno et al. (2014a) used a vegetable based, NO_3^- rich diet for 7 days. Ten hour ambulatory BP along with home and office based measurements were used to assess BP. They found no reductions in BP or arterial stiffness. There are key differences around the supplementation protocol and timing and method of blood pressure measurement. In Kelly et al's study the office based blood pressure measurement was timed to coincide with the plasma NO_2^- peak post NO_3^- ingestion. In the Bondonno et al study measurements took place outside this window.

It is also worth noting the differing BMI's in these studies and our present manuscript. Kelly et al's cohort of older adults are the only group in the normal range ($24 \pm 3 \text{ kg/m}^2$). Our present cohort had a mean BMI of $29 \pm 8 \text{ kg/m}^2$, Bondonno et al's cohort were overweight $27 \pm 4 \text{ kg/m}^2$, and in our previous study of subjects with type 2 diabetes the group mean BMI was $30.8 \pm 3.2 \text{ kg/m}^2$. This raises the possibility that adiposity may attenuate the response to inorganic NO_3^- by an as yet unknown mechanism.

One factor which may have had an impact is that subjects in the present study were taking multiple classes of drugs including antihypertensives. It is possible that the scope for reductions in BP subsequent to NO_3^- supplementation is significantly reduced when individuals are already taking antihypertensive medication. It is noteworthy that in studies where subjects were taking antihypertensives (current study - 38% prescribed antihypertensives; Gilchrist et al. (2013) - 98% prescribed antihypertensives), no reductions in BP have been reported (see table 1 for drug classifications). Alternatively, the healthy older adults, on no medications, studied by Kelly et al. (2013a) showed a significant reduction in BP following NO_3^- supplementation. It is possible that antihypertensive agents mitigate the NO -mediated reduction in BP.

There is conflicting evidence to suggest that ACEi/ARBs can alter the bioavailability of NO_x with some studies showing reduction (Cacciatore et al., 2011) and others proposing increases (Ceconi et al., 2007, Jacoby and Rader, 2003). Therefore the direction in which ACEi/ARBs may alter the bioavailability of NO remains unclear. β_2 -adrenergic receptor agonists are known to increase endothelial NO production and are at least, in part, responsible for their vasodilatory effects (Yong-Xiang et al., 1993). β_2 -agonists are the most common treatment for individuals with COPD and

thus we could not reasonably exclude individuals who were prescribed this medication. We cannot exclude the possibility that prescribed medications which modulate NO· bioavailability may attenuate a beneficial effect from dietary NO₃⁻ supplementation. This study is a crossover design and therefore both treatment arms will be equally affected. Further study is required to better understand the possible interaction of different medications and inorganic NO₃⁻ and NO₂⁻.

Berry et al. (2014) recently examined the effect of NO₃⁻ rich beetroot juice vs. prune juice (as a placebo) in individuals with COPD. Plasma NO₂⁻ concentration was significantly higher post beetroot juice compared to post prune juice, which indicates that the entero-salivary pathway is operational in people with COPD. The authors reported reductions in resting systolic BP, iso-time (defined as: last 60s of the shortest exercise time during either active or placebo visits compared with the same time point from the longer exercise time) BP, end exercise diastolic BP and an improvement in exercise tolerance (i.e. lengthened time to exhaustion during submaximal constant rate). Whilst the increase in exercise time is of interest, there are significant limitations in this study. Firstly, the design utilises prune juice as the placebo, which is likely to have a substantially different antioxidant content which could alter NO· bioavailability (Bondonno et al., 2012). Secondly, and related to the lack of a 'true' placebo where the participant did not know whether active or placebo juice was being taken (as used in the present study), the widely reported (in the national press as well as in scientific literature) beneficial effects of beetroot juice on exercise performance/tolerance may have given rise to a placebo effect in informed volunteers. The authors do not show a reduction in the O₂ cost of exercise which is consistent with the present study. However, with no reduction in the O₂ cost of

exercise, it is not immediately clear what mechanism underpins the improved time to exhaustion reported by Berry et al. (2014).

This is the first double blind, randomised, placebo, controlled, crossover design study to examine the effects of NO_3^- supplementation on the O_2 cost of exercise, walking performance and BP in individuals with COPD. The study had a robust experimental design (double-blind, placebo-controlled, randomised, cross-over study). A limitation is that we were not able to ascertain whether or not the increase in plasma NO_3^- concentration lead to an increase in plasma NO_2^- concentration, as we were not able to measure the latter due to logistical constraints. However, a recent study examining NO_2^- levels in individuals with COPD did show elevated plasma NO_2^- concentrations (Berry et al., 2014) which suggests the entero-salivary pathway is operational.

3.2.8: Conclusion

In contrast to findings in healthy young individuals, and despite a statistically significant and physiologically meaningful rise in plasma NO_3^- concentration, 2.5 days of beetroot supplementation with 6.77 mmol of NO_3^- twice daily did not reduce the O_2 cost of cycling exercise, improve functional capacity or reduce resting blood pressure. Potential explanations for the lack of effect include a reduced P/O ratio due to systemic ROS generation associated with oxidative stress, or a reduced conversion of NO_3^- to NO_2^- in this population.

3.3: *Experimental chapter 3 (Nitrate supplementation, hepatic diffusion and glucose homeostasis)*

Title: Nitrate supplementation, hepatic diffusion and glucose homeostasis: A double-blind, placebo controlled, randomised control trial.

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3.3.3: *Abstract*

Background:

Type 2 diabetes mellitus is a growing burden on the NHS budget predominantly due to an increased cardiovascular and diabetes risk factors. Nitrates derivatives (nitrite and nitric oxide) have been shown to alter gastric blood flow, improve vascular function and mediate glucose uptake within the intestines and skeletal muscle. Dietary NO₃⁻ appears to be a potential low cost therapy that may help maintain glucose homeostasis.

Methods:

In a randomised, double blind, placebo-controlled cross-over study, 31 participants (16 young adults and 15 healthy older adults) arrived fasted overnight and had a standardised breakfast, supplemented with either NO₃⁻ rich beetroot juice (11.91 mmol NO₃⁻) or NO₃⁻ depleted beetroot juice as placebo (0.01 mmol NO₃⁻). A

minimum 7 day washout period was employed. Magnetic resonance imaging was used to assess apparent diffusion coefficient (ADC), portal vein flux and velocity. Plasma glucose concentrations and blood pressure were also assessed. On each visit outcome variables were measured at baseline and again every hour for 3 subsequent hours. Repeated measures ANOVA were used to assess the interaction effect of time and supplement.

Results:

Compared with a placebo, beetroot juice resulted in a physiologically meaningful and significant elevation in plasma NO_3^- and plasma nitrite concentration in both cohorts. No differences were seen between placebo and beetroot juice for ADC (young adults: $F_{(3, 45)} = 0.25$, $P = 0.74$; older adults; $F_{(3, 42)} = 1.3$, $P = 0.28$), portal vein flux (young adults: $F_{(3, 45)} = 0.339$, $P = 0.79$; older adults; $F_{(3, 42)} = 1.65$, $P = 0.19$), however, there was an interaction effect in the young adults: ($F_{(3, 45)} = 2.9$, $P = 0.04$) but not in the older adults; ($F_{(3, 42)} = 1.8$, $P = 0.16$) between visits for portal vein velocity. Nitrate supplementation did not reduce plasma glucose concentrations (young adults: $F_{(3, 45)} = 0.96$, $P = 0.42$; older adults; $F_{(3, 42)} = 0.04$, $P = 0.99$). Nitrate supplementation did not reduce systolic blood pressure (young adults: $F_{(3, 45)} = 0.20$, $P = 0.89$; older adults; $F_{(3, 42)} = 1.7$, $P = 0.18$) or diastolic blood pressure (young adults: $F_{(3, 45)} = 0.25$, $P = 0.86$; older adults; $F_{(3, 42)} = 0.45$, $P = 0.72$).

Conclusion:

This was the first study to examine the hepatic blood flow response to NO_3^- supplementation. Despite a large and physiologically meaningful elevation in plasma NO_2^- concentration following an acute dose of 11.91 mmol of NO_3^- , there was no

effect on hepatic blood flow, plasma glucose concentrations or systolic blood pressure.

3.3.4: Introduction

The total cost of direct and indirect care of individuals with T2DM in the UK is £20.5 billion and equates to 8% of the annual NHS budget (Hex et al., 2012). It is anticipated that by 2030, 439 million individuals will have type 2 diabetes (T2DM) worldwide (Shaw et al., 2010). This represents a serious challenge to global healthcare systems (Wild et al., 2004). T2DM is a disease of progressive hyperglycemia. Hyperglycemia is strongly associated with cardiovascular disease and microvascular complications such as retinopathy, neuropathy, nephropathy (Stratton et al., 2000). Reductions in glycated hemoglobin (HbA_{1c}) have been associated with reduced risk of both micro and macrovascular complications (Stratton et al., 2000) and events (Patel et al., 2008).

Diets rich in vegetables have been shown to have beneficial effects on cardiovascular health (Gilchrist et al., 2010), morbidity and mortality (Joshi et al., 2001) and reduces the risk of developing T2DM (Carter et al., 2010). These diets are rich in inorganic NO₃⁻. There is growing evidence to suggest NO₃⁻ is at least in part responsible for these beneficial effects (Bryan and Hord, 2010). Recent reports have shown NO₃⁻ supplementation reduces systolic and diastolic blood pressure in healthy older adults (Jajja et al., 2014, Kapil et al., 2015, Kelly et al., 2013a) and in clinical cohorts with elevated cardiovascular risk factors (Berry et al., 2014, Kerley et al., 2015, Kenjale et al., 2011). This may in part be due to its effects on vascular tone and prevention of vasospasm (Cosby et al., 2003, Pluta et al., 2005).

One of NO_3^- derivatives, nitric oxide (NO) has been shown to mediate glucose uptake from the intestines (Guan et al., 2003) and facilitate its disposal into skeletal muscle in animal models (Merry et al., 2010) and in individuals with T2DM (Kingwell et al., 2002). Whilst another derivative of NO_3^- , nitrite (NO_2^-) when supplemented in eNOS deficient mice for 10 weeks has shown to reduce glycated hemoglobin concentrations, lower baseline and postprandial glucose concentrations (Carlström et al., 2010). An vivo study in young adults has shown a reduction in plasma glucose concentrations following nitrate supplementation following exercise (Wylie et al., 2013b). Recently, another study has described reduction in baseline plasma glucose concentrations 2.5h after supplementation with pharmacological sodium NO_3^- in individuals with T2DM whilst there was no effect on post prandial glucose concentrations following an oral glucose tolerance test (Cermak et al., 2015).

Incretins are a group of gastrointestinal hormones which stimulate $\text{NO}\cdot$ production (Hattori et al., 2010, Ding et al., 2004). Upon ingestion of food, plasma incretin concentrations rise following release from the small intestines (Holst, 2007) and concurrently, $\text{NO}\cdot$ increases gastric blood flow (Björne et al., 2004, Petersson et al., 2007). Incretins are intrinsically linked with insulin and are a key component in glucose homeostasis via their insulinotropic effect. Incretins mediate the uptake of glucose in the intestines in a $\text{NO}\cdot$ dependent fashion (Guan et al., 2003). Incretins have also been shown to expedite the production of $\text{NO}\cdot$ within the portal vein (Ding et al., 2004).

$\text{NO}\cdot$ is produced within the body via two independent pathways. The first pathway in which $\text{NO}\cdot$ is produced is via the amino acid L-arginine combining with an enzyme, nitric oxide synthase (NOS) and is O_2 dependent (Alderton et al., 2001). The second pathway, known as the entero-salivary pathway is O_2 independent. Nitrate from the

diet is ingested, absorbed through the stomach wall and proximal small intestine (Bartholomew and Hill, 1984, Florin et al., 1990) into the circulation where it is concentrated in the salivary glands. A reduction of NO_3^- to NO_2^- via facultative anaerobic bacteria occurs within the mouth (Duncan et al., 1995). Nitrite is then swallowed where some NO_2^- is converted to $\text{NO}\cdot$ in the acidic environment of the stomach (Benjamin et al., 1994) whilst some of the NO_2^- is absorbed into the circulation and acts as a storage pool for subsequently conversion to $\text{NO}\cdot$ (Lundberg et al., 2008). Another possible mechanism exists for the conversion of NO_3^- to NO_2^- , whereby hepatic xanthine oxidoreductase reduces NO_3^- to NO_2^- (Jansson et al., 2008). This mechanism in conjunction with the uptake of NO_2^- into the portal circulation may explain why one of the highest concentrations of nitrite in any organ are found within the liver (Totzeck et al., 2012, Bryan et al., 2005). Subsequent increases in the bioavailability of $\text{NO}\cdot$ within the liver may be expedited by a number of nitrite reductases which are in relatively high concentrations such as; xanthine oxidoreductase (Li et al., 2008b, Martin et al., 2004), aldehyde oxidase (Li et al., 2008a, Kundu et al., 2012), neuroglobin (Fordel et al., 2007, Tiso et al., 2011), cytoglobin (Burmester et al., 2002, Fordel et al., 2007, Li et al., 2012), deoxyhaemoglobin (Cosby et al., 2003) and myoglobin (Cossins et al., 2009). The potential increase of $\text{NO}\cdot$ within the hepatic vasculature may lead to vasodilation of the parenchyma and lead to greater surface area for glucose uptake to occur. Increases in the bioavailability of $\text{NO}\cdot$ has also been shown to stimulate insulin secretion (Nystrom et al., 2012) and increase GLUT4 translocation (Li et al., 2004).

Purpose / hypothesis

By reducing post-prandial hyperglycaemia it may be possible to reduce the associated micro and macrovascular complications (Stratton et al., 2000). A

reduction in hyperglycaemic exertions is a long term goal in the treatment of T2DM in order to protect against cardiovascular events and microvascular damage (Shamoon et al., 1993, Del Prato et al., 2005) and even the risk of morbidity and mortality. Therefore, dietary NO_3^- may offer a simple, low cost means of modifying diabetes and cardiovascular risk factors.

The aim of this study was to assess if inorganic NO_3^- modulates portal vein flux and velocity and hepatic microvascular diffusion and secondly; does NO_3^- lower postprandial plasma glucose concentrations and blood pressure in healthy young and older adults. We hypothesised that supplementation of the diet with dietary NO_3^- would increase blood flow to the liver and vasodilate the microvasculature causing improved postprandial glucose uptake. We also hypothesise that dietary nitrate supplementation would reduce blood pressure.

3.3.5: Methods

Volunteers

37 individuals (17 healthy young individuals and 20 healthy older adults) provided written informed consent to participate in this double-blind, placebo-controlled, cross-over design study (see table 12 for subject characteristic). The healthy young individuals were recruited via word of mouth. The older adults were recruited via the NIHR Exeter Clinical Research Facility, Exeter 10,000 cohort. This is a database of individuals who have been pre-screened and consented to be contacted as research volunteers. The trial commenced in July 2014 and ended in April 2015. Ethical approval was obtained from the Exeter NRES Committee (14/SW/0092). This trial was registered on the ClinicalTrials.gov website (NCT02195856). Healthy young individuals were recruited if they were aged between 18 and 35. Older adults were

recruited if they were aged between 50 and 75. Exclusion criteria were incapacity to consent, on any regular vasoactive medications, uncontrolled hypertension, antibiotic therapy within the preceding two weeks, on regular organic NO₃⁻, thiazolidinidiones or nicorandil, severe claustrophobia, were smokers (smoked within past 3 months), presence of chronic kidney disease (estimated glomerular filtration rate (eGFR) <30 ml/min/1.73 m²), myocardial infarction or cerebro-vascular event within the preceding 3 months, previous brain surgery, cardiac pacemaker, metal fragments in the eye or larger metal objects that would interfere with data collection or analysis. Volunteers, who had medical interventions where metal implants (including some plates, clips, staples or sutures) were inserted, were thoroughly assessed to determine safety in the scanner.

Table 11. Participant characteristics included in the final analysis.

	Young adults	Older adults
n	16	15
Age (years)	26.6	59.2
Male (%)	68.8	53.3
Height (m)	1.75	1.69
Weight (kg)	76.2	75.3
BMI (kg/m ²)	24.6	26.3
Baseline SBP (mmHg)	121.0	130.0
Baseline DBP (mmHg)	70.0	79.0

Experimental Overview

Screening and consent took place at the Diabetes and Vascular Research Centre at the NIHR Exeter Clinical Research Facility. Following arrival, volunteers completed informed consent followed by a medical history, anthropometric measures and a

resting ECG were taken. Venous blood samples were taken and checked for markers of undiagnosed liver disease, lipid profile, eGFR and glycated haemoglobin. Following the screening checks the volunteers were randomly assigned to a double-blind crossover experimental design to consume 140 ml of NO_3^- rich beetroot juice (beetroot juice; containing 11.91 mmol of NO_3^- ; Beet it, James White Drinks Ltd., Ipswich) or NO_3^- depleted beetroot juice (placebo; NO_3^- depleted beetroot juice containing 0.01 mmol of NO_3^- ; Beet it, James White Drinks Ltd., Ipswich). The placebo production has been detailed previously (Gilchrist et al., 2014); the final product is identical in appearance, odour taste, colour and texture.

Volunteers were asked to fast overnight (from 10pm) although water consumption was allowed to ensure they arrived in a hydrated state. Volunteers were asked to refrain from antibacterial mouthwash throughout the study and for at least 7 days prior to the experimental visits. Antibacterial mouthwash has been demonstrated to reduce the concentration of oral bacterial anaerobes responsible for the reduction of NO_3^- in the entero-salivary pathway (Govoni et al., 2008). Volunteers were also asked to avoid caffeine for 12 hours, alcohol and strenuous activity for 24 hours and NO_3^- rich foods on the day prior to their visits..

Volunteers arrived at the Peninsula Magnetic Resonance Research Centre at the University of Exeter. A 30 minute acclimatisation period was implemented prior to the magnetic resonance imaging (MRI) scans. During this acclimatisation period a cannula was inserted in order to take baseline plasma concentrations for glucose (fluoride & EDTA tubes; Sarstedt, S-Monovette, Nümbrecht, Germany). Plasma NO_3^- and NO_2^- were collected (lithium heparin tubes; Sarstedt, S-Monovette, Nümbrecht, Germany) and analysis was performed as previously described (Gilchrist et al., 2013). Immediately prior to the baseline MRI scan, 5 resting blood pressure (BP)

measurements were taken (Schiller Medical, Wissembourg, France) and an average of the final 3 recorded.

Following the baseline MRI scans the volunteers were provided with either the NO₃⁻ rich beetroot juice or the placebo with two slices of toast, butter and jam. The combined quantity of carbohydrate equated to 76 grams and is equivalent to that that would be consumed during an oral glucose tolerance test (Bartoli et al., 2011a). Every hour, for three subsequent hours, from the consumption of the beetroot juice another set of scans were performed. Immediately prior to each scan brachial artery blood pressure and venous blood samples were taken and processed as previously described. A minimum 7 day washout period between the crossover was employed.

MRI scans

A 1.5 T (Philips, Amsterdam, The Netherlands) magnetic resonance imaging (MRI) scanner was used in order to examine changes in velocity and flux in the portal vein and microvascular diffusion in the posterior right lobe of the liver.

Initial structural images were obtained to orientate the portal vein and an 8mm slice was selected perpendicular to the long axis of the vein. To determine flux and velocity a cardiac triggered velocity sensitive phase encoding imaging sequence (Gatehouse et al., 2005) was employed which obtained image data at 20 time points throughout the cardiac cycle. Analysis of the portal vein was subsequently undertaken using a package supplied as part of the general scanner software. For each separate measurement the circumference of the vessel was manually drawn and recorded for each of the 20 time points to establish a defined region of interest (ROI). Within this ROI, flow and velocity were automatically calculated to give

profiles throughout the cardiac cycle. A mean across the cardiac cycle for flux and velocity was created.

To examine the microvascular diffusion in the posterior right lobe of the liver, a magnetic resonance sequence sensitive to flow was employed via the application of magnetic field gradients in three orthogonal directions. Microvascular diffusion was averaged over all directions within the region of interest and is known as the apparent diffusion coefficient (ADC). Day to day repeatability was assessed in 6 individuals for ADC (1 ROI: ADC = 1.15 ± 0.12, CV = 9.77) and with multiple ROI. One ROI away from any major vessels had greater repeatability than 2, 3 and 6 sites (2 ROI: ADC = 1.11 ± 0.21, CV = 15.29; 3 ROI: ADC = 1.10 ± 0.23, CV = 21.34; 6 ROI : ADC = 1.09 ± 0.33, CV = 30.32). To calculate ADC within the posterior left lobe of the liver, a ROI (typically 2500mm³) was manually drawn using the scanner software and the signal intensity within determined. ADC was subsequently calculated based upon the ratio of signal intensity from the two images generated from the MR sequence employed, one of which was sensitive to flow, whereas the other had a low sensitivity to flow, where:

$$ADC = -1/(b_1 - b_0) \ln (S_0/S_1)$$

S₀ signal intensity in low flow sensitivity image

S₁ signal intensity in flow sensitive image

b₀ magnetic field gradient used in low flow sensitivity image = 250 s/mm²

b₁ magnetic field gradient used in flow sensitive image = 750 s/mm².

Sample size and randomisation

For 90% power with an α -level set at $P = 0.05$ (two tailed), to detect a 1 SD difference, 13 volunteers were required to compare between placebo and active conditions. A computer program was used to generate a randomisation schedule for both groups.

Data and statistical analysis

All data were tested for normality. Data are presented as means \pm standard deviation (*SD*). Statistical analyses were performed on SPSS software version 21.0 (Chicago, IL, USA). Statistical difference was accepted when $P < 0.05$. Statistical differences were assessed using paired samples t-tests.

3.3.6: Results

37 individuals (17 healthy young individuals and 20 healthy older adults) gave written informed consent to participate. Post screening and consent 6 individuals were excluded from the trial. 1 individual had abnormal liver function, 1 had a metal pin (in an area which would interfere with data collection) and 4 had previously undiagnosed claustrophobia. 31 individuals (16 healthy young individuals and 15 healthy older adults) were randomised to start in either the NO_3^- rich beetroot arm or the placebo arm. No differences between dietary intake or exercise patterns were recorded prior to both study visits. The beetroot juice was well tolerated and no adverse events were reported.

Plasma NO_3^- concentration:

Repeated measures ANOVA revealed a significant main effect for time, supplementation and an interaction for plasma NO_3^- concentration in the young adults and the older adults; $P < 0.001$. Post hoc analysis revealed no significant

differences at baseline (prior to any supplementation) for plasma NO_3^- concentration (young adults: mean difference; $2 \pm 4.4 \mu\text{M}$, $P = 0.64$, 95% CI -7.4, 11.5; older adults: mean difference; $1.5 \pm 4.1 \mu\text{M}$, $P = 0.72$, 95% CI -10.4, 7.4) Post hoc analysis revealed a significant increase when beetroot juice was compared with placebo at 1 hour post supplementation (young adult: mean difference; $543 \pm 37 \mu\text{M}$, $P < 0.001$, 95% CI 463, 624; older adult: mean difference; $505 \pm 39 \mu\text{M}$, $P < 0.001$, 95% CI 420, 590), 2 hours post (young adult: mean difference; $645 \pm 29 \mu\text{M}$, $P < 0.001$, 95% CI 581, 707; older adult: mean difference; $632 \pm 35 \mu\text{M}$, $P < 0.001$, 95% CI 556, 710) and 3 hours post (young adult: mean difference; $598 \pm 31 \mu\text{M}$, $P < 0.001$, 95% CI 530, 665; older adult: mean difference; $616 \pm 26 \mu\text{M}$, $P < 0.001$, 95% CI 559, 673).

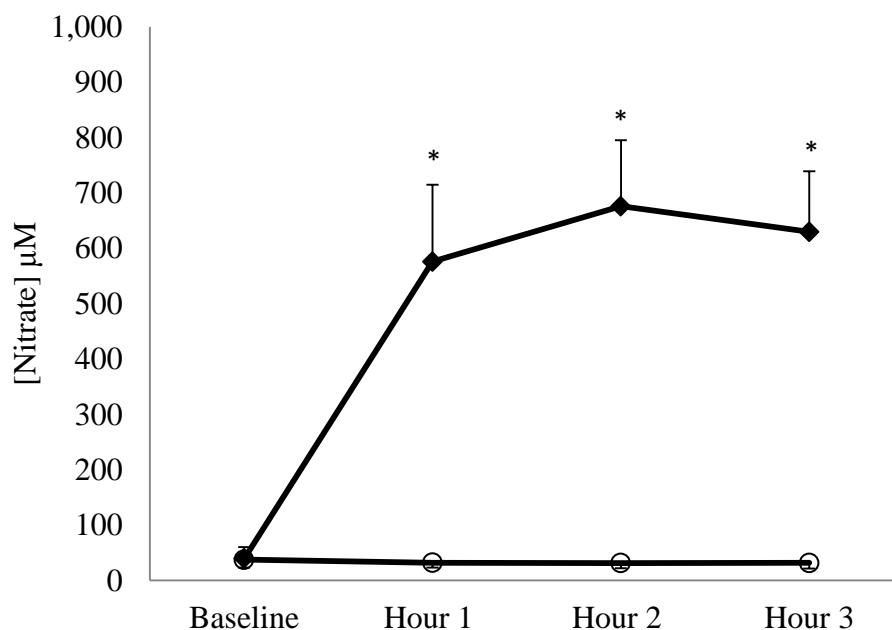


Figure 16. Plasma NO_3^- concentration in a young adult cohort.

This figure shows plasma NO_3^- concentration for the young adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot juice. The * represents a significant difference when beetroot juice is compared with placebo < 0.001 .

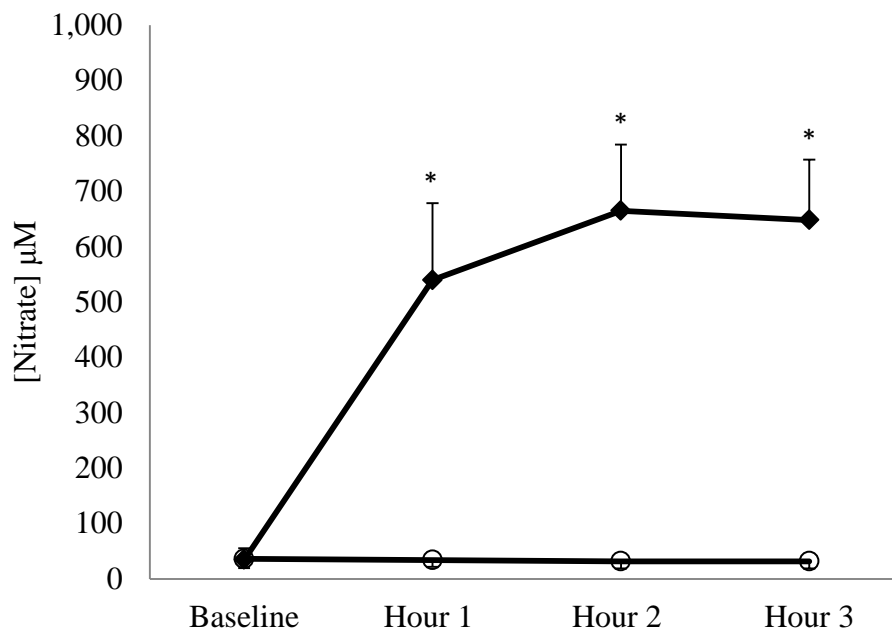


Figure 17. Plasma NO_3^- concentration in a older adult cohort.

This figure shows plasma NO_3^- concentration for the older adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot juice. The * represents a significant difference when beetroot juice is compared with placebo < 0.001 .

Plasma NO_2^- concentration:

Repeated measures ANOVA revealed a significant main effect for time, supplementation and an interaction for plasma NO_2^- concentration in the young adults and the older adults; $P < 0.001$. Post hoc analysis revealed no significant differences at baseline (prior to any supplementation) for plasma NO_2^- concentration (young adults: mean difference; -3.3 ± 74 nM, $P = 0.86$, 95% CI -43, 36; older adults: mean difference; 26.5 ± 78 nM, $P = 0.21$, 95% CI -17, 69) Post hoc analysis revealed a significant increase when beetroot juice was compared with placebo at 1 hour post supplementation (young adults: mean difference; 283 ± 201 nM, $P < 0.001$, 95% CI 176, 391; older adults: mean difference; 471 ± 381 nM, $P < 0.001$, 95% CI 260, 682), 2 hours post (young adults: mean difference; 497 ± 259 nM, $P < 0.001$,

95% CI 353, 640: older adults: mean difference; 545 ± 325 nM, $P < 0.001$, 95% CI 364, 325) and 3 hours post (young adults: mean difference; 559 ± 201 nM, $P < 0.001$, 95% CI 442, 675: older adults: mean difference; 797 ± 525 nM, $P < 0.001$, 95% CI 493, 1100). There was also a significant increase at peak plasma nitrite concentration (hour 3), compared with hour 2 (mean difference; 201 ± 344 nM, $P = 0.039$, 95% CI 11, 392).

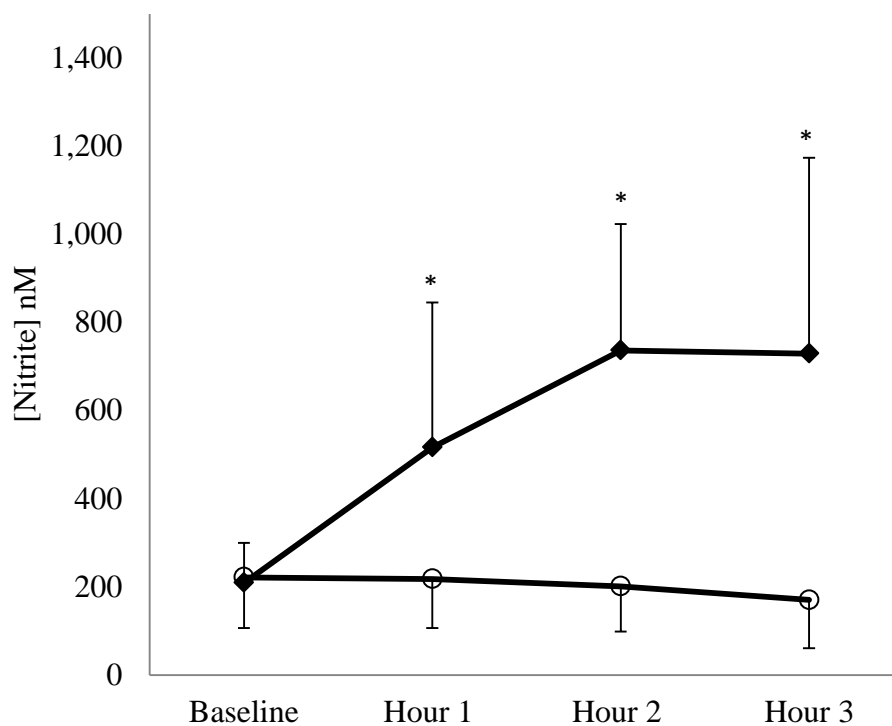


Figure 18. Plasma NO_2^- concentration in a young adult cohort.

This figure depicts plasma NO_2^- concentration for the young adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot juice. The * represents a significant difference when beetroot juice is compared with placebo < 0.001 .

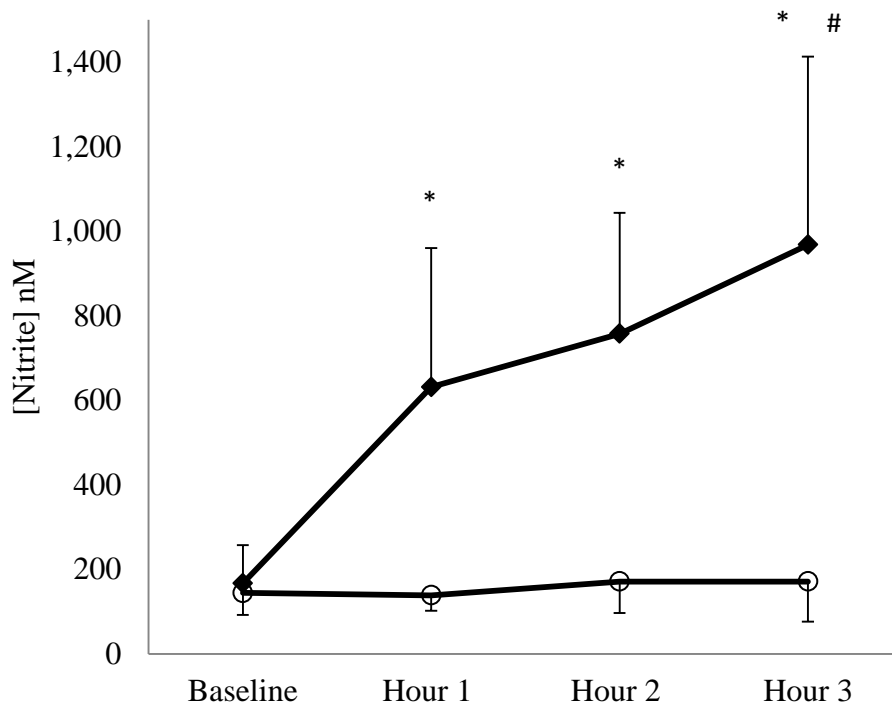


Figure 19. Plasma NO_2^- concentration in a older adult cohort.

This figure depicts plasma NO_2^- concentration for the older adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot juice. The * represents a significant difference when beetroot juice is compared with placebo < 0.001 . The # shows a significant difference in the beetroot condition when hour 2 is compared to hour 3.

ADC:

Repeated measures ANOVA revealed no significant main effect for time (young adults: $F_{(3, 45)} = 0.96$, $P = 0.42$; older adults; $F_{(3, 42)} = 0.27$, $P = 0.85$), supplementation (young adults: $F_{(1, 15)} = .314$, $P = 0.58$; older adults; $F_{(1, 14)} = 1.65$, $P = 0.22$) or an interaction (young adults: $F_{(3, 45)} = 0.25$, $P = 0.74$; older adults; $F_{(3, 42)} = 1.3$, $P = 0.28$) between visits for ADC index.

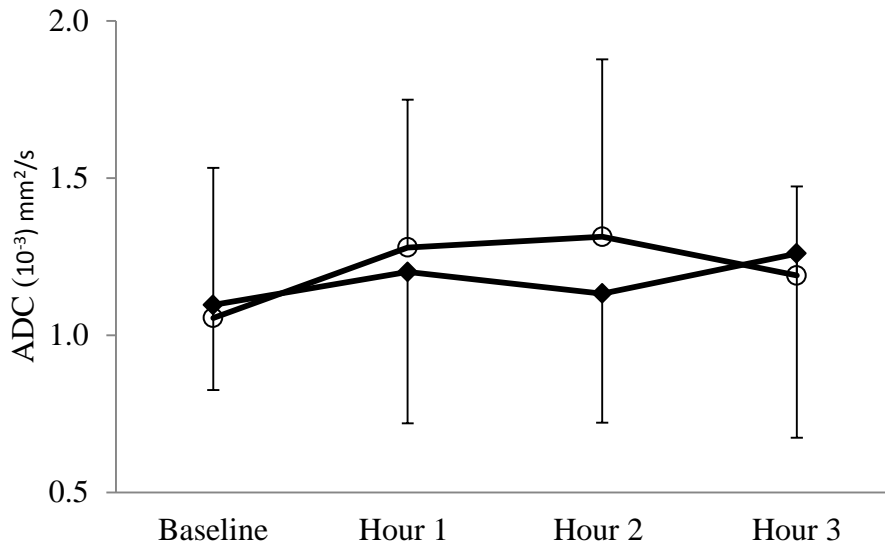


Figure 20. ADC in a young adult cohort across time for beetroot and placebo juice.

The figure shows ADC (microvascular diffusion) for the young adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot juice.

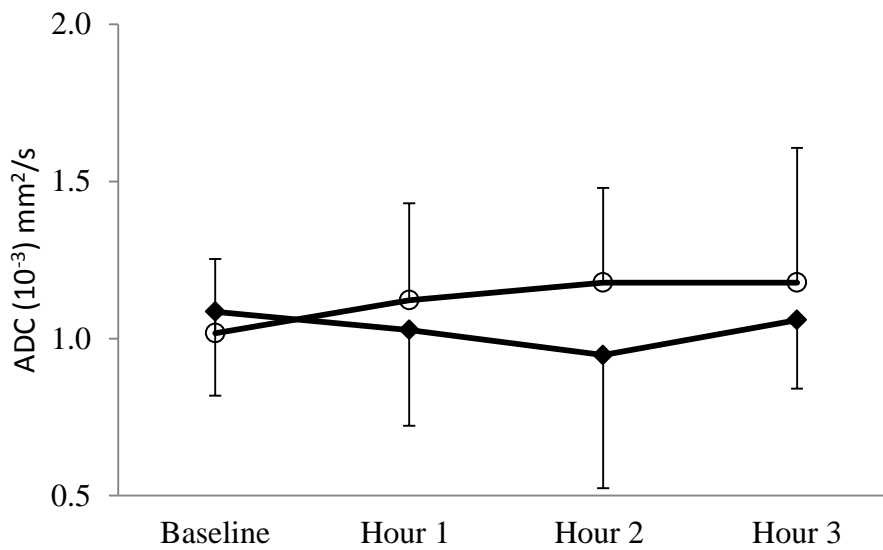


Figure 21. ADC in an older adult cohort across time for beetroot and placebo juice.

This table shows ADC (microvascular diffusion) for the older adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot.

Portal Vein Flux

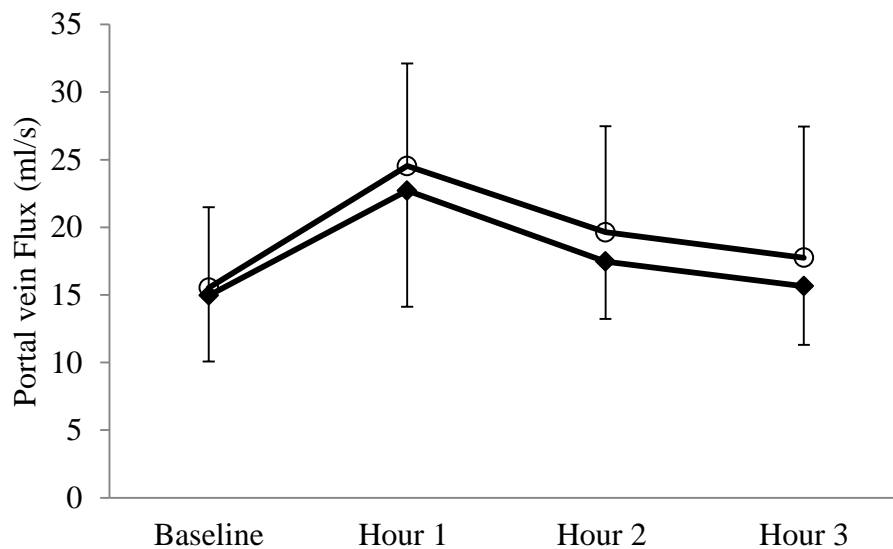


Figure 22. Portal vein flux in a young adult cohort across time for beetroot and placebo juice.

This figure shows portal vein flux for the young adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot.

Repeated measures ANOVA revealed a significant main effect for time (young adults: $F_{(3, 45)} = 14.07$, $P < 0.001$; older adults; $F_{(3, 42)} = 32.84$, $P < 0.001$), however, no significant effect for supplementation (young adults: $F_{(1, 15)} = 1.00$, $P = 0.33$; older adults; $F_{(1, 14)} = 3.04$, $P = 0.10$) or interaction (young adults: $F_{(3, 45)} = 0.339$, $P = 0.79$; older adults; $F_{(3, 42)} = 1.65$, $P = 0.19$) between visits for portal vein flux. However, there was a baseline difference for older adults (older adults: placebo; 14.6 ± 4.3 vs. beetroot juice; 11.7 ± 2.9 ml/s, $P = 0.04$, 95% CI -5.67, -0.13).

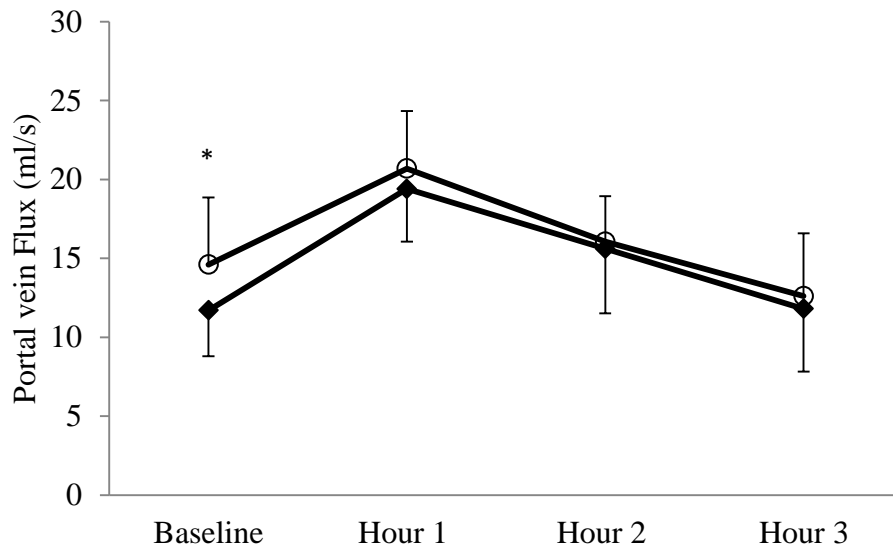


Figure 23. Portal vein flux in an older adult cohort across time for beetroot and placebo juice.

This table shows portal vein flux for the older adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot. The * represents a statistically different baseline portal vein flux between conditions.

Portal Vein Velocity

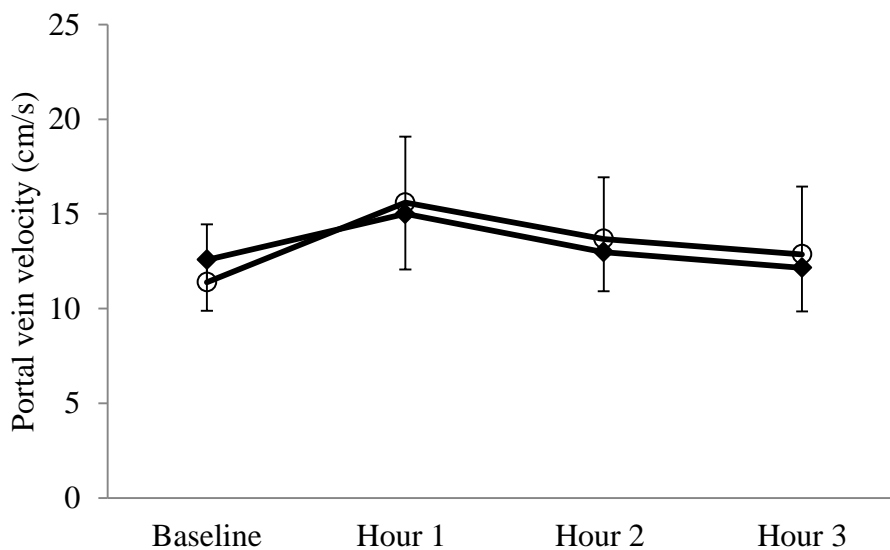


Figure 24. Portal vein velocity in a young adult cohort across time for beetroot and placebo juice.

This figure shows portal vein velocity for the young adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot. The * represents a statistically different baseline portal vein velocity between conditions.

Repeated measures ANOVA revealed a significant main effect for time (young adults: $F_{(3, 45)} = 10.7, P < 0.001$; older adults; $F_{(3, 42)} = 17.7, P < 0.001$), however, no significant effect for supplementation (young adults: $F_{(1, 15)} = 0.1, P = 0.75$; older adults; $F_{(1, 14)} = 2.3, P = 0.15$) however, there was an interaction effect in the young adults: ($F_{(3, 45)} = 2.9, P = 0.04$) but not in the older adults; $F_{(3, 42)} = 1.8, P = 0.16$) between visits for portal vein velocity. Post hoc analysis of the interaction revealed no statistical differences. However, there was a baseline difference for older adults (older adults: placebo; 13 ± 3.4 vs. beetroot; 11.1 ± 3 cm/s, $P = 0.04$, 95% CI -3.7, -1.1).

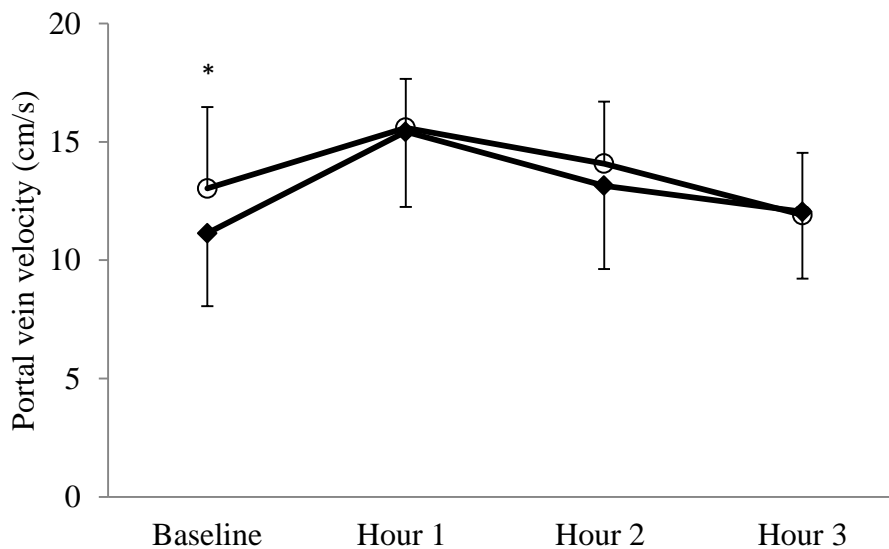


Figure 25. Portal vein velocity in a older adult cohort across time for beetroot and placebo juice.

This figure shows portal vein velocity for the older adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot. The * represents a statistically different baseline portal vein velocity between conditions.

Plasma glucose concentration:

Repeated measures ANOVA revealed a significant main effect for time (young adults: $F_{(3, 45)} = 7.3$, $P < 0.001$; older adults; $F_{(3, 42)} = 17.7$, $P < 0.001$), however, no significant main effect on supplementation (young adults: $F_{(1, 15)} = 0.96$, $P = 0.35$; older adults; $F_{(1, 14)} = 1.4$, $P = 0.26$) or an interaction (young adults: $F_{(3, 45)} = 0.96$, $P = 0.42$; older adults; $F_{(3, 42)} = 0.04$, $P = 0.99$) between visits for plasma glucose concentration.

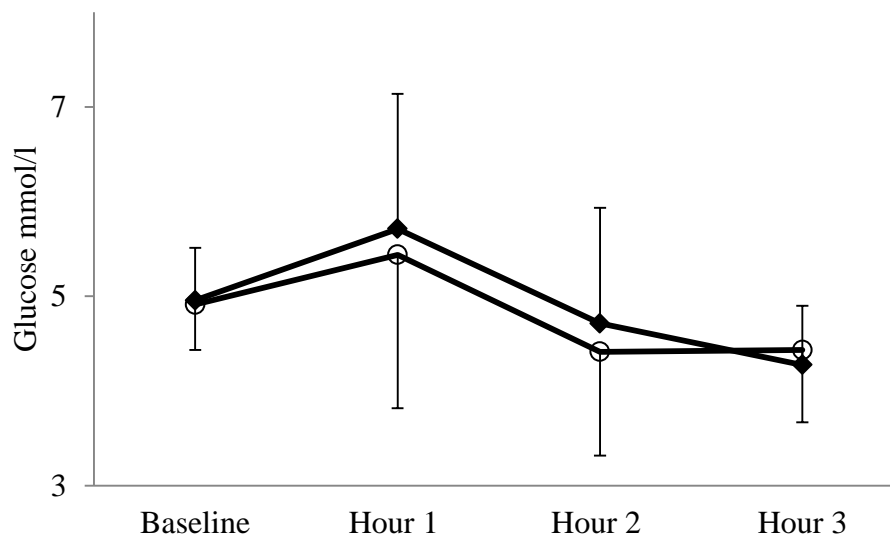


Figure 26. Plasma glucose concentrations in a young adult cohort across time for beetroot and placebo juice.

Figure shows plasma glucose concentration for the young adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot.

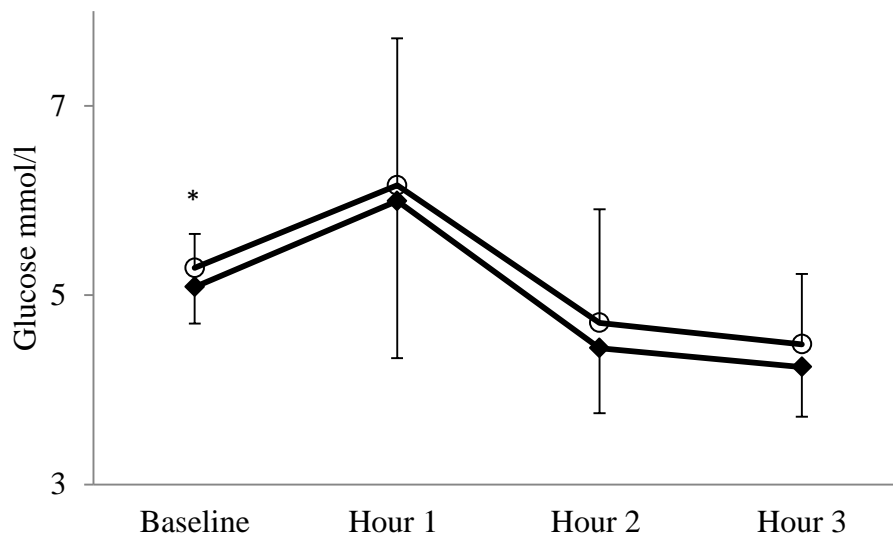


Figure 27. Plasma glucose concentrations in a older adult cohort across time for beetroot and placebo juice.

This figure shows plasma glucose concentration for the older adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot. The * represents a statistically different baseline glucose concentration between conditions.

Effects on resting blood pressure:

Repeated measures ANOVA revealed a significant main effect for time (young adults: $F_{(3, 45)} = 4.5, P = 0.008$; older adults; $F_{(3, 42)} = 13.3, P < 0.001$), however, no significant main effect on supplementation (young adults: $F_{(1, 15)} = 1.2, P = 0.28$; older adults; $F_{(1, 14)} = 1.7, P = 0.20$) or an interaction (young adults: $F_{(3, 45)} = 0.20, P = 0.89$; older adults; $F_{(3, 42)} = 1.7, P = 0.18$) between visits for systolic blood pressure.

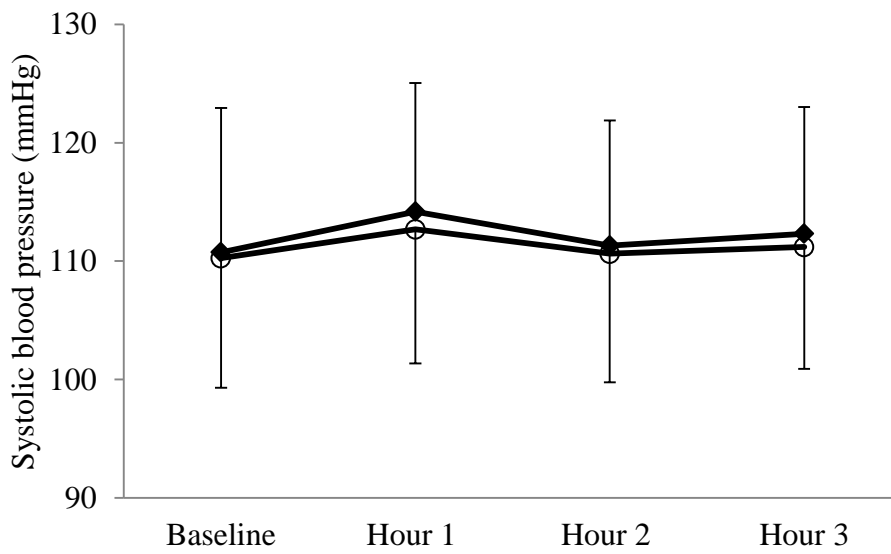


Figure 28. Systolic blood pressure in a young adult cohort across time for beetroot and placebo juice.

Depicts systolic blood pressure for the young adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot.

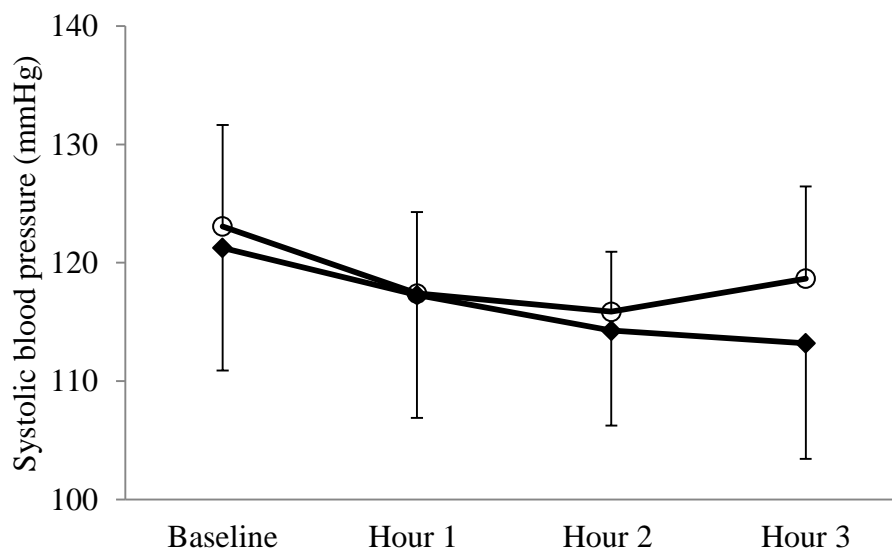


Figure 29. Systolic blood pressure in an older adult cohort across time for beetroot and placebo juice.

Depicts systolic blood pressure for the older adult cohort with changes across time for beetroot and placebo juice.

Repeated measures ANOVA revealed a non-significant main effect on diastolic blood pressure for time in young adults ($F_{(3, 45)} = 2.4, P = 0.08$) and a significant main effect of time in older adults ($F_{(3, 42)} = 18.8, P < 0.001$), however, no significant main effect on supplementation in young adults ($F_{(1, 15)} = 2.6, P = 0.13$). There was however, a trend for a reduction as a main effect of supplementation in older adults ($F_{(1, 14)} = 4.0, P = 0.06$). No effect on interaction (young adults: $F_{(3, 45)} = 0.25, P = 0.86$; older adults; $F_{(3, 42)} = 0.45, P = 0.72$) was found between visits for diastolic blood pressure.

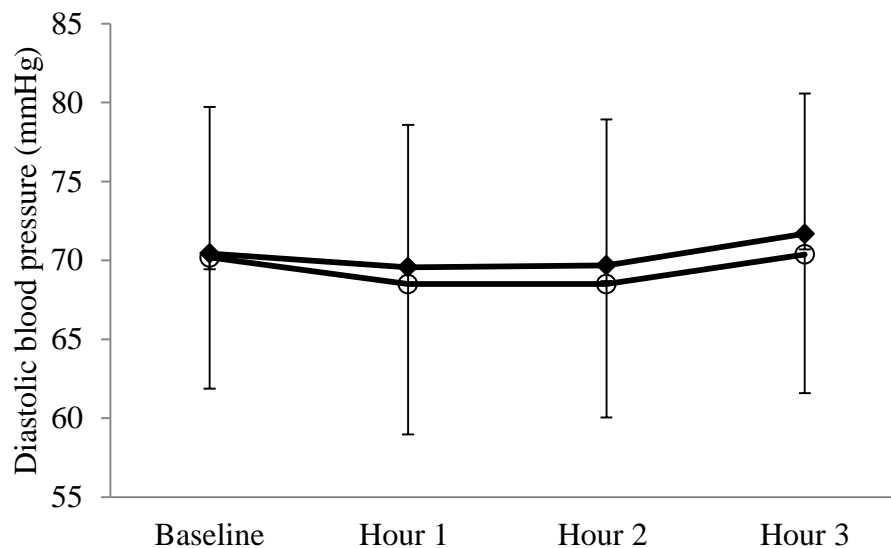


Figure 30. Diastolic blood pressure in a young adult cohort across time for beetroot and placebo juice. Depicts diastolic blood pressure for the young adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot.

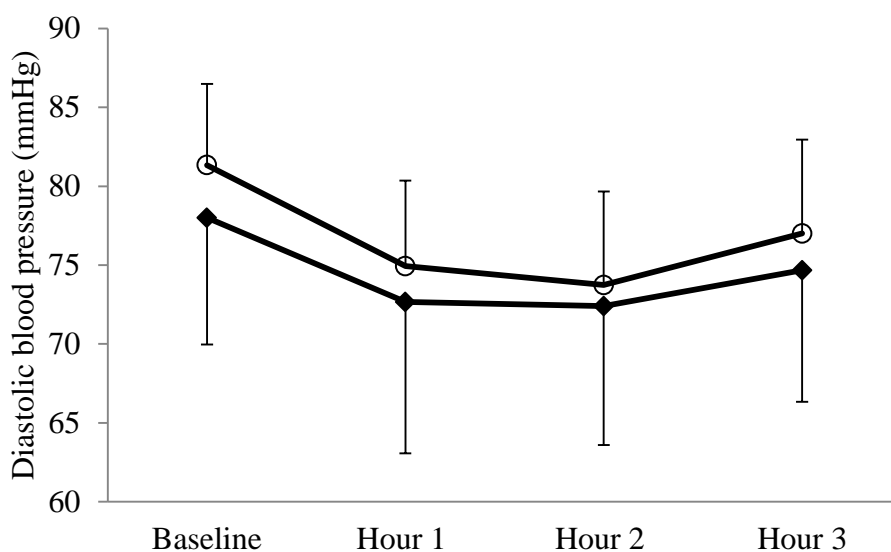


Figure 31. Diastolic blood pressure in a older adult cohort across time for beetroot and placebo juice.

Depicts diastolic blood pressure for the older adult cohort with changes across time for beetroot and placebo juice. The open circles are placebo and the closed circles beetroot.

3.3.7: Discussion

This is the first study to investigate the effects of dietary NO_3^- supplementation on hepatic blood flow and glucose homeostasis in a healthy older adult population. The primary outcomes were to assess changes in microvascular diffusion (ADC), portal vein flux and velocity. Nitrate supplementation did not alter portal vein flux, velocity or affect ADC. Secondary outcomes were to assess plasma glucose concentration and blood pressure changes. Nitrate supplementation did not alter plasma glucose concentration. Nitrate supplementation did not lower systolic or diastolic blood pressure.

Nitrate supplementation and plasma NO_3^- and plasma NO_2^- concentration.

Plasma NO_3^- concentration peaked post NO_3^- rich beetroot juice compared with placebo for both the young (30.9 vs. 664.3 μM) and older adult (32.1 vs. 633.8 μM)

groups at 2 hours by ~2000%. Similarly, plasma NO_2^- concentration rose following NO_3^- rich beetroot juice compared to the placebo and peaked at 2 hours for the young group (218.3 vs. 715.5 nM) and 3 hours for the older adult group (171 vs. 967.9 nM) representing a ~230 and 460% increase, respectively. The pharmacokinetic response for both plasma NO_3^- and NO_2^- concentrations of both cohorts are similar to a previously reported does response study in young healthy individuals (Wylie et al., 2013a). However, the pharmacokinetic response of plasma NO_3^- and NO_2^- concentrations have not previously been reported in a group of healthy older adults. Kelly et al. (2013a) recently reported a rise in plasma NO_2^- concentration of a similar magnitude by 418% (248 to 1037 nM) in a group of healthy older adults. The pharmacokinetic response in the healthy older adult group shows a peak at 3 hours. This is much higher than at hour 2 (757 nM) and therefore may still have been rising at 3 hours.

The lengthened time to peak plasma NO_2^- concentration in the older adult cohort may be associated with the commensal oral bacteria which play a fundamental role in the entero-salivary pathway. Serum antibodies to these oral bacteria decrease in concentration as we age (Percival et al., 1996, Challacombe et al., 1995, Percival et al., 1997). These reductions can lead to increased risk of infection and disease from pathogens (Marsh and Percival, 2006). However, this increase in oral microflora may lead to a greater increase in the conversion of NO_3^- to NO_2^- , potentially changing the bacteria's contribution as a rate limiting factor which may explain why plasma NO_2^- concentrations were still rising 3 hours after NO_3^- supplementation. Resting salivary flow rates are not different between young and older healthy adults. Therefore salivation is unlikely to be a contributory factor leading to the delayed peak in plasma

NO_2^- concentration (Ship and Fischer, 1997). This suggests that more NO_3^- will be converted to NO_2^- , however, due to the flow rate of saliva the peak is delayed.

Nitrate supplementation and hepatic blood flow

Despite a statistically significant and physiologically meaningful rise in plasma NO_2^- concentration, this did not lead to an increase in portal vein flux, velocity or microvascular diffusion (ADC). The portal vein supplies 75% of inflow, with the remainder supplied by the hepatic artery (Rappaport, 1979) and even though the blood it supplies is partially deoxygenated, the portal vein supplies ~50% of the O_2 delivery (See Hwang., 2011 and Vollmar and Menger., 2009 for reviews). A higher proportion of deoxyhaemoglobin may lead to a faster conversion of NO_2^- to $\text{NO}\cdot$ (Cosby et al., 2003) in the portal vein. It is therefore likely that the highest concentrations of $\text{NO}\cdot$ and plasma glucose concentrations would be found in the liver.

The hepatic artery has the capacity to dilate in response to changes in portal vein flux however, the portal vein cannot dilate in response to changes in hepatic arterial flow (Vollmar and Menger, 2009, Legare and Lutt, 1987). Thus if the portal vein flow decreases the hepatic artery will dilate to increase, flow and if the portal vein increases flow the hepatic artery will constrict to reduce flow (Lutt et al., 1990). This is known as the known as the 'hepatic arterial buffer response' (Hwang, 2011). This buffering response is limited and may be able to compensate for 25 - 60% of change in flow from the portal vein (Lutt, 1980, Lutt, 1977). The underlying reason behind this is to enable a steady perfusion rate in order to manage changes in blood flow from splanchnic organs and the associated hormones and nutrients (Lutt, 1980, Lutt, 1977). This could, in part explain the lack of effect of an increased plasma

NO₂⁻ concentration on portal vein blood flow. As expected, there was an increase in portal vein flux and velocity from baseline to the liver regardless of condition.

There was, however, a statistical difference in baseline portal vein flux (placebo: 14.6 and active 11.7 cm/s; mean difference of 2.9 cm/s) and velocity (placebo: 13 and active 11.1 cm/s; mean difference of 1.9 ml/s) on the two visits. The day to day coefficient of variation for portal vein flux and velocity was 13% and 16% respectively. The mean differences are outside our repeatability and are likely to be genuine differences between visits. This suggests that portal vein flux and velocity were faster on the placebo visit at baseline. This may cloud any differences between the active condition and placebo later in the time course. Participants were fasted overnight, having avoided caffeine, strenuous exercise and were in a hydrated state. Therefore it is unclear why there is a baseline difference. It may be due to a type 2 error.

Nitrate supplementation and plasma glucose concentrations

There was no significant difference between active or placebo juice for plasma glucose concentration. It should be noted that in the older adult cohort there was statistically significant baseline difference in plasma glucose concentration (mean difference = -0.2 mmol/l or 3.8%). On the active testing days plasma glucose concentration was already ~4% lower than placebo. Mechanisms for the uptake of glucose have been partially elucidated. NO[•] is known to mediate glucose uptake from the intestines and skeletal muscle (Merry et al., 2010, Guan et al., 2003, Kingwell et al., 2002). Potentially via elevated NO[•] bioavailability which has been shown to stimulate insulin secretion (Nystrom et al., 2012) and increase GLUT4 translocation (Li et al., 2004). The potential to modifying this diabetes risk factor via

reductions in plasma glucose concentrations would likely reduce micro and macrovascular complications which are a severe cause of reduced quality of life, morbidity and mortality (Sprafka et al., 1991, Jaffe et al., 1984). Larger samples sizes that are appropriately powered are required to assess the effect of nitrate supplementation on plasma glucose concentrations.

Nitrate supplementation and resting blood pressure.

There was no significant difference in systolic or diastolic BP following NO_3^- rich beetroot juice supplementation (at peak plasma NO_2^- concentration) compared to placebo in the healthy young adult cohort. This is similar to some acute NO_3^- supplementation studies (Wilkerson et al., 2012, Coles and Clifton, 2012, Sandbakk et al., 2014). However, most studies have shown hypotensive effects with acute supplementation regimens (Webb et al., 2008a, Vanhatalo et al., 2010, Vanhatalo et al., 2011, Lansley et al., 2010, Lansley et al., 2011a). Larger sufficiently powered trials are required to determine the effect of NO_3^- as a hypotensive agent in young individuals. There was no significant reduction in systolic BP following NO_3^- rich beetroot juice compared to placebo in the healthy older adult cohort. We report a non significant 5 mm Hg drop in systolic BP. Kelly et al. (2013a) report a statistically significant 5 mmHg drop in systolic BP, whilst another groups reported larger reductions (Jajja et al., 2014). Another study in healthy older adults reported no effect of nitrate supplementation on systolic BP (Bondonno et al., 2014b). Kelly et al. reported diastolic BP reductions of 3 mmHg. Whereas we report a non-significant change in diastolic BP of 2 mmHg. A detailed pharmacokinetic study in healthy older adults over a prolonged period may elucidate at what time peak plasma NO_2^- concentrations occur. If the pharmacokinetic response of plasma NO_2^-

concentrations in the healthy older adult group continued to rise past 3 hours, a greater hypotensive effect than measured here may have occurred.

Strengths and limitations.

This is the first study to examine the effect of NO_3^- supplementation on hepatic blood flow. This study has a robust experimental design as a double-blind, placebo controlled, crossover trial. A limitation to this study is that we did not measure plasma NO_2^- concentration beyond 3 hours supplementation. Future research should aim to elucidate if plasma NO_2^- concentration rises beyond 3 hours in healthy older adults.

3.3.7: Conclusion

This was the first study to examine the hepatic blood flow response to NO_3^- supplementation. Despite a large and physiologically meaningful elevation in plasma NO_2^- concentration following an acute dose of 11.91 mmol of NO_3^- , there was no effect on hepatic blood flow, plasma glucose concentrations or systolic blood pressure. .

4.0: General discussion

This body of work has made a number of novel contributions in this area of research; it was the first study to investigate the effects of dietary NO_3^- supplementation on the exercise responses of individuals with T2DM. Dietary NO_3^- supplementation did not reduce the O_2 cost of walking in individuals with T2DM or increase the distance covered in the six minute walk test. In addition this work confirms previous findings that NO_3^- supplementation has no effect on resting blood pressure in individuals with T2DM.

This was the third peer reviewed article to assess the effects on individuals with COPD (all published in *Nitric Oxide* within 6 weeks of each other). Dietary NO_3^- supplementation did not reduce the O_2 cost of cycling in individuals with COPD, or increase the distance covered in the six minute walk test. In contrast to the other reports in individuals with COPD, no hypotensive effect was found.

This is the first study to examine the liver diffusion or delivery. The data shows no increase in liver diffusion or delivery. The data shows no effect of nitrate in blood glucose concentrations or blood pressure.

Dietary NO_3^- supplementation in the form of beetroot juice (in an older adult population) is unlikely to reduce the O_2 cost of exercise, improve walking performance or reduce BP in individuals with T2DM and COPD. Moreover, it does not alter glucose homeostasis or affect blood pressure in healthy adults.

I will begin the discussion with an update of the studies published since the beginning of this body of work. Table 13 describes each trial published and each of the key outcome measures assessed. I will then describe and critically appraise my findings in the context of recent literature.

Table 12: A, B and C. Publications on NO₃⁻ supplementation from 2012 – April 2015 (split via age and disease status).

A, Healthy young individuals

B, Healthy older individuals

C, Clinical populations

Studies in table 13, B and C (that are not abstracts), will be critically reviewed in the discussion below. Please see marked with a *.

Table A: Nitrate supplementation studies in healthy young individuals

Author	Participants & Protocol	NO ₃ ⁻ supplement	Plasma NO ₃ ⁻ & NO ₂ ⁻ concentration (BR vs. PL respectively)	BP changes	Exercise efficiency or performance and/or clinical relevance	Primary outcome successful?
Wilkerson et al. (2012)	8 well-trained male cyclists. A minimum of a 7 day washout was given. 4BP measurements were taken on each visit. Exercise involved a 50 mile TT.	500ml of BR juice (NO ₃ ⁻ =6.2mmol) or PL (NO ₃ ⁻ =0.005mmol) 2.5 hours prior to exercise.	NO ₂ ⁻ = 379 ± 94 and 472 ± 96 nM	↔SBP, DBP or MAP	↔ TT performance	No
Masschelein et al. (2012)	15 healthy volunteers. With a 14 day washout period. Exercise included cycling in normoxia and hypoxia.	500ml of BR (0.07 Kg ⁻¹ day ⁻¹ of body weight) for 6 days or PL (apple and blackcurrant).	NO ₂ ⁻ = 3.8 ± 0.1 and 5.3 ± 0.3 μM NO ₃ ⁻ = 41 ± 3 and 147 ± 7 μM	N/A	BR negates some negative effects of hypoxia. ↑Arterial and muscle oxygenation.	Mixed

Engan et al. (2012)	12 healthy subjects. Tests; vital capacity, apnoeic duration and arterial O ₂ saturation. Washout period not stated.	70ml of BR juice (NO ₃ ⁻ =5mmol) or PL (NO ₃ ⁻ =0.003mmol) 2.5h prior to each test.	Figures not given.	↓MAP	↓S _a O ₂ ↑ Max apnoeic duration by 11%	Yes
Hobbs et al. (2012).	14 subjects (sex and disease status unknown). 24h ambulatory BP was measured.	Subjects consumed BR juice (0, 100, 250 or 500g) or water (PL) and bread (PL) or BR bread (white and red beets).	Abstract only, figures not presented.	N/A	↓BP in a ~ dose dependant manor. ↔ BP for white BR bread	Yes
Bond et al. (2012). (Abstract only)	14 well-trained junior male rowers. A 7 day washout period was employed. Exercise included 6 maximal 500m ergometer reps.	500ml of BR or PL daily for 6 days.	Abstract only, figures not presented.	N/A	↑ average repetition time	Yes
Coles and Clifton (2012)	30 healthy subjects (15 males). 14 day washout was employed. 24h ambulatory blood pressure was taken	500ml of BR juice (NO ₃ ⁻ =15 mmol) or PL (apple juice matched for colour and sweetness)	Figures not given.	Trend for ↓SBP	↓SBP in males at 6h post supplement	Yes
Krajka-Kuzniak et al. (2012).	24 male 6 week old rats. Split into 4 independent groups. DNA damage and liver injury induced by hepatocarcinogenic N-nitrosodiethylamine (NDEA).	Two groups fed beetroot juice and one of two different enzymes. Groups ¾ had PL and water.	Figures not published.	N/A	Protects against oxidative stress Protects against liver damage	Yes

Klewicka et al. (2012)	Caco-2 cell line (Rat cells/colons acquired from 32 rats, 4 groups of 8).	Group 1 had a basal diet. Group 2 was fed a basal diet and BR juice for 30 days. Group 3 was fed BR and a carcinogen. Group 4 had BR and a different carcinogen.	N/A	N/A	Protect against precancerous aberrant crypt formation. ↓Oxidative stress	Yes
Ferguson et al. (2013)	Rats were fed BR or PL (H ₂ O) for 5 days. Microvascular PO ₂ was measured during 180s of electorally induced muscle contractions.	BR (NO ₃ ⁻ = ~0.8 mmol/kg/day) or PL (H ₂ O)	↑ NO ₂ ⁻ ↑ NO ₃ ⁻ Values not provided	N/A	↔ in steady-state Microvascular or PO ₂ , however, it did slow the decline. BR ↑ O ₂ driving pressure during the resting phase	Mixed
Christensen et al. (2013)	10 highly trained male cyclists. An approximate 14 day washout was given. The exercise involved a 120 min preload followed by a 400-kcal TT.	500ml/day of BR juice (~0.5g of NO ₃ ⁻) per day for 6 days. PL was a blackcurrant cordial. Day 6 was consumed 3h prior to exercise.	NO _x = 159 ± 103 and 40 ± 7 μM	N/A	↔ $\dot{V}O_2$ kinetics ↔ TT performance	No
Fulford et al. (2013)	8 healthy male subjects. A minimum of a 14 day washout. Exercise involved two tasks. Repeated MVC and short bouts of continuous exercise.	500ml of BR juice (NO ₃ ⁻ =10.2 mmol) or PL (NO ₃ ⁻ =0.17 mmol) per day for 15 days. Participants arrived 2.5h, 5 and 15 days post supplementation.	NO ₂ ⁻ = 408 ± 131 and 232 ± 49 nM	N/A	↔ Force output ↔ Peak contraction ↔ Mean or End force ↔ Time effect Trend for ↓ Pcr cost as an average (0.06) and a ↓ Pcr cost for end exercise	Mixed

Wylie et al. (2013b)	14 healthy male subjects. A minimum 72h washout period was used. Exercise involved a Yo-Yo IR1.	BR juice ($\text{NO}_3^- = 4.1$ mmol) or PL ($\text{NO}_3^- = 0.04$ mmol). Participants consumed two shots in the morning and two in the evening the day before testing. On the day two shots were consumed 2.5h and 1 shot 1.5h prior to exercise.	$\text{NO}_2^- = 584 \pm 343$ and 118 ± 44 nM $\text{NO}_3^- = 768 \pm 180$ and 25 ± 9 μM . NO_2^- values declined by 20% in PL and 54% in BR. NO_3^- rose by 17% in PL and 10% in BR.	N/A	\uparrow Yo-Yo performance (4.2%) \uparrow Plasma potassium \downarrow Blood glucose levels	Yes
Wylie et al. (2013a)	10 healthy male subjects. A minimum of 3 days washout was given. Exercise involved a 2 MI and 1 SI bout on each of 6 visits (1 visit per condition).	Participants consumed 70ml, 140ml and 280ml BR juice ($\text{NO}_3^- = 4.2$ mmol) or PL ($\text{NO}_3^- = 0.04$ mmol) per shot).	$\text{NO}_2^- = 425 \pm 225$ and 150 ± 73 nM $\text{NO}_3^- = 580 \pm 89$ and 130 ± 17 μM . \uparrow in a dose dependant manor.	\downarrow SBP (peak at 4h) \downarrow DBP (peak at 4h) \downarrow MAP (peak at 1h)	140 & 280 \downarrow steady state O_2 and time to task failure.	Yes
Kelly et al. (2013b)	9 healthy active males. Exercise involved 4 SI bouts at different % of peak power to exhaustion.	500ml of BR juice ($\text{NO}_3^- = 8.2$ mmol) or PL ($\text{NO}_3^- = 0.006$ mmol)	$\text{NO}_2^- = 297 \pm 98$ nM and 98 ± 41 nM	N/A	\uparrow at 60%, 70% and 80% peak power but not 100%. \leftrightarrow CP or W'	Yes
Breese et al. (2013)	9 (4 males) healthy subjects. A 7 day washout period was utilised. NIRS, muscle oxygenation and $\dot{V}\text{O}_2$ kinetics were assessed.	Participants consumed 70ml, BR juice ($\text{NO}_3^- = 4$ mmol) or PL ($\text{NO}_3^- = 0.002$ mmol) per shot) in the morning and the evening for 6 days.	$\text{NO}_2^- = 348 \pm 170$ nM and 65 ± 32 nM	N/A	\uparrow speed of $\dot{V}\text{O}_2$ kinetics \uparrow deoxyhaemoglobin kinetics (derived from NIRS) \uparrow time to task failure	Yes

Hoon et al. (2013)	10 highly trained male rowers. Exercise involved a 2000m TT.	Participants consumed 70ml, BR juice ($\text{NO}_3^- = 4.2 \text{ mmol}$ or 8.4 mmol) or PL ($\text{NO}_3^- = 0 \text{ mmol}$).	Abstract only, figures not presented.	N/A	\leftrightarrow in rowing performance	No
Muggeridge et al. (2013a)	8 healthy male kayakers. Exercise included 15m paddling at 60% max WR and 5x10s all out sprints and a 1km TT.	Participants consumed 70ml, BR juice or PL.	$\text{NO}_2^- = 687.9 \pm 20$ and $519.8 \pm 25.8 \text{ nM}$ $\text{NO}_3^- = 152 \pm 3.5$ and $33.8 \pm 1.9 \mu\text{M}$.	N/A	\leftrightarrow TT performance \leftrightarrow Peak power \downarrow steady state $\dot{V}\text{O}_2$	No
Muggeridge et al. (2013b)	9 male trained cyclists. Exercise included a 15 ride at 60% max WR and a 16.1 km TT at 2500m simulated altitude.	Participants consumed 70ml, BR juice or PL 3h prior to exercise.	$\text{NO}_2^- = 678.1 \pm 103.5$ and $289.8 \pm 27.9 \text{ nM}$ $\text{NO}_3^- = 150.5 \pm 9.3$ and $39.1 \pm 3.5 \mu\text{M}$.	N/A	\downarrow steady state $\dot{V}\text{O}_2$ \uparrow TT performance	Yes
Velmurugan et al. (2013)	24 healthy subjects (12 males). With a 1-4 week washout period. In vivo and in vitro methods were used.	250ml BR juice or K NO_3^- ($\text{NO}_3^- = 8 \text{ mmol}$) vs. PL.	Figures not published.	N/A	\downarrow Platelet reactivity in males but not in females. Males have a greater dependence on the NO- sGC pathway.	Yes
Lane et al. (2013)	24, Trained cyclists completed 4 experimental conditions. BR, caffeine, BR & caffeine and control. Ergometer TT.	Caffeine = $3 \text{ Kg}^{-1} \text{ day}^{-1}$ of body weight and BR (8.4 mmol) supplementation 2h prior to testing.	Abstract only, figures not presented.	N/A	\leftrightarrow in TT performance following BR vs. PL.	No
Bond Jr et al. (2013) (Abstract only)	12 healthy young african americans. Protocol, rest and 40, 60 and 80% peak $\dot{V}\text{O}_2$.	BR and orange juice as placebo.	Abstract only, figures not presented.	\downarrow SBP \leftrightarrow DBP	\downarrow $\dot{V}\text{O}_2 \leftrightarrow$ in RQ, minute ventilation and HR	Yes

Schiffer et al. (2013)	15 healthy young males. Indirect calorimetry.	Three days ($\text{NO}_3^- = 0.1 \text{ mmol Kg}^{-1} \text{ day}^{-1}$) or PL (NaCl)	Published abstract, not data presented.	N/A	↓ metabolic rate Plasma NO_2^- correlates ($r 0.7$) with BMR.	Yes
Murphy et al. (2014) (<i>Abstract only</i>)	13 recreationally active males. 10 day washout. Protocol, 10 x 6s repeated sprints with 30s recovery.	BR (5 mmol) supplementation vs. PL 2.5h prior to exercise.	Abstract only, figures not presented.	N/A	↔ $\dot{V}\text{O}_2$ or fatigue	No
Hoon et al. (2014)	26 well-trained cyclists. 2 x 4min TT, separated by 75 mins. 6 day washout.	70ml BR 75 or 150 mins prior to exercise with 35ml top up following first bout.	Figures not published, but peaked at 75 mins.	N/A	↔ in TT performance following BR vs. PL. Potential negative effects.	No
Boorsma et al. (2014)	8 elite male 1500m runners. 7+ day washout. Protocol includes a submaximal run and 1500 m TT run.	210ml BR juice for 8 days ($\text{NO}_3^- = 19.5 \text{ mmol}$) vs. PL.	$\text{NO}_3^- = 870 \pm 259$ and $37 \pm 15 \mu\text{M}$.	N/A	↔ $\dot{V}\text{O}_2$ or improvement in TT performance.	No
Peeling et al. (2014)	Study A: 6 male national level kayakers completed a 7 x 4 min lab based step test TT. Study B: 5 Females national level kayakers completed a 500 m 'on water' TT.	Study A: 70 ml BR (4.8 mmol) or PL (not a true PL) 2.5h prior to exercise. Study B: 140 ml BR (9.6 mmol) or PL (not a true PL). 2 h prior to exercise.	Abstract only, figures not presented.	N/A	↓ $\dot{V}\text{O}_2$ (mod) ↔ in TT performance in the laboratory but ↑ TT in the field.	Primary outcome not stated.
Richards et al. (2014)	7 young adults performed hand grip strength at 5, 15 and 25% of MVC. Doppler ultrasound was used to assess changes in blood flow.	12.6 mmol BR post baseline. Exercise 2 hours post ingestion.	Abstract only, figures not presented.	N/A	↑ forearm blood flow and sig more as % MVC went up and also in hypoxia	Yes

Thompson et al. (2014)	16 recreationally active males. 20 min stages at 50%, 70% $\dot{V}O_{2\text{ peak}}$ and exercise to volitional exhaustion at 90% $\dot{V}O_{2\text{ peak}}$. 7 day washout.	450ml BR (5 mmol) or blackcurrant PL.	$\text{NO}_2^- = 222 \pm 61$ and 124 ± 10 nM	\downarrow SBP \leftrightarrow DBP	\uparrow exercise tolerance at 90% $\dot{V}O_{2\text{ peak}}$. \leftrightarrow in RPE, energy levels or cognitive function.	Yes
Martin et al. (2014)	16 recreationally active male (9) and females (7). Repeated 8s sprints with 30s recovery until volitional exhaustion.	70 ml (0.3 g of NO_3^-) or PL 2h prior to exercise.	NOx not measured.	N/A	\leftrightarrow repeated sprint performance. \downarrow total work	No
Sandbakk et al. (2014)	9 elite male cross-country skiers. Sub maximal running economy and 5km TT.	A; L-arginine (6g) & NO_3^- (614 mg). B; NO_3^- (614 mg) & PL. C; L-arginine and PL.	$\text{NO}_2^- = 328 \pm 107$ nM and 149 ± 64 nM	\leftrightarrow MAP -	L-arginine did not increase NO_2^- concentration. \leftrightarrow economy or TT.	No
Bond et al. (2014)	13 female African Americans. Washout not specified. Measures were taken at baseline, 40 & 80% $\dot{V}O_{2\text{ peak}}$. HR variability measured via SD of RR intervals of an ECG. Collofello et al. (2014)	500 ml BR (750 mg), PL = orange juice. 2h 20min prior to exercise.	$\text{NO} = 21.1 \pm 4.9$ and 4.4 ± 1 nM	\downarrow SBP	\uparrow Heart rate variability during exercise.	Yes
Collofello et al. (2014)	12 healthy females. 20s submaximal static apnoea with 90s rest followed by a max effort apnoea.	70ml BR juice for 8 days ($\text{NO}_3^- = 5$ mmol) vs. PL ($\text{NO}_3^- = 0.003$ mmol). 2.5 post ingestion the measurement begun.	NOx not measured / reported.	\downarrow SBP \leftrightarrow DBP \leftrightarrow MAP	\leftrightarrow maximal static apnoea	No
Pinna et al. (2014)	14 moderately trained male masters swimmers performed two incremental swimming tests.	500ml BR juice for 6 days ($\text{NO}_3^- = 5.5$ mmol) vs. no supplementation.	NOx not measured / reported.	N/A	\downarrow $\dot{V}O_2$ during swimming. \uparrow anaerobic threshold	Yes

Bentley et al. (2014)	9 healthy males. fMRI was performed at 15% & 25% MVC.	700 ml BR 2.5 h prior to exercise scan vs. no juice.	NO _x not measured / reported.	N/A	↓ tissue oxygenation maximal change (more effective at lower intensities) ↔ recovery rate	Yes
Puype et al. (2014)	22 healthy males in independent groups (n=11). 6 week training program. 5 x 30mins a week sustaining a BLA of 4-6 mmol.	NO ₃ ⁻ = 0.07 mmol Kg ⁻¹ day ⁻¹ or PL (apple-blackcurrant juice) 2.5 h prior to each training session.	NO ₃ ⁻ concentrations not given. Estimate from figure = 165 and 35 μM.	N/A	16% ↑ PO & TT. ↔ activated AMP, glycogen breakdown or mRNA variables.	Yes
Ferguson et al. (2014)	20 rats were split into independent groups. Microvascular PO ₂ was measured during 180s of electorally induced twitches	BR juice (NO ₃ ⁻ = 0.08 mmol Kg ⁻¹ day ⁻¹) or water day ⁻¹)	NO ₃ ⁻ & NO ₂ ⁻ concentrations but given. Estimate from figure = NO ₃ ⁻ = 140 and 22 μM. NO ₂ ⁻ = 640 and 200 nM	N/A	↔ Microvascular PO ₂ ↓ O ₂ driving pressure	No
Ashmore et al. (2014)	40 male wister rats. Exposed to chronic hypoxia (13%).	0.7 mmol l ⁻¹ NaCl (as control) or 0.7 mmol l ⁻¹ NaNO ₃ ,	Elevated plasma NO ₃ ⁻ levels by 80%. Figures not given but graphical representation is.	N/A	Nitrate maintains redox state (compared to hypoxia) ↑ ETC protein levels ↓ Respiration rate, ↑ ATP ↑ L-arginine	Yes
Muggeridge et al. (2014)	9 trained cyclists. 10 mins sub max followed by a 10 mile TT. 4 conditions, NO ₃ ⁻ GEL and UV-A , UV-A and PL, NO ₃ ⁻ GEL and sham light and PL and sham light	2 gels (=16.2 mmol), 2.5 h prior to exercise	Plasma NO ₂ ⁻ was higher for GEL + SHAM 332; and NIT + UV-A 456 nM; PL + SHAM 215. PL 282 nM	↓ SBP ↔ DBP ↓ MAP	UV-A light and NO ₃ ⁻ alters response to exercise. UV-A does not improve exercise performance alone.	No

Kim et al. (2014) abstract only	12 young healthy adults. 3h post supplementation, brachial artery diameter, flow, and blood velocity were measured (Doppler ultrasound) at rest and during 6 exercise intensities.	140 ml of BR (= 12.9 mmol) or PL.	NO ₃ ⁻ supplementation raised plasma NO ₃ ⁻ (19.5-fold) and NO ₂ ⁻ (1.6-fold) concentrations. Values not given.	N/A	↓ PWV. ↔ brachial artery diameter. ↔ brachial artery flow ↔ brachial artery shear stress	Yes
Carpentier et al. (2014)	13 recreationally active males. 20 min at 85% $\dot{V}O_2$ max	450 mg potassium NO ₃ ⁻ or PL for 7 days.	Not measured.	N/A	↔ kidney function	No
Porcelli et al. (2014)	21 young male individuals (28.2 to 81.7 ml. kg ⁻¹ . min ⁻¹). Sub max running and a incremental running test. + 3k TT	5.5 mmol of NO ₃ ⁻ per day for 6 days.	NO ₂ ⁻ = 462.3 ± 249.5 and 265.7 ± 163.7 nM NO ₃ ⁻ = 181 ± 32.7 and 21.3 ± 3.4 μM.	N/A	A strong negative correlation (-0.8) between reduction of O ₂ cost of submaximal exercise and individual aerobic fitness level was observed. Strong positive correlation (0.75) for aerobic fitness and increase in NO ₂ ⁻ concentration.	Yes
Keen et al. (2014)	6 healthy adults. Microdialysis fibers on ventral forearm. L-NAME inhibitor. Laser-Doppler	70 ml (5 mmol) one a day for 3 days	Not measured.	↓ MAP ↓ DBP	↑ NOS independent vasodilation to local heating	Yes
Casey et al. (2015)	13 young and 12 older adults. 20% MVC during normoxia and hypoxia. Forearm vascular conductance was measured	140 ml BR or PL (topped up with water to 500ml). NO ₃ ⁻ content not assessed.	Precise figures not given. State that it increased significantly P < 0.0001.	N/A	↑ compensatory vasodilatory response to hypoxia in older but not young adults	Yes

Levitt et al. (2015)	7 healthy males. Microdialysis fibers on ventral forearm. L-NAME inhibitor. Whole body heating	70 ml (5 mmol) one a day for 3 days	Not measured.	↓ MAP ↓ SBP ↔ DBP (during heat stress)	↔ skin blood flow ↑ vasodilation in the skin	No
Rocha et al. (2015)	2 healthy adults and male Wistar rats (n not specified). Gastric head space was sampled for ethyl NO ₂ ⁻ and smooth muscle relaxation in the rats	50g of lettuce and wine or whiskey	NO particles per billion. Figures not given but in graphical representation.	N/A	Nitrosation of ethanol produced ethyl NO ₂ ⁻ in the stomach after wine and whiskey when consumed with NO ₃ ⁻ rich foods.	Yes
Kocoloski and Crecelius (2015)	6 young, moderate active males. Performed a ramp test and 45 min moderate exercise (38% max).	70 ml 0.4g NO ₃ ⁻ 2 hours prior to exercise. Placebo was created via the use of mouthwash.	Abstract only, figures not presented.	↓ MAP	↔ O ₂ cost	No

Table 13, B. Nitrate supplementation studies in healthy older adults.

Author	Participants & Protocol	NO ₃ ⁻ supplement	Plasma NO ₃ ⁻ & NO ₂ ⁻ concentration (BR vs. PL respectively)	BP changes	Exercise efficiency or performance and/or clinical relevance	Primary outcome successful?
Treichler et al. (2015) abstract only	13 young and 12 older normotensive adults. Aortic BP and wave reflection were assessed.	Data not available. Looks like the same cohort as Casey et al., 2015.	Precise figures not given. State that it increased significantly P < 0.0001	↓ SBP ↓ DBP	↓ central aortic pressure	Yes
Jajja et al. (2014)*	21 older healthy participants had BP measured over a 4 week period with 3 different measures. Resting clinic BP, 24 hour ambulatory BP and home monitoring.	70 ml (300 mg of NO ₃ ⁻) or PL (blackcurrant juice) 2h prior to exercise.	NO ₃ ⁻ concentrations not given. Estimate from salivary figure = 6 mmol/l and 1.2 mmol/l.	↔ Resting clinic BP, 24 hour ambulatory BP. ↓ Home SBP		Yes
Kelly et al. (2013a)*	12 healthy older adults (64 ± 4 years). Minimum of a 72h washout. Exercise involved a 6MWT, cognitive function tests and a symptom limited ramp test.	70ml BR juice (NO ₃ ⁻ =4.8 mmol) or PL (NO ₃ ⁻ =0.01 mmol) twice a day for 2.5 days.	NO ₂ ⁻ = 1,037 ± 627 and 248 ± 182 nM	↓SBP ↓DBP ↓MAP	↔ O ₂ cost of exercise ↔Cognitive function ↔ 6MWT ↔Brain metabolite concentrations ↑ $\dot{V}O_2$ kinetics	No
Miller et al. (2012). (Abstract only)	13 older (72.5 ± 4.7 years) healthy adults (8 males & 5 females). BP and NO ₃ ⁻ & NO ₂ ⁻ were measured before each meal and hourly for 3h post.	Supplementation involved 3 days of LNF and BR juice, HNF and HNF & BR juice.	↑ NO ₃ ⁻ & NO ₂ ⁻ with foods and supplement	↓SBP	All treatment arms ↓SBP in older adults.	Yes

Bondonno et al. (2014b)*	38 (60.6 ± 7 years) healthy older adults. Ambulatory, home and office BP measured. Arterial stiffness	Dietary intervention. Low and high NO ₃ ⁻ diets. High diets = > 300mg/day. Low diet < 100 mg/day.	NO ₂ ⁻ = 2 ± 1.5 and 8 ± 6.5 μmol/L.	↔ SBP ↔ DBP	Ambulatory BP ↔ Home BP ↔ office BP ↔	No
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Table 13, C. Nitrate supplementation studies in clinical populations.

Author	Participants & Protocol	NO ₃ ⁻ supplement	Plasma NO ₃ ⁻ & NO ₂ ⁻ concentration (BR vs. PL respectively)	BP changes	Exercise efficiency or performance and/or clinical relevance	Primary outcome successful?
Kapil et al. (2015)*	68 hypertensive adults. 2 week washout. BP measured at clinic, ambulatory and home (34 in independent groups, BR and PL).	250ml BR or PL. 6.4 mmol. Once per day for 4 weeks.	Figures not given.	↓SBP 24h ambulatory and home.	Improved endothelial function. Measured via FMD. PWV not altered.	Yes
Gilchrist et al. (2014)*	27 individuals with T2DM. Protocol includes a battery of 5 cognitive function tests.	250 ml (7.5 mmol) vs. PL (0.002 mmol) daily for 2 weeks.	NO ₂ ⁻ = (IQR) 390 and 232 nM NO ₃ ⁻ = 150 and 31µM.	N/A	↓ simple reaction time	No
Zamani et al. (2015)*	17 individuals with heart failure. Supine $\dot{V}O_2$ max with measures of cardiac output and skeletal muscle oxygenation.(5 day washout period)	A single dose of NO ₃ ⁻ 3 hours prior to exercise (12.9 mmol).	Data not given.	N/A	↑ ejection fractions ↔ O ₂ cost of exercise ↓ arterial wave reflections Tendency to improve mitochondrial efficiency.	

Gilchrist et al. (2013)*	27 (18 males). T2DM subjects. A four week washout period was utilised. 24h ambulatory BP was taken. Macro & Microvascular endothelial function measures were assessed	250ml of BR juice ($\text{NO}_3^- = 7.5 \text{ mmol}$) or PL ($\text{NO}_3^- = 0.002 \text{ mmol}$) a day for 14 days. BR & PL were consumed with their evening meal to prevent hyperglycaemic events.	$\text{NO}_2^- = 537$ and 390 nM $\text{NO}_3^- = 150$ and $31 \mu\text{M}$.	\leftrightarrow SBP \leftrightarrow DBP \leftrightarrow MAP	\leftrightarrow Micro or Macro vascular function.	No
Shepherd et al. (2015)* Experimental chapter 2.	13 individuals with COPD. 80% GET. BP and 6MWT	70 ml of 6.77 mmol twice daily for 2 days. Plus 70 ml ~3h prior to exercise.	$\text{NO}_3^- = 215$ and $48 \mu\text{M}$	\leftrightarrow SBP \leftrightarrow DBP	\leftrightarrow O_2 cost \leftrightarrow 6MWT	No
Berry et al. (2014)*	15 individuals with COPD. Sub-maximal 75% max work rate.	140ml BR juice ($\text{NO}_3^- = 7.58 \text{ mmol}$) vs. PL (prune juice) 2.5h prior to exercise.	NO_2^- increased by 379% accurate values not presented. 375nM and 175 nM (estimates from fig)	\downarrow SBP \leftrightarrow DBP	\uparrow exercise iso-time \leftrightarrow sub-max $\dot{V}\text{O}_2$ \leftrightarrow dyspnoea \leftrightarrow dynamic hyperinflation	Yes
Kerley et al. (2015)*	11 individuals with COPD. Incremental shuttle walk test and BP	140 ml (12.9 mmol) 3h prior to test plus blackcurrant juice. Vs PL = water and blackcurrant juice	$\text{NO}_2^- = 751$ and 139 nM $\text{NO}_3^- = 508$ and $56 \mu\text{M}$.	\downarrow SBP \downarrow DBP \downarrow MAP	\uparrow Incremental shuttle walk test	Yes

\uparrow = statistical significant increase; \leftrightarrow = no statistical reduction; \downarrow = statistical significant reduction; $\dot{V}\text{O}_2$ = oxygen uptake; $\dot{V}\text{O}_{2\text{peak}}$ = peak oxygen uptake; WR = work rate; GE = gross efficiency; DE = delta efficiency; SBP = systolic blood pressure, DBP = diastolic blood pressure; MAP = mean arterial blood pressure; IR = ischemic reperfusion, FMD = flow-mediated dilation; PWV = pulse wave velocity; MI = moderate intensity; RPE = rating of perceived exertion ; SI = severe intensity; LI = low intensity; HI = high intensity; PCr = phosphocreatine; Pi = inorganic phosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; GET = gas exchange threshold; PAD = peripheral arterial disease; cGMP = cyclic guanosine monophosphate; S_{aO_2} = arterial O_2 saturation; Tlim = limit of tolerance; P/O ratio = phosphorylation efficiency; PO = power output; TT = time trial; TTE = time to exhaustion; LNF = low nitrate food; HNF = high nitrate food; PO_2 = partial pressure of oxygen; DMBA = dimethoxybenzaldehyde (a powerful organ-specific laboratory carcinogen); NIRS = near infrared spectrometry and sGC = Soluble guanylate cyclase; RQ = respiratory quotient; MVC = maximal voluntary contraction; ETC = electron transport chain. Where NO_3^- & NO_2^- values are given it is from the final supplementation period if multiple doses given.

General issues in the NO₃⁻ literature

A major limitation of the majority of the inorganic NO₃⁻ supplementation literature ($n=53$) regardless of the outcome measure is that they have small sample sizes (mean of 14.2 ± 9.4 , with a range of 7 - 68 as of 01/12/14). Moreover, Kapil et al. (2015) who had the largest n (68) used a parallel group design. The largest crossover design (apart from experimental chapter 1; $n = 48$) examined blood pressure changes following NO₃⁻ in older adults. The cohort size was 38 (Bondonno et al., 2014b). Reporting of power calculations and confidence intervals are rare. This makes determination of effect sizes difficult. The lack of power in assessing BP changes following NO₃⁻ supplementation has been somewhat explored by meta-analysis by Siervo et al. (2013). His findings showed an association for a significant reduction in systolic blood pressure of 4.4 mmHg. However, larger sample sizes would elucidate more definitive conclusions.

Assay variation is still an issue within the NO₃⁻ research community. Kapil et al., (2011) was the first study to report plasma NO₂⁻ concentration in the μM range in an unsupplemented or placebo condition. Since the beginning of this body of work two further studies have reported plasma NO₂⁻ concentration in the μM range (Bondonno et al., 2014b, Masschelein et al., 2012). The values reported in these studies (Bondonno et al., 2014b, Masschelein et al., 2012, Bescos et al., 2011) are unlikely to be accurate given that dietary intake accounts for $\sim 1 - 2$ mmol of NO₃⁻ per day (ECETOC., 1988). Moreover, these studies gave a much lower dose of NO₃⁻ than Webb et al. (2008a) Wylie et al. (2013b) Kapil et al. (2011) who gave the largest doses of NO₃⁻ (~ 20 mmol) to date and did not reach the μM range post NO₃⁻ rich beetroot juice supplementation.

Nitrate supplementation and effects on plasma NO₂⁻ concentration

Baseline plasma NO₂⁻ concentration varied markedly between cohorts in this body of work. The healthy young individuals had a mean baseline plasma NO₂⁻ concentration of 218 nM while the healthy older adults had a mean of 171 nM. These concentrations rose following NO₃⁻ rich beetroot juice to 715 and 968 nM, respectively. These concentrations are within the expected range given the peer reviewed literature (Kelly et al., 2013a, Sandbakk et al., 2014, Muggeridge et al., 2013b). Moreover, the pharmacokinetic responses and peak plasma NO₂⁻ concentration were similar to a profile from a cohort of young healthy individuals previously published (Wylie et al., 2013a). Interestingly, the baseline plasma NO₂⁻ concentrations of individuals with T2DM were significantly elevated (see figure 32). A detailed discussion of the possible ramifications and explanations of an elevated baseline are explored in the discussion of experimental chapter 1. However, previous reports reviewed by Vanhoutte (2009) suggest that NO· concentration is reduced in individuals with T2DM. This is brought about via a reduced production of NO· via eNOS (Vanhoutte, 2009). The reduction in eNOS activity is likely due to endopenetration by macrophages, cellular growth and atherosclerosis (Voetsch et al., 2004, Li et al., 2002). If eNOS activity is reduced, explanations for the high concentrations of plasma NO₂⁻ found in experimental chapter 1 consist of; an habitual up-regulation of iNOS activity in this population (Krause et al., 2012), the elevated oxidative stress prevalent in individuals with T2DM (Stadler, 2013) which would be expected to result in an increase in the scavenging of NO·, leading to higher NO₂⁻ concentrations or medications which increase NOS activity (Davis et al., 2006, Ceconi et al., 2007, Andrade et al., 2013).

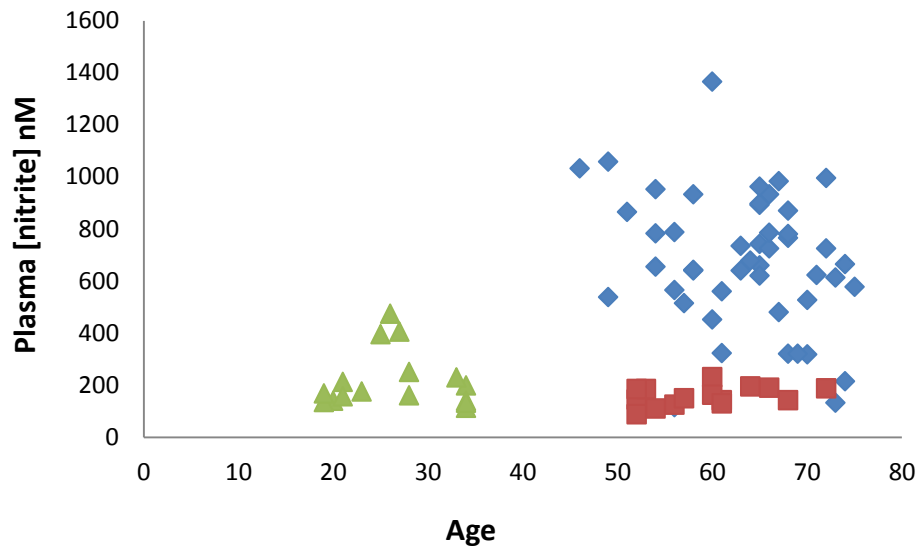


Figure 32. Baseline plasma NO_2^- concentration for young adults, older adults and individuals with T2DM.

Shows the baseline plasma NO_2^- concentration for all samples collected from this body of work for young adults (green triangles), older adults (red squares) and individuals with T2DM (blue diamonds).

NO_3^- supplementation and its effect on exercise in individuals with T2DM.

The O_2 requirement of exercise is a key determinant of an individual's ability to tolerate exercise (Whipp et al., 1981). By reducing the O_2 cost of exercise the individual will be further away from the maximal O_2 uptake and thus, will feel less physiological stress and will fatigue slower (Regensteiner et al., 1998, Regensteiner et al., 1995a). The aetiology of fatigue in individuals with T2DM is predominantly due to deficiencies in O_2 delivery (Regensteiner et al., 1998, Regensteiner et al., 1995b, Scognamiglio et al., 1998). These deficiencies in delivery of O_2 include; transportation from the lungs (Scognamiglio et al., 1998), reduced stroke volume (Gusso et al., 2008), cardiac autonomic function (Gerritsen et al., 2001) and reduced O_2 extraction in the mitochondria (Baldi et al., 2002; MÅrin et al., 1994; O. Mathieu-Costello et al., 2005; Padilla et al., 2007; J. Regensteiner, G et al., 1998; J. Regensteiner, G et al., 1995).

Reducing the O₂ cost of exercise or improving the kinetics of O₂ uptake in any clinical population would likely have important implications. By reducing the O₂ cost of exercise it may be possible to improve the quality of life in these patients. An individual's $\dot{V}O_2$ kinetics and the O₂ deficit are key determinants to the individual's ability to tolerate exercise (Jones and Poole, 2005). For example, by speeding the uptake of O₂ so the individual attains a steady state $\dot{V}O_2$, the greater the contribution of a oxidative metabolism compared to an anaerobic contribution (Jones and Poole, 2005). By using oxidative metabolism compared to an anaerobic metabolism the speed that lactic acid concentrations build up is slowed (Bruton et al., 1998, Pate et al., 1995, Westerblad et al., 1997, Wiseman et al., 1996).

We reported no difference between the active and placebo conditions for the $\dot{V}O_2$ responses (kinetics, O₂ deficit or the O₂ cost of exercise) to moderate intensity walking exercise in experimental chapter 1. This is in contrast to much of the literature which has described a reduction in steady state O₂ cost of moderate intensity exercise (Bailey et al., 2009, Vanhatalo et al., 2010, Lansley et al., 2010). No reduction in the O₂ cost of exercise has been reported in any clinical population to date (Berry et al., 2014, Zamani et al., 2015) (including experimental chapters 1 and 2) whilst other studies examining the effect of NO₃⁻ supplementation in clinical populations have not looked at the O₂ cost of exercise and have focused on exercise capacity in terms of walking performance (Kenjale et al., 2011, Kerley et al., 2015). Nitrate supplementation has also been shown to improve cardiac output and stroke volume in heart failure patients (Zamani et al., 2015) but not reduce the O₂ cost of exercise.

There was no difference between the active and placebo juice conditions for the distance covered in the 6MWT. Walking performance, however, has been improved following NO_3^- supplementation in other clinical populations such as peripheral artery disease and COPD (Kenjale et al., 2011, Kerley et al., 2015, Berry et al., 2014). Potential reasons for the lack of effect in individuals with T2DM include: an elevated baseline plasma NO_2^- concentrations, reduced $\text{NO}\cdot$ bioavailability due to higher levels of oxidative stress or pathological consumption of NO_2^- during exercise and medications.

This cohort was split for drug classification (ACEi/ARB, metformin, insulin and statins; other drug sample sizes too small), no effect on plasma NO_2^- concentration, walking performance, O_2 cost of exercise or blood pressure were found. It is therefore unlikely that medication is having an effect on these outcomes (note: we were not powered to find these effects). Unfortunately, in experimental chapter 1, plasma samples were only taken prior to exercise. A detailed pharmacokinetic study of NO_2^- concentration levels during exercise (Rassaf et al., 2010) may elucidate the speed of change plasma NO_2^- in this cohort. Diminished $\text{NO}\cdot$ bioavailability due to oxidative stress maybe the reason for the lack of effects following NO_3^- supplementation in individuals with T2DM. Restoring balance to the redox state in individuals with T2DM (prior to nitrate supplementation) may prolong the half-life and thus, bioavailability of $\text{NO}\cdot$.

Nitrate supplementation in individuals with COPD and A critical appraisal of the literature.

Recently Berry et al. (2014) and Kerley et al. (2015) examined the effect of NO_3^- rich beetroot juice supplementation vs. prune juice or water (respectively: as a placebo) in individuals with COPD. In experimental chapter 2 (NO_3^- and COPD) logistical

restraints prevented the measurement of plasma NO_2^- concentration. Berry et al. (2014) and Kerley et al. (2015) reported significantly elevated plasma NO_3^- and NO_2^- concentrations compared with placebo. This indicates that the entero-salivary pathway is operational in people with COPD.

Berry et al., reported reductions in resting systolic BP, iso-time BP, end exercise diastolic BP. Whilst Kerley et al., report reductions in systolic, diastolic blood pressure and MAP following NO_3^- supplementation compared to placebo. Following placebo (water) Kerley et al., showed a statistically significant increase in systolic (3 mmHg) diastolic (9 mmHg) blood pressure and MAP (7 mmHg). Berry et al., reported an improvement in exercise tolerance (i.e. lengthened time to exhaustion during submaximal constant rate).

Kerley et al., report improved distance covered in an incremental shuttle walk test following NO_3^- rich beetroot juice compared to placebo. The placebo condition significantly decreased walking performance by 7.6% from baseline. The significant change in outcome measures (blood pressure and exercise performance) following placebo in the Kerley et al cohort is difficult to explain given the 'double-blind' nature of the trial. The O_2 cost of exercise was not assessed in this study. Berry et al., do not show a reduction in the O_2 cost of exercise which is consistent with experimental chapter 2. However, with no reduction in the O_2 cost of exercise, it is not clear what mechanism underpins the improved time to exhaustion reported by Berry et al. (2014) and Kerley et al. (2015).

A potential reason for the improvement seen in walking performance that does not reflect a reduction in the O_2 cost of exercise maybe an increase in the delivery of O_2 to the working muscle (Ferguson et al., 2014, Ferguson et al., 2013). This would

mask any reduction in the O_2 cost of exercise and help to improve exercise performance. However, given the lack of evidence in the experimental chapters 1 and 2, we were either underpowered to detect this or this is not the mechanism for the improvement in walking performance.

Although these studies in the literature highlight the potential for NO_3^- supplementation to be beneficial to individuals with COPD in terms of blood pressure reduction and improved walking performance these studies have major limitations. Of paramount importance with both studies is a lack of a true placebo as used in experimental chapter 2. Given the substantial media attention given in the scientific literature, written press and on the television in recent years describing the beneficial effects of beetroot juice for exercise performance/economy and blood pressure it is impossible to rule out a placebo effect for knowledgeable and enthused volunteers. In informal discussions with the cohort of volunteers in the present trials, many of the participants in this body of work were aware of the putative beneficial effects of beetroot prior to receiving the participant information sheets. An example published on the 29/06/2010 in the daily mail online (DailyMail, 2013) had a headline which read: beetroot juice 'could save your life'. Secondly, the antioxidant content of prune juice and water are likely to be significantly different to beetroot juice (Donovan et al., 1998). This change in antioxidant content may affect $NO\cdot$ bioavailability (Bondonno et al., 2012) due to reduced scavenging of $NO\cdot$. This may increase the half-life on $NO\cdot$ in diseased populations. Berry et al., matched the calorie content of the different juice supplementation to control for the performance benefits of carbohydrate availability. Kerley et al., did not match this and had a 120 kcal difference between the conditions. However, this is unlikely to be the reason for the improvement given

evidence from Vermeeren et al. (2001) who demonstrated a 250 kcal supplement did not improve submaximal cycling performance in individuals with COPD.

Berry et al., used an iso-time to established the O₂ cost of exercise. This method takes an average of the last minute of O₂ consumption of the shorter exercise period and compares to the longer exercise period. These averages were then compared. A methodological weakness exists in Berry et al's assessment of the O₂ cost of exercise. Using one minute of data is not a robust measure of the O₂ cost of exercise. Towards the end of an exercise session coughing and spluttering as you fatigue is common (Lamarra et al., 1987). Typically, 6 minutes of steady state $\dot{V}O_2$ is used with multiple transitions to improve repeatability of the measure. This cohort of individuals have severe respiratory dysfunction as described in the introduction. This will increase the variability of data and given the short period of assessment this will magnify the variability. Given this, it is unlikely that this method of assessment of iso-time assessment of O₂ uptake is appropriate in this cohort of participants.

While the findings from Berry and Kerley were of interest, they were not replicated in a truly double blind trial and, in part, do not utilise the most robust measurement for O₂ uptake dynamics.

Although many mechanisms were explored for the lack of effect on the O₂ cost of exercise in the discussion of experimental chapter 2, a further possible explanation exists. In individuals with COPD, increasing the bioavailability of NO· (NO· gas in this study) may increase venous return and caused elevated pulmonary artery pressure in heart failure patients (Ichinose et al., 2004). By elevating pulmonary artery pressure, gas exchange may be slowed which may prevent the reduction in the O₂ cost of exercise that was hypothesised. However, the inorganic molecule GTN (in

dogs), has been shown to reduce pulmonary artery hypertension (Kim et al., 2010). More research is needed in this area to establish whether increasing NO· bioavailability increases or decreases pulmonary artery hypertension. Conversely, it may be due to the differing effects of the pharmacokinetic and dynamic responses of inorganic vs. organic NO₃⁻/ NO₂⁻ and not the bioavailability of NO· (Omar et al., 2012).

Nitrate supplementation and blood pressure in other clinical populations.

The gold standard measurement of blood pressure is 24h ambulatory blood pressure monitoring. For ease of measurement repeated rested brachial artery BP was used for all experimental chapters. This method can be susceptible to the white coat syndrome (Pickering, 1996). Given the timing of blood pressure measurement (described in the experimental chapters) it coincided with peak plasma NO₂⁻ (Wylie et al., 2013a) which would give the optimal chance for observing any blood pressure reductions.

Recently, the results from a 24h ambulatory blood pressure monitoring protocol following NO₃⁻ supplementation or placebo were reported in a group of individuals with T2DM. The authors reported no reductions in blood pressure (Gilchrist et al., 2013). The mean change in systolic blood pressure was 0.5 mmHg. This is similar to the effect shown in systolic blood pressure in experimental chapter 1 (-2 mmHg). A recently reported phase II trial in hypertensive adults following NO₃⁻ supplementation was published. The trial by Kapil et al. (2015) is the largest NO₃⁻ supplementation study, in any population to date (apart from experimental chapter 1). Clinic BP, home BP and 24h ambulatory blood pressure monitoring was employed. They showed in a phase 2, randomised, parallel group design that 4 weeks of NO₃⁻ supplementation causes a reduction in diastolic (2.4 – 3.8 mmHg depending on method) and systolic

blood pressure (7.7 – 8.1 mmHg depending on method). Following withdrawal of NO_3^- , BP returned to baseline levels. One study has looked at the effect of NO_3^- supplementation in 8 individuals with peripheral artery disease. Kenjale et al. (2011) reported reductions in diastolic blood pressure at rest and during exercise. No reductions in systolic blood pressure or MAP were present.

Nitrate supplementation and blood pressure in healthy individuals.

Varying reports of the blood pressure lowering effects of NO_3^- supplementation have been reported in young healthy individuals. In experimental chapter 3 we report no reductions in systolic or diastolic blood pressure following acute supplementation of NO_3^- rich beetroot juice in young healthy individuals. This is similar to some of the studies (Wilkerson et al., 2012, Coles and Clifton, 2012, Sandbakk et al., 2014) whilst most have reported reductions (Webb et al., 2008a, Vanhatalo et al., 2010, Vanhatalo et al., 2011, Lansley et al., 2010, Lansley et al., 2011a, Engan et al., 2012, Wylie et al., 2013b, Thompson et al., 2014, Muggeridge et al., 2014, Keen et al., 2014, Levitt et al., 2015, Treichler et al., 2015, Kocoloski and Crecelius, 2015, Bond Jr et al., 2013, Bond et al., 2014). There does not appear to be obvious differences in age, BMI, disease status, ethnicity or physical activity level between the studies who found a reduction and those that did not.

In experimental chapter 3 systolic and diastolic blood pressure were assessed as a secondary outcome measure. These data confirms the findings described by Bondonno et al. (2014b) who found no effect of NO_3^- supplementation on systolic blood pressure. Kelly et al. (2013a), Jajja et al. (2014) and two other studies have shown reductions in systolic blood pressure in an healthy older adult population however, only abstracts are available online (Miller et al., 2012, Treichler et al., 2015). However, these studies are the only other studies to examine the effects of

NO₃⁻ in an older adult cohort. Kelly et al., and the data from experimental chapter 3 report a 5 mmHg drop whilst Jajja et al. (2014) report a 7 mmHg drop in systolic blood pressure compared to baseline. It should be noted that Jajja et al., did not use a true placebo and did not find reduction using 24 hour blood pressure monitoring or clinical blood pressure assessment. Bondonno et al., cohort had a mean age of ~60 but there range was from 38-70 years old. Moreover some participants were on antihypertensives. A discussion on drug therapy and its effects on NO₃⁻ supplementation (and study outcomes) can be found in the discussion sections of experimental chapters 1 and 2. Bondonno et al., also used (predominantly) green leafy vegetables to increase the NO₃⁻ dose in each individual's diet. Although it is relatively easy to estimate the NO₃⁻ content of foods, it is much harder to standardise the dose. NO₃⁻ content of different crops could have different concentrations depending on growth rate (Rodgers and Barneix, 1988) although this is not always the case (Mattsson et al., 1991). Kelly et al., report a statistically significant 3 mmHg drop in diastolic blood pressure whilst we report a non-significant 2 mmHg drop. Jajja et al. (2014) report no change in diastolic blood pressure. A larger trial examining the effect in older adults is required to examine the efficacy of NO₃⁻ supplementation in normotensive older adults. If NO₃⁻ lowers blood pressure it may prevent/delay the onset of hypertension.

Nitrate supplementation, portal vein flux, velocity and hepatic diffusion and glucose concentration

Camilleri et al. (1985) described the uptake of solids and liquid foods from the stomach (see figure 33). At 1 hour the food has started to be absorbed with ~ 60% of the fluids taken up and 20% of the solids. At 2 hours 65% of the solids have been taken up and 90% of the fluids. At 3 hours both the fluids and solids have been

absorbed. Thus it is likely that hepatic blood flow and importantly, peak glucose concentration would occur at 2 hours.

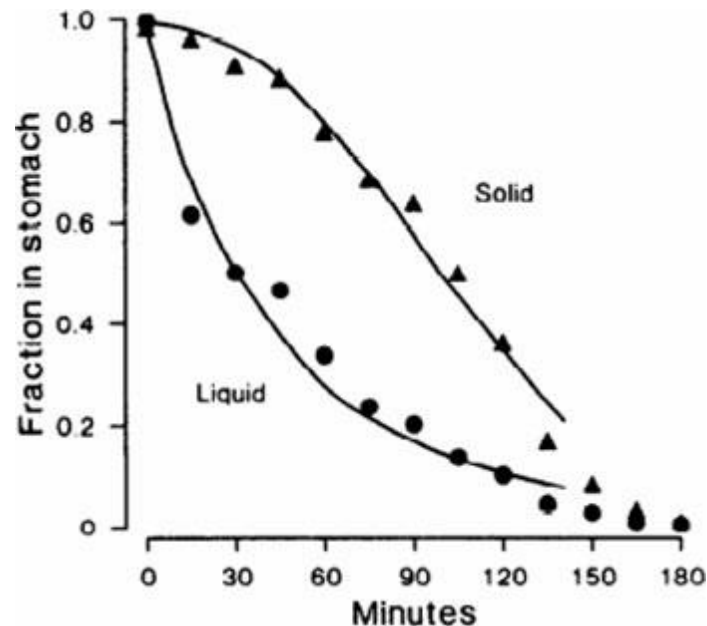


Figure 33. Uptake of liquids and solids from the stomach.

This figure is from Camilleri et al. (1985) with the appropriate rights from the publishers.

As glucose and other micro nutrients are absorbed through the stomach and proximal small intestine, nitrite is simultaneously absorbed. Peak plasma nitrite concentrations occur at 2 – 2.5 hours (Wylie et al., 2013a, Webb et al., 2008a). The nitrite along with the nutrients will meet within the portal vein from tributaries of the stomach, proximal small intestine and the spleen. Blood from the portal vein, having already been through vascular beds will be deoxygenated. Deoxyhaemoglobin is a nitrite reductase (Cosby et al., 2003). Therefore, $\text{NO}\cdot$ bioavailability would be expected to increase and thus vasodilate the portal vein leading to an increase in flux.

The liver has many nitrite reductases. Neuroglobin and cytoglobin are in small concentrations and therefore may not have a meaningful effect (Reeder, 2010, Omar and Webb, 2014). However, xanthine oxidase and aldehyde oxidase are in much higher concentrations (Martin et al., 2004, Li et al., 2008b). Increase in the bioavailability of NO \cdot has the potential to have significant biological effects within the liver. NO \cdot has previously been shown to mediate the uptake of glucose into skeletal muscle (Merry et al., 2010) and mediate its uptake from the intestines via incretins (Guan et al., 2003) into the circulation. Moreover, a study by Carlstrom et al., showed that in eNOS deficient mice that 10 weeks of nitrite supplementation in their drinking water, reduced HbA1c, baseline glucose concentrations and post prandial glucose concentrations (Carlström et al., 2010). In healthy humans and in individuals with T2DM, a NOS inhibitor has shown a reduction in the uptake of glucose within the exercising skeletal muscle (Kingwell et al., 2002). It is therefore possible or even probable (given the elevated NO \cdot and glucose concentrations) that it will help mediate the uptake of glucose in to hepatic tissue along with skeletal muscle. In vitro work on liver cells may help to elucidate this mechanism. GLP-1 and incretins in general appear to be interlinked in many biological processes such as increasing glucose utilisation (in an NO \cdot dependant mechanism) (Chai et al., 2012) and production of NO \cdot in the portal vein (Ding, Zhong, Xu, & Isales, 2004). By elevating the NO \cdot bioavailability we may increase GLP-1 and vice versa (Post et al., 2010) and thus facilitate uptake of glucose (Bhashyam et al., 2010) into cells leading to lower blood glucose concentrations.

Experimental chapter 3 shows that dietary NO $_3^-$ supplementation did not alter portal vein flux, velocity or hepatic diffusion. The hepatic arterial buffer response described by Hwang (2011) suggests that the hepatic artery can vasodilate in response to the

portal vein. Therefore if the portal vein changes in flow the hepatic artery responds with constriction or relaxation (Hwang, 2011, Lautt et al., 1990). The buffer response can only compensate for 25-60% of alterations in blood flow from the portal vein (Lautt, 1980, Lautt, 1977). The primary reason behind this mechanism is to maintain a constant perfusion rate from organs such as the stomach, spleen and intestines (Lautt, 1980, Lautt, 1977). Therefore dietary NO_3^- has the capacity to affect up to 75% of flow to the liver. This may be a potential reason why we saw no effect in this study.

Methodological issues / limitations with experimental chapters.

The link between NO^- , glucose uptake, insulin and incretin release has been discussed in depth with in experimental chapter 3 and the general discussion. Samples for incretin and insulin quantification for experimental chapter 3 have been stored. Unfortunately, we did not secure the funding to analyse these samples and this should be recognised as a limitation of the study and thesis in general.

Hepatic ADC analysis

From repeatability analysis involving multiple regions of interest it appears that to gain an accurate and repeatable ADC, the region of interest needs to be away from large vessels such as the right branch of the hepatic vein and the anteroinferior branch of the portal vein. The closer the ROI to the vessel the more unpredictable the ADC becomes. Potentially due to a higher variability of flow in larger blood vessels compared with a constant flow in the liver parenchyma.

The area in red and its close surrounding allow for reproducibility. The closer you get to the black box the more variable the data becomes.

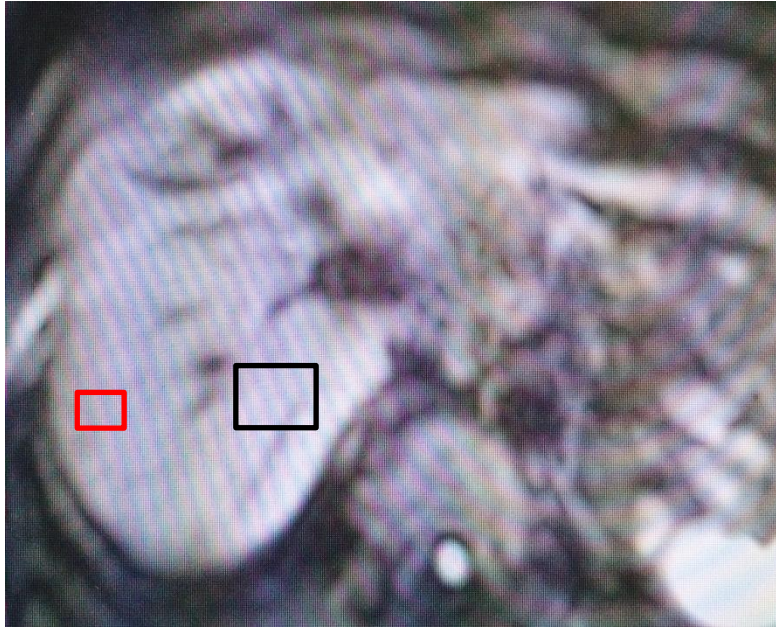


Figure 34. Depicts a slice of a liver from a representative participant. The highlighted boxes shows the regions of interest with the best and worst repeatability.

Future work

The results from this body of work have helped to answer many questions and has raised others. Notably, the pharmacokinetic response of older adults, the potential for blood pressure reductions in older adults, antioxidants in individuals with T2DM and critically the effect of dietary NO_3^- on plasma glucose concentration.

Antioxidants in clinical populations.

Previously it has been demonstrated that the beneficial effects of beetroot juice can be predominantly explained by its large NO_3^- content (Lansley et al., 2010). It has been suggested that polyphenols (in vitro) may have a beneficial antioxidant effects (Kanner et al., 2001). Given the pro-oxidant state of many clinical populations a supplement that could help to re-balance the redox state could help to prolong the $\text{NO}\cdot$ half-life. If this is possible then the hypothesis in experimental chapter one with improved efficiency in individuals with T2DM could be revisited. Thus enabling individuals with T2DM to gain from the mitochondrial adaptations that healthy young adults appear to benefit from.

Pharmacokinetic response in older adults and a phase II trial.

In experimental chapter 3 the data show that plasma NO_2^- concentrations are continuing to rise after 2 hours and peak at 3. This suggests that plasma NO_2^- concentration may rise beyond 3 hours which has only been shown once before (Kapil et al., 2011) with a peak at 6 hours. Future work should try to elucidate the precise pharmacokinetic response to dietary NO_3^- . In experimental chapter 3, systolic blood pressure was reduced. This coincided with peak plasma NO_2^- concentration. If the conversion of NO_3^- to NO_2^- continues past 3 hours this hypotensive effect may continue.

Three small studies to date have consistently shown a reduction in blood pressure of normotensive, drug naive older adults. A larger phase II trial in normotensive adults would help to characterise the blood pressure reductions shown in this work and by Kelly et al. (2013a) and Jajja et al. (2014). Dietary NO_3^- supplementation could slow the onset of hypertension in healthy older adults. The potential to save the NHS money on treatment of hypertension and the associated diseases caused via hypertension is vast. A health economist should also be used to assess any potential savings.

Plasma glucose concentration

The potential to reduce the plasma glucose concentration following a NO_3^- rich meal compared to baseline has been shown in experimental chapter 3. Nitrate-rich beetroot juice reduced plasma glucose concentration levels where placebo did not. However, this experiment did not show a reduction at two hours post supplementation and a standardised meal between conditions. Although the data are not unequivocal it does raise the possibility of a reduction in postprandial glucose concentrations and should be assessed again in larger numbers of healthy older

adults. This could help to prevent delay the onset of insulin resistance and even T2DM. If the findings are replicated then a trial in individuals with T2DM would be of interest. The potential to reduce plasma glucose concentrations in this cohort could help to manage the disease and has the potential to save the NHS vast amounts of money in treatment costs. Although this would require a significant increase in the nationwide uptake of NO_3^- supplementation. A phase II trial would be the next step in this process. This may be easier to achieve via pharmacological means i.e. sodium or potassium NO_3^- than NO_3^- rich beetroot juice or increasing the intake of NO_3^- rich vegetables.

Summary and conclusion of findings in this body of work

This is the first body of work to assess the effect of dietary NO_3^- supplementation on the O_2 cost of exercise in individuals with T2DM and COPD with a true placebo. Supplementation of the diet with dietary NO_3^- does not reduce the O_2 cost of walking (in individuals with T2DM) or cycling (in individuals with COPD), improve distance covered in the 6MWT or reduce BP in individuals with T2DM or COPD. This body of work is the first to assess the effect NO_3^- rich beetroot juice on hepatic blood flow. Dietary NO_3^- does not affect liver diffusion, portal vein flux or velocity, reduce systolic, diastolic BP or plasma glucose concentrations in healthy adults. By modifying an individual's diet with NO_3^- supplementation it is unlikely to reduce the O_2 cost of exercise, improve walking performance or reduce BP. Potential reasons for this are; an elevated baseline plasma NO_2^- concentration in the placebo condition, and/or reductions in the bioavailability of $\text{NO}\cdot$ in individuals with T2DM. In individuals with COPD likely reasons for the lack of effect include; NO_3^- dosage, oxidative stress, ageing and efficacy of NO_3^- reduction to NO_2^- . A hypothesis for the lack of effect in microvascular diffusion is the hepatic arterial buffer response.

Modifying an individual diet with supplementary NO_3^- does not appear to reduce the O_2 cost of exercise, improve walking performance or reduce BP in individuals with T2DM or COPD. Nitrate supplementation does not affect systolic, diastolic blood pressure or plasma glucose concentrations in healthy young and older adults.

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