Investigation of the effect of inorganic nitrate on the cardiovascular system in humans

Vikas Kapil

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Supervisor:

Prof Amrita Ahluwalia (QMUL)

Centre for Clinical Pharmacology,
William Harvey Research Institute,
Barts and the London School of Medicine and Dentistry,
Queen Mary University of London,
Charterhouse Square,
London EC1M 6BQ

(t) 020 7882 8931
(e) v.kapil@qmul.ac.uk

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for better health

Dedication

This thesis is dedicated to:

Nanaji & Naniji, who saw me start this path but sadly are not here to see गधा finish.

My parents, biological (VMK & UK) and otherwise (SSA & NKA), for never understanding why but forever and always being positive, encouraging and loving.

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Abstract

Fruit and vegetable-rich diets reduce blood pressure and risk of ischaemic stroke and ischaemic heart disease. Whilst the cardioprotective effects of a fruit and vegetable-rich diet are unequivocal, the exact mechanisms of this effect remain uncertain. Recent evidence has highlighted the possibility that dietary nitrate, an inorganic anion found in large quantities in vegetables (particularly green leafy vegetables), might have a role to play. This beneficial activity lies in the processing *in vivo* of nitrate to nitrite (a process that has been traditionally viewed as detrimental) and thence to the pleiotropic molecule nitric oxide. Ingestion of dietary (inorganic) nitrate elevates circulating and tissue levels of nitrite via bioconversion in the entero-salivary circulation. In addition, nitrite is a potent vasodilator in humans; an effect thought to underlie the blood pressure lowering effects of dietary nitrate ingestion.

In a series of randomized, cross-over, placebo controlled studies in healthy and hypertensive subjects (n=6-20), I show that single-dose supplementation with either inorganic nitrate capsules (4-24 mmol KNO_3) or dietary nitrate (as beetroot juice, 3.3-5.5 mmol nitrate) elevated plasma nitrite levels and reduced blood pressure in a dose-dependent manner. In a separate study, interruption of the entero-salivary circulation with antiseptic mouthwash use for 7 days reduced plasma nitrite levels and elevated blood pressure significantly. Stratification of results by sex revealed important differences in the entero-salivary circulation of nitrate to nitrite that had consequences on resting blood pressure and response to nitrate supplementation.

In conclusion, these studies challenge the current dogma that inorganic nitrate is only detrimental, and on the contrary suggests that dietary nitrate is important for cardiovascular health. It may be that sufficient supply of nitrate through the diet together with functioning, oral microflora is essential for normal cardiovascular homeostasis and may be a contributing factor to the lower blood pressure and vasoprotective phenotype of pre-menopausal women. Lastly, the importance of the oral microflora to maintain plasma nitrite levels intimates that oral hygiene treatments may disturb nitrite/nitric oxide homeostasis with potential deleterious effects.

Publications

Publications related to this thesis

Original Research Papers

Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. **Kapil V**, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, MacAllister R, Hobbs AJ, Webb AJ, Ahluwalia A. *Hypertension* 2010; 56, 274-81.

Control of blood pressure by oral nitrate-reducing bacteria. **Kapil V**, Haydar SMA, Pearl V, Lundberg JO, Weitzberg E, Ahluwalia A. *Free Radical Biology and Medicine* 2012; *epub ahead of press: http://dx.doi.org/10.1016/j.freeradbiomed.2012.11.013.*

Enhanced vasodilator activity of nitrite in hypertension: translational potential and role for xanthine oxidoreductase. Ghosh SM*, **Kapil V***, Fuentes-Calvo I*, Bubb KJ, Pearl V, Milsom AB, Khambata R, Maleki-Toyserkani S, Yousuf M, Benjamin N, MacAllister R, Webb AJ, Hobbs AJ, Ahluwalia A. *(in review).*

Invited Review

Inorganic nitrate and the cardiovascular system. **Kapil V**, Webb AJ, Ahluwalia A. *Heart* 2010; 96; 1703-1709.

Correspondence

Response to: Inorganic Nitrate for Blood Pressure Lowering? **Kapil V**, Benjamin N, Hobbs AJ, Ahluwalia A. *Hypertension*, 2011; 57: e3.

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Disruption of oral-nitrate reducing bacteria elevates blood pressure in healthy volunteers. **Kapil V**, Syed MF, Pearl V, Weitzberg E, Lundberg JO, Ahluwalia A. *Journal of Hypertension* 2012; 30: e198.

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Mechanisms underlying nitrite reduction in hypertension: Role for erythrocyte xanthine oxidoreductase. Ghosh S, **Kapil V**, Bubb KJ, Fuentes-Calvo I, Ahluwalia A. *Nitric Oxide* 2012; 27: S30-31.

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Table of Contents

List of Figures

List of Tables

List of Equations

Abbreviations

Abbreviation	Full name
Å	Ångstrom (=10^{-10} metres)
ABP	Ambulatory blood pressure
ACh	Acetylcholine
ADI	Acceptable daily intake
ADMA	Asymmetric dimethylarginine
AE-1	Anion-exchange transporter-1
ALDH-2	Aldehyde dehyrogenase-2
Akt	Protein kinase B
AO	Aldehyde oxidase
B/B0	% bound
BK	Bradykinin
BMI	Body mass index
BP	Blood pressure
bpm	beats per minute
Ca^{2+}	Calcium
Cam	Calmodulin
cAMP	Cyclic adenosine monophosphate
Cl^-	Chloride
cGMP	Cyclic guanosine monophosphate
CNG	Cyclic nucleotide-gated ion channel
C-PTIO	2-(4-Carboxyphenyl)-4,4,5-teramethylimidazoline-1-oxyl-3-oxide

Cu	Copper
CVD	Cardiovascular disease
DASH	Dietary Approaches to Stop Hypertension
DBP	Diastolic blood pressure
deoxyHb	Deoxyhaemoglobin
deoxyMb	Deoxymyoglobin
DIDS	Diidothiocyanotostilbene disulfonate
e^-	Electron
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
FAD	Flavin adenine dinucleotide
FBF	Forearm blood flow
Fe	Iron
FMD	Flow mediated dilatation
FMN	Flavin mononucloetide
GDN	Glyceryl dinitrate
GFR	Glomerular filtration rate
GI	Gastro-intestinal
GTN	Glyceryl trinitrate
GTP	Guanosine triphosphate
GPCR	G-protein coupled receptor
h	Hour(s)

H^+	Proton
Hb	Haemoglobin
HbNO	Iron-nitrosyl-haemoglobin
H_2B	Oxidized biopterin
H_4B	Tetrahydrobiopterin
HCO_3^-	Bicarbonate
H_2O	Water
HNO_2	Nitrous acid
HO-1	Haem oxygenase 1
HR	Heart rate
hv	Chemiluminescence
IBMX	3-isobutyl-1-methylxanthine
i.v.	Intravenous
IHP	Inositol hexaphosphate
IR	Ischaemia-reperfusion
K^+	Potassium
KCl	Potassium chloride
KI	Potassium iodide
K_M	Michaelis–Menten constant
KNO_2	Potassium nitrite
KNO_3	Potassium nitrate
LDL	Low-density lipoprotein
L-NAME	L-N^G-nitroarginine methyl ester

L-NMMA	L-N^G-monomethyl-arginine
MAP	Mean arterial pressure
Mb	Myoglobin
MbNO	Iron-nitrosyl-myoglobin
MDRD	Modification of Diet in Renal Disease formula
metHb	Methaemoglobin
MI	Myocardial infarction
min	Minute(s)
mRNA	Messenger ribonucleic acid
N_2	Nitrogen gas
NaCl	Sodium chloride
$NADP^+/H$	Nicotinamide adenine dinucleotide phosphate
$NaNO_2$	Sodium nitrite
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NO_2	Nitrogen dioxide
NO_2^*	Nitrogen dioxide in excited state
NO_x	Nitrite/Nitrate
NO_2^-	Nitrite
NO_3^-	Nitrate
N_2O_3	Dinitrogen trioxide

NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
$ONOO^-$	Peroxynitrite
O_2	Oxygen
O_2^-	Superoxide
O_3	Ozone
oxyHb	Oxyhaemoglobin
PDE	Phosphodiesterase
PI3K	Phosphoinositide 3-kinase
pK_a	Acid dissociation constant
PKG	Protein kinase G
PWV	Pulse wave velocity
RCT	Randomized clinical trial
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of the mean
sGC	Soluble guanylyl cyclase
SOD	Superoxide dismutase
$t_{1/2}$	Half-life
VASP	Vasodilator-stimulated phosphoprotein
VCl_3	Vanadium (III) Chloride
XOR	Xanthine oxidoreductase

Materials

Solutions/drugs	Manufacturer/City/Country
Beetroot Juice (250mL)	James White Drinks Ltd, Ipswich, UK
Corsodyl™ (0.2% chlorhexidine)	GlaxoSmithKilne Company, Stevenage, UK
Glacial acetic acid	Sigma-Aldrich Company Ltd., Dorset, UK
Hydrochloric acid	Prolabo VWR, Lutterworth, UK
3-isobutyl-1-methylxanthine	Sigma-Aldrich Company Ltd., Dorset, UK
KCl capsules (300mg)	Martindale Pharmaceuticals, Ipswich, UK
KNO_3 capsules (400mg)	Martindale Pharmaceuticals, Ipswich, UK
Low NO_3^- containing mineral water	Zepbrook Ltd, London, UK
MilliQ NO_x-free water	Millipore Corporation, Billerica, USA
Potassium iodide	Sigma-Aldrich Company Ltd., Dorset, UK
Sodium hydroxide	Sigma-Aldrich Company Ltd., Dorset, UK
Sodium nitrate	Sigma-Aldrich Company Ltd., Dorset, UK
Sodium nitrite	Sigma-Aldrich Company Ltd., Dorset, UK
Vanadium (III) chloride	Sigma-Aldrich Company Ltd., Dorset, UK

CHAPTER 1

Introduction

Despite major advances over the past two decades in the pharmacotherapy of cardiovascular diseases (CVDs), CVDs have still become the biggest killer worldwide with incidence likely to increase further as the non-western world adopts a western lifestyle (World Health Organization, 2011). This rise in the incidence of CVDs is associated with a huge economic cost with direct and indirect health costs in 2006 estimated to total €192 billion within the EU and £31 billion in the UK alone (Allender *et al.*, 2008). Such figures clearly support an urgent need to develop novel and cost-effective strategies to combat these diseases. This imperative, in part, underlies the major emphasis in the western world, over the last decade, to increase the public consumption of fruit and vegetables (World Health Organization, 2003; Lichtenstein *et al.*, 2006). In the UK, this has taken the form of a large-scale multimedia '5-a-day' campaign (Department of Health, 2003) run by the Department of Health. This approach has been initiated as a consequence of the substantial body of evidence accumulated over the last century that fruit and vegetable-rich diets reduce the incidence of many modern-day diseases, not least the broad spectrum of CVDs.

1.1 Beneficial effects of a fruit and vegetable-rich diet

In the early 20th century, nutritionists noted that vegetarian diets were associated with lower blood pressure (BP) (Donaldson, 1926), a finding corroborated by further observational studies in different populations including American macrobiotists (Sacks *et al.*, 1974), Australian 7th day Adventists (Armstrong *et al.*, 1977, 1979) and Sephardic Israelis (Ophir *et al.*, 1983). This finding was tested in a number of small-scale controlled studies (Rouse *et al.*, 1983; Lindahl *et al.*, 1984; Margetts *et al.*,

1986; John *et al.*, 2002), including the landmark Dietary Approaches to Stop Hypertension (DASH) study (Appel *et al.*, 1997), providing further evidence supporting the thesis that a diet rich in fruit and vegetables lowers BP. The DASH study enrolled 459 drug-naïve subjects (systolic BP (SBP) <160 mmHg and diastolic BP (DBP) 80-95 mmHg) and provided a control diet for 3 weeks. Subjects were then randomized to a receive a further dietary pattern for 8 weeks: control diet; fruit and vegetable-rich diet; or a 'combination' diet rich in fruits and vegetables and low in saturated fat (Appel *et al.*, 1997). The remarkable findings from the study included the demonstration that provision of a fruit and vegetable-rich diet (~5 portions of fruit and vegetables more than the control diet per day) caused significant reductions in both SBP (~2.8 mmHg) and DBP (~1.1 mmHg) in a population with high-normal BP (mean BP ~131/85 mmHg), with even larger reductions (SBP ~7.2 mmHg; DBP ~2.8 mmHg) in the subset of subjects with a diagnosis of hypertension (SBP >140 mmHg or DBP >90 mmHg) (Appel *et al.*, 1997).

Although these reductions in BP may at first glance seem trivial, it is important to appreciate the large reductions in vascular mortality that could occur if these changes were applied to a whole population. In treated hypertensive patients, every 2 mmHg increase in SBP results in a 7% increase in the risk of mortality due to ischaemic heart disease and a 10% increase in the risk of mortality due to stroke (Lewington *et al.*, 2002). Separately, it has been estimated that lowering SBP by 10 mmHg or DBP by 5 mmHg reduces the risk of ischaemic heart disease events by ~20% and ischaemic stroke by 35-40% (Law *et al.*, 2003, 2009). Further, larger cohort studies have extended these observations to demonstrate that fruit and

vegetable-rich diets reduce cardiovascular morbidity and mortality. Willett and colleagues have published a series of papers detailing the effects of dietary fruit and vegetable intake in a cohort of over 114,000 American health professionals with a minimum of 8 years follow up who were all free of CVD at baseline. Their analyses demonstrated that those in the highest quintile for daily fruit and vegetable consumption had ~30% reduced risk for ischaemic stroke (Joshipura *et al.*, 1999), ~20% reduced risk for ischaemic heart disease (Joshipura *et al.*, 2001) and ~30% reduced risk of total CVD (Hung *et al.*, 2004) compared to those in the lowest quintile. Also, each additional daily serving of fruits and vegetables was associated with an incremental 6% lower risk of ischaemic stroke (Joshipura *et al.*, 1999) and 4% lower risk of ischaemic heart disease (Joshipura *et al.*, 2001). These results have been replicated in other even larger cohorts, including most recently the European-based *EPIC* cohort, which analysed data from over 310,000 persons over mean 8.4 years. In this study, there was a similar 4% risk reduction for ischaemic heart disease deaths for 1 additional daily serving of fruits and vegetables (Crowe *et al.*, 2011). Similar estimates for cardiovascular risk reduction with fruit and vegetable-rich diets have also been confirmed in very large *meta* analyses (> 230,000 participants) for both ischaemic heart disease (Dauchet *et al.*, 2006; He *et al.*, 2007) and ischaemic stroke (Dauchet *et al.*, 2005; He *et al.*, 2006). Given the strong evidence base for the inverse relationship between fruit and vegetable intake and CVD risk described in the studies above, it may be no surprise that the global burden of CVD due to low consumption of fruit and vegetables has been estimated to account for 31% of all CVD leading to an excess of 2.6 million CVD deaths annually (Lock *et al.*, 2005).

1.2 Proposed mechanisms of the beneficial effects of fruit and vegetable-rich diets

Whilst the BP-lowering and cardioprotective effects of a fruit and vegetable-rich diet are unequivocal, the exact mechanisms of this effect remain uncertain (Fraser, 2009). Specific, individual components found in abundance in fruits and vegetables, including potassium (K^+) and other cations, dietary fibre, and non-nutrient phytocemicals such as anti-oxidant vitamins have been proposed to underlie the benefits of such diets (Berkow and Barnard, 2005). One of the major problems in this strand of research is the ability to provide an adequate blinded, placebo control to the dietary intervention and also the heterogeneity between ostensibly similar interventions in different studies.

1.2.1 Dietary fibre

Dietary fibre is classed as the indigestible fraction of plant foodstuffs. Fruits and vegetables are, along with nuts and legumes, the primary dietary sources of fibre (Bingham *et al.*, 1979). Dietary fibre intake is therefore higher in those who consume more fruits and vegetables, such as vegetarians (Haddad and Tanzman, 2003). A recent *meta* analysis of 22 short-term (4-8 weeks) randomized clinical trials (RCTs) investigating the effects of soluble fibre supplementation (in particular, with psyllium or oat β-glucan) in ~1000 total patients revealed significant reductions in low-density lipoprotein (LDL) cholesterol of ~5.5% (Anderson *et al.*, 2009). Dietary fibre may also reduce BP, though this effect was only apparent in recent *meta* analyses in hypertensive subjects, with pooled estimates suggesting 10-15g/day of soluble fibre supplementation may reduce BP in hypertensive individuals by ~2.5-6/2-4 mmHg in short-term RCTs (Streppel *et al.*, 2005; Whelton *et al.*, 2005). The

mechanism of dietary fibre modulation of cholesterol is thought to be related to binding of bile acids in the gastro-intestinal (GI) tract and increasing excretion in the faeces (Kirby *et al.*, 1981). However, there is no current consensus on the potential mechanisms by which dietary fibre may lower BP, with some researchers suggesting beneficial modulation of insulin resistance (Fukagawa *et al.*, 1990), whilst others suggest it is secondary to associated weight loss that occurs with fibre-rich diets (Solum *et al.*, 1987).

1.2.2 K^+ and other cations

Fruit and vegetables contain large amounts of K^+ and other cations such as magnesium (Food and Nutrition Board, 2005). Population studies have shown an inverse relationship between K^+ intake and BP (Intersalt Cooperative Research Group, 1988) and small scale clinical studies have demonstrated that dietary K^+ depletion both increases BP in normotensive subjects (Krishna *et al.*, 1989) and worsens hypertension in hypertensive patients (Krishna and Kapoor, 1991). The mechanisms involved in the detrimental effects of K^+ depletion are complex and involve alterations in sodium reabsorption in the kidney, with concomitant increases in circulatory volume, and the activation of both the renin-angiotensin-aldosterone and sympathetic nervous systems (Adrogué and Madias, 2007). In addition, it has been recognized for 3 decades that short-term K^+ supplementation, as potassium chloride (KCl) or potassium citrate, ameliorates raised BP (MacGregor *et al.*, 1982; He *et al.*, 2005) and indeed a large *meta* analysis including 33 RCTs of K^+ supplementation revealed a pooled estimate BP reduction of ~3/2 mmHg (Whelton *et al.*, 1997).

However, a more recent *meta* analysis failed to find conclusive overall evidence of significant BP reduction with K^+ supplementation due, in part, to large heterogeneity between trials and the short duration of many of the trials included in the previous analysis (Dickinson *et al.*, 2006). In addition, it has been shown recently that the provision of magnesium, K^+ and fibre in hypertensive subjects does not fully account for the beneficial effects of a fruit and vegetable-rich diet. Hypertensive subjects were fed both the standard DASH diet and a control diet that was supplemented with magnesium, K^+ and fibre to match the DASH diet for 3 weeks in a randomized, cross-over design. The standard DASH diet was more effective at lowering BP (~6/4 mmHg) than the control-supplemented diet. The control-supplemented diet did not lower BP on its own, suggesting alternative nutritional factors must play a significant beneficial role in the DASH diet (Al-Solaiman *et al.*, 2010).

1.2.3 Anti-oxidant vitamins

Oxidative stress has long been established to be a critical part of the initiation and progression of atherosclerotic disease. (Steinberg and Witztum, 1990). The oxidative-modification hypothesis of atherosclerosis implicates superoxide (O_2^-) and other oxidative free radicals in the oxidation of LDL-cholesterol (Hessler *et al.*, 1983) within the vessel wall, representing a master step in the accumulation of LDL-cholesterol in recruited macrophages that eventually become foam cells in the plaque (Brown and Goldstein, 1983; Steinbrecher *et al.*, 1984; Heinecke *et al.*, 1986). The corollary of such a paradigm is that anti-oxidants should be beneficial at some point in the pathological process. This has spurred much interest and research

on the candidature of non-nutrient phytochemicals (Liu, 2003), such as anti-oxidant vitamins (Bruckdorfer, 2008) as a potential mediator of beneficial effects and are particularly relevant to this thesis as fruit and vegetables are the primary dietary source of anti-oxidant vitamins (Halvorsen *et al.*, 2002).

Prospective studies in which nutrient status was measured many years before disease onset provided some evidence that there is an inverse relationship between dietary and supplementary intake of such anti-oxidant vitamins, including vitamin E (α-tocopherol), β-carotene and vitamin C (reviewed in Stanner *et al.*, 2004). A few RCTs have purported to show that supplementation with anti-oxidant vitamins have beneficial effects on risk of CVDs. For example, the CHAOS trial involved ~2000 patients with ischaemic heart disease and randomized them to receive vitamin E or placebo and participants were followed for up to 2 years. In this study, vitamin E use was associated with 47% reduction in risk of CVD events and mortality (Stephens *et al.*, 1996). However recent observational cohort studies have failed to replicate these same results (Rautiainen *et al.*, 2010; Mursu *et al.*, 2011). Similarly, several large RCTs, including the Heart Protection Study (Heart Protection Study Collaborative Group, 2002), HOPE (Yusuf *et al.*, 2000) and HOPE-TOO (Lonn *et al.*, 2005) studies have failed to show a beneficial effect of anti-oxidant vitamin supplementation. Indeed, recent *meta* analyses, involving 80,000-300,000 participants, have challenged this anti-oxidant hypothesis, with the pooled results of large-scale RCTs of several different antioxidant vitamins failing to replicate the same cardioprotective effects as a fruit and vegetable-rich diet (Vivekananthan *et al.*, 2003; Bjelakovic *et al.*, 2007), with some suggestion of a 3-4% increased risk of

mortality in those who were supplemented with anti-oxidant vitamins (Bjelakovic *et al.*, 2008, 2012).

1.2.4 Dietary nitrate (NO_3^-)

These failures have spurred many others on to identify alternative, likely candidates. One constituent, of particularly vegetables, that has been proposed recently is inorganic NO_3^- (Lundberg *et al.*, 2006). The majority of NO_3^- intake (~80%) in humans comes from vegetable consumption (Table 1.1), with a minor portion coming from drinking water, animal products and grain (World Cancer Research Fund/American Institute for Cancer Research, 2007). Daily intakes of NO_3^- in European populations are estimated to be 1.5-2 mmol (European Food Safety Authority, 2008).

NO_3^- intake is regulated and there is an acceptable daily intake (ADI) that is set by the World Health Organization and the European Food Safety Authority at 3.7 mg/kg per day (Speijers and van den Brandt, 2003; European Food Safety Authority, 2008), which is equivalent to ~4.2 mmol per day in a 70 kg person. Water supplies in the European Union are legally required by directive 91/676/EEC (European Union Council, 1991) to have less than 50 mg/L NO_3^- (=0.8 mM [NO_3^-]) due to 2 major concerns: methaemoglobinaemia and carcinogenesis.

NO_3^- content (mg kg^{-1})				
Very low (<200)	Low (200-500)	Medium (500-1000)	High (1000-2500)	Very high (>2500)
Artichoke	Broccoli	Cabbage	Celeriac	Celery
Asparagus	Carrot	Raddichio	Fennel	Lettuce
Mushroom	Cauliflower	Turnip	Kohlrabi	Radish
Pepper	Cucumber		Chicory	Beetroot
Potato	Pumpkin		Leek	Spinach
Tomato				Swiss chard

Table 1.1 *Classification of vegetables according to NO_3^- content (NO_3^-=nitrate). (data adapted from Santamaria, 2006).*

1.2.4.1 NO_3^- and methaemoglobinaemia

Initial concerns regarding the aetiological role of NO_3^- and methaemoglobinaemia were first reported in the early 1900s in children treated with bismuth subnitrate, (cited in English by Beck, 1909). It was recognized by these early clinician-scientists that many people could tolerate very large doses of NO_3^- with no ill effects (Beck, 1909). However, children, and those will intestinal infections, were particularly prone to methaemoglobinaemia. It was discovered that NO_3^- was metabolized to nitrite (NO_2^-) and that the problems of methaemoglobinaemia after NO_3^- ingestion were synonymous with that of NO_2^- ingestion, as first described by Gamgee (see section 1.5.3), which could lead to death especially in young infants (Roe, 1933). Surveys of NO_3^- levels in well water and associated cases of infant methaemoglobinaemia after World War II (Comly, 1945; Walton, 1951) led to the

establishment of regulatory frameworks to control the NO_3^- level in such water (U.S. Public Health Service, 1962) at levels <50 mg/L that we still currently have as discussed above. The reactions of NO_2^- with haemoglobin (Hb) to induce methaemoglobinaemia will be discussed in section 1.5.3.

1.2.4.2 NO_3^- and carcinogenesis

NO_3^- itself is not thought to be carcinogenic (Speijers and van den Brandt, 2003) but requires the endogenous conversion to the chemically related anion, NO_2^- (see section 1.6.4) and the further reaction of NO_2^- with secondary amines to form N-nitrosamines. N-nitrosoamines were first recognized to be carcinogenic more than 60 years ago. Dimethylnitrosoamine (50 *ppm*) was found to produce large, necrotic hepatocellular carcinoma in rats after 6 months feeding (Magee and Barnes, 1956) and other related N-nitrosoamines have been shown to cause malignant tumours of the liver, kidney, stomach and oesophagus in rats when given orally over prolonged periods (Magee and Barnes, 1967). Further studies have revealed there are over 38 animal species in which N-nitrosoamines are directly carcinogenic (Bogovski and Bogovski, 1981). N-nitrosoamines can be formed in humans *in vitro* by incubating gastric juice, NO_2^- and secondary amines (Sen *et al.*, 1969) and *in vivo* after dietary ingestion of NO_2^--containing foods (Fine *et al.*, 1977). Although there has been much epidemiological research aimed at exploring the link between NO_3^- intake and human cancer, much of it is equivocal (as reviewed in McKnight *et al.*, 1999) and a comprehensive review by the World Health Organization and other parties in 2003 concluded that there was no evidence that NO_3^- ingestion was associated with carcinogenesis in humans (Speijers and van den Brandt, 2003). Moreover, those

with the highest NO_3^- intake in large (>100,000 subject) cohorts, have no increased cancer incidence or mortality (Hung *et al.*, 2004); in fact the converse may be true (World Cancer Research Fund/American Institute for Cancer Research, 2007; Boffetta *et al.*, 2010).

1.2.4.3 Potential beneficial role of inorganic NO_3^-

Despite these negative views of inorganic NO_3^-, possible clues that inorganic NO_3^- may have beneficial effects on the cardiovascular system were provided by Willett and colleagues in their large cohort (>100,000 subjects) studies, in which they suggested that the greatest protection against ischaemic heart disease, ischaemic stroke and CVD was afforded by a diet particularly rich in green leafy vegetables (Joshipura *et al.*, 1999, 2001; Hung *et al.*, 2004). Although green-leafy vegetables contain numerous nutrients, they are also particularly rich in inorganic (dietary) NO_3^- content (Table 1.1). This observation has led some to consider the possibility that inorganic NO_3^- may contribute to the vasoprotective effects of fruits and vegetables (Lundberg *et al.*, 2006; Webb *et al.*, 2008a; Ralt, 2009), since recently described reductive pathways in humans (Gladwin *et al.*, 2005) suggest that physiologically relevant production of NO_3^--derived nitric oxide (NO) can be attained that may have beneficial effects on human vascular homeostasis.

1.3 NO

NO was discovered by the pre-eminent British chemist, Joseph Priestley, at the same time as he discovered oxygen (O_2) in 1776. For the next 200 years, NO was largely thought of as an atmospheric pollutant (Spicer, 1977). The discovery of mammalian NO production and the protean systems it had effects on radically changed this view.

1.3.1 Discovery of NO as an endogenous gasotransmitter

In 1998, Furchgott, Ignarro and Murad shared the Nobel Prize in Physiology or Medicine (http://nobelprize.org/nobel_prizes/medicine/laureates/1998/illpres) for their seminal roles in the discovery "concerning NO as a signalling molecule in the cardiovascular system". The critical role of the endothelium in permitting vasorelaxation of blood vessels was elucidated by Robert Furchgott. He was intrigued by the observation that acetylcholine (ACh) reduced BP *in vivo*, yet contracted isolated spiral aortic preparations. On changing methodology to the use of isolated aortic rings, he observed ACh-induced vasorelaxation for the first time, in accord with its potent vasodilating action *in vivo* (Furchgott and Zawadzki, 1980) because of preservation of intact endothelium during the preparation of aortic rings. Furthermore, elegant 'sandwich' experiments demonstrated that an endothelium-denuded aortic strip (that would not dilate on its own to ACh) could be made to relax in response to ACh if it was placed in contact with an intact aortic strip (Furchgott and Zawadzki, 1980). Furchgott coined the term endothelium-derived relaxing factor (EDRF) (Cherry *et al.*, 1982) to describe the unknown, soluble entity produced by the endothelium that caused smooth muscle relaxation. EDRF

was subsequently demonstrated to activate the enzyme soluble guanylyl cyclase (sGC) to elevate tissue levels of the signalling molecule, cyclic guanosine monophosphate (cGMP), in various vascular tissues, including bovine coronary artery (Holzmann, 1982), rat (Rapoport and Murad, 1983; Rapoport *et al.*, 1983) and rabbit aorta (Diamond and Chu, 1983), and bovine pulmonary artery preparations (Ignarro *et al.*, 1984).

By this time, it was already established that 'nitrovasodilators' (such as glyceryl trinitrate (GTN) and sodium nitroprusside) and NO itself activated sGC and were associated with the accumulation of cGMP in tissue supernatants (Arnold *et al.*, 1977; Katsuki *et al.*, 1977b). This was followed by the demonstration that nitrovasodilators and NO caused accumulation of cGMP in, and relaxation of, non-vascular (Katsuki *et al.*, 1977a) and vascular (Gruetter *et al.*, 1979) smooth muscle preparations. One of the key pieces of evidence demonstrating the link between EDRF and the nitrovasodilators/NO was the finding that their effect on smooth muscle and subsequent cGMP accumulation in the tissues could be inhibited by both methylene blue (Gruetter *et al.*, 1981; Ignarro *et al.*, 1984, 1986) and Hb (Furchgott *et al.*, 1984).

In further cascade-perfusion bioassay experiments using known inhibitors of EDRF, the short half-life ($t_{1/2}$) of EDRF was not only demonstrated, but also prolonged by superoxide dismutase (SOD), suggesting a key role for the *redox* properties of EDRF and for O_2^- in terminating the effects of EDRF (Gryglewski *et al.*, 1986; Moncada *et al.*, 1986).

Further experiments in Furchgott's group using acidified sodium nitrite ($NaNO_2$) as a NO donor and electron paramagnetic resonance (EPR) spectroscopy experiments by Ignarro's group revealed the incredibly close similarity between EDRF and NO. This similarity was first suggested simultaneously at a conference in 1987 (Ignarro *et al.*, 1987; Furchgott, 1988). The first direct measurement of NO production was conducted by Moncada's group. They used the chemiluminescent signal produced by the reaction of ozone (O_3) and NO (Downes *et al.*, 1976) and detected authentic concentration-dependent NO release from bradykinin (BK)-stimulated endothelial cells in sufficient quantities to account for the action of EDRF (Palmer *et al.*, 1987).

1.3.2 NO and NO-related chemistry

NO is a small diatomic, amphipathic, free radical molecule that is freely-diffusible and membrane-permeable and has been demonstrated to have important effects in almost every physiological system in the body, including in neurotransmission, GI physiology, genito-urinary function, innate immunity as well as on the cardiovascular system.

NO is a highly reactive radical that can be oxidized or reduced to generate a variety of different nitrogen species in biological systems (Figure 1.1). This reactivity underlies the complexity of NO functions, endowing NO with a potent ability as a free-radical agent to react with other biological species.

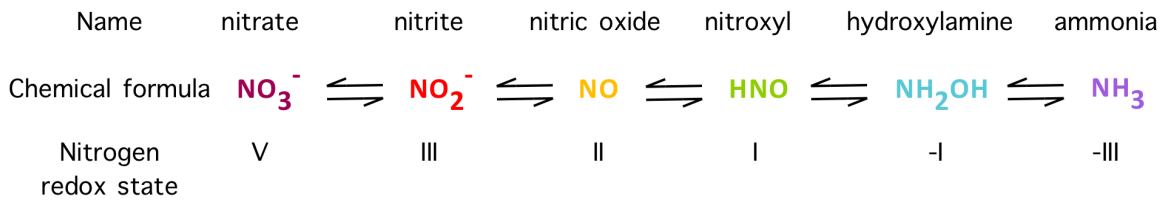


Figure 1.1 *Redox relationship of NO and other nitrogen species (NO=nitric oxide).*

NO rapidly reacts with species that possess unpaired electrons (e^-), such as O_2, which is the main oxidative mechanism to form NO_3^- in aqueous solutions (Bonner and Hughes, 1988; Ignarro *et al.*, 1993), and radicals such as O_2^- (Blough and Zafiriou, 1985). In addition, NO quenches radical-type reactions, such as lipid peroxidation (Rubbo *et al.*, 1996).

NO reacts with a variety of metals to form nitrosyl complexes, most commonly with iron (Fe) but also copper (Cu) (Fukuto *et al.*, 2000). One classic Fe-nitrosyl reaction that occurs has already been described above; namely, the activation of haem-containing protein, sGC. However, there are other haem-containing proteins that have important interactions with NO, including cytochrome P450 and Hb itself. The facileness of these reactions means that NO can be produced in separate cells or even tissues to the target protein (Fukuto *et al.*, 2000).

Thiols represent a third chemical group that reacts with NO to generate nitrosothiols in biological systems. These nitrosothiols have been proposed to act as NO donors, releasing NO when coming into contact with other thiols (Ignarro and Gruetter, 1980; Ignarro *et al.*, 1980a, b). There has also been some suggestion that

nitrosothiols have direct effects binding to an, as yet, unidentified receptor (Ohta *et al.*, 1997; Lipton *et al.*, 2001).

The termination of action of NO *in vivo* is achieved through its oxidation. The reaction of NO and oxyhaemoglobin (oxyHb) was first described more than 140 years ago by Hermann in 1865 (as cited in Gladwin *et al.*, 2005) and produces NO_3^- and methaemoglobin (metHb) (Equation 1.1).

oxyHb	***nitric oxide***		***metHb***	***nitrate***
$Fe(II)HbO_2$	**+ NO**	**⇒**	**Fe(III)Hb**	**+ NO_3^-**

Equation 1.1 *Oxidative termination of NO activity with Hb. (Hb=haemoglobin; NO=nitric oxide).*

It is now understood that this reaction represents an incredibly fast and avid sink (6-8 x 10^7 M^{-1} s^{-1}) for NO (Feelisch and Noack, 1987; Eich *et al.*, 1996; Herold *et al.*, 2001). This reaction is so fast that there were concerns that NO could not be a functional *in vivo* EDRF (Lancaster, 1994). However, the discovery of a cell-free layer of blood flowing adjacent to the endothelium (Liao *et al.*, 1999), and the recognition that the encapsulation of Hb within the erythrocyte limits the speed of the reaction due to the need for diffusion through the erythrocyte membrane (Liu *et al.*, 1998; Vaughn *et al.*, 1998, 2000; Han *et al.*, 2002) have allayed these concerns for the most part.

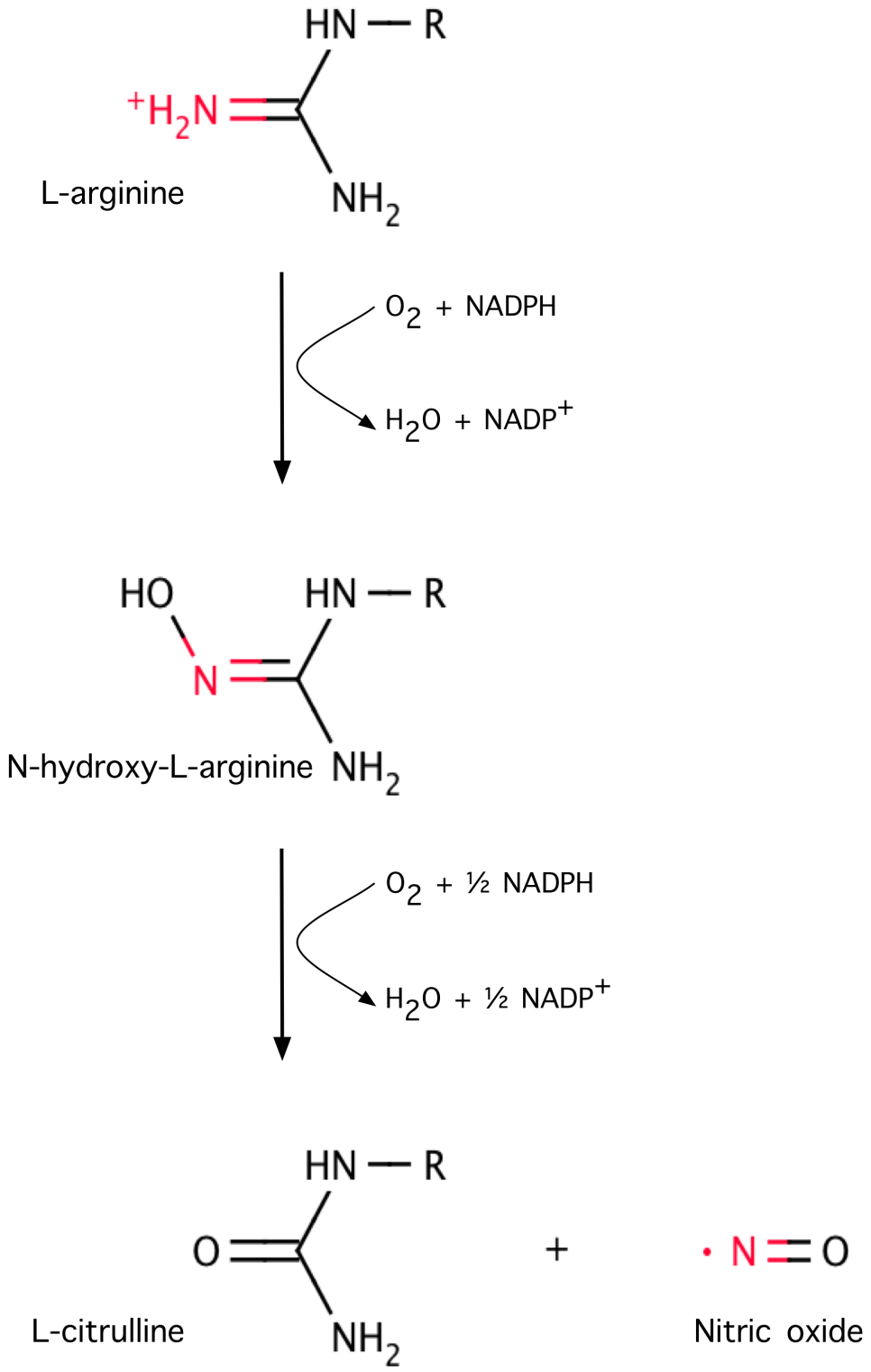
In pure aqueous solutions, the oxidation of NO occurs slowly and the primary product is NO_2^- (Ignarro *et al.*, 1993), however, with the addition of oxyhaemoproteins, such as oxyHb, then the predominant product is NO_3^- (Ignarro *et al.*, 1993) and thus NO_3^- is the predominant oxidative metabolite of NO in biological systems. There is recent evidence of a NO oxidase/NO_2^- synthase function of the multi-function Cu-containing enzyme, caeruloplasmin (Shiva *et al.*, 2006) but the importance of this pathway in terminating NO activity and regulating basal NO levels is unclear at this time.

1.3.3 Classical NO production and canonical signalling through the sGC/cGMP pathway

Pioneering work on rat brain and neuroblastoma cell homogenates revealed that there was a soluble activator of sGC whose activity could be blocked by Hb (Deguchi, 1977). Its identity was later confirmed as the amino acid, L-arginine, but not D-arginine, by EPR spectroscopy and chromatography (Deguchi and Yoshioka, 1982). Further work on macrophage cell lines revealed that L-arginine was the critical factor required for the production of NO_2^- and NO_3^- (collectively termed NO_x) (Hibbs *et al.*, 1987b; Iyengar *et al.*, 1987) and that this process could be inhibited by a chemically similar structure to L-arginine; L-^{N}G-monomethyl-arginine (L-NMMA) (Hibbs *et al.*, 1987a, b). Further experiments in cultured endothelial cells in the absence of L-arginine (using bioassays and chemiluminescence to detect authentic NO production) revealed a decrease in NO production that could be restored with L- but not D-arginine; and could be blocked by L-NMMA (Palmer *et al.*, 1988). Similarly, use of ^{15}N-labeled L-arginine and mass spectroscopy revealed that NO was

produced from the terminal guanido N-atom of L-arginine (Palmer *et al.*, 1988). The obligatory role of calcium (Ca^{2+}) in the so-called 'L-arginine:NO pathway' (Moncada *et al.*, 1989) was discovered by the use of Ca^{2+} chelators (Knowles *et al.*, 1989; Palacios *et al.*, 1989) and the importance of molecular O_2 confirmed by the incorporation into both NO and L-citrulline from $^{18}O_2$ (Kwon *et al.*, 1990; Leone *et al.*, 1991; Stuehr *et al.*, 1991) (Equation 1.2).

NO has multiple downstream targets to transduce its biological activity (Figure 1.2). NO regulates gene expression and messenger ribonucleic acid (mRNA) transcription via binding to Fe response elements (Pantopoulos and Hentze, 1995; Khan *et al.*, 1996). NO also causes post-translational modifications to proteins via S-nitrosation, via peroxynitrite ($ONOO^-$) as an intermediate (Ridnour *et al.*, 2004). However, the main molecular target for the beneficial effects of NO had been elucidated even before the identification of EDRF as outlined previously. sGC is a haem-containing protein that can form Fe-NO adducts and activate the enzyme to catalyse the conversion of guanosine triphosphate (GTP) to cGMP, which is the canonical intracellular secondary messenger for NO (Hobbs and Stasch, 2010). cGMP itself has a molecular target identified more than 35 years ago as a cGMP-dependent protein kinase (now commonly known as protein kinase G (PKG)) (Greengard, 1975). There are various substrates identified for PKG of which an important few are summarized in Table 1.2 below.



Equation 1.2 *The oxidation of L-arginine to produce NO. (NO=nitric oxide; H_20=water; O_2=oxygen; $NADP^+$/NADPH=nicotinamide adenine dinucleotide phosphate).*

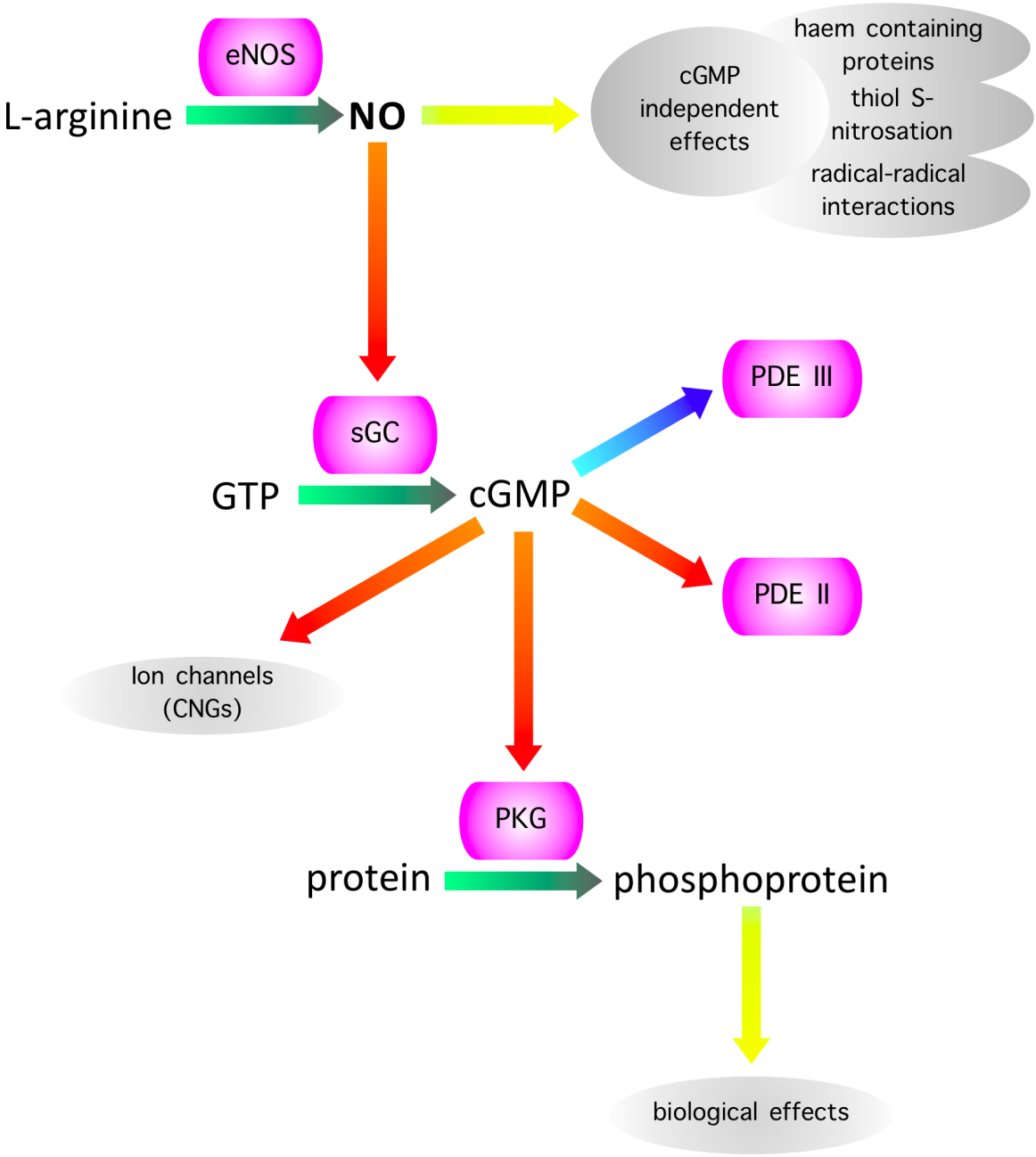


Figure 1.2 *NO-sGC-cGMP and downstream pathways. Coloured arrows indicate: **inhibition**; **stimulation**, **catalysis**. (cGMP=cyclic guanosine monophosphate; CNG=cyclic nucleotide-gated ion channel; eNOS=endothelial nitric oxide synthase; NO=nitric oxide; PDE=phosphodiesterase; PKG=protein kinase G; sGC=soluble guanylyl cyclase).*

Substrate	Function
Vasodilator-stimulated phosphoprotein (VASP)	Cell adhesion/motility Integrin signal transduction
Inositol phosphate receptor	Modulation of Ca^{2+} release in smooth muscle cells
Myosin binding subunit	Modulation of myosin light chain phosphatase
Heat-shock protein 20	Modulation of contractility of smooth muscle cells
Thromboxane receptor	Inhibition of G-protein coupled receptor (GPCR)-mediated inositol phosphate formation in platelets

Table 1.2 *Important substrates for PKG. (adapted from Lincoln and Komalavilas, 2000). (Ca^{2+}=calcium; GPCR=G-protein coupled receptor; PKG=protein kinase G).*

cGMP also acts directly on non-specific cation ion channels, particularly in retinal and olfactory tissues, though there is some suggestion for a role of cGMP-mediated ion channel activity in spermatozoa and kidney tubular cells in vertebrates (for a detailed review of this subject see Kaupp and Seifert, 2002). These channels are termed cyclic nucleotide-gated ion channels (CNGs).

Lastly, cGMP has effects on cGMP-dependent phosphodiesterases (PDEs) that can cross talk with another biological secondary messenger, cyclic adenosine monophosphate (cAMP). PDE II and III (of 11 so far discovered) are regulated by cGMP and decrease (Martinez *et al.*, 2002) and increase cAMP (Degerman *et al.*, 1997) respectively.

1.3.4 NO synthase (NOS) enzymes

Garthwaite and colleagues demonstrated that in cell suspensions of rat cerebella, glutamate stimulation of the N-methyl-D-aspartate (NMDA) receptor released a diffusible factor that was Ca^{2+} dependent and was responsible for cGMP elevations both in the cell preparations themselves and also in detector cells perfused in the medium from the stimulated cerebellar cells (Garthwaite *et al.*, 1988). All these features pointed to NO release from the neural tissues. This was quickly followed by the isolation by affinity chromatography of the first enzyme capable of producing NO from L-arginine, which was called at the time NO synthetase (Bredt and Snyder, 1990). We now know this enzyme as NOS-1 or neuronal (n)NOS. Within this first report of nNOS was the elucidation of the critical co-factor, calmodulin (Cam) that acts as a Ca^{2+}-binding messenger protein for all NOSs (EC 1.14.13.39) and is required for catalytic activity (Bredt and Snyder, 1990). This nNOS protein was successfully cloned in 1991 (Bredt *et al.*, 1991) and noted to be structurally similar to nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase. Since then, there has been the identification of 2 further NOSs, named after the tissues in which there were discovered and purified: inducible (i)NOS (NOS-2) (Stuehr *et al.*, 1991) and endothelial (e)NOS (NOS-3) (Pollock *et al.*, 1991) (see Table 1.3 for comparisons of the NOS isoforms).

Enzymatic Property	nNOS	iNOS	eNOS
Expression	Constitutive	Inducible	Constitutive
Ca^{2+}-dependency	Dependent	Independent	Dependent
Localization	Nervous system; skeletal muscle	All cells, especially immune cells	Endothelium; cardiac myocytes, renal tubular cells
Function	Neurotransmission; vasodilation	Pathogen cytotoxicity	Vasodilation; inhibition of platelet aggregation; inhibition of leucocyte-endothelium adhesion
Role in disease	Stroke; excitotoxic neurodegeneration	Septic shock; auto-immune disease	Endothelial dysfunction, CVDs

Table 1.3 *Properties of mammalian NOS isoforms (Ca^{2+}=calcium; CVD=cardiovascular disease; **eNOS=endothelial nitric oxide synthase**; **iNOS=inducible nitric oxide synthase**; **nNOS=neuronal nitric oxide synthase**; NOS=nitric oxide synthase) (adapted from Masters, 2000; Forstermann and Sessa, 2012).*

All isoforms of NOS are functional as homodimers (Klatt *et al.*, 1996; List *et al.*, 1997) and use L-arginine and molecular O_2 as previously described above. In addition, however, a number of other cofactors are also required (Figure 1.3 A). NADPH is essential and acts as an e^- donor (Knowles *et al.*, 1989; Palacios *et al.*, 1989; Stuehr *et al.*, 1989). Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are present in 1:1 stoichiometry (Bredt *et al.*, 1991; Mayer *et al.*, 1991) and are important for e^- transfer the reductase domain on one monomer (Klatt *et al.*, 1992; Stuehr and Ikeda-Saito, 1992; White and Marletta, 1992), to the haem/oxygenase domain on the opposite NOS monomer (Abu-Soud and Stuehr, 1993; Brunner *et al.*, 1998). Finally the pterin co-factor, tetrahydrobiopterin (H_4B) (Tayeh and Marletta, 1989; Mayer *et al.*, 1991) is essential for coupling of haem, O_2 and e^- transfer to NO production (Vásquez-Vivar *et al.*, 1998). In its absence, NOS is said to be uncoupled, and can produce O_2^- instead (Vásquez-Vivar *et al.*, 1998; Landmesser *et al.*, 2003) (Figure 1.3 B).

Each NOS monomer contains a C-terminal reductase (binding site for FMN, FAD and NADPH) and an N-terminal oxygenase domain (binds haem, O_2, L-arginine and H_4B). In the functional homodimeric configuration, these monomers run antiparallel so that the reductase domain of one monomer is adjacent to the oxygenase domain of the other monomer (Masters *et al.*, 1996) (Figure 1.3).

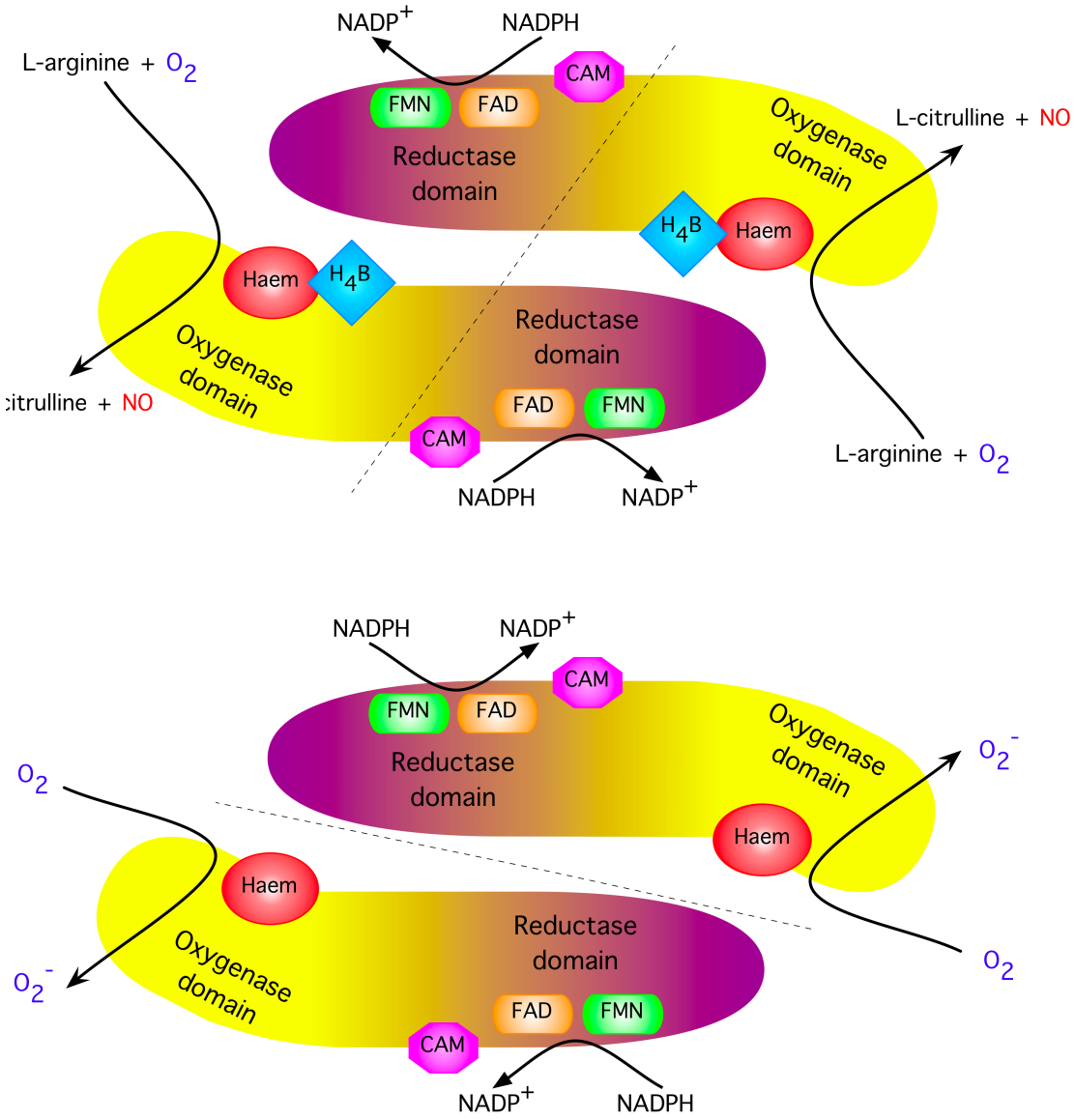


Figure 1.3 *Coupled and uncoupled dimeric eNOS. Representation of the domains and cofactors required for (A) coupled eNOS synthesis of NO and (B) the uncoupled production of O_2^-. In the presence of H_4B (A), e^- flow from the reduction of NADPH through FAD and FMN and are transferred to oxidase domain of the corresponding monomer and are coupled to the oxidation of L-arginine to liberate NO. In the absence of H_4B (B), e^- no longer flow to the corresponding monomer and instead facilitates the production of O_2^-. (Cam=calmodulin; e^- =electron; eNOS=endothelial nitric oxide synthase; FAD=flavin adenine dinucleotide; FMN=flavin mononucleotide; H_4B=tetrahydrobiopterin; $NADP^+$/H=nicotinamide adenine dinucleotide phosphate; NO=nitric oxide; O_2=oxygen; O_2^-=superoxide).*

1.3.4.1 eNOS

NO generation within the circulation is thought to be largely produced largely from eNOS that resides within the endothelial cell. eNOS-derived NO is generated in response to circulating hormones such as ACh and BK (Furchgott and Zawadzki, 1980; Cherry *et al.*, 1982); and in response to mechanical shear stress (Pohl *et al.*, 1986; Rubanyi *et al.*, 1986; Joannides *et al.*, 1995). The former results in eNOS activation following GPCR activation (Flavahan *et al.*, 1989; Liao and Homcy, 1992), triggering Ca^{2+} influx (Busse *et al.*, 1988; Danthuluri *et al.*, 1988) and further release of intracellular Ca^{2+} stores (Freay *et al.*, 1989). Shear stress, sensed by mechanoreceptors on the endothelial cell surface (Lansman *et al.*, 1987), triggers eNOS activity primarily through protein kinase B (Akt)-dependent phosphorylation of eNOS, independent of intracellular Ca^{2+} rises (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999) (Figure 1.4).

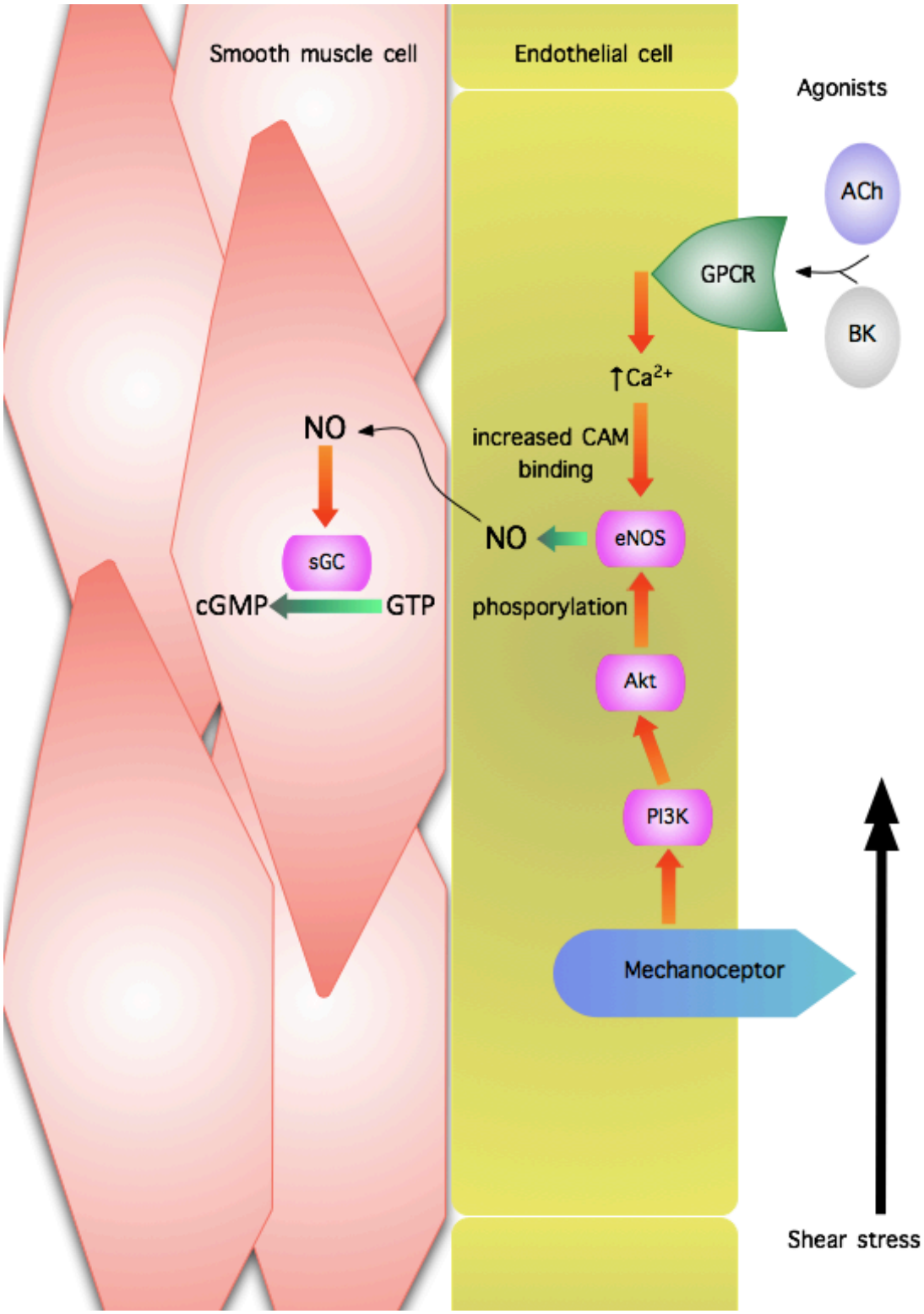


Figure 1.4 *Agonist-dependent and -independent stimulation of eNOS activity. (ACh=acetylcholine; Akt=Protein kinase B; BK=bradykinin; CAM=calmodulin; Ca^{2+}=calcium; cGMP=cyclic guanosine monophosphate; eNOS=endothelial nitric oxide synthase; GTP=guanosine triphosphate; GPCR=G-protein coupled receptor; NO=nitric oxide; PI3K= phosphoinositide 3-kinase; sGC=soluble guanylyl cyclase).*

However, it was largely with the advent and use of the NOS inhibitor, L-NMMA, that the fundamental roles of endogenous NO were elucidated within the cardiovascular system. Experiments using rabbit aorta *ex vivo* demonstrated that application of L-NMMA caused dose-dependent, endothelium-dependent smooth muscle contraction, suggesting for the first time, continuous NO-release was responsible in part for regulating vascular tone (Rees *et al.*, 1989a). These seminal findings were confirmed in whole animal studies. Infusion of L-NMMA caused dose-dependent increases in BP in anaesthetized rabbits that were reversed with co-infusion of L-arginine, but not D-arginine (Rees *et al.*, 1989b). Similarly, L-NMMA pre-treatment prevented the expected BP reduction with an endothelium-dependent agonist, ACh, but did not change the response an endothelium-independent vasodilator, GTN (Rees *et al.*, 1989b).

Similar studies were then conducted in healthy subjects where infusion of L-NMMA into the brachial artery caused a reduction in forearm blood flow (FBF) and, similarly, attenuated the increase in FBF produced in response to ACh (Vallance *et al.*, 1989). These studies demonstrated the dependence of baseline blood flow and vessel tone in humans on endothelial production of NO. The development of the eNOS knockout mouse has demonstrated that the dependence of the vasculature on eNOS-derived NO ultimately impacts on BP, with the demonstration of a raised mean arterial pressure (MAP) of 117±10 mmHg compared to 97±8 mmHg in littermate controls, as well as a complete absence of vasodilation in response to ACh (Huang *et al.*, 1995). Conversely, mice with eNOS over-expression have constitutively low BP (Ohashi *et al.*, 1998).

In addition to vasodilation, NO possesses several other potentially advantageous properties. Cascade-perfusion experiments of ACh-stimulated aortic strips demonstrated that the effluent of such circuits (i.e. eNOS-derived NO) was not only able to relax endothelium-denuded aortic tissues (as had been previously demonstrated) but also attenuated the aggregation response of washed platelets to arachidonic acid (Azuma *et al.*, 1986). Furthermore, stimulation of bovine endothelial cell monolayer cultures with the endothelium-dependent agonist BK, or by superfusion of exogenous NO, attenuated platelet adhesion to the monolayers (Radomski *et al.*, 1987).

In addition to preventing platelet adhesion to the endothelium, which is increasingly recognized as a fundamental primary event in atherogenesis (Davì and Patrono, 2007), further studies have also revealed the critical role of eNOS-derived NO on leucocyte adhesion to the endothelium. Superfusion of cat mesenteric preparations with L-NNMA and other NOS inhibitors substantially increased the leucocyte adherence and emigration detected by intravital microscopy (Kubes *et al.*, 1991), suggesting that basal eNOS-derived NO might be involved in producing an anti-inflammatory endothelial phenotype.

1.3.4.2 Endothelial dysfunction

Traditional risk factors for CVD including smoking, hypertension, male sex and diabetes are all associated with impaired ability to produce adequate amounts of bioavailable endogenous NO (reviewed in Brunner *et al.*, 2005), suggesting that reduced bioavailable NO is a key first step in the development of CVDs. This concept

is now synonymous with the term endothelial dysfunction (Moncada and Higgs, 2006). A variety of mechanisms have been proposed to underlie this reduced NO bioavailability, in particular, increased reactive oxygen species (ROS) production, such as O_2^-. Increased ROS production can come from many sources, such as NADPH oxidases (Warnholtz *et al.*, 1999), xanthine oxidoreductase (XOR) (Ohara *et al.*, 1993) and the mitochondrial respiratory chain (Turrens *et al.*, 1985). However, O_2^- can also be produced from eNOS uncoupling (Xia *et al.*, 1998).

A number of different mechanisms themselves have been implicated in bringing about eNOS uncoupling. Perhaps the most important pathway relates to insufficient levels of H_4B. Although NOS dimers can form in the absence of H_4B (Rodríguez-Crespo *et al.*, 1996; Ghosh *et al.*, 1997), H_4B stabilizes and promotes the activity of NOS dimers through structural means (Giovanelli *et al.*, 1991; Baek *et al.*, 1993; Klatt *et al.*, 1995) as well as improving affinity for L-arginine binding to NOS (Klatt *et al.*, 1994). Whilst oxidized biopterin (H_2B) does bind eNOS and stabilizes the eNOS dimer, it is unable to couple e^- transfer to NO production (Ghosh *et al.*, 1997; Presta *et al.*, 1998) and increasing H_2B levels leads to eNOS uncoupling and O_2^- production (Vásquez-Vivar *et al.*, 2002; Crabtree *et al.*, 2009).

The rate of NO production in endothelial cells is closely correlated to intracellular H_4B levels (Werner-Felmayer *et al.*, 1993; Rosenkranz-Weiss *et al.*, 1994). In animal models, depletion of H_4B, by pre-treating with an inhibitor of H_4B synthesis, 2,4-diamino-6-hydroxypyrimidine, produced endothelial dysfunction *in vivo* (Yamashiro *et al.*, 2002). Conversely, *i.v.* supplementation with H_4B improved endothelial

function, as measured by the increases in FBF to endothelium-dependent stimuli, in human subjects with diabetes (Heitzer *et al.*, 2000) hypertension (Higashi *et al.*, 2002), hypercholesterolaemia (Stroes *et al.*, 1997) and intra-arterial H_4B prevented ACh-induced vasoconstriction in coronary arteries of patients with ischaemic heart disease (Maier *et al.*, 2000). However, more recently, the highly oxidative environment that is apparent in CVD states has proved challenging to the therapeutic exploitation of H_4B. In patients waiting to undergo coronary artery bypass grafting for ischaemic heart disease, oral supplementation of H_4B (400mg-700mg /day for 2-6 weeks) elevated plasma and venous tissue H_4B levels but was associated with a similar increase in H_2B as well, thus not altering the overall H_2B:H_4B ratio. This was associated with no change in vascular function or vascular *redox* state (Cunnington *et al.*, 2012), suggesting alternative strategies to target endothelial function in established CVDs may be required.

Other potential causes of reduced bioavailable NO production in endothelial dysfunction include accumulation of asymmetric dimethylarginine (ADMA), which is an endogenous inhibitor of eNOS (Vallance *et al.*, 1992a, b) and may lead to eNOS uncoupling (Antoniades *et al.*, 2009) as well as directly inhibiting NO synthesis from functional eNOS (reviewed in Sydow and Münzel, 2003). An interesting concept in this field is the 'arginine paradox'. This is the unexpected phenomenon that exogenous provision of L-arginine increases endothelium-dependent vasodilation in many human equivalents of endothelial dysfunction (Drexler *et al.*, 1991; Hishikawa *et al.*, 1993). This is despite normal intracellular L-arginine levels being several fold higher (~100μM) (Closs *et al.*, 2000) than the K_M of eNOS for L-arginine (~3μM)

(Pollock *et al.*, 1991) It has been suggested that the paradox may be explained by an increased arginase activity situated in close proximity to eNOS in disease states (Bachetti *et al.*, 2004; Ming *et al.*, 2004) but also more recently to the activity of arginosuccinate lyase that co-localizes to eNOS in a complex protein structure (Erez *et al.*, 2011).

Recently, S-glutathionylation of eNOS (Chen *et al.*, 2010b) has been shown to be an important down-regulator of eNOS function that is particularly activated by ROS and leads to further eNOS uncoupling and eNOS-derived O_2^- production, which itself can scavenge NO and further reduce bioavailable NO (Chen *et al.*, 2010b).

As stated earlier, endothelial dysfunction is synonymous with the reduced production of eNOS-derived NO. Over the last 30 years, there have been a series of different techniques to study this phenomenon in humans *in vivo*. One of the earliest demonstrations of endothelial dysfunction in CVD was in patients undergoing invasive epicardial coronary angiography due to suspected ischaemic heart disease (Ludmer *et al.*, 1986). In this study, in patients with no evidence of coronary artery disease at angiography, it was shown that infusion of both the endothelium-dependent agonist ACh and the non-endothelium-dependent NO-donor GTN, both effected concentration-dependent coronary artery dilation. However, in patients with either mild (<20% angiographic stenoses) or severe (>50% stenoses) atherosclerosis, ACh caused concentration-dependent constriction (Ludmer *et al.*, 1986), similar to that observed by Furchgott in *ex vivo* denuded aortic strips (Furchgott and Zawadzki, 1980). GTN still produced concentration-

dependent relaxation in patients with angiographic stenoses (Ludmer *et al.*, 1986). These findings in patients with atherosclerotic stenoses demonstrate that the ability of the smooth muscle to respond to NO was not impaired, whilst endothelial dysfunction was apparent in both early and late stages of atherosclerosis (Ludmer *et al.*, 1986). These findings were further explored in patients who had received allogeneic cardiac transplantation (Fish *et al.*, 1988), who commonly develop accelerated atherosclerosis in the allogeneic epicardial arteries (Nitkin *et al.*, 1985). In this study, the angiographically normal epicardial arteries of allogeneic heart transplant recipients demonstrated vasoconstriction to ACh but normal dilation to GTN, revealing the presence of endothelial dysfunction in the arteries of patients known to be at much higher risk of atherosclerosis (Fish *et al.*, 1988). It has now also been established in patients free of angiographic atherosclerosis that epicardial coronary endothelial dysfunction is associated with all the classical risk factors for CVD such as age, male sex, hypercholesterolaemia, positive family history of ischaemic heart disease (Vita *et al.*, 1990) and hypertension (Treasure *et al.*, 1992); again suggesting that endothelial dysfunction is both pathogenic in and precedes CVD. Furthermore, it is also now accepted that epicardial coronary endothelial dysfunction is an independent predictor of the future likelihood of developing coronary events in patients with ischaemic heart disease but also in those free of disease (Halcox *et al.*, 2002; Targonski *et al.*, 2003).

Whilst this coronary angiography-based technique has been very useful in articulating the presence of endothelial dysfunction, the invasive nature of the method has inevitably limited mechanistic studies. One technique that overcomes

some of these issues is venous occlusion plethysmography. Venous occlusion plethysmography was first described by Brodie and Russell in 1905 to determine the rate of flow of blood into an organ or limb by preventing venous blood escape by the means of a pneumatic cuff inflated to sub-diastolic levels (Brodie and Russell, 1905). This technique has since been modified with the introduction of mercury-in-silastic stretch gauges (Hokanson *et al.*, 1975) and has been coupled with cannulation of the brachial artery to permit the application of endothelium-dependent agonists, such as ACh and NOS-inhibitors, such as L-NMMA (Vallance *et al.*, 1989). Through this moderately less-invasive technique, it has been demonstrated that endothelium-dependent increases in FBF are attenuated in patients with hypertension (Linder *et al.*, 1990; Panza *et al.*, 1993) and hypercholesterolaemia (Chowienczyk *et al.*, 1992). Moreover, prospective studies show that the magnitude of the changes in ACh-induced FBF is predictive of future events in hypertensive patients (Perticone *et al.*, 2001) and patients with established ischaemic heart disease (Heitzer *et al.*, 2001; Fichtlscherer *et al.*, 2004).

Whilst again this technique is useful, the invasive nature of the method is less than ideal. The advent of ultrasound assessment of brachial artery diameter has now superseded both the above techniques due to its reproducibility and non-invasive nature, and is currently accepted as a *gold*-standard technique for the assessment of endothelial dysfunction (Deanfield *et al.*, 2005). This technique uses ultrasound to detect brachial artery dilation in response to reactive hyperaemia (flow-mediated dilatation, FMD). By taking advantage of the response of the endothelium to produce NO in response to flow/shear stress (Pohl *et al.*, 1986; Rubanyi *et al.*,

1986), Deanfield and Celermajer developed an entirely non-invasive test of endothelial function by using ultrasound detection of arterial dilation after a brief period of arterial occlusion by a pneumatic cuff (Celermajer *et al.*, 1992). Using this technique, they established that FMD was diminished (i.e. there was endothelial dysfunction) in children and adults with risk factors for atherosclerosis, such as hypercholesterolaemia and smoking, prior to anatomically-evident atherosclerosis, and also in patients with ischaemic heart disease (Celermajer *et al.*, 1992). Further studies revealed that FMD was impaired in individuals expressing all of the known traditional cardiovascular risk factors and that the magnitude of the impairment of endothelial function increased with an increased number of independent risk factors (Celermajer *et al.*, 1994). Importantly, experiments using brachial artery infusion of the NOS inhibitor, L-NMMA, abolished radial artery FMD, confirming the requirement for endothelial NO production to produce the FMD response (Joannides *et al.*, 1995; Mullen *et al.*, 2001). Furthermore, just as with coronary angiographic and forearm plethysmographic techniques above, ultrasound assessment of FMD has been demonstrated to be predictive of CVD outcomes in prospective patient populations with peripheral arterial disease, hypertension (Gokce *et al.*, 2003) and ischaemic heart disease (Neunteufl *et al.*, 2000).

Whilst all the studies and results described above confirm the importance of endothelial dysfunction as an entity that precedes CVD and is also part of the pathogenesis of CVD, additional evidence for the importance of eNOS-derived NO comes from intervention trials. Treatment of major CVD risk factors which improve CVD mortality, such as LDL-cholesterol lowering with hydroxymethylglutaryl-

coenzyme A reductase inhibitors (commonly known as 'statins') and BP lowering with drugs that interfere with the renin-angiotensin-aldosterone system, reverse endothelial dysfunction with prolonged treatment (Treasure *et al.*, 1995; Mancini *et al.*, 1996; John *et al.*, 1998) and increase bioavailable NO (Imanishi *et al.*, 2008).

Therefore, other strategies to replace the 'lost' NO in CVD have obvious therapeutic potential (Herman and Moncada, 2005). The organic nitrates, such as GTN and isosorbide mononitrate, represent the first class of NO donors to reach the clinical setting, although a number of issues have limited their clinical utility (see section 1.4). Currently there is a trend towards the invention of novel, hybrid small chemical entities with NO-releasing properties and also combining drugs already used clinically with the added improvement of an NO-releasing moiety such as the NO-non steroidal anti-inflammatory drugs (NSAIDs) (del Soldato *et al.*, 1999). More recently, it has been hypothesized that provision of inorganic NO_3^-, given either by dietary or inorganic supplementary route may obviate the need for these costly drugs and this class of 'NO donor' is the focus of this thesis.

1.4 Use of organic NO-releasing compounds on the cardiovascular system

Strategies that provide exogenous, synthesized sources of NO have been used for medical purposes for more than 120 years before the discovery of endogenous NO synthesis. The advent of organic chemistry and the new scientific discipline of detailing physiological processes, aided through the invention of devices such as the sphygmograph, led to an *explosion* in the field of organic NO therapeutics in the late 19th century, which eventually led to physicians studying the effects of related inorganic compounds.

1.4.1 Organic nitrites and nitrates

The first compounds that we would now recognize to be nitrovasodilators that were synthesized and used for medical purposes were the organic nitrites and nitrates. Guthrie's seminal observations of the 'flushing' (vasodilation) and 'acceleration' (tachycardia) after exposure to amyl nitrite (Guthrie, 1859) led Gamgee and Brunton to prescribe amyl nitrite (Brunton, 1867), and Murrell to prescribe GTN (Murrell, 1879a, b, c, d) to patients with angina with good results. However, despite these early successes, the utility of organic nitrites and nitrates has been limited to some degree by their chemistry.

1.4.1.1 Chemistry and bioactivation of organic nitrites and nitrates

Organic nitrites and nitrates are distinguished by the presence of an -ONO_2 group (or in the isolated case of ethyl nitrite, -ONO), which is the pharmacophore of their actions by liberating NO (Thatcher *et al.*, 2004). These groups can be bound to any organic residue and it is the stereochemistry and complexity of the organic residue

that determines lipophilicity and potency of the organic nitrites and nitrates (Thatcher *et al.*, 2004; Koenig *et al.*, 2007) (Figure 1.5).

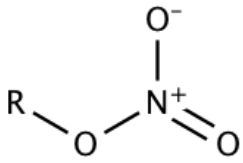


Figure 1.5 *Typical organic nitrite and nitrate structure*

Since the pharmacophore is covalently bound to the organic residue, bioactivation of its nitrosovasodilating action requires enzymatic conversion to release the NO from the $–ONO_2$ moiety. There are thought to be 2 main pathways for organic nitrate bioactivation: the low and high potency pathways. The low potency pathway produces measurable NO from GTN at high doses (Kleschyov *et al.*, 2003) and is largely thought to be mediated by enzymes of the cytochrome P450 system (McDonald and Bennett, 1990). More recently, however, a high potency pathway for GTN bioactivation, involving the mitochondrial isoform of the enzyme, aldehyde dehydrogenase-2 (ALDH-2), was elucidated by using the specific ALDH-2 inhibitors, chloral hydrate and cyanamide (Chen *et al.*, 2002). The ALDH-2 inhibitors blocked the production of NO_2^- and glyceryl dinitrate (GDN) from GTN, which had previously been shown to be important intermediates in producing NO-related vasodilation (Sage *et al.*, 2000). ALDH-2 inhibitors also attenuated the vasodilating and hypotensive properties of GTN in anaesthetized rabbits (whilst having no effect on NO released from donors such as sodium nitroprusside) (Chen *et al.*, 2002) and

attenuated increases in cGMP and phosphoVASP as indicators of reduced throughput of the NO-dependent sGC/PKG signalling pathways (Daiber *et al.*, 2004).

However, one of the major problems with the organic nitrates is the occurrence of tolerance or tachyphylaxis. This is the progressive reduction in the haemodynamic effects of organic nitrates with prolonged treatment (Elkayam *et al.*, 1987), which also leads to endothelial dysfunction. Endothelial dysfunction can be observed in humans after prolonged exposure to organic nitrates. In one such study, patients with ischaemic heart disease were randomized to receive 5 days of continuous GTN transdermally or nothing. ACh caused greater coronary artery vasoconstriction in the GTN-treated group, revealing the development of endothelial dysfunction in response to prolonged GTN administration (Caramori *et al.*, 1998). The same group revealed similar results in the forearm circulation of healthy individuals using venous occlusion plethysmography (Gori *et al.*, 2001). The exact mechanisms of organic nitrate tolerance are debated but there are some compelling theses. The molecular mechanisms underlying organic nitrate-induced tolerance and endothelial dysfunction are largely thought to relate to increased production of ROS, including O_2^-. Rabbit aortae from animals treated with transdermal GTN for 3 days have double the amount of O_2^- (as measured by lucigenin chemiluminescence) as control animals (Münzel *et al.*, 1995a), and elevated levels of $ONOO^-$ (assayed by nitrotyrosine accumulation) (Skatchkov *et al.*, 1997). $ONOO^-$ is a powerful oxidant product of reaction between NO and O_2^- (Huie and Padmaja, 1993) and oxidizes the critical factor H_4B (Laursen *et al.*, 2001) and therefore can uncouple eNOS and cause increased production of eNOS-derived O_2^- (Münzel *et al.*, 2000) and increase

oxidative stress in a positive feedback mechanism. These increases in O_2^- and concomitant endothelial dysfunction could be detected *ex vivo* in segments of graft arteries of patients (pre-treated with GTN) undergoing coronary artery bypass operations (Schulz *et al.*, 2002). Another mechanism that has been proposed is the increased sensitivity to receptor dependent vasoconstrictors. In animals treated with GTN for prolonged periods there was increased sensitivity to vasoconstrictor substances such as angiotensin-II and phenylephrine (Münzel *et al.*, 1995b). Similarly, in the forearm circulation of patients with ischaemic heart disease treated for 48 h continuously with GTN, the vasoconstrictor responses to angiotensin-II and phenylephrine were enhanced (Heitzer *et al.*, 1998). It is possible, however, that this effect is simply due to the loss of bioavailable NO that normally opposes vasoconstriction.

These problems of tolerance and endothelial dysfunction have largely limited the utility of organic nitrates and perhaps partly explains the lack of efficacy of organic nitrates in large scale clinical trials (ISIS-4 Collaborative Group, 1995). The use of organic nitrates has diminished over time and they are now predominantly restricted in terms of clinical utility to symptomatic relief in angina and heart failure (British Medical Association and the Royal Pharmaceutical Society of Great Britain, 2012). Whilst these limiting effects of organic nitrites and nitrates have been disappointing, recent proposals suggest that exploiting NO donors in CVD may still be an option using inorganic NO_2^- and NO_3^-. This possibility has been raised as potentially offering a better option since their clinical utility seems not to be limited by either tolerance or the development of endothelial dysfunction.

1.5 Inorganic NO_2^- and NO_3^-

The monographs published on the excellent anti-anginal properties of amyl nitrite by Lauder Brunton and GTN by Murrell led other physicians of the time to explore related chemicals, including inorganic NO_2^- salts.

1.5.1 Historical uses of inorganic NO_2^-

Reichert and Mitchell published an extensive treatise on the physiological actions of potassium nitrite (KNO_2) in 1880 (Reichert and Mitchell, 1880). Reichert started this monograph with these words to explain why he was using a related substance to amyl nitrite (Reichert and Mitchell, 1880):

> "The very great value of amyl nitrite in warding off impending paroxysms of epileptic convulsions, angina pectoris, and asthma, has been so generally recognized by the profession, that the discovery of a new salt whose physiological action is identical with… that of amyl nitrite, but whose effects would be more permanent and therefore suitable for maintaining a continuous systemic influence, we would have an addition to our *materia medica* which would fill a very apparent therapeutic void."

Whilst much of the monograph is filled with experimental detail of the effects of KNO_2 on the central and peripheral nervous system, respiration and muscles, there was also extensive reporting on the effects of KNO_2 on the pulse and arterial tension. Mitchell's human experiments revealed that the effects of KNO_2 were of similar effect to that of amyl nitrite, whilst Reichert's experiments on dogs and cats revealed that large doses of KNO_2 could reduce the BP so drastically and over extended periods as to cause death of the animal (Reichert and Mitchell, 1880). These observations were followed by a detailed comparative analysis of the effects of $NaNO_2$ and organic nitrites, including amyl nitrite (Wallace and Ringer, 1909) and GTN (Matthew, 1909; Wallace and Ringer, 1909). In subjects with normal BP, the

hypotensive effects of organic nitrites and nitrates was observed for up to 7-30 min post-administration but a similar level of BP reduction was maintained for up to 60-65 min following inorganic NO_2^- dosing (Matthew, 1909; Wallace and Ringer, 1909). The effects were much larger (25% reduction in SBP) in patients with severely elevated BP (SBP> 170mmHg) compared to healthy individuals (13% reduction in SBP) (Wallace and Ringer, 1909), with maximal SBP reductions in hypertensive patients of up to 50 mmHg (Matthew, 1909; Wallace and Ringer, 1909). The use of inorganic NO_2^- for the treatment of BP was established in the early part of the 20th century and appeared in *materiae medicae* and were produced by several pharmaceutical suppliers for use in hypertension (in Butler and Feelisch, 2008).

1.5.2 Historical uses of inorganic NO_3^-

Inorganic NO_3^- has been used to treat CVD for over a millennium in traditional Chinese medicine. The following passage is a translation from an 8th century CE manuscript that was discovered in the Mogao caves near to Dunhuang in Gansu province of China (Butler and Moffett, 2005):

"Putting under the tongue to cause heart *qi* to flow freely for treating symptoms such as struck by evil, acute heart pains and cold in the hands and feet which can kill a patient in an instant. Look at the patient's fingers and those with greenish-black nails are such cases. Take saltpeter [*xiaoshi*, potassium nitrate] (5 measures of a *bi* spoon) and realgar [*xiongshi*, arsenic sulphide] (1 measure of a *bi* spoon) and combine the two into a fine powder. Lift the patient's tongue and sprinkle one measure of a *bi* spoon under the tongue. If saliva is produced, have the patient swallow it. This is a certain cure."

What is most interesting in this description of a medical condition that is thought to represent symptomatic angina is the command in the last line regarding the production of saliva and the importance of swallowing of it, as this relates to an important bioactivation step for inorganic NO_3^- (section 1.6.4).

It took a great deal of time before a modern, Western physician would be interested in the same chemical entities as the pre-medieval Chinese physicians. Stieglitz produced a body of work in the 1920-30's relating to his studies and treatments of patients with bismuth subnitrate (chemical formula: $Bi_5O(OH)_9(NO_3)_4$). Bismuth subnitrate was already an established and recommended treatment for peptic ulcer disease and diarrhoea but prolonged use was cautioned against due to the risk of hypotension (Frick, 1924). Stieglitz noted in his first monograph on the treatment of hypertension with bismuth subnitrate his reasoning for use of this medication (Stieglitz, 1927):

"The idea to use bismuth subnitrate as an auxiliary to break the vicious circle of vascular fatigue arose from the observation of three cases of nitrate poisoning resulting from the liberal use of bismuth subnitrate in severe diarrhoeas."

Stieglitz was aware of the discovery that symbiotic colonic bacteria, such as *E. Coli* (at that time known as *B. Coli*), were able to metabolize inorganic NO_3^- to NO_2^- (Salen, 1925; Zobell, 1932). He thus surmised the following (Stieglitz, 1927):

"Therefore, theoretically, small frequent doses should lead to the liberation of small amounts of nitrite, uniformly and continuously absorbed. The effect of this is quite different from the violent, very transient vasodilatory effect of other forms of nitrite, such as nitroglycerol, amyl nitrite and sodium nitrite. The action to be expected is a gradually increasing vascular relaxation, with localized physiological rest to the arteriolar musculature."

His reports of the sustained hypotensive effects in almost 1000 patients (Stieglitz, 1927, 1928, 1930, 1932) were followed by methodological improvements to the colorimetric techniques available at the time to measure NO_2^- levels in biological fluids (Stieglitz and Palmer, 1934). He was then able to determine that the basal levels of NO_2^- in whole blood as 0.5-1.0 µg NO_2^-/100 mL blood (~110-220 nM) (Stieglitz and Palmer, 1937). Additionally, he demonstrated that NO_2^- was not a

normal constituent of fresh urine (Stieglitz and Palmer, 1937) but was detectable after an inorganic NO_3^- load (Stieglitz and Palmer, 1937), in keeping with significant elevations in blood NO_2^- after ingestion of inorganic NO_3^- (Stieglitz and Palmer, 1936). Lastly, he expended much effort into *in vitro* determination of the rate of NO_2^- formation from NO_3^- under the action of NO_3^--reducing bacteria to hypothesize that bacterial reduction of NO_3^- could be responsible for significant physiological effects *in vivo* (Stieglitz and Palmer, 1936).

1.5.3 Traditional views of the chemistry of inorganic NO_2^- and NO_3^-

Despite these early studies, the view of NO_3^- has been as a chemically inert metabolite of endogenous NO metabolism that does not participate in any important chemical reactions (Bonner and Hughes, 1988; Ignarro *et al.*, 1993), whilst there are a number of *in vivo* chemical reactions that occur with NO_2^-. The most widely described reaction of NO_2^- in mammalian systems is its reaction with oxyHb to generate metHb and NO_3^- with 1:1 stoichiometry of all the constituents of the reaction (Kosaka *et al.*, 1979) (Equation 1.3).

oxyHb		***nitrite***		***metHb***		***nitrate***
$Fe(II)HbO_2$	**+**	**NO_2^-**	**$\Rightarrow$**	**$Fe(III)Hb$**	**+**	**NO_3^-**

Equation 1.3 *Reaction of NO_2^- with oxyHb (NO_2^-=nitrite; oxyHb=oxyhaemoglobin).*

Gamgee made the first reports of this reaction (Gamgee, 1868):

"My attention was directed to the peculiar action of nitrites upon the blood-colouring matter by observing that the blood of mice poisoned by exposure to an atmosphere impregnated with the vapour of nitrite of amyl presented a chocolate-colour."

In a series of detailed experiments involving fractional gas determination and optical characteristics of blood exposed to inorganic NO_2^- (both $NaNO_2$ and KNO_2), Gamgee was able to determine that the reactions of NO_2^- with oxyHb produced changes in the Hb species that although had the same crystalline structure as oxyHb, had different optical characteristics (i.e. dark brown) but more importantly had now lost the ability to bind and release O_2 (Gamgee, 1868). The Fe of metHb is in the ferric (Fe^{3+}) form that is unable to carry O_2 (Darling and Roughton, 1942). MetHb is physiologically reduced back to ferrous (Fe^{2+}) Hb predominantly by the enzyme, cytochrome b5 reductase (Gibson, 1948) which physiologically maintains normal metHb levels at less than 1% (Wright *et al.*, 1999). However, in situations where metHb levels rise >10%, the O_2 content of the Hb becomes insufficient, thus provoking symptomatic hypoxaemia despite adequate oxygenation (Skold *et al.*, 2011). This reaction of NO_2^- with ferrous oxyHb to form metHb is used clinically to treat cyanide poisoning (British Medical Association and the Royal Pharmaceutical Society of Great Britain, 2012). Cyanide is a potent inhibitor of cytochrome c oxidase in the mitochondrial respiratory chain (Antonini *et al.*, 1971) but preferentially binds to metHb (Smith *et al.*, 1977). Thus NO_2^- can be used to increase metHb levels to act as a cyanide sink. Nevertheless, concerns about organic nitrite and inorganic NO_2^--induced acquired methaemoglobinaemia continue to this day and have been a large part of the reduced use of these medications in clinical practice (Pierce and Nielsen, 1989; Finan *et al.*, 1998; Modarai *et al.*, 2002).

1.6 The fate of NO_3^- in humans

The view that the oxidation of NO to NO_2^- and NO_3^- represents a termination of the pathway has needed revision with the publication of numerous studies demonstrating bioactivity of a reductive pathway prevalent *in vivo* whereby NO_3^- is reduced to NO_2^- and thence to the biologically active NO.

NO_3^- is found in the plasma of healthy individuals, concentrations measured ranging between 20-40 μM (Gladwin *et al.*, 2000; Lundberg and Govoni, 2004; Webb *et al.*, 2008a). *In vivo*, NO_3^- originates from two potential sources i.e. from the oxidative metabolism of NO, but also from NO_3^- ingested orally in the diet as previously discussed in section 1.2.4. Nitrogen-balance studies (lasting up to 14 weeks), in which participants were provided all food and fluid intake by the investigators (providing <180 μmol NO_3^- per day) have estimated from 24 h urine NO_3^- collections that endogenous biosynthesis of NO_3^- is ~0.6-0.9 mmol per day in healthy people (Green *et al.*, 1981), though this may be much higher when iNOS has been induced (Stichtenoth *et al.*, 1994). Estimates of average daily ingestion of NO_3^-, which is predominantly due to vegetable intake (World Cancer Research Fund/American Institute for Cancer Research, 2007), suggest that total daily NO_3^- intake is between 1.5-2 mmol across different European countries (European Food Safety Authority, 2008), though individual intake may vary depending on the types of vegetables consumed. The WHO recommends a daily intake of 400 g of mixed vegetables which would lead to an estimated daily NO_3^- intake from all food sources of ~2.5 mmol (European Food Safety Authority, 2008), whilst the DASH study diet has been estimated to contain up to ~20 mmol NO_3^- (Hord *et al.*, 2009).

1.6.1 Pharmacokinetics of NO_3^-

Orally ingested NO_3^- is rapidly absorbed across the upper GI tract (Hawksworth and Hill, 1971; Witter *et al.*, 1979; Miyoshi *et al.*, 2003) and bypasses first-pass metabolism with close to 100% bioavailability (van Velzen *et al.*, 2008). Significant elevations of plasma NO_3^- levels are noted within 15 min of oral ingestion of an inorganic NO_3^- (as a salt) (McKnight *et al.*, 1997; Lundberg and Govoni, 2004; van Velzen *et al.*, 2008) or dietary NO_3^- load (Lundberg and Govoni, 2004; van Velzen *et al.*, 2008; Webb *et al.*, 2008a). After a single oral dose of inorganic or dietary NO_3^-, peak plasma levels seem to be reached between 30-60 min (Lundberg and Govoni, 2004; van Velzen *et al.*, 2008; Webb *et al.*, 2008a). The effective $t_{1/2}$ for NO_3^- in the plasma after consumption of different vegetable sources of NO_3^- (spinach, lettuce and beetroot) has been calculated to be 5.7-6.7 h (van Velzen *et al.*, 2008).

1.6.2 Urinary excretion of NO_3^-

The fate of NO_3^- in the plasma is two-fold. Studies with $^{15}NO_3^-$ supplementation reveal that most (50-65%) is excreted in the urine (with peak excretion occurring ~6 h following supplementation) (Green *et al.*, 1981; Packer *et al.*, 1989; Wennmalm *et al.*, 1993; Pannala *et al.*, 2003), with small amounts accounted for in sweat (up to 10%) or faeces (<1%) (Wagner *et al.*, 1983; Bartholomew and Hill, 1984). NO_3^- is freely filtered at the glomerulus and clearance from the plasma has been estimated as ~20 mL/min in healthy subjects (Wennmalm *et al.*, 1993). This relatively low rate of clearance (taking into consideration that normal glomerular filtration rate (GFR) is between 100-125 mL/min) suggests that much of the filtered NO_3^- is reabsorbed (Kahn *et al.*, 1975; Rahman *et al.*, 2001). As such, in anaesthetized dogs, renal

reabsorption of NO_3^- increases with increasing filtered NO_3^- level with no clear transport limit (Godfrey and Majid, 1998). Using clearance and stop-flow methods in anaesthetized dogs, Abe and colleagues utilized 2 diuretics with different sites of action, mannitol (that works in the proximal convoluted tubule) and furosemide (that works in the ascending limb of Henle), to delineate the sites of NO_3^- reabsorption (Rahman *et al.*, 2001). In clearance experiments, both mannitol and furosemide inhibited tubular reabsorption of NO_3^-, suggesting that NO_3^- reabsorption occurs across the whole tubule. Stop-flow experiments also suggest that NO_3^- is avidly reabsorbed at the same location as sodium in the distal tubule, suggesting co-transport (Rahman *et al.*, 2001).

1.6.3 The entero-salivary circulation of NO_3^-

The entero-salivary circulation of NO_3^- relates to the concentration from the circulation to the salivary glands of NO_3^-, and then the secretion of NO_3^- into the oral cavity resulting in NO_3^--rich saliva and its further metabolism (Duncan *et al.*, 1995). Much of the early work in this area was performed by the Japanese groups of Ishiwata, Maruyuma and Sasaki. Ishiwata's group first demonstrated that ingestion of both dietary NO_3^- (as Chinese cabbage) and inorganic NO_3^- (as sodium nitrate, $NaNO_3$) caused significant elevations in NO_3^- measurable in the saliva within 1 h after NO_3^- consumption (Harada *et al.*, 1974; Ishiwata *et al.*, 1975b), though more recent work has demonstrated that salivary NO_3^- levels increase within 20 min of oral NO_3^- ingestion (McKnight *et al.*, 1997). It has since been estimated that 25% of ingested NO_3^- (taking into account urinary and other excretory routes) is actually concentrated in the salivary glands (Spiegelhalder *et al.*, 1976; Tannenbaum *et al.*,

1976; Kortboyer *et al.*, 1994) and when secreted into the saliva results in NO_3^- levels that are 10-fold greater than plasma NO_3^- levels (Spiegelhalder *et al.*, 1976; Lundberg and Govoni, 2004). The mechanism that relates to NO_3^- uptake in the salivary glands was initially thought to be related to competitive inhibition of the anionic iodide transporter in the salivary glands (Edwards *et al.*, 1954) but very recently, sialin (a sialic acid transporter), has been identified as a $2NO_3^-/H^+$ co-transporter in human cells (Qin *et al.*, 2012).

1.6.4 Oral NO_3^- reduction

Although there was no suggestion at the time that the discovery of the entero-salivary circulation of NO_3^- may represent an important physiological mechanism, it was demonstrated that rises in salivary NO_3^- concentrations were similarly associated with rises in chemically related but distinct anion, NO_2^-. Indeed, the measurement of NO_2^- levels post-NO_3^- ingestion was the primary aim of these investigations, to determine whether NO_3^- ingestion might result in sufficient NO_2^- generation and thus derivation of N-nitroso compounds that were linked to carcinogenesis (Harada *et al.*, 1974; Tannenbaum *et al.*, 1974, 1976; Eisenbrand *et al.*, 1980) as discussed in section 1.2.4.2.

Analysis of saliva taken directly from salivary gland ducts, as opposed to mixed saliva in the oral cavity, revealed that within the salivary gland there is no NO_2^-, suggesting that conversion of NO_3^- to NO_2^- within the oral cavity was responsible for salivary NO_2^- levels (Ishiwata *et al.*, 1975d). Ishiwata was the first to suggest that the appearance of NO_2^- in the saliva may be due to the presence of NO_3^--reducing

bacteria (Ishiwata *et al.*, 1975d) similar to the symbiotic, NO_3^- reducing bacteria identified in the GI tract (Salen, 1925; Zobell, 1932; Stieglitz and Palmer, 1936). It is recognized that some anaerobic bacteria can use NO_3^- as a terminal e^- donor in respiration instead of O_2 and, thereby, reduce NO_3^- to NO_2^- (as reviewed in Moreno-Vivian *et al.*, 1999; Lundberg *et al.*, 2004).

Ex vivo studies with human saliva at 37^oC demonstrated an apparent consumption of NO_3^-, with corresponding increases in NO_2^- levels (Goaz and Biswell, 1961; Ishiwata *et al.*, 1975a). These increases in NO_2^- levels were prevented by heating the saliva to 100^oC or by passing the saliva through a filter (Goaz and Biswell, 1961; Ishiwata *et al.*, 1975a). If the filter residue was returned to the saliva filtrate, the changes in salivary NO_3^- and NO_2^- levels were restored (Goaz and Biswell, 1961; Ishiwata *et al.*, 1975a). These findings suggested that there was a denaturable, biological element in the residue fraction that was necessary for NO_3^- reduction. Tannenbaum and colleagues used a commercially available anti-bacterial mouthwash to determine whether bacteria might underlie this oral NO_3^- reduction. In this study, it was shown that after a single instillation of anti-bacterial mouthwash, there were significantly lower (80-90% reduction) salivary NO_2^- levels after a dietary NO_3^- (celery juice) load when compared to basal levels (Tannenbaum *et al.*, 1976).

Following these indirect evidences of oral bacterial NO_3^- reduction, Maruyuma's group isolated the first species of human NO_3^--reducing bacteria in the oral cavity by growing saliva samples anaerobically on NO_3^--containing blood agar. The first

species identified was *Bacillus coagulans* (Maruyuma *et al.*, 1976) and later, *Veilonella, Lactobacillus, Micrococcus, Corynebacterium, Propionibacterium, Neisseria* (Murumatsu *et al.*, 1979), *Actinomyces,* commensal *Staphylcoccus and Rothia spp.* (Doel *et al.*, 2005) were identified. Sasaki and Matano used a filter-paper technique to identify particular areas of the oral cavity that were responsible for NO_3^- reduction (Sasaki and Matano, 1979). They impregnated small 1.5 cm^2 pieces of filter paper with potassium nitrate (KNO_3) and placed them on particular areas of the oral cavity for 90 s. On removal, they agitated the paper squares with distilled water and determined NO_2^- accumulation colorimetrically (Sasaki and Matano, 1979). Their investigations revealed that only the posterior, dorsal aspect of the tongue was a location that was associated with significant NO_3^- reduction (Sasaki and Matano, 1979). They further developed their techniques to investigate NO_3^- reduction in whole saliva *ex vivo* (Sasaki and Matano, 1980) and also whole cavity oral NO_3^- reduction (Sasaki *et al.*, 1981). This latter technique involved the instillation of a fixed volume and concentration of inorganic NO_3^- solution in the oral cavity for a fixed time. At the end of this time, the entire mouth contents were expelled and NO_2^- levels were determined. A control experiment was performed with distilled water for each subject so that the extra NO_2^- production due to inorganic NO_3^- instillation could be determined (Sasaki *et al.*, 1981).

The importance of oral microflora in effecting NO_3^- reduction has been confirmed in more recent investigations. Both chlorhexidine-based antiseptic mouthwash use (Duncan *et al.*, 1995; Govoni *et al.*, 2008) and systemic antibiotic use (amoxicillin) (Dougall *et al.*, 1995) reduced salivary NO_2^- levels in response to an inorganic NO_3^-

load. The location of NO_3^- reduction was confirmed to take place almost exclusively in the posterior third of the dorsal aspect of the tongue in rats (Duncan *et al.*, 1995). This activity was completely absent in any part of the oral cavity of rats raised in a germ free environment (Duncan *et al.*, 1995). Incubation of rat tongue sections with NO_3^- solutions *ex vivo* revealed abundant NO_3^- reduction (Duncan *et al.*, 1995; Li *et al.*, 1997) that could be totally attenuated by boiling the tongue sections (Duncan *et al.*, 1995) and histological examinations of such sections demonstrated the presence of abundant bacteria in the deep, interpapillary sulci in the posterior third of the tongue and relatively less elsewhere (Duncan *et al.*, 1995), reflecting the distribution of NO_3^- reductase activity (Sasaki and Matano, 1979; Duncan *et al.*, 1995; Li *et al.*, 1997). Recently, it has been confirmed that bacteria in human dental plaque are capable of NO_3^- reduction (Schreiber *et al.*, 2010), but the relative contribution from this site is not thought to be of overall importance to total oral cavity NO_3^- reduction.

Although it has been largely accepted that mammals lack the enzymes capable of undertaking NO_3^- reduction to NO_2^- and are therefore reliant on functioning oral microflora for this crucial initial step in NO_3^- bioactivation, there is some evidence that mammalian NO_3^- reductases may have a role. Using metHb as a bioassay for NO_2^- production, Walker and colleagues demonstrated NO_3^- reduction in germ-free rats and showed in tissues that NO_3^- reduction was inactivated by heat (Ward *et al.*, 1986). Indeed, recent evidence in rats *in vivo* suggests XOR is a functional, if slow, mammalian NO_3^- reductase (Jansson *et al.*, 2008). XOR demonstrates NO_3^- reduction activity *in vitro* under anoxic conditions that can be abrogated by the XOR inhibitor,

oxypurinol (Li *et al.*, 2003). In normoxic wild-type rats, administration of NO_3^- resulted in elevated NO_2^- levels and this rise was inhibited by allopurinol (another XOR inhibitor) (Jansson *et al.*, 2008). Similar results were demonstrated in eNOS-deficient and germ-free mice (Jansson *et al.*, 2008), thereby excluding bacterial NO_3^- reduction and vascular NOS activation as the source of NO_2^-. Germ-free mice exhibited greater tissue levels of XOR, suggesting that this may represent a functional compensatory response to uphold NO_2^- production in the absence of a commensal microflora (Huang *et al.*, 2010). These studies seem to confirm much earlier studies on *ex vivo* mammalian liver/muscle homogenates (Bernheim and Dixon, 1928), suggesting that XOR may exhibit NO_3^- reducing capability. However, the presence and importance of putative NO_3^- reductases in human physiology is yet to be established.

1.7 Bioactivation of NO_2^-

The prevailing view until recently on the above salivary processes was that oral reduction of NO_3^- to NO_2^- served no physiological role and was harmful due to concerns over oral and intra-gastric N-nitrosamine formation and consequent carcinogenesis (Harada *et al.*, 1974; Tannenbaum *et al.*, 1974, 1976; Eisenbrand *et al.*, 1980). However, remarkable investigations in the early 1990s revealed that this view might be presumptive. Two groups simultaneously demonstrated that following a dietary NO_3^- load, some of the swallowed NO_2^- is directly protonated in the acidic environment of the stomach to release free NO (Benjamin *et al.*, 1994; Lundberg *et al.*, 1994), thus describing non-enzymatic NO production for the first time. NO_2^- reduction to NO represents a 1 e^- reduction step (Equations 1.4-1.6) and it is hypothesized dinitrogen trioxide (N_2O_3) is formed as an intermediate that is unstable and dissociates to nitrogen dioxide (NO_2) and NO (Benjamin *et al.*, 1994).

nitrite	***proton***		***nitrous acid***
NO_2^-	+ H^+	$\Leftrightarrow$	HNO_2

Equation 1.4 *NO_2^- acidification to form HNO_2. (NO_2^-=nitrite, HNO_2=nitrous acid).*

nitrous acid		***water***	***dinitrogen trioxide***
$2\ HNO_2$	$\Leftrightarrow$	H_2O +	N_2O_3

Equation 1.5 *Dehydration of HNO_2. (HNO_2=nitrous acid).*

dinitrogen trioxide		***nitric oxide***	***nitrogen dioxide***
$N_2O_3^-$	$\Leftrightarrow$	NO +	NO_2

Equation 1.6 *N_2O_3 dissociation to NO. (N_2O_3=dinitrogen trioxide; NO=nitric oxide).*

Benjamin and colleagues demonstrated that after ingestion of 2 mmol KNO_3, salivary NO_2^- levels were elevated 10-fold above basal levels (~1 mM). *In vitro* studies incubating NO_2^- with acid at pH 2 (approximate to stomach pH) revealed concentration-dependent NO generation, with ~600 nM NO generated from 200 µM NO_2^- (Benjamin *et al.*, 1994), a level far in excess of NO levels required for biological effects (Palmer *et al.*, 1987). This group also demonstrated anti-microbial effects of NO_2^- under acidic conditions on both yeast (*Candida albicans*) and bacterial (*E. Coli*) growth (Benjamin *et al.*, 1994). The authors suggested that the direct chemical acidification of NO_2^- to NO may be a useful process for controlling the risk of GI infection (Benjamin *et al.*, 1994) and decreasing pathogen growth (Benjamin *et al.*, 1994; Dykhuizen *et al.*, 1996, 1998) and it has subsequently been demonstrated that NO_2^--derived NO is bacteriocidal (Björne *et al.*, 2006). The Karolinska group of Weitzberg and Lundberg simultaneously published their results showing almost identical effects (Lundberg *et al.*, 1994). In their experiments, they used NO_3^--rich lettuce as a NO_3^- source and demonstrated that authentic NO production could be detected in expelled gastric air after ingestion of lettuce. Further *in vitro* tests on acidified saliva or chewed lettuce revealed pH dependent production of NO, with very little NO produced with pH>3 (Lundberg *et al.*, 1994). To confirm that this was biologically relevant, they demonstrated that pre-treatment with a proton pump inhibitor, omeprazole, prevented NO production after ingestion of NO_3^--rich lettuce (Lundberg *et al.*, 1994).

In addition to protection against micro-organisms, intra-gastric NO generation from NO_2^- may have other important, local gastroprotective effects that are dependent

on elevations in cGMP, including increasing mucus formation and mucosal blood flow (Björne H *et al.*, 2004; Petersson *et al.*, 2007). Furthermore, it has been substantiated that NO_2^--derived NO protects against animal models of stress-induced and NSAID-induced gastric ulceration (Miyoshi *et al.*, 2003; Jansson *et al.*, 2007).

1.7.1 Pathways for systemic NO_2^- absorption

Although NO_2^- that is produced in the oral cavity is swallowed and may be directly acidified to increase local NO levels, a significant proportion of swallowed NO_2^- appears to survive passage into the stomach and enters the systemic circulation. The disappearance of NO_2^- from the gastric lumen is a rapid process with decline of NO_2^- levels by half within 10 min, with most of this estimated to be related to absorption rather than chemical reactions of NO_2^- or gastric emptying (Licht *et al.*, 1986). Exactly how NO_2^- enters the circulation is uncertain. As a charged anion, an active uptake mechanism is likely to be involved but this is yet to be proven. Whilst little work has been done looking at active uptake mechanism in gastric environs specifically, there have been some studies attempting to discern uptake mechanisms across membranes in erythrocytes or erythrocyte ghosts. In erythrocyte ghosts, NO_2^- exchange with bicarbonate (HCO_3^-) was inhibited by the non-specific anion-exchange inhibitor, diidothiocyanotostilbene disulfonate (DIDS), suggesting that at least for erythrocytes, there may be a role for the anion-exchange transporter-1 (AE-1) for the movement of NO_2^- across the membrane (Shingles *et al.*, 1997; Vitturi *et al.*, 2009; Jensen and Rohde, 2010). A similar AE-1 dependency for NO_2^- transport has been demonstrated in pancreatic acini (Zhao *et*

al., 1994). However, other researchers have failed to demonstrate the same effect of DIDS (Zavodnik *et al.*, 1999; May *et al.*, 2000; Jensen, 2005). Additionally, May and colleagues demonstrated that NO_2^- uptake was dependent upon sodium and phosphate levels and therefore suggested that the sodium-dependent phosphate transporter was important (May *et al.*, 2000).

However, in the gastric lumen, pH is between 1.0-2.5 (Evans *et al.*, 1988). The pK_a of nitrous acid (HNO_2) in aqueous solutions is 3.3-3.4 (Butler and Ridd, 2004) and therefore most of the NO_2^- will be in the neutral, lipophilic HNO_2 form. It has been hypothesized that the passive movement of HNO_2 from the acidic gastric environment into the neutral circulation may underlie the apparent transport of NO_2^- across the gastric wall (Webb and Ahluwalia, 2010), a possibility demonstrated to occur across the erythrocyte membrane (Samouilov *et al.*, 2007).

The exact contribution of passive diffusion of HNO_2 and/or active uptake of NO_2^- is still to be fully determined and quantified. However, what is apparent is that ingestion of inorganic NO_3^-, via the entero-salivary circulation and bacterial NO_3^- reduction, eventually leads to increases in plasma and tissue NO_2^- levels. Interestingly, it is now apparent that tissue levels of NO_2^- can be widely different and that plasma NO_2^- levels are in general the lowest *in vivo*, with potentially most of 'blood' NO_2^- carried inside the erythrocyte (Dejam *et al.*, 2005) and much higher NO_2^- levels in certain tissues of the body, particularly the blood vessel wall (Bryan *et al.*, 2005).

1.7.2 The entero-salivary circulation modulates plasma NO_2^- levels

Elevation of systemic NO_2^- levels after ingestion of inorganic NO_3^- has been demonstrated to occur across a range of species as well as in healthy subjects. For example, provision of 1 g/L inorganic NO_3^- (=16.2 mM [NO_3^-]) in the drinking water in wild-type mice increased steady state plasma NO_2^- levels by ~50% (from ~0.8 µM to 1.2 µM) but increased tissue levels (for example heart tissue) by 500% (from ~3 µM to 18 µM) (Bryan *et al.*, 2007), highlighting that plasma NO_2^- levels may not be indicative of tissue-specific levels or changes in levels. Interestingly, supplementation of drinking water in Sprague-Dawley rats with much lower amounts of NO_3^- (to provide total daily NO_3^- intake of 0.1 mmol/kg and 1 mmol/kg) for 7 days increased plasma NO_2^- levels from 0.4 µM to 0.6 µM and 2.6 µM respectively (Jansson *et al.*, 2007). The differences reflected in these two studies reveal that the pharmacokinetics and handling of NO_3^- may be species dependent. Thus, studies in humans are important to determine the impact of NO_3^- supplementation on systemic NO_2^- levels. In these studies, systemic NO_2^- levels have largely been only examined in saliva, plasma and urine as, understandably, it is difficult to get tissue samples in healthy subject studies. Although Stieglitz had shown more than 70 years ago that inorganic NO_3^- supplementation acutely (within 1 h) and chronically (up to 14 days) elevated blood NO_2^- levels (Stieglitz and Palmer, 1936), it is only more recently that researchers have studied systemic NO_2^- elevations after inorganic NO_3^- supplementation in depth, trying to elucidate if sufficient NO_3^--derived NO_2^- from the oral cavity reaches the circulation in humans. One of the first explorations of this phenomenon was conducted in healthy subjects who were supplemented with ~120 µmol/kg $NaNO_3$ (equivalent to ~8 mmol $NaNO_3$

for a 70 kg person) in a single dose (Lundberg and Govoni, 2004). Salivary, plasma and urinary NO_x levels were assayed for up to 3 h post-ingestion. In contrast to the very rapid appearance of NO_3^- in the plasma (within 15 min as previously discussed above) and early plateau phase (within 1 h), plasma NO_2^- levels were only significantly detected above basal values after 30 min and were still rising at 90 min. In this experiment, some subjects were asked to refrain from swallowing their saliva (by spitting all saliva out) to interrupt the entero-salivary circulation (Lundberg and Govoni, 2004). In these subjects, whilst there was no difference in plasma NO_3^- levels compared to control (swallowing saliva), plasma NO_2^- levels did not rise (Lundberg and Govoni, 2004). However, following resumption of swallowing saliva after 1 h, plasma NO_2^- levels started to rise again (Lundberg and Govoni, 2004). Weitzberg and Lundberg also demonstrated that plasma NO_2^- levels were significantly higher (219±105 nM) after 3 days supplementation with 0.1 mmol/kg $NaNO_3$, compared to after 3 days of matched placebo (0.1 mmol/kg sodium chloride, NaCl) supplementation (138±38 nM) (Larsen *et al.*, 2006). The ~1.5-fold increases in plasma NO_2^- levels from baseline after supplementation with 0.1 mmol/kg in humans (Larsen *et al.*, 2006) was similar to that observed in rats described above (Jansson *et al.*, 2007). Furthermore, Ahluwalia and colleagues demonstrated similar effects after the ingestion of NO_3^- in a dietary form of beetroot juice (Webb *et al.*, 2008a). Although beetroot is purple-red, it is a green-leafy vegetable and contains significant NO_3^- levels (Santamaria, 2006). In this study, ingestion of 500 mL beetroot juice beetroot juice (mean $[NO_3^-]$= 45.0 mM) provided ~22.5 mmol NO_3^- (~0.32 mmol/kg NO_3^-) and was associated with an elevation of plasma NO_2^- levels from 0.4 µM to 0.6 µM (Webb *et al.*, 2008a). Plasma NO_2^- levels

were slower to rise than plasma NO_3^- levels, with peak plasma NO_2^- levels apparent 3 h after ingestion, compared to peak plasma NO_3^- levels at 90 min (Webb *et al.*, 2008a). Similarly, interruption of the entero-salivary circulation by avoidance of swallowing saliva prevented the rises in plasma NO_2^-, but not plasma NO_3^-, levels (Webb *et al.*, 2008a) confirming the critical importance of oral NO_3^- reduction in modulating systemic NO_2^- levels after dietary NO_3^- ingestion. Further evidence for the importance of the oral microflora in facilitating this process is taken from studies that have sought to impair the bacteria responsible for NO_3^- reduction. Prior treatment with a chlorhexidine anti-septic mouthwash in animals (Petersson *et al.*, 2009; Jädert *et al.*, 2012) and humans (Govoni *et al.*, 2008) prevented the increases in plasma NO_2^- levels associated with NO_3^- ingestion.

In summary, in humans ~1 h after a dietary NO_3^--load, plasma NO_2^- levels rise in a slow and sustained manner, peaking in the circulation after 2.5-3 h, reflecting the ingestion and entero-salivary processing of inorganic NO_3^- (Lundberg and Govoni, 2004; Webb *et al.*, 2008b).

1.7.3 Sources and pharmacokinetics of NO_2^- in the circulation

Plasma NO_2^- levels in the circulation are much lower than NO_3^-, with measurements from several different research groups falling in the 0.2-0.5 µM range under basal, fasting conditions (as reviewed by Grau *et al.*, 2007). NO_2^- is estimated to have a very short $t_{1/2}$ in the circulation, ranging between ~15-45 min (Dejam *et al.*, 2007; Hunault *et al.*, 2009). In addition, NO_2^- levels measured in the plasma are likely to reflect multiple different sources.

NO_2^- is formed by oxidation of NO as previously described above and has been suggested to sensitively reflect regional eNOS activity. In the human forearm circulation, stimulation of eNOS with ACh or inhibition with L-NMMA rapidly altered venous plasma NO_2^- levels up or down respectively but without changes in plasma NO_3^- levels (Lauer *et al.*, 2001). Furthermore basal plasma NO_2^- levels in several species were found to be similar, suggesting similar levels of constitutive NOS activity, and application of several different NOS inhibitors demonstrated cross-species increases in vascular resistance that correlated to decreases in plasma NO_2^- levels but not plasma NO_3^- levels (Kleinbongard *et al.*, 2003). Genetically eNOS deficient mice had 70% lower plasma NO_2^- levels than wild-type, littermate controls, which could be closely approximated in wild-type controls by the systemic application of NOS inhibition activity (Kleinbongard *et al.*, 2003). These findings suggest that 70% of plasma NO_2^- is likely derived from eNOS. Similarly, in humans with CVD risk factors, it has been shown that plasma NO_2^- concentration inversely correlates to increasing number of cardiovascular risk factors and correlates directly to brachial artery FMD, suggesting that plasma NO_2^- levels could be used as a surrogate for endothelial function in those with CVD risk factors (Kleinbongard *et al.*, 2006b).

However, in addition to eNOS activity, plasma NO_2^- levels will also be determined by dietary NO_2^- and NO_3^- consumption. Dietary NO_2^- intake levels in humans are not as substantial as dietary NO_3^-, with the major contributions from cured meats, cereals and vegetables, and with minimal intake occurring from drinking water (Schuddeboom, 1993). Estimates of daily NO_2^- intake are relatively similar across

Europe and North America at 0.3-0.9 mg/day (Schuddeboom, 1993), which equates to 6-20 µmol NO_2^- ingested per day.

However, mean salivary volume swallowed is estimated to be ~1.5 L/day (Dawes, 1972; Lagerlöf and Dawes, 1984) and mean basal salivary NO_2^- levels are ~100-250 µM (Lundberg and Govoni, 2004; Govoni *et al.*, 2008). Thus, swallowing of saliva could be responsible for total amounts of intra-gastric NO_2^- in the order of 150-300 µmol daily. Mean plasma NO_2^- levels as stated are 0.2-0.5 µM, suggesting a total circulatory NO_2^- levels of 1-2.5 µmol. Thus the same total amount of NO_2^- would enter the stomach every 10-25min. Although not all of the swallowed NO_2^- survives to appear in the circulation, even if a fraction entered the circulation, it would make an important contribution to plasma NO_2^- levels under basal conditions. It is possible that this salivary NO_2^- may account for the other 30% of plasma NO_2^- concentration that cannot be accounted for by eNOS activity (Lauer *et al.*, 2001; Kleinbongard *et al.*, 2003).

1.8 Mechanisms of NO_2^- bioactivity

It had been suggested from the early work of Reichert, Mitchell and Stieglitz described earlier that elevations in systemic NO_2^- levels after ingestion of inorganic NO_2^- or NO_3^- (via bacterial NO_3^- reduction) was potentially useful in hypertension and other CVDs. However, their mechanism of action was not known at the time. With the discovery that direct chemical acidification can release authentic NO from NO_2^- in the stomach (Benjamin *et al.*, 1994; Lundberg *et al.*, 1994), substantial research effort has focused on identifying other biochemical pathways that may be involved in mammalian NO_2^- reduction to NO.

1.8.1 Non-enzymatic NO_2^- reduction

As stated previously, NO_2^- can be directly acidified to NO in the stomach as described in Equations 1.4-1.6. Whilst this process relies on high proton concentrations that are readily available in the stomach, this pathway may not be that apparent in other healthy tissues that do not attain such levels of acidaemic stress. However, in ischaemic insults, acidosis accompanies significant hypoxia, with pH levels in ischaemic rat Langendorff heart preparations measuring as low as pH 5.5 (Zweier *et al.*, 1995; Gabel *et al.*, 1997). Since eNOS activity is O_2 dependent (Leone *et al.*, 1991; Abu-Soud *et al.*, 1996), under ischaemic conditions one would suspect NO generation would decrease. Indeed, global ischaemia in rat Langendorff heart preparations reduces eNOS activity by ~75% (Giraldez *et al.*, 1997). However, some NO generation still occurs.

In 1995, Zweier and colleagues published a seminal paper in the NO_2^- field investigating this residual NO generation. Using EPR spectroscopy, they surprisingly demonstrated a 10-fold increase in NO production from NO_2^- (1 mM) in the ischaemic Langendorff rat heart preparation compared to the normoxic control (Zweier *et al.*, 1995). This NO production could not be fully inhibited by NOS inhibition (only ~65% inhibition), suggesting there was non-NOS mediated NO production (Zweier *et al.*, 1995). They performed further experiments demonstrating that NO_2^- levels in the heart were ~20 µM but also that treatment with $^{15}NO_2^-$ produced ^{15}NO (Zweier *et al.*, 1995). This study was the first demonstration of NO_2^--derived NO production within the cardiovascular system (Zweier *et al.*, 1995). The authors of this paper suggested that the reduction of NO_2^- to NO was simply a consequence of disproportionation due to the acidosis.

It has also been suggested that several other dietary constituents may modulate the chemical acidification of NO_2^- to NO. Vitamin C is a potent reducing agent that reduces nitrite-induced nitrosation in the stomach (Licht *et al.*, 1988) and increases NO generation from NO_2^- in human urine at pH between 4.5-6.0 (Carlsson *et al.*, 2001) and also increases salivary NO_2^--derived NO *in vitro* (Peri *et al.*, 2005). In addition, dietary polyphenols, from red wine and other fruit and vegetable sources, similarly increase NO generation from salivary NO_2^- i*n vitro* (Peri *et al.*, 2005), in simulated gastric juice supplemented with NO_2^- and polyphenols (Gago *et al.*, 2007) and in expelled gastric air in healthy human subjects who have consumed polyphenol-containing drinks (Gago *et al.*, 2007; Rocha *et al.*, 2009). NO_2^--derived NO that is accentuated by polyphenolic contents under acidic conditions has local

effects on gastric physiology, including gastric smooth muscle relaxation *ex vivo* (Rocha *et al.*, 2009).

Whilst acidosis is essential for all of these pathways, it is now clear that NO_2^- reduction can occur under physiological conditions and that acidosis cannot fully account for all NO_2^- reduction. We now know that NO_2^- reduction is also a phenomenon dependent upon the presence of mammalian NO_2^- reductases.

1.8.2 Enzymatic NO_2^- reduction to NO

The discovery of significant human artery-vein gradients of NO_2^- in the forearm circulation of healthy subjects suggested that there was consumption of NO_2^- within the human circulation under physiological conditions and suggested a possible role in the regulation of vascular tone (Gladwin *et al.*, 2000). Further evidence was provided by the apparent endocrine activity of inhaled NO. In healthy human subjects, inhalation of NO led to increases in FBF under continuous NOS inhibition (Cannon *et al.*, 2001), an effect that could not be related to NO directly inhaled into the lungs due to its incredibly short $t_{1/2}$ as mentioned previously. These effects of inhaled NO were paralleled by increases in both plasma NO_2^- and Fe-nitrosyl-Hb (HbNO) levels (Cannon *et al.*, 2001). In this paper, it was suggested that these NO-adducts could serve as intravascular, endocrine sources of NO, distant to the site of production (i.e. in this case, the pulmonary circulation) (Cannon *et al.*, 2001). However, for NO_2^- to be bioactive in the human circulation, there would have to be other mechanisms for NO_2^- reduction to NO that are not solely dependent on acidosis, or NO_2^- would have to be bioactive by non-NO dependent means.

Confirmation that NO_2^- did affect vascular tone under physiological conditions was provided by (Cosby *et al.*, 2003) and will be discussed in section 1.9.

There is also some evidence that NO_2^- may have some direct effects that are not mediated via conversion to NO. Application of NO_2^- to tissue homogenates in the presence of NO scavengers, oxyHb and 2-(4-carboxyphenyl)-4,4,5-teramethylimidazoline-1-oxyl-3-oxide (C-PTIO), did not alter NO_2^--mediated S-nitrosation reactions with thiol-containing proteins or haem, suggesting that NO was not an obligate intermediate of this reaction (Bryan *et al.*, 2005). In the same manuscript, data was presented showing NO_2^- induced dose-dependent decreases in haem oxygenase-1 (HO-1) activity (Bryan *et al.*, 2005), in contrast to the recognized upregulatory effects of NO on HO-1 activity (Motterlini *et al.*, 2002).

However, in the main it is now accepted that the bioactivity of NO_2^- is reliant on its reduction to NO and that this process can be facilitated by a number of different NO_2^- reductases discussed below.

1.8.2.1 XOR

One of the first enzymes proposed to act as a mammalian NO_2^- reductase was XOR. Studies with purified bovine XOR incubated with NO_2^- and the reducing agent NADH in almost total anoxia (aqueous O_2 levels <1%) for 20 h, produced NO detectable by gas-phase chemiluminescence in the headspace, that was inhibited by the XOR inhibitor, allopurinol, (Zhang *et al.*, 1998). Whilst XOR-mediated NO_2^--derived NO production was greater in anoxia/hypoxia, significant NO was produced from the

same aqueous experiment under ambient O_2 tensions (Zhang *et al.*, 1998). Later studies in cell-free preparations, and in rat and human tissues have provided further evidence that XOR is a NO_2^- reductase whose activity is greatly enhanced in acidosis and hypoxia. Zweier and colleagues incubated $^{15}NO_2^-$ with purified XOR or rat heart sections under different O_2 tensions, in the presence of XOR co-factors and L-N^G-nitroarginine methyl ester (L-NAME, a NOS inhibitor), and demonstrated that O_2 behaved as a competitive inhibitor to XOR-mediated NO_2^- reduction but that XOR-mediated NO_2^- reduction occurred under aerobic conditions (Li *et al.*, 2004).

In rat and human heart homogenates, addition of NO_2^- (10-100 µM) increased NO production in a concentration–dependent manner, as measured by gas phase chemiluminescence. This NO_2^--derived NO production was enhanced by acidosis (pH 6 vs. pH 5) and also in anoxia (bubbled with nitrogen gas (N_2)) compared to ambient O_2 tension (bubbled with room air) (Webb *et al.*, 2004). These effects were prevented by boiling the tissue, confirming the enzymatic nature of the process (Webb *et al.*, 2004). Pre-incubation of samples for 30 min with L-NAME (300 µM) had no effect, whilst allopurinol (100 µM), or another XOR inhibitor that works at a different site on the enzyme, (-)BOF-4272 (10µM), attenuated NO_2^--derived NO production by ~50% (Webb *et al.*, 2004). Incubation of rat heart sections with NO_2^- (10 µM) under 2% O_2 demonstrated 50% greater NO_2^- reduction than under 5% O_2 and both effects could be largely attenuated by pre-incubation with the XOR inhibitor, oxypurinol (Li *et al.*, 2004). NO_2^- infusions (10-1000 µM) caused time and concentration dependent increases in NO production (sampled in the head space and measured by gas-phase chemiluminescence) in the presence of global

ischaemia in Langendorff isolated heart preparations, which could be abrogated by XOR inhibition (Webb *et al.*, 2004). Further studies in hypoxia and acidotic conditions have revealed similar effects of XOR inhibition on NO_2^--derived NO production in rat liver (Liu *et al.*, 2007) and kidney (Tripatara *et al.*, 2007) homogenates.

Removal of the myocardial endothelium in the Langendorff heart preparation also attenuated NO_2^--derived NO (Webb *et al.*, 2004), suggesting XOR-dependent NO_2^- reduction in the heart at least in part occurred at the level of the endothelial cell. Further studies have demonstrated XOR-dependent NO_2^- reduction in homogenates of rat aortae and vena cavae, human internal mammary artery segments and human erythrocytes under hypoxia and acidosis (Webb *et al.*, 2008b). The most abundant source of XOR is the liver and during periods of physiological stress, XOR is released into the circulation (Terada *et al.*, 1992) and can bind to distant cell types via heparan sulphate proteoglycans, such as on erythrocytes (Webb *et al.*, 2008b) and the vascular endothelium (Spiekermann *et al.*, 2003; Kelley *et al.*, 2004), where it may be responsible for NO_2^- reduction in conditions of acidosis and hypoxia, reflecting its role as potentially the main NO_2^- reductase in pathophysiological conditions (Webb and Ahluwalia, 2010).

1.8.2.2 Aldehyde oxidase (AO)

AO has 95% exonic homology, 86% amino acid sequence homology and similar tertiary structure homology to XOR (Calzi *et al.*, 1995; Turner *et al.*, 1995; Terao *et al.*, 1998) and accordingly has been identified as a NO_2^- reductase in various tissues.

The AO inhibitor, raloxifene (50 μM) was shown to attenuate NO_2^--derived NO production by ~35% in rat liver and heart homogenates, under anoxia and acidosis (pH 7.0) (Li *et al.*, 2008). Furthermore, this group demonstrated that combination of AO and XOR inhibition abrogated the vast majority of tissue NO_2^- reduction to NO, suggesting that XOR and AO may be the key NO_2^- reductases in tissues, especially with decreasing O_2 tension and pH (Li *et al.*, 2008).

1.8.2.3 Mitochondrial respiratory chain enzymes

Over the last decade, it has been established that various elements of the mammalian mitochondrial respiratory chain function as NO_2^- reductases. Incubation of rat liver isolated mitochondria in anoxic conditions under mild acidosis (pH 7.25) with NO_2^- (50 μM) produced NO, detected by EPR spectroscopy, that was partially abrogated by complex I inhibition with rotenone (concentration not stated) and fully attenuated by complex III inhibition with myxothiazole (concentration not stated) (Kozlov *et al.*, 1999; Nohl *et al.*, 2000). The importance of complex I-mediated NO_2^- reduction in the presence of complex III inhibition has not been studied.

Incubation of rat liver mitochondria at pH 6.5 with NO_2^- (100-1000 μM) produced NO (measured by NO electrode) that could be enhanced by pre-bubbling with N_2 instead of ambient air (Castello *et al.*, 2006). Although inhibition of the respiratory chain with complex III inhibitors prevented NO generation, giving an e^- donor that provided reducing power directly to cytochrome c oxidase (complex IV) caused a

resumption in NO generation that could be inhibited by carbon monoxide, suggesting that complex IV is a NO_2^- reductase under anoxia (Castello *et al.*, 2006).

1.8.2.4 Globins

The globin family, including Hb, myoglobin (Mb), neuroglobin and cytoglobin, have all been described as potential NO_2^- reductases.

The reactions of globins with NO_2^- have been studied for over 100 years. Haldane discovered that NO bound to Mb from the interaction of NO_2^-, Mb and reducing agents (vitamin C in citrus juice) was responsible for the bright red colour of cured meats (Haldane, 1901). Brooks in the 1930s and Doyle in the 1980s further characterized this reaction and were able to determine that the reaction of NO_2^- and deoxyhaemoglobin (deoxyHb) produces NO, metHb and HbNO (Equation 1.7) (Brooks, 1937; Doyle *et al.*, 1981). More recent characterization of this reaction suggests that the majority of the NO may be weakly bound to ferric haem rather than avidly bound to ferrous haem, allowing for the potential facile release of free NO (Nagababu *et al.*, 2003).

deoxyHb		***nitrite***		***metHb***		***iron-nitrosyl-Hb***		***nitric oxide***
2 Fe(II)Hb	**+**	**2 NO_2^-**	**⇒**	**Fe(III)Hb**	**+**	**Fe (II)Hb-NO**	**+**	**NO**

Equation 1.7 *Reaction of NO_2^- with deoxyHb. (NO_2^-=nitrite; deoxyHb=deoxyhaemoglobin).*

Gladwin and colleagues have demonstrated that addition of NO_2^- (100-200 μM) to deoxygenated erythrocytes in-line with a gas-phase chemiluminescence analyzer

produces bursts of NO production, whilst NO_2^- on its own has no effect (Cosby *et al.*, 2003). In further experiments, they modulated the oxygenation state of cell-free Hb (at pO_2= 15 mmHg) with the addition of inositol hexaphosphate (IHP). In the absence of IHP, Hb at such O_2 tension would be fully oxygenated. However, with the addition of IHP, Hb remains in a deoxygenated state. The addition of IHP to keep Hb in the deoxyHb state reduced the concentration of NO_2^- that was required to facilitate relaxation of pre-contracted aortic rings by 3 orders of magnitude compared to that which was required in control preparations of oxyHb (100 nM vs. 100 µM), suggesting a physiologically relevant model for NO_2^- reduction to NO by its reaction with erythrocyte Hb (Cosby *et al.*, 2003).

The kinetics of this reaction are complicated and reflect differential rates of NO_2^- reduction and affinities of tetrameric Hb for NO depending on the individual R (oxy-haem) and T (deoxy-haem) co-ordination of each of the Hb tetramers within the erythrocyte (Huang *et al.*, 2005; Grubina *et al.*, 2007). This process leads to NO_2^- reduction by erythrocytic Hb being fastest when 50% of haem groups are bound to O_2, which is the p50 for oxy-haem (Nagababu *et al.*, 2003; Huang *et al.*, 2005; Crawford *et al.*, 2006).

However, one of the major problems of such chemistry is that any NO produced intra-erythrocytically by such reductive chemistry should be scavenged by oxyHb to form NO_3^- (Equation 1.3) as previously mentioned, especially as NO_2^- reduction is maximal at p50, when there will be abundant oxyHb.

One suggestion to this challenge has been the proposal that there is concomitant generation of an NO intermediate that is less reactive than NO that is able to diffuse away from the site of production (Robinson and Lancaster, 2005). One idea that has gained popularity is the thought that Hb may act as a NO_2^- anhydrase (Basu *et al.*, 2007; Hopmann *et al.*, 2011). The overall stoichiometry suggested of such a reaction reveals that Hb is dehydrating 2 molecules of NO_2^- with the addition of a proton and is thus an anhydrase reaction. N_2O_3 could therefore escape the cellular confines of the erythrocyte and dissociate to NO and NO_2 or participate in S-nitrosation of thiols to export NO-like activity (Robinson and Lancaster, 2005; Basu *et al.*, 2007). This reaction has recently been shown in theoretical computational chemical terms to be energetically favourable and therefore potentially relevant *in vivo* (Hopmann *et al.*, 2011).

Mb is another globin protein that can bind O_2 and NO (Flögel *et al.*, 2001) and has attracted much interest as a potential NO_2^- reductase. Deoxymyoglobin (deoxyMb) forms Fe-nitrosyl-Mb (MbNO) adducts after incubation with NO_2^- in a concentration-dependent manner, synonymous with the reactions noted above for Hb (Rassaf *et al.*, 2007). NO_2^--derived NO production ([NO_2^-] 100 µM at pH 5) was 65% lower in heart homogenates from Mb knockout compared to wild-type (Rassaf *et al.*, 2007). In the same Mb knockout tissues, NO_2^--derived NO production could be restored to control levels by the addition of exogenous Mb (Rassaf *et al.*, 2007). Mb-dependent NO_2^- reduction increases with decreasing pH and O_2 tension (Shiva *et al.*, 2007a) and may be particularly important in the response to ischaemic

myocardial injury due to abundant Mb (Shiva *et al.*, 2007a) and NO_2^- stores (Bryan *et al.*, 2005).

Recently, neuroglobin has been investigated for its NO_2^- reductase activity. Incubation of purified mouse neuroglobin under hypoxic conditions also facilitated NO_2^- reduction to NO measured by EPR spectroscopy, though the related cytoglobin did not (Petersen *et al.*, 2008).

1.8.2.5 eNOS

A surprising addition to the list of functional mammalian NO_2^- reductases is eNOS itself, suggesting not all NO_2^--derived NO is NOS-independent as once thought. Slama-Schwok and colleagues demonstrated that eNOS in anoxic conditions could produce NO (Gautier *et al.*, 2006). Incubation of eNOS at physiological pH (7.6) in anoxia, with NADPH and NO_2^- (500 μM) revealed Fe-nitrosyl formation of the haem group within eNOS measured by EPR spectroscopy (Gautier *et al.*, 2006). Simultaneous recording with a NO electrode demonstrated a burst of NO production that did not occur in the absence of NADPH or eNOS respectively (Gautier *et al.*, 2006). Further experiments with $^{15}NO_2^-$ demonstrated ^{15}NO production, confirming that NO was produced from NO_2^- rather than ^{14}N-labelled L-arginine that was also present (Gautier *et al.*, 2006). This reaction is specific for the eNOS isoform and is not evident with iNOS or nNOS, and geminate recombination studies have revealed that NO_2^- reduction occurs at a site 2-5 Å adjacent to the haem site (Mikula *et al.*, 2009). Furthermore, confocal microscopy (Kleinbongard *et al.*, 2006a) and Western blotting (Webb *et al.*, 2008b) have demonstrated the

presence of eNOS on human erythrocytes and incubation of erythrocytes with NOS inhibitors (L-NAME and L-NMMA, 300 µM) attenuated NO_2^- (10-100 µM)-derived NO production detected by gas-phase chemiluminescence (Webb *et al.*, 2008b).

The relative contribution of each of these pathways may vary dependent upon the prevalent conditions. It has been suggested that under physiological pH and pO_2 the globins predominate as the primary NO_2^- reductase in the cardiovascular system, however, as conditions progressively move into the pathological realm of acidosis and hypoxia that other reductases predominate such as XOR (Gladwin *et al.*, 2005; van Faassen *et al.*, 2009; Webb and Ahluwalia, 2010).

In summary, it is now clear that the conversion of NO_2^- to NO increases with increasing acidosis and hypoxia (Zweier *et al.*, 1995; Cosby *et al.*, 2003; Webb *et al.*, 2004; Li *et al.*, 2008), environments in which the classical L-arginine/eNOS pathway is dysfunctional (Giraldez *et al.*, 1997). This particular finding has led to the proposal that this alternative pathway for NO generation may act as a complementary, back-up system (Figure 1.6) when conventional NO generation has been compromised (Lundberg *et al.*, 2008). In addition, a number of *in vivo* enzymatic NO_2^- reductases have now been identified that are functional in physiological conditions.

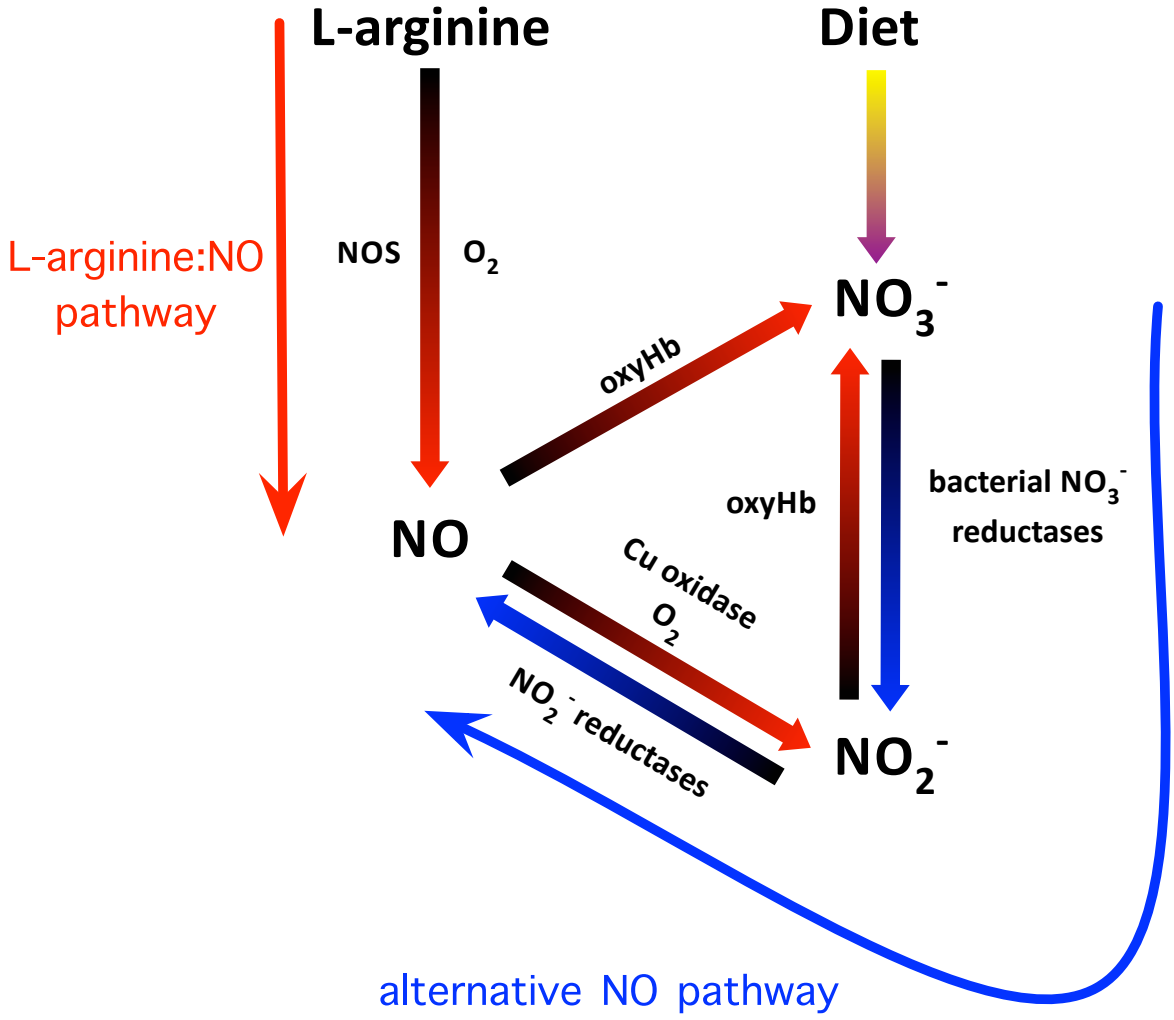


Figure 1.6 *NO cycle in humans. Coloured arrows indicate:* ***oxidation*** *and* ***reduction****. (Cu=copper; NO=nitric oxide, NO_3^-=nitrate; NO_2^-=nitrite, NOS=nitric oxide synthase, O_2=oxygen, oxyHb=oxyhaemoglobin).*

1.9 Therapeutic utility of the NO_3^--NO_2^--NO pathway

Initially these pathways were dubbed non-NOS but since the discovery noted above of eNOS-mediated NO_2^--derived NO, these pathways are best described as an alternative *pathway* for NO generation, or the *NO_3^--NO_2^--NO pathway* (Lundberg *et al.*, 2008). Overwhelming evidence supporting the existence of this alternative endogenous pathway for NO generation has raised interest with respect to determining the potential of exploiting this pathway to 'rescue' levels of NO in diseases, particularly CVD, where decreased L-arginine-derived NO generation is thought to contribute to pathology. In this respect a number of studies have evaluated the potential of provision of inorganic NO_x, via the diet or by systemic application, to serve to increase intravascular NO_2^- to modulate the cardiovascular system and CVD risk factors.

NO_2^- has been used as a cyanide antidote for more 70 years (as reviewed in Butler and Feelisch, 2008) and NO_2^- in food preservation prevents oxidation of fatty acids to prevent rancidity and malodour formation (Honikel, 2008), as well as critically controlling *Clostridium botulinum* growth and toxin production (Hustad *et al.*, 1973). Inorganic NO_x have been used in the cardiovascular system for over 85 years (Reichert and Mitchell, 1880; Stieglitz, 1927) but there has been a recent renewed interest in these anions in both animal and human studies seeking to explore the *new* biology of NO_2^--derived NO production.

It was in 2001 that the view that NO_2^- was inert at physiological levels within the cardiovascular system was finally eroded. In rat aortic rings, application of low µM

NO_2^- (2.5 μM), whilst inactive under physiological pH, were shown to relax contracted rat aorta under acidic (pH 6.6) conditions (Modin *et al.*, 2001). The significance of this finding was finally appreciated with the relatively recent demonstration by Gladwin and colleagues that infusion of $NaNO_2$ into the human forearm causes vasorelaxation with concomitant increased FBF; a phenomenon augmented by the mildly hypoxic conditions formed during exercise (Cosby *et al.*, 2003).

Following these first demonstrations of physiological activity of NO_2^-, there are now numerous studies showing the benefits of physiologically-relevant NO_2^- concentrations in the context of ischaemia-reperfusion (IR) injury of solid organs, including notably heart (Webb *et al.*, 2004; Duranski *et al.*, 2005; Bryan *et al.*, 2007; Gonzalez *et al.*, 2008), brain (Jung *et al.*, 2006), kidney (Tripatara *et al.*, 2007; Milsom *et al.*, 2010) and the liver (Duranski *et al.*, 2005; Shiva *et al.*, 2007b).

Understandably, it is not possible to perform mechanistic studies in human myocardial IR injury so easily. Hence, models of temporary IR injury on the endothelium of the human circulation have been developed (Kharbanda *et al.*, 2001, 2002). Utilizing this model of IR injury, Ahluwalia and colleagues investigated the effect of dietary NO_3^- supplementation (in the form of beetroot juice) on IR-induced endothelial dysfunction (Webb *et al.*, 2008a) compared to water placebo. In 10 healthy subjects, IR caused reductions in brachial artery FMD that could be prevented by ingestion of a single dose of dietary NO_3^- (22.5 mmol dose) 2 h prior to the IR injury (Webb *et al.*, 2008a).

However, the focus of this thesis is on the effect of NO_3^--NO_2^--NO pathway on BP and the evidence for this to date is discussed below.

1.9.1 NO_2^-, NO_3^- and BP

The reported effects of NO_2^- on vascular tone raise the possibility that systemic NO_2^- administration or dietary NO_3^- supplementation may be useful in the treatment of hypertension. Intra-arterial infusion of $NaNO_2$ (36 µmol/min for 15 min) into the forearm of 18 healthy subjects achieved local intravascular NO_2^- concentrations of ~200 µM and caused NO-dependent vasodilation of the forearm vasculature and an increase of ~175% in FBF (Cosby *et al.*, 2003). Systemic NO_2^- levels increased from 0.2-0.4 µM to 16 µM and was associated with a 7 mmHg drop in MAP (Cosby *et al.*, 2003). Infusion of approximately 100-fold less $NaNO_2$ (400 nmol/min) to achieve lower, physiologically relevant concentrations of NO_2^- (local venous concentrations of ~2.5 µM) in 10 healthy subjects increased FBF by ~30% (Cosby *et al.*, 2003). These effects were augmented by exercise, and were all associated with the detection of increased HbNO as a marker of intravascular NO production (Cosby *et al.*, 2003). This publication was followed by another from the same group demonstrating that infusion into the brachial artery of increasing doses of $NaNO_2$ (2-fold stepped increases in 5 min intervals, with starting dose 0.1 µmol/kg/min up to 1.6 µmol/kg/min) resulting in local venous plasma NO_2^- levels of 25-30 µM, caused a ~10 mmHg drop in MAP that persisted for up to 3 h (Dejam *et al.*, 2007).

The effect of exercise to increase NO_2^--induced vasodilation (Cosby *et al.*, 2003) suggested to the authors that relative hypoxia might increase NO_2^--derived NO, in

accordance with the activity of cell-free enzyme systems already described. This thesis was further developed more recently by Frenneaux and colleagues (Maher *et al.*, 2008). Utilizing radio-labelled, autologous blood and standard forearm plethysmographic techniques, they were able to determine arterial and venous vasodilation separately. Interestingly, under normoxic conditions, intra-arterial delivery of increasing doses of NO_2^- (314 nmol/min-7.84 µmol/min), decreased venous tone in a dose-dependent manner, by up to 20-35% with the highest doses used after 20min of NO_2^- infusion (Maher *et al.*, 2008). However, in this study, low dose NO_2^- infusions (314 nmol/min), that approximated doses used previously that were associated with ~30% increases in FBF (Cosby *et al.*, 2003) did not dilate the arterial side of the circulation (Maher *et al.*, 2008). Indeed, arterial dilation was only apparent (increasing FBF by 60-80%) at much higher doses (3.14-7.84 µmol/min) (Maher *et al.*, 2008). These findings replicate much earlier work that used detailed tilt-table testing to elucidate the role of dilation of the venous circulation (and not arterial circulation) as the cause of NO_2^- induced cardiovascular collapse (Weiss *et al.*, 1937; Wilkins *et al.*, 1937).

However, to get the arterial side of the circulation 'hypoxic', subjects repeated the protocol after arterial saturations were maintained at SpO_2 of 83-88% by breathing 12% O_2 (Maher *et al.*, 2008). In this situation, whilst there was no augmentation of the effects on venodilation, or of hypoxia on its own, infusion of 314 nmol/min NO_2^- (which had no effect in normoxic conditions) increased FBF by ~40% (Maher *et al.*, 2008). Such a finding is consistent with the idea that the vasodilator potential of NO_2^- is not solely limited to anoxia or extreme ischaemia but is proportional to the

extent of 'hypoxia' in the tissues and blood as it deoxygenates from arterial to venous sides.

These demonstrations of the vasodilator potential of NO_2^- are also concordant with the effects of NO_2^- supplementation in wild-type animals and animal models of hypertension. In anaesthetized Wistar rats, *i.v.* NO_2^- (10-1000 µmol/kg over 5 min) caused dose-dependent reductions in MAP measured invasively over 30 min. (Vleeming *et al.*, 1997). In free-moving Wistar rats, implantation of telemetric BP recorders revealed that supplementation of drinking water with NO_2^- (36 mM) reduced BP, and that this effect was reversed immediately on changing drinking water supplementation to NaCl control (Vleeming *et al.*, 1997). Similarly, in spontaneously hypertensive rats, prolonged (up to 1 year) oral administration of large amounts of NO_2^- in the drinking water (50-100 mM) ameliorated the BP phenotype in a dose-dependent fashion (Beier *et al.*, 1995; Haas *et al.*, 1999). The authors of these articles were aware of the bacterial conversion of NO_3^- to NO_2^- and postulated that the effect of dietary NO_3^- should be investigated to see whether elevation of systemic NO_2^- levels from NO_3^- would reduce BP (Classen *et al.*, 1990; Vleeming *et al.*, 1997). Indeed, there is now a substantial body of evidence in both animal models and humans to support this proposal.

BP was measured either in anaesthetized (by cannulation) or in conscious (telemetrically) Sprague-Dawley rats that were supplemented with NO_3^- (10 mM) or matched control in drinking water for 1 week. MAP was reduced by ~20 mmHg and ~5 mmHg in anaesthetized and conscious animals respectively after treatment with

NO_3^- only (Petersson *et al.*, 2009). Importantly, in this study, the significance of bacterial NO_3^- reduction for bioactivity of NO_2^- was confirmed. Treatment with antibacterial mouthwash (chlorhexidine 0.2%) twice daily during the same week as NO_3^- supplementation prevented the reductions in BP noted above, but had no effect on rats supplemented with NO_2^- (1 mM) (Petersson *et al.*, 2009).

Further evidence for the utility of NO_3^- in BP control comes from animal models of hypertension. For example, in high-salt fed, uni-nephrectomized Sprague-Dawley rats, an established model for hypertension in rats (Carlström *et al.*, 2007), supplementation of the diet with 1 mmol/kg inorganic NO_3^- ameliorated salt-induced hypertension and reduced associated cardiac and renal fibrosis (Carlström *et al.*, 2011).

Such effects of dietary NO_3^- have been translated into the clinical setting. The first demonstration of this was published by the Karolinska group using short-term supplementation (3 days) with $NaNO_3$ (0.1 mmol/kg daily) compared to matched NaCl control in 17 healthy subjects. They noted increased plasma NO_2^- levels in NO_3^- -supplemented subjects (209±105 nM) compared to control (138±38 nM) and demonstrated reductions in DBP by 3.7mmHg compared to placebo (Larsen *et al.*, 2006).

Later, Ahluwalia and colleagues, in an open-label study, used a dietary source of NO_3^-, in the form of beetroot juice, compared to a matched volume of water (Webb *et al.*, 2008a). A single ingestion of a dietary NO_3^- (500mL, $[NO_3^-]$= 45 mM) reduced

SBP and DBP over 24 h with peak reductions of ~10.4 and ~8 mmHg respectively occurring at 2.5-3 h post-ingestion; effects coinciding with peak elevations in plasma NO_2^-, but not NO_3^-, levels (Figure 1.7). SBP was still significantly reduced at 24 h post-ingestion by ~6 mmHg. Furthermore, the changes in SBP were not correlated to changes in either plasma NO_3^- or K^+ levels but were significantly inversely correlated to changes in plasma NO_2^- levels, suggesting that the change in plasma NO_2^- level was the determining effect in changing BP (Webb *et al.*, 2008a). Changes in plasma NO_2^- levels and reductions in BP were abolished if subjects refrained from swallowing their saliva, thereby interrupting the entero-salivary circulation and preventing the rises in plasma NO_2^- levels in the circulation (Webb *et al.*, 2008a) which again confirms the functional importance of the entero-salivary circulation and further identifies NO_2^- as the bioactive effector in this study (Webb *et al.*, 2008a).

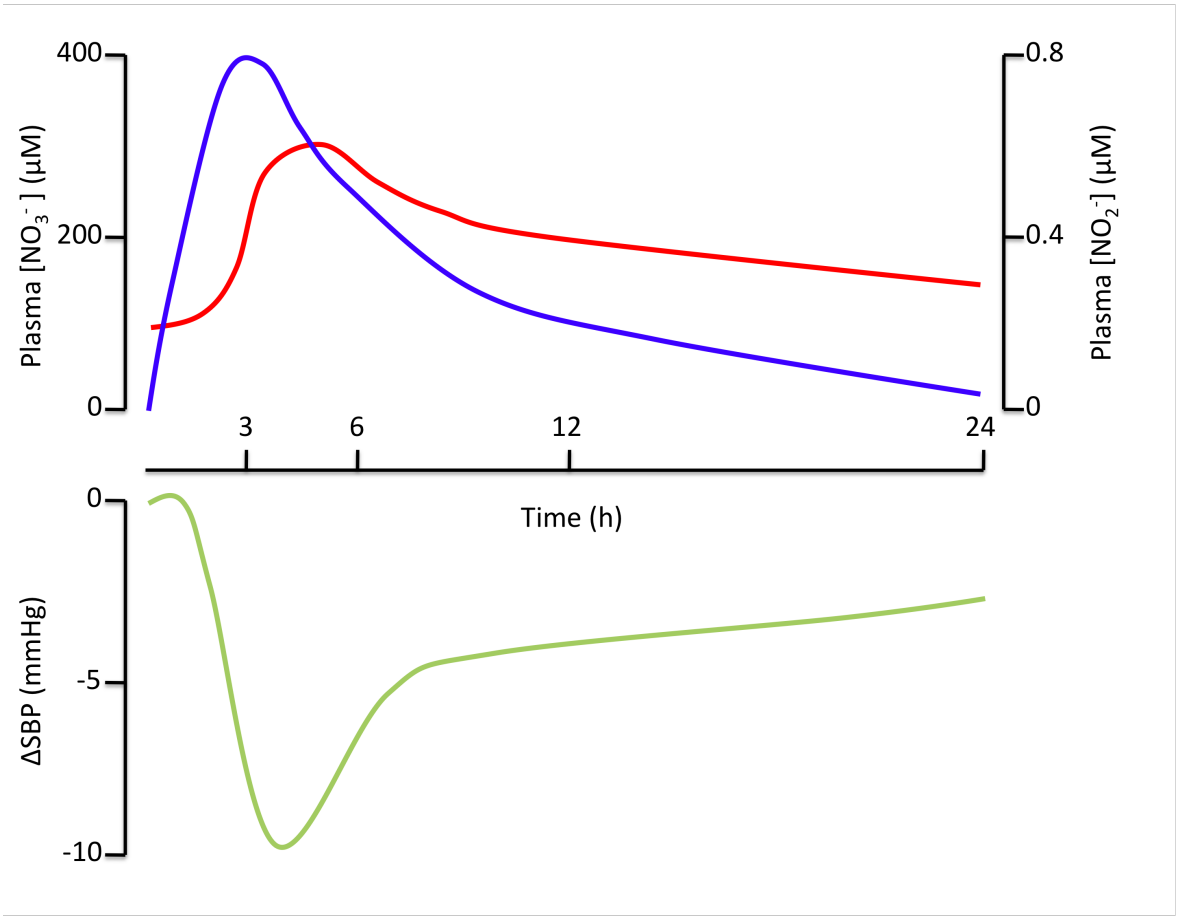


Figure 1.7 *Association of changes in plasma NO_x levels with SBP. Changes in plasma NO_x levels and SBP over 24 h after dietary NO_3^- ingestion (~22.5 mmol) showing changes in SBP to baseline (lower y- axis), plasma NO_2^- (upper right y-axis) and plasma NO_3^- (upper left y-axis) levels. (Data adapted with permission from Webb et al., 2008a). (NO_3^-=nitrate; NO_2^- =nitrite; SBP=systolic blood pressure).*

NO_3^-, NO_2^-, *SBP*.

Importantly, these effects of dietary NO_3^- appear to be sustained over longer periods of time in healthy subjects. Comparison of the effects of a traditional Japanese diet, which is commonly rich in NO_3^- (~18 mmol/day NO_3^-), with a relatively low NO_3^- containing control diet (~3.5 mmol/day NO_3^-) in a cross-over study lasting 10 days for each dietary intervention, demonstrated significantly lower DBP (decrease of 4.5mmHg) on the high-NO_3^- diet in 25 healthy Japanese subjects (Sobko *et al.*, 2010). Similarly, dietary NO_3^- (as beetroot juice, 500 mL providing ~5.2 mmol/day NO_3^- for 15 days) lowered BP acutely in healthy subjects, and the BP-lowering effects were sustained up to 15 days compared to control (Vanhatalo *et al.*, 2010).

Whilst these studies suggest there is little tolerance to the effect of dietary NO_3^-, this has not been established definitely. However, there is supportive evidence that there is no tolerance to systemic elevations of NO_2^- in the circulation, which is of critical importance as the effects of NO_3^- are mediated by the NO_2^- anion.

In non-human primates that received a continuous background infusion of NO_2^- (0.18 µmol/kg/hr), repeated daily bolus administration of a relatively high dose of $NaNO_2$ (0.17 mmol/kg) caused decreases in MAP of ~18 mmHg that did not diminish over 2 weeks (Dejam *et al.*, 2007) confirming a lack of tachyphylaxis to the effects of sustained elevation of NO_2^-. This lack of tolerance to the vasodilatory and BP-lowering effects of sustained elevations of NO_2^- levels confirms earlier reports of a lack of tolerance to prolonged NO_2^- administration. In hypertensive patients who were treated with stable doses of NO_2^-, thrice daily, for periods of more than 2

weeks, there was no diminution of the hypotensive effects thereof (Matthew, 1909). Similarly, in healthy subjects, oral bolus doses of NO_2^- (2.2-7 mmol $NaNO_2$) produced similar BP reductions before and after a period of sustained oral administration of NO_2^- (4-5 days) (Crandall Jr *et al.*, 1931).

These effects contrast to the tolerance that affects the clinical utility of organic nitrites and nitrates already discussed (section 1.4.1.1). However, in the previous report, once tolerance to the headache-inducing properties of an organic nitrate (ethylene glycol dinitrate) was established through prolonged administration, this prevented BP lowering with an inorganic NO_2^- bolus that was apparent prior to the acquired tolerance (Crandall Jr *et al.*, 1931). This suggests that tolerance formation to organic nitrates could affect cross-tolerance to the effects of NO_2^-. However, more detailed and modern investigations of the potential cross-tolerance between organic nitrites and nitrates and inorganic NO_2^- have not been performed to date.

Thus, the accumulation of these evidences suggest that there is a functional pathway for the sequential reduction of NO_3^- to NO_2^- and thence to NO which is bioactive in the circulation and can effect changes in vasoregulation and thence BP (Figure 1.8).

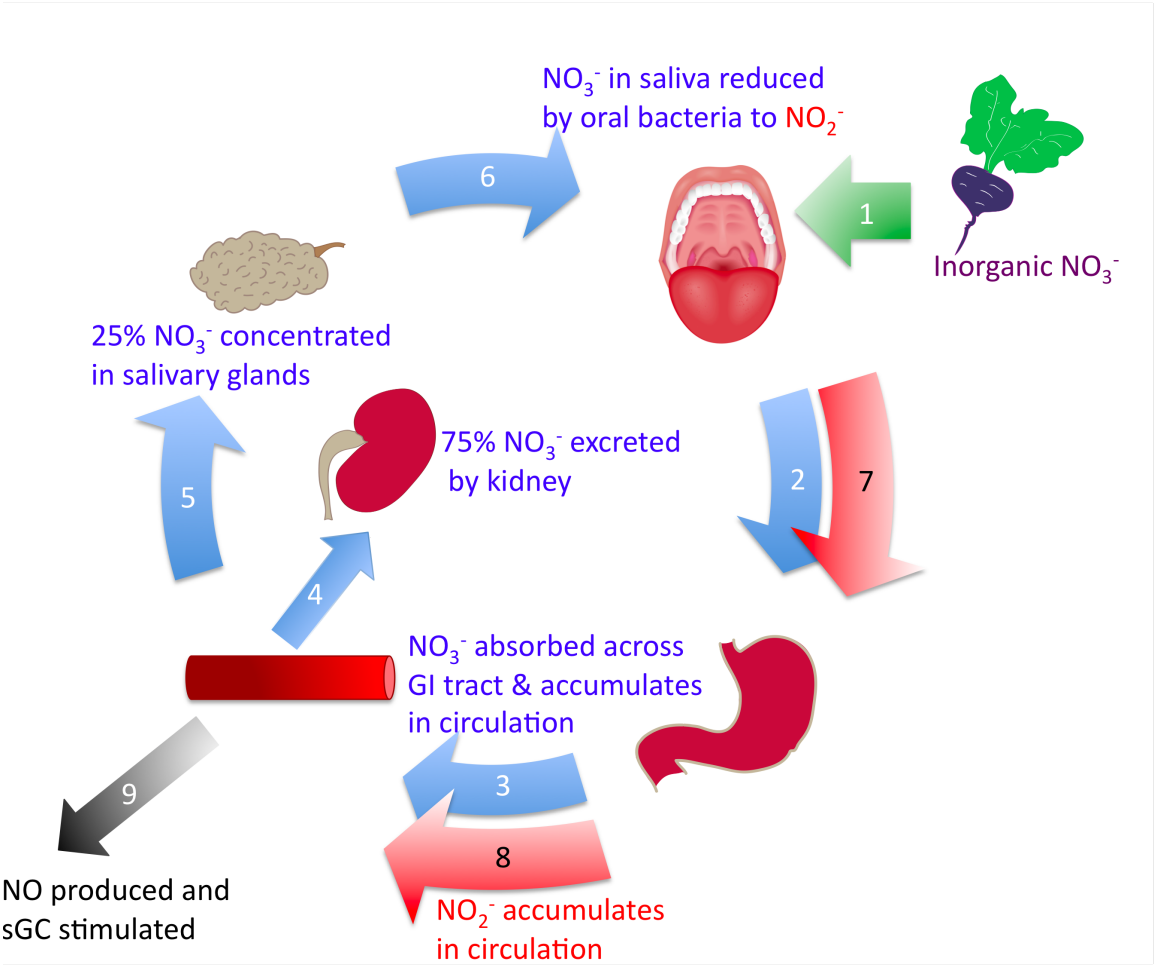


Figure 1.8 *Entero-salivary circulation. After ingestion of oral inorganic NO_3^- (1), NO_3^- is swallowed and enters the GI tract (2), where it is then absorbed and accumulates in the circulation (3). Approximately 75% of ingested NO_3^- is excreted via the kidneys (4) and the remaining 25% concentrated in salivary glands (5). This NO_3^- enters the saliva where it is reduced to NO_2^- by oral bacteria (6). NO_2^- is then swallowed thereby entering the GI tract (7) and subsequently resulting in the accumulation of NO_2^- in the circulation (8). Once within the circulation, NO_2^- is reduced to NO in the vasculature where it activates sGC resulting in bioactivity. (GI=gastro-intestinal; NO_3^-=nitrate; NO_2^-=nitrite; NO=nitric oxide; sGC=soluble guanylyl cyclase).*

1.10 Aims

However, what is currently unknown is whether the effects of NO_3^- either given via dietary or supplementary means are dose-dependent in terms of BP decreases; or whether NO actually mediates these effects. In addition, although the importance of the entero-salivary circulation after NO_3^- supplementation has been demonstrated, NO_3^- formed from NO and NO_2^- oxidation will also undergo recirculation, and thus may be contributing to vasoregulation and BP control under basal conditions (i.e. without supplemental inorganic or dietary NO_3^-) and this has not been studied to date. Although the first clinical applications of NO_3^- therapy for BP control in man have taken place in healthy subjects, it is currently unclear whether dietary or supplementary NO_3^- would function similarly in hypertensive subjects.

Thus the aims of this thesis are to investigate:

1. Whether inorganic NO_3^- supplementation acts similarly to dietary NO_3^- in healthy subjects, with respect to changes in plasma NO_x levels and BP
2. Whether the BP-lowering effects of inorganic NO_3^- are dose-dependent
3. The importance of the entero-salivary circulation on basal plasma NO_2^- levels and BP in healthy subjects.
4. Whether dietary NO_3^- ingestion has similar effects, as in healthy subjects, on plasma NO_x levels and BP in hypertensive subjects.

CHAPTER 2

General methods

All studies were performed with local Research Ethics Committee approval (05/Q0512/145 & 08/H0703/91) and were conducted in the clinical research facility of the William Harvey Research Institute and were conducted in a temperature-controlled environment (22-24^{o}C). For all studies, subjects attended clinic appointments following an overnight fast and adherence to a low-NO_x containing diet for the preceding 24 h prior to the visit, except for any interventions provided. Subjects were provided with a dietary information sheet to aid food choices. Subjects were given a low-NO_x containing lunch (consisting of a hard-cheese sandwich on brown bread) midway through the study if it was longer than 4 h.

2.1 Subjects

All subjects gave informed, written consent.

2.1.1 Healthy subjects

Healthy subjects were recruited by means of word of mouth, by poster advertising and by advertising on the Vascular Pharmacology webpage of the William Harvey Research Institute website (http://www.whri.qmul.ac.uk/staff/Ahluwalia.html).

The *inclusion criteria* for healthy subjects in all studies were:

1. Healthy male or female adult of 18-45 years of age.
2. Body mass index (BMI) between 18-40 kg/m^2.
3. No systemic medication (other than the oral contraceptive pill).
4. Non-smoker.
5. Normotensive.

2.1.2 Hypertensive subjects

Hypertensive subjects were recruited from the outpatient department of Barts and the London Centre of Excellence in Hypertension and by means of poster and newspaper advertising.

The *inclusion criteria* for hypertensive patients were:

1. Male and females between 18 and 85 years of age, inclusive.
2. To be eligible, female subjects required to state that they are not pregnant, and will not become pregnant during the course of the study.
3. BMI between 18 and 40 kg/m^2.
4. Able to understand and comply with protocol requirements, instructions and protocol-stated restrictions.
5. Grade 1 hypertensive defined as: SBP 140-159 or DBP 90-99 mmHg (determined by 24 h ambulatory BP (ABP) monitoring).
6. No systemic medication (other than the oral contraceptive pill).

The *exclusion criteria* for hypertensive patients were:

1. Evidence on examination or electrocardiography of hypertensive target organ damage.
2. History of symptomatic ischaemic heart disease, stroke, or other known atherosclerotic disease.
3. History of chronic viral hepatitis (including presence of hepatitis B surface antigen or hepatitis C antibody), or other chronic hepatic disorders.

4. History of increased liver function tests due to acute or chronic liver conditions (any liver enzymes >3x above the upper limit of normal or bilirubin >1.5x above the upper limit of normal).
5. Chronic kidney disease, with estimated GFR of <50 mL/min.
6. Current poorly controlled diabetes mellitus, defined as glycated Hb >10% at screening.
7. Subjects with LDL-cholesterol >7.5 mmol/L or fasting triglyceride level >6 mmol/L.
8. History of heart failure defined as New York Heart Association class II-IV or those with known severe left ventricular systolic dysfunction (ejection fraction <30% on echocardiography) regardless of symptomatic status.
9. History of malignancy within the past 5 years, other than non-melanoma skin cancer.
10. Subjects with rheumatoid arthritis, connective tissue disorders and other conditions known to be associated with chronic inflammation (e.g. inflammatory bowel disease).
11. Current life-threatening conditions (e.g., very severe chronic airways disease, life-threatening arrhythmias etc.) that may prevent a subject from completing the study.
12. Subjects with any acute infection, or significant trauma (burns, fractures).
13. Subjects who have donated more than 500 mL of blood within 56 days prior to the study commencement.
14. Use of an investigational device or investigational drug within 30 days or 5 $t_{1/2}$ (whichever is the longer) preceding the first dose of study medication.

15. Subjects who will commence or who are likely to commence treatment with NSAIDs from screening until study completion.
16. Any non-stable dosing of ongoing medication regimens throughout the study trial.
17. History of alcohol or drug abuse within the past 6 months.
18. The subject has a three-month prior history of regular alcohol consumption exceeding an average weekly intake of >28 units (or an average daily intake of greater than 3 units) for males, or an average weekly intake of >21 units (or an average daily intake of greater than 2 units) for females (1 unit is equivalent to a half-pint (284 mL) of beer/lager; 25mL measure of spirits or 125 mL of wine); or a positive alcohol breath test at the screening visit.
19. A positive urine test for drugs of abuse at screening or prior to study medication administration.
20. Any other subject whom the investigator deems unsuitable for the study (e.g., due to either medical reasons, laboratory abnormalities, expected study medication non-compliance, or subject's unwillingness to comply with all study-related study procedures).

2.2 Interventions

All study interventions were kept in a temperature-controlled refrigerator (4-7^{o}C) as per manufacturers' recommendations. All capsules were taken with 500 mL low-NO_x containing water (purchased from Zepbrook Ltd, London, UK) and a slice of wholemeal toast.

2.3 Randomization

Subjects were randomly assigned to either intervention using a random binary number table (http://www.random.org).

2.4 BP measurement

BP and heart rate (HR, measured as beats per min (bpm)) were measured in all studies using digital, oscillometric devices that had undergone British Hypertension Society (BHS) validation (http://www.bhsoc.org/blood_pressure_list.stm).

2.4.1 Clinic BP

BP and HR were measured according to BHS guidelines (Williams *et al.*, 2004). Subjects were seated in a quiet, temperature-controlled environment and BP and HR readings were taken in triplicate using a calibrated Omron 715IT (Omron Corporation, Tokyo, Japan) and an appropriately sized cuff. The 2^{nd} and 3^{rd} readings were averaged to determine clinic BP and HR at each time-point.

For establishment of all baseline measurements, subjects were seated for 5 min followed by BP and HR measurements in triplicate every 15 min for 1 h. The mean of the averaged values from each of the 5 time-points within this 1 h period was taken to represent baseline BP and HR. In studies where an intervention was employed, further measurements were again taken in triplicate at specified time-points. Both the investigator and subject were blind to these measurements by means of laminating coverings attached to the printer and machine (Figure 2.1).

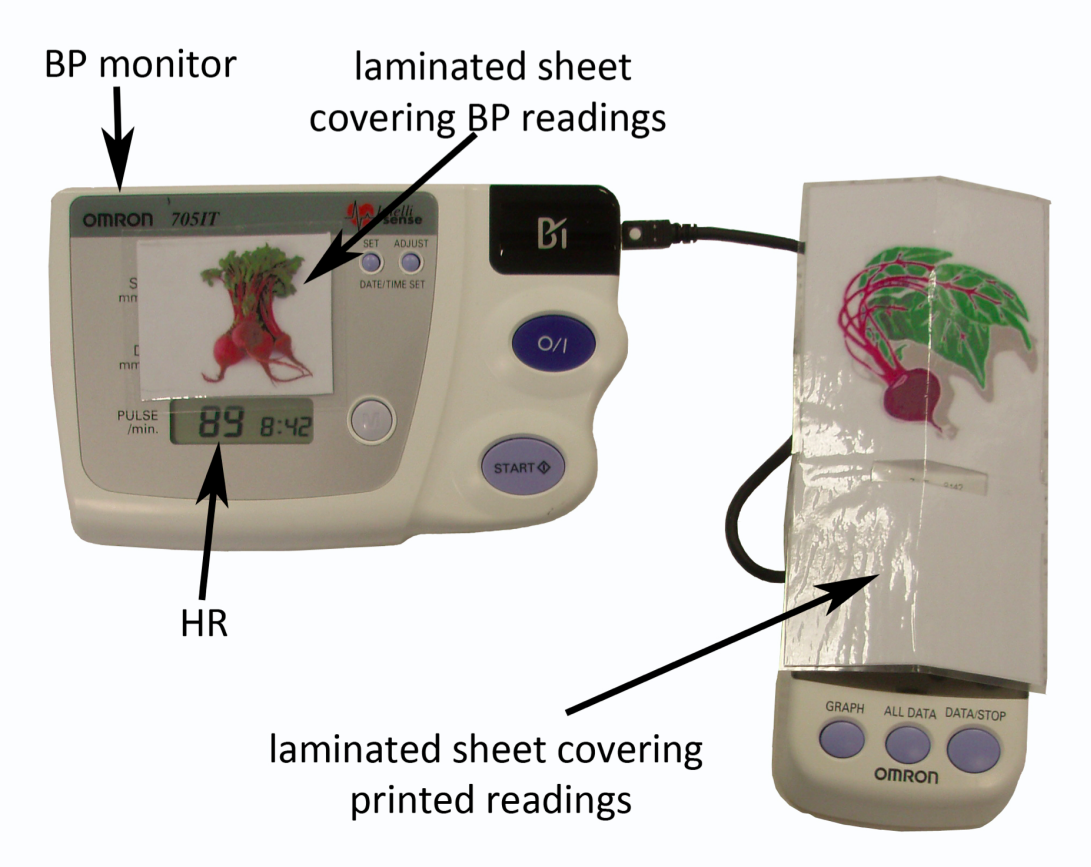


Figure 2.1 *Photograph of modified BP machine and printer; to obscure readings from investigator and subject. (BP=blood pressure; HR=heart rate).*

2.4.2 24 h ABP

24 h ABP and HR were determined using a calibrated Spacelabs 90207 ABP monitor (Spacelabs Healthcare, Issaquah, USA). The standard protocol used for all 24 h ABP measurements was used of 1 reading every 20 min between 0700-2300 and 1 reading every 1 h between 2300-0700. Proprietary software was used to download readings and produce 24 h, daytime (0700-2300) and nighttime (2300-0700) raw and mean ABP and HR readings (90256 ABP Report Management System, Spacelabs Healthcare, Issaquah, USA).

ABP measurements reported for hypertensive subjects at screening have been adjusted upwards by 10 mmHg systolic and 5 mmHg diastolic as per BHS guidelines (Williams *et al.*, 2004). Subjects were defined for all purposes as having hypertension if the corrected daytime mean systolic ABP was >140 mmHg or diastolic ABP was >90 mmHg as per BHS guidelines (Williams *et al.*, 2004).

ABP results reported in the results section in healthy subjects in the oral NO_3^- reductase study (chapter 4) have not been adjusted.

2.4.3 Home BP

Subjects were instructed, in person, on the proper use of a calibrated Omron 715IT (Omron Corporation, Tokyo, Japan) and were provided with an appropriately sized cuff. Subjects were instructed to perform home BP measurements in triplicate at the same time daily. Results were self-recorded into provided diaries. The 2nd and 3rd readings at each time-point were averaged to determine mean daily home BP.

2.5 Measurement of arterial stiffness

A Vicorder device (Skidmore Medical Limited, Bristol, UK) was used to simultaneously record the pulse wave from the carotid and femoral site using an oscillometric method (Hickson *et al.*, 2009). A small, inflatable neck pad was placed directly over a single carotid artery and secured around the neck by a Velcro™ tab. A further cuff was similarly placed around the subject's ipsilateral upper thigh. Both carotid and femoral cuffs were inflated automatically to 65 mmHg and the corresponding oscillometric signal from each cuff was digitally analysed to extract the pulse time delay. The distance between the sternal notch and the thigh cuff was measured and used as a standard estimate for the aortic length. From these measurements aortic pulse wave velocity (PWV), which is the *gold*-standard measurement for arterial stiffness (Laurent *et al.*, 2006), was derived according to the following formula PWV=aortic distance/pulse time delay (Figure 2.2).

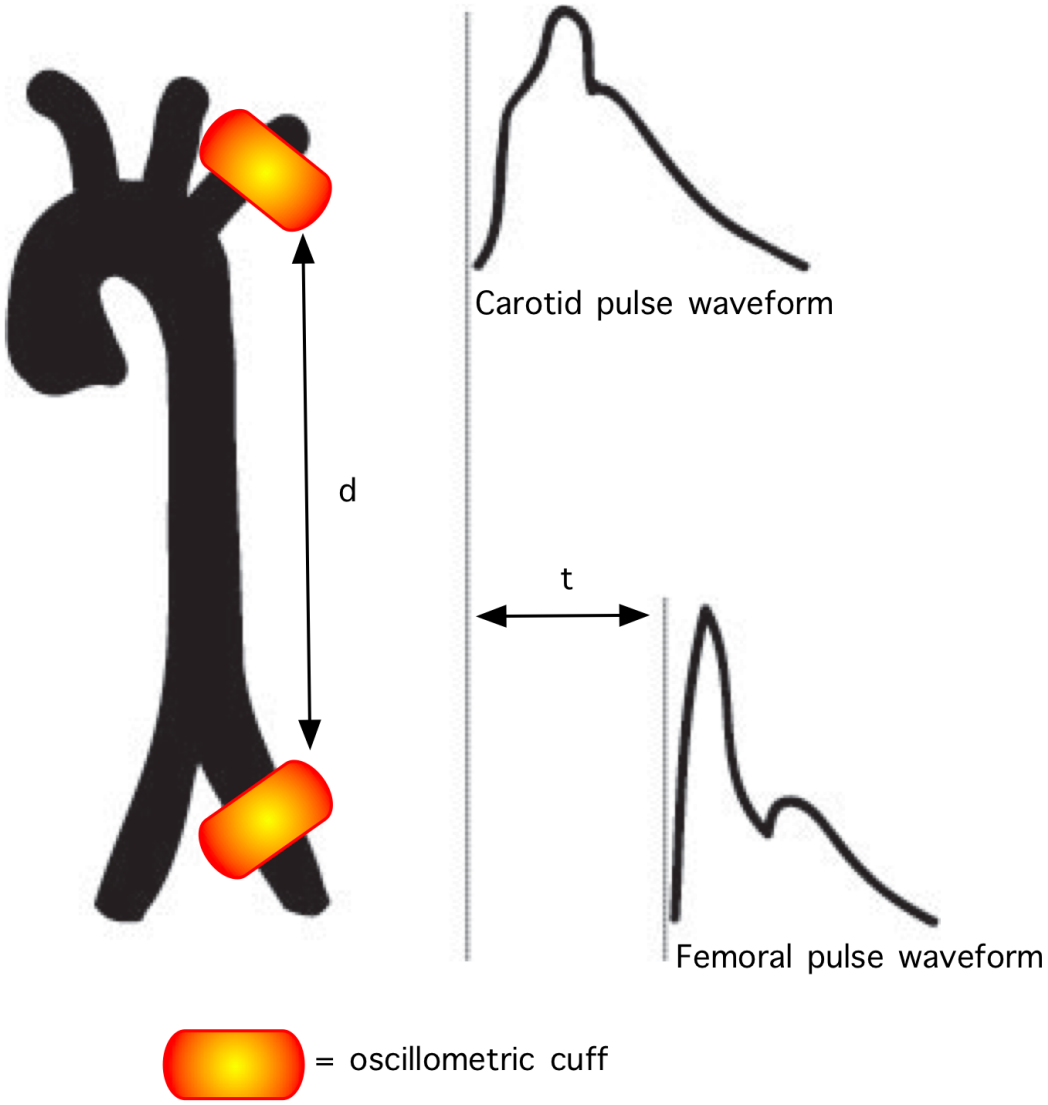


Figure 2.2 *Determination of PWV using Vicorder device. Carotid and femoral oscillometric signals are detected and the time delay from the foot of carotid pulse waveform to foot of femoral pulse waveform is used as the pulse transit delay (t). Estimation of aortic length by sternal-femoral distance is used as distance (d). Aortic distance/ pulse transit delay = aortic PWV. (PWV=pulse wave velocity).*

2.6 Blood sampling

In subjects in whom more than one blood sample was to be taken in the same day, a 21-gauge Y-can cannula (Beldico, Marche-en-Famenne, Belgium) was inserted into an antecubital or dorsal hand vein prior to any intervention, and again at 24 h if required, and secured to skin. Blood samples were taken atraumatically, via the cannula or by standard venepuncture, into pre-chilled Lithium-heparin vacutainer tubes (Becton, Dickinson & Co, Franklin Lakes, USA) and immediately centrifuged (Eppendorf chilled centrifuge 5702R, Eppendorf UK Limited, Cambridge, UK) (1300*g*, 4^{o}C, 10 min). Plasma was separated and stored at -80^{o}C until measurement of NO_x and cGMP levels were undertaken (see section 2.10). Bloods taken at screening for entry into the hypertensive study (i.e. fasting lipids, glycated Hb) were sent to Dr Phillip Miall, Consultant in Clinical Biochemistry, Department of Biochemistry, Barts and the London NHS Trust and were processed as per standard clinical laboratory methods.

2.7 Saliva sampling

Unstimulated, whole saliva samples were collected into ice-chilled, sterile eppendorfs and stored at -80^{o}C until analysis at a later date for assessment of NO_x levels by ozone chemiluminescence (see section 2.10).

2.8 Urine sampling

Mid-stream urine samples were collected into sterile containers and an aliquot stored at -80^{o}C until analysis at a later date for assessment of NO_x levels by ozone chemiluminescence (see section 2.10).

2.9 Measurement of oral NO_3^- reductase activity

Oral NO_3^- reductase activity was assessed by modification to a previously established protocol (Sasaki *et al.*, 1981; Doel *et al.*, 2005). Subjects were instructed to hold 10mL of NO_x-free water (Millipore Corporation, Billerica, USA) for 1, 3 and 5 min to establish baseline NO_3^- reductase activity. Following this, matched volumes of KNO_3 solutions (Martindale Pharmaceuticals, Ipswich, UK) were held in the oral cavity for 1, 3 and then 5 min in a randomized order. At the specified time (1-5 min), the total oral contents were collected into a sterile ice-chilled falcon tube, centrifuged (5500*g*, 4^{o}C, 10min) and the supernatant collected and stored at -80^{o}C until NO_2^- levels were determined by ozone chemiluminescence. Oral cavities were rinsed with 10mL NO_x-free water in between every experimental solution. The concentrations of NO_3^- solutions used were 800 µM, reflecting ~near physiological salivary [NO_3^-] (Lundberg and Govoni, 2004) and 8 mM, reflecting a 10-fold higher salivary [NO_3^-] that approximates the salivary [NO_3^-] after ingestion of a NO_3^--rich meal (Lundberg and Govoni, 2004; Bahra *et al.*, 2012).

2.10 Measurement of NO_x levels by ozone chemiluminescence

Prior to ozone chemiluminescence, plasma samples were filtered by centrifugation (15000*g*, 4^{o}C, 60min) using Microcon® Ultracel YM-3KDa filters (Millipore Corporation, Billerica, USA) that had been washed 2 times in NO_x-free water to remove any potential NO_x contamination.

NO_x levels in the plasma filtrate, saliva and urine samples were determined by liquid phase ozone chemiluminescence (Downes *et al.*, 1976); a powerful, quantitative

analytical technique for measuring NO species in biological fluids (Cox, 1980; Ignarro *et al.*, 1993).

The apparatus consists of two distinct components (see Figure 2.3 below): the purge vessel (reaction chamber) and the NO chemiluminescence analyser. Within the purge vessel, gaseous N_2 is continuously bubbled to render the chamber anoxic. Here, standards and biological samples containing NO_x are reduced to NO in an equimolar fashion. The NO produced by these reactions is carried in the gaseous phase through the vessel into the NO chemiluminescence analyser (NOA 280i, Sievers, Manchester, UK), where it reacts with ozone. Light is emitted as a result of the chemical reaction (Equation 2.1), and is detected by the analyser, thence producing a digital signal corresponding to NO concentration (therefore NO_x levels as appropriate); NO_x levels were calculated by comparison to a standard curve generated daily from known standards.

ozone	***nitric oxide***		***oxygen***	***nitrogen dioxide****	***nitrogen dioxide***	***light***
O_3	**+ NO**	**⇒**	**O_2**	**+ NO_2***	**⇒ NO_2**	**+ hv**

Equation 2.1 *Reaction of ozone and NO to chemiluminescent light. (NO=nitric oxide; NO_2*=nitrogen dioxide in an excited state).*

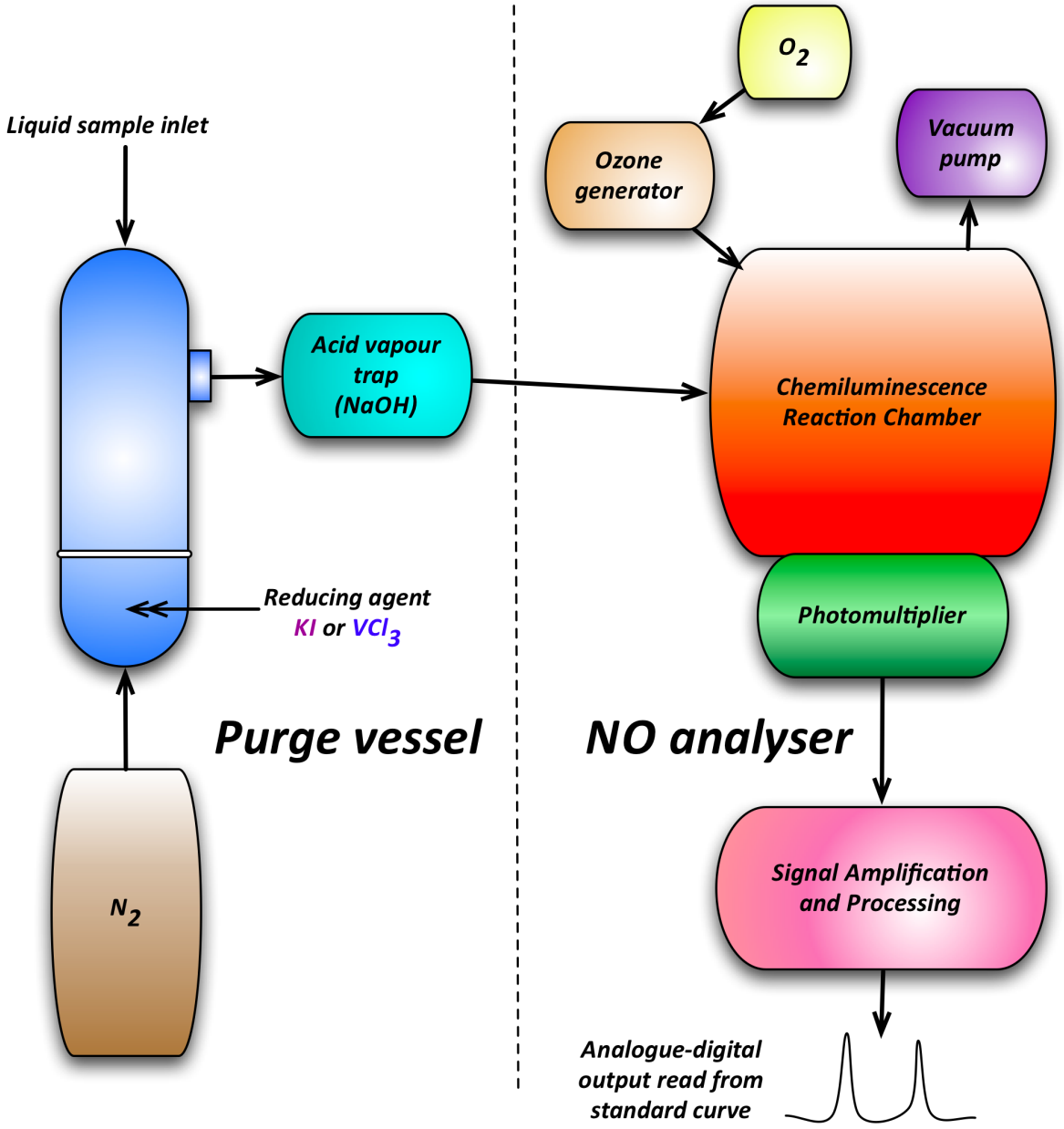


Figure 2.3 *Schema of ozone chemiluminescence apparatus. (KI=potassium iodide; N_2=nitrogen; NaOH=sodium hydroxide; NO=nitric oxide; O_2=oxygen; O_3=ozone; VCl_3=vanadium (III) chloride).*

To determine total [NO_x], samples were incubated in a strongly reducing environment using 0.1 M vanadium (III) chloride (VCl_3) in 1 M hydrochloric acid refluxing at 95^{o}C under N_2 which results in a sequential reduction of all NO_3^- to NO_2^-, and then all NO_2^- to NO (Cox, 1980) (Equations 2.2-2.3).

nitrate		*protons*		*electrons*		*nitric oxide*		*water*
NO_3^-	+	$4\ H^+$	+	$3\ e^-$	$\Rightarrow$	NO	+	$2\ H_20$

Equation 2.2 *Reduction of NO_3^- by VCl_3 to produce NO. (NO=nitric oxide; NO_3^-=nitrate; VCl_3=vanadium (III) chloride).*

nitrite		*protons*		*electron*		*nitric oxide*		*water*
NO_2^-	+	$2\ H^+$	+	e^-	$\Rightarrow$	NO	+	$2\ H_20$

Equation 2.3 *Reduction of NO_2^- by VCl_3 to produce NO. (NO=nitric oxide; NO_2^-=nitrite; VCl_3=vanadium (III) chloride).*

[NO_2^-] of samples was determined by addition of samples to milder reducing conditions, 1.5% potassium iodide (KI) in glacial acetic acid under N_2 at room temperature (equation 2.4), which is unable to reduce NO_3^- (Cox, 1980).

nitrate		*protons*		*electron*		*nitric oxide*		*water*
NO_3^-	+	$2\ H^+$	+	e^-	$\Rightarrow$	NO	+	H_20

Equation 2.4 *Reduction of NO_2^- by KI to produce NO (NO=nitric oxide; NO_2^-=nitrite; KI=potassium iodide).*

[NO_3^-] was calculated by subtraction of [NO_2^-] from total [NO_x] determined by the above reactions. Typical standard curves for the analysis of total [NO_x] and [NO_2^-] are shown in Figure 2.4-2.5.

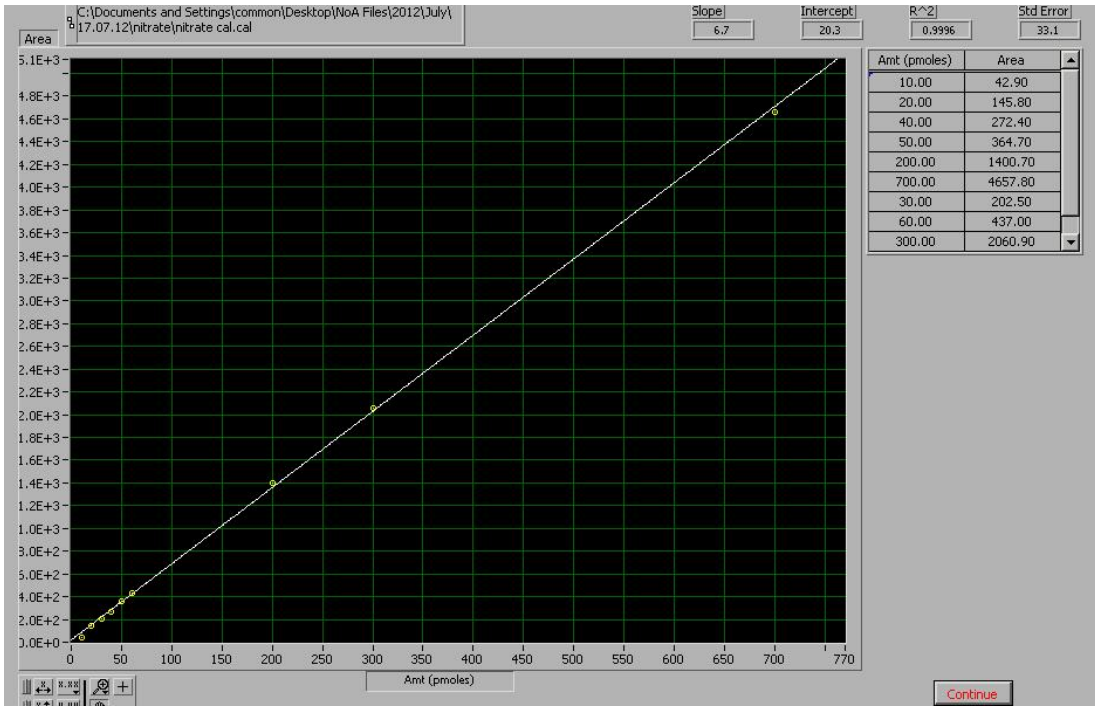


Figure 2.4 *Standard curve for determination of total NO_x levels by ozone chemiluminescence. Volumes (10-50 µL) of known standards of $NaNO_3$ (1-100 µM) were analysed to create a standard curve daily. ($NaNO_3$=sodium nitrate; NO_x=nitrite/nitrate$^-$).*

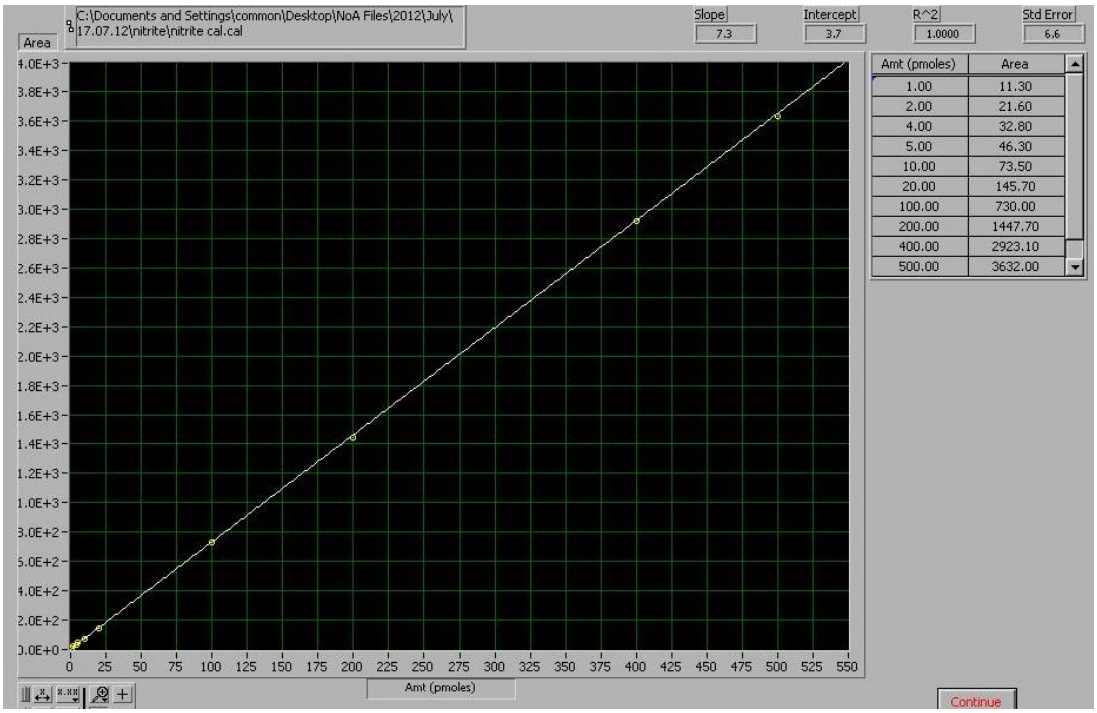


Figure 2.5 *Standard curve for determination of NO_2^- levels by ozone chemiluminescence. Volumes (10-50 µL) of known standards of $NaNO_2$ (100 nM-10 µM) were analysed to create a standard curve daily. ($NaNO_2$=sodium nitrite; NO_2^-=nitrite).*

In addition, I determined the levels of contamination of NO_x as a consequence of use of the commercially available blood tubes and ascertained a [NO_2^-] contamination of 102.0±10.3 nM and [NO_3^-] contamination of 1.7±0.1 µM (n=20 tubes).

2.11 Measurement of cGMP levels

cGMP levels in samples were determined using an enzyme immunoassay (cGMP Enzymeimmunoassy Biotrak System RPN226) according to the manufacturer's instructions (GE Healthcare, GE Healthcare, Little Chalfont, UK), using a 96-well plate spectrophotometer. Thawed samples were incubated with a competitive, non-selective PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 100µM) to prevent cleavage of cGMP in plasma samples. The assay is based on competition between unlabelled cGMP and a fixed quantity of peroxidase-labelled cGMP, for binding sites on a cGMP-specific antibody (Figure 2.6). Samples measured were compared to a standard curve generated that day from known standards (Figure 2.7).

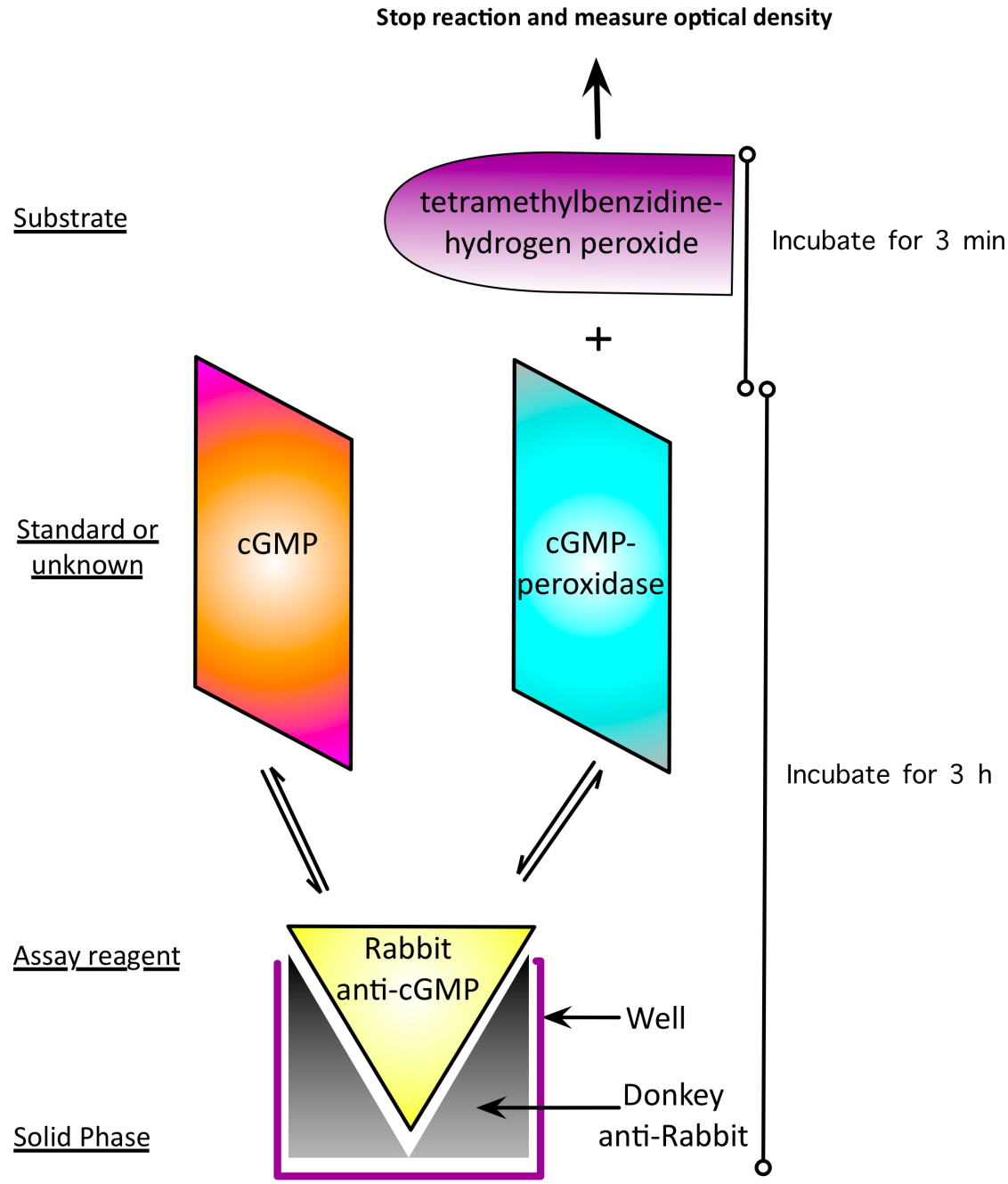


Figure 2.6 *Schema of enzyme immunoassay for measurement of cGMP levels. (cGMP=cyclic guanosine monophosphate).*

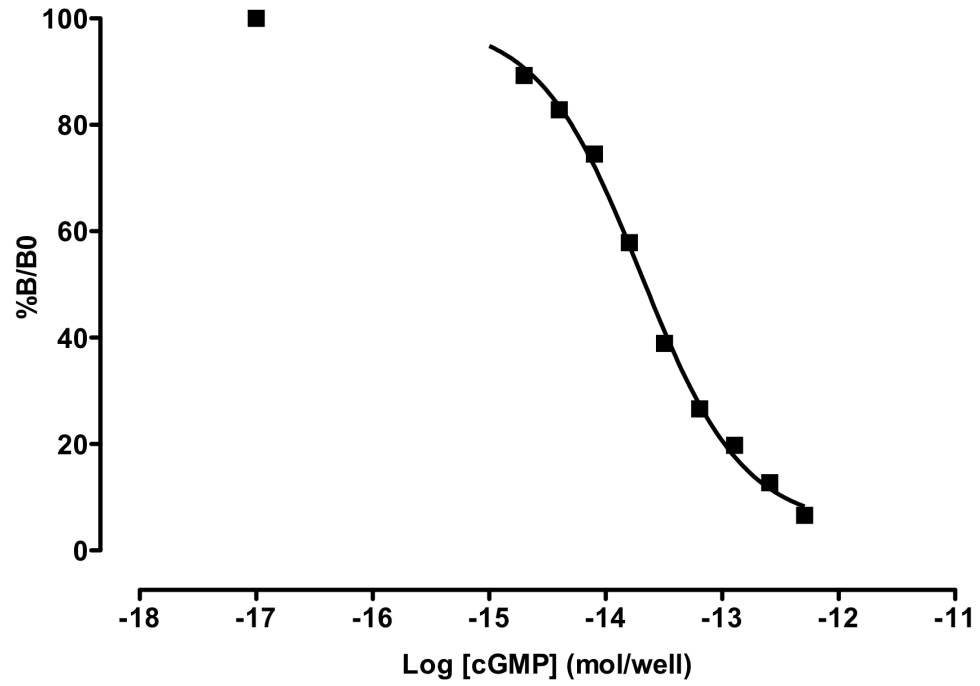


Figure 2.7 *Standard curve for determination of cGMP levels. (%B/B0=% bound; cGMP=cyclic guanosine monophosphate).*

2.12 Measurement of biochemical indices

An aliquot of separated plasma was sent for commercial analysis (Quest Diagnostics, UK) for determination of K^+, chloride (Cl^-), HCO_3^-, urea and creatinine levels.

2.13 Statistical analyses

All data are expressed as mean±standard error of the mean (SEM) and significance accepted at $p<0.05$. Analysis was performed using GraphPad™ Prism software version 5.0 for Mac OsX. Statistical analyses were conducted blind to the treatment groups. Power calculations were performed using G*Power v3.0 and are presented in the experimental protocols in each chapter.

For BP measurements and plasma NO_x levels (primary end-points), paired Student's t-tests and repeated-measures 2-way ANOVA were used where appropriate. Dunnett's post-hoc test was used for comparison to baseline measurements (mean of 1^{st} h BP measurements or baseline plasma NO_x levels) and Bonferroni post-hoc tests for comparison between groups at individual time-points following either 1-way or 2-way ANOVA as appropriate. For post-hoc and primary analyses by sex, unpaired Student's t-tests were used for baseline differences and one-sample Student t-tests used for analysis of changes from baseline.

For PWV and cGMP level measurements, repeated-measures one-way ANOVA followed by Bonferroni post-hoc tests for individual group comparisons were used.

In all studies, determinations of correlations between baseline and changes in plasma NO_x, cGMP and K^+ levels with baseline and changes in BP were performed using the Pearson's correlation coefficient analysis of least-squares and are expressed ±95% confidence intervals.

CHAPTER 3

Investigation of the effect of inorganic NO_3^- salt supplementation on plasma NO_x levels and BP in healthy subjects

3.1 Introduction

Recently, our group has established in an open-label study in healthy subjects that a single dietary NO_3^- load (as beetroot juice, 500mL, NO_3^- dose 22.5 mmol) lowers BP over 24 h compared to water control, with peak BP reductions coinciding with peak plasma NO_2^- levels at 2.5-3 h post-ingestion (Webb *et al.*, 2008a). Changes in BP did not correlate to changes in plasma NO_3^- levels but did correlate to changes in plasma NO_2^- levels, implicating the NO_2^- anion as the functional mediator of the dietary NO_3^--induced changes in BP. However, beetroot juice also contains a number of other potentially beneficial nutrients and, in particular, abundant K^+ (in this study, $[K^+]$= 93mM) (Webb *et al.*, 2008a) and there is evidence that short-term K^+ supplementation can cause reductions in BP (He *et al.*, 2005).

Thus, the aim of the first study described in this chapter was to establish whether administration of a NO_3^- salt might mimic the effect of beetroot juice. Secondly, for inorganic or dietary NO_3^- to be useful as a potential treatment for BP or other CVDs, it is important to determine whether the effects of NO_3^- are dose-dependent. Therefore, I also investigated the dose-dependency of inorganic NO_3^- supplementation or dietary NO_3^- supplementation in healthy subjects.

3.2 Protocols

There were 3 distinct sub-studies with independent subject recruitment; each sub-study requiring 2 visits with a minimum of 7 days between each visit. Healthy subjects were entered into one of 3 different sub-studies described below.

3.2.1 Investigation of whether KNO_3 supplementation mimics the effects of dietary NO_3^- on plasma NO_x levels and BP (24 mmol KNO_3 vs. KCl study)

Design: To assess whether KNO_3 supplementation mimics dietary NO_3^- supplementation, a randomized, double-blind, cross-over study of KNO_3 compared to matched amount of KCl placebo control was conducted. Subjects were randomized to receive 24 mmol KNO_3 or 24 mmol KCl capsules with 500 mL low-NO_x containing water ($[NO_3^-]$ 61.2±1.9 μM; $[NO_2^-]$ 0.2±0.03 μM), with at least 7 days between each limb of the study. This dose was chosen to approximate the NO_3^- dose used in a previous dietary NO_3^- intervention (Webb *et al.*, 2008a).

Power analysis: Power calculations for the 24 mmol KNO_3 vs. KCl study were determined from the previous study with dietary NO_3^- (Webb *et al.*, 2008a). In this study ~22.5 mmol dietary NO_3^- lowered BP at 24 h by ΔSBP= 4.4 mmHg (standard deviation (SD)= 5.5 mmHg). Based on these values, with an α=0.05 and 1-β=0.90, n=19 is required. 2 extra subjects were recruited to account for potential drop-outs, thus the final number recruited was n=21.

Measurements: Following arrival in the clinic, clinic BP and HR were measured for 1 h to establish baseline values (section 2.4.1). Following this, subjects ingested the randomized intervention with a light breakfast and 500 mL of low-NO_x containing water. BP and HR were measured, and blood samples for plasma NO_x and cGMP analysis collected at specific time-points over the following 6 h (section 2.6). After this time, the subjects left and then returned the following morning for 24 h measurements (see Figure 3.1).

3.2.2 Investigation of the dose-response of inorganic NO_3^- supplementation (Inorganic NO_3^- dose-response study)

Design: To establish the dose-dependent effects of inorganic NO_3^- supplementation, a randomized, single-blind (investigator blind), cross-over study of different doses of KNO_3 was conducted in healthy subjects for 3 h after capsule ingestion. This duration was chosen since previous studies indicated that the peak effects occur by this time after NO_3^- ingestion (Webb *et al.*, 2008a). Subjects were randomized to receive either 4 mmol or 12 mmol KNO_3 capsules with 500 mL low-NO_x containing water ($[NO_3^-]$ 61.2±1.9 μM; $[NO_2^-]$ 0.2±0.03 μM), with at least 7 days between each limb of the study. These doses were chosen to reflect half the dose used above and a much lower NO_3^- dose that approximates to the ADI of NO_3^- (3.7 mg/kg per day (Speijers and van den Brandt, 2003; European Food Safety Authority, 2008) for a 70 kg person ~4.2 mmol).

Power analysis: For the dose-response study, the expectation was that the lowest dose may not have any significant effects. It was hypothesized that with half the dose given in the first 24 mmol cross-over study (i.e. 12 mmol), that there would be half the expected rise in plasma NO_2^- levels (expected peak $\Delta[NO_2^-]$ at 3 h would be 1.2 μM, SD= 0.8). Based on these values, with an α=0.05 and 1-β=0.90, n=6 is required.

Measurements: Following arrival in the clinic, clinic BP and HR were measured for 1 h to establish baseline values (section 2.4.1). Following this, subjects ingested the randomized intervention with a light breakfast and 500 mL of low-NO_x containing

water. BP and HR were measured, and blood samples for plasma NO_x analysis collected at specific time-points over the following 3 h (section 2.6) (see Figure 3.1).

3.2.3 Investigation of the effect of a reduced dose of dietary NO_3^- on BP (Dietary NO_3^- study)

Design: To investigate the dose-dependent effects of dietary NO_3^- on BP in healthy subjects, a randomized, open label, cross-over study was conducted in healthy subjects for 3 h after intervention, as this was the time take for peak effects to occur after dietary NO_3^- ingestion (Webb *et al.*, 2008a). Subjects were randomized to receive either 250 mL dietary NO_3^- (reflecting half the dose of beetroot juice used in Webb *et al.*, 2008a) or matched volume of low-NO_x containing water (placebo, $[NO_3^-]$ 61.2±1.9 μM; $[NO_2^-]$ 0.2±0.03 μM), with at least 7 days between each limb of the study.

Power analysis: Peak ΔSBP at 2.5-3h in Webb *et al.,* 2008a was 10.4 mmHg, SD=8. A reduced dose of dietary NO_3^- (~half of the dose) should theoretically provide ~half the change in SBP and SD with an α=0.05 and 1-β=0.90, n=9 is required for the dietary NO_3^- study in healthy subjects.

Measurements: Following arrival in the clinic, BP and HR were measured for 1 h to establish baseline values (section 2.4.1). Following this, subjects ingested the randomized intervention with a light breakfast. BP and HR were measured, and blood samples for plasma NO_x and cGMP analysis collected at specific time-points over the following 3 h (section 2.6) (see Figure 3.1).

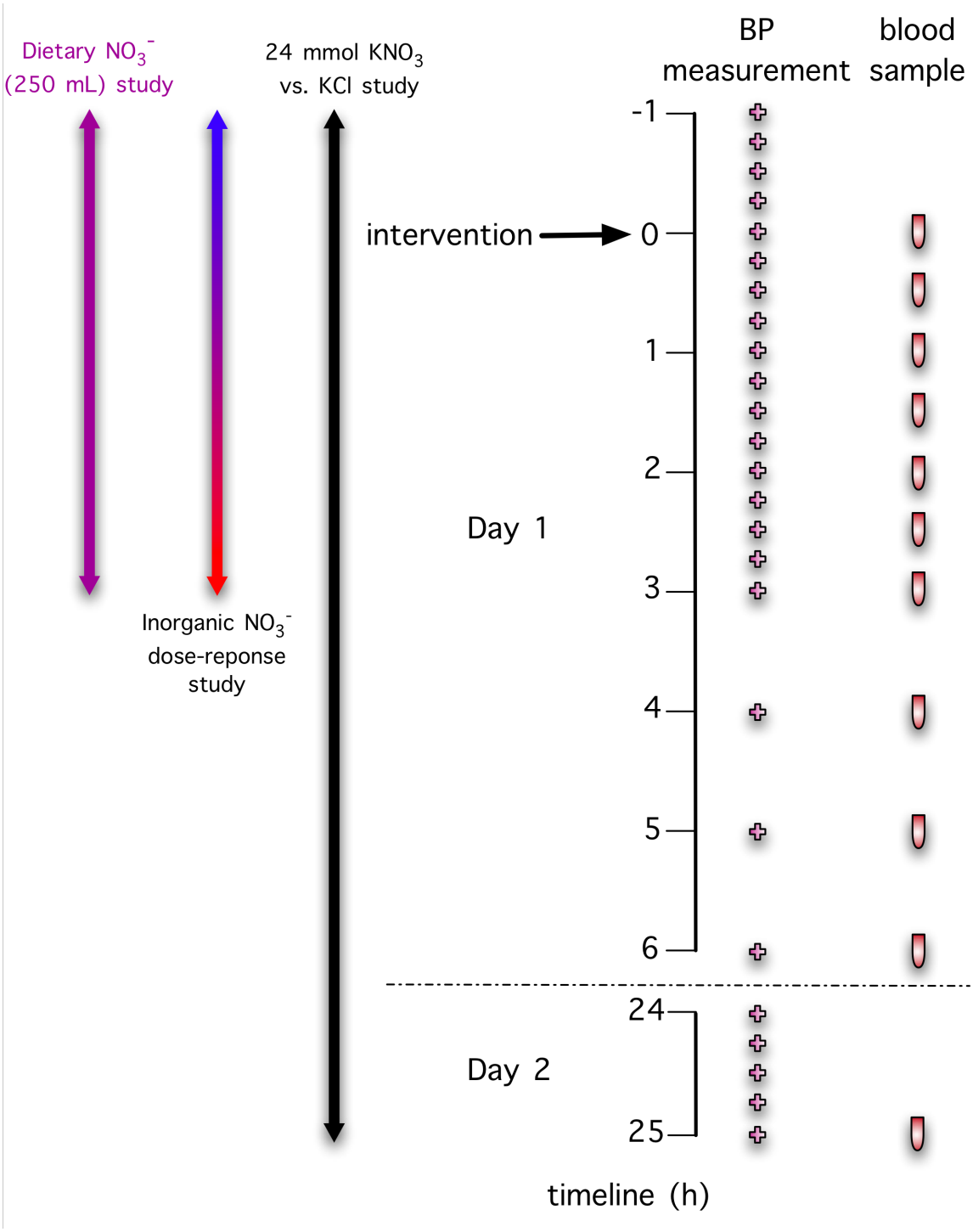


Figure 3.1 *Timelines for healthy subject BP studies. (BP=blood pressure; KCl=potassium chloride; KNO_3=potassium nitrate, NO_3^-=nitrate).*

3.3 Results

3.3.1 Baseline characteristics

There were no significant differences in the general characteristics of the individuals recruited for the 3 separate sub-studies or between the baseline BP in each limb of each sub-study (Table 3.1).

Study	24 mmol KNO_3 vs. KCl	Inorganic NO_3^- dose-response	Dietary NO_3^-
Subjects (n)	20	6	9
Age (years)	22.5±0.9	28.8±1.7	25.1±1.1
BMI (kg/m^2)	22.5±0.6 (range 19.6-30.0)	24.5±1.6 (range 18-29.3)	26.5±0.9 (range 23.1-30.7)
Baseline SBP (mmHg) (control limb)	112.9±3.9	116.7±5.6	120.7±3.0
Baseline SBP (mmHg) (active limb)	110.1±3.4	114.5±4.6	120.6±4.1
Baseline DBP (mmHg) (control limb)	68.6±2.0	70.2±2.2	71.3±2.2
Baseline DBP (mmHg) (active limb)	70.1±2.3	71.0±2.2	70.9±2.5

Table 3.1 *Demographics of recruited healthy subjects and baseline haemodynamic parameters for the 3 distinct sub-studies. Data are expressed as mean±SEM. (For inorganic NO_3^- dose-response study: control=4mmol KNO_3; active=12mmol KNO_3 (BMI=body mass index; DBP=diastolic blood pressure; KCl=potassium chloride; KNO_3=potassium nitrate; NO_3^- =nitrate; SBP=systolic blood pressure).*

3.3.2 24 mmol KNO_3 vs. KCl study

Capsules were well tolerated in general, although one subject, who had not taken toast with the capsules, was treated for gastritis after consumption of capsules. This individual was unblinded and withdrawn from the study. Upon unblinding it was discovered that gastritis occurred after taking KCl capsules. All subsequent subjects were made to take toast with the capsules and there were no further adverse effects.

3.3.2.1 Plasma NO_x and cGMP levels

Following ingestion of KNO_3 capsules (24mmol), there was a rapid (within 30 min) increase in plasma NO_3^- levels. These levels peaked at 3 h, ~35-fold higher than basal values, and remained significantly elevated at 24 h post-ingestion (Figure 3.2 A). In contrast, the rise in plasma NO_2^- levels was moderate and followed a slower time-course (Figure 3.2 B). Significantly raised levels were first evident at 1.5 h (though plasma NO_2^- levels were elevated at 1 h post-ingestion of inorganic NO_3^- capsules) and appeared to reach a plateau at ~2.5 h. Levels were sustained at this plateau to 6 h and peaked ~4-fold higher than basal plasma NO_2^- levels. These levels remained significantly elevated at 24 h (Figure 3.2 B). Plasma cGMP levels were significantly raised compared to baseline at 3h and 24h after ingestion of KNO_3 capsules (24mmol) (Figure 3.2 C). There were no significant changes in plasma NO_x or cGMP levels after KCl (24 mmol) ingestion (Figures 3.2 A-C)

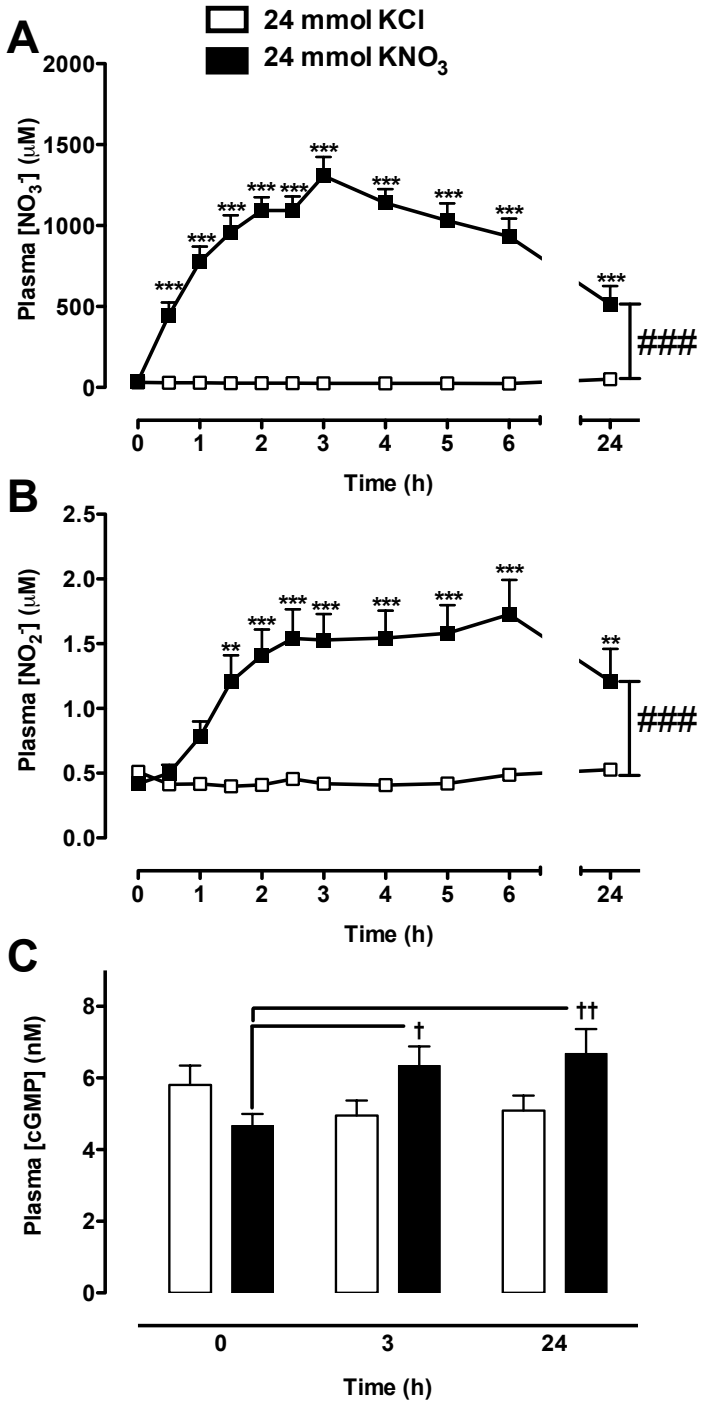


Figure 3.2 *Effect of inorganic NO_3^- supplementation on plasma NO_x levels. The effects of 24 mmol KNO_3 and KCl (control) capsules on plasma (A) NO_3^-, (B) NO_2^- and (C) cGMP levels (n=20). Data are expressed as mean±SEM. Significance shown for comparisons between groups as ###p<0.001 for 2-way ANOVA followed by **p<0.01 and ***p<0.001 for Bonferroni post-hoc tests; and †p<0.05 and ††p<0.01 for Dunnett's post-hoc tests comparison to baseline (t= 0 h) following 1-way ANOVA. (cGMP=cyclic guanosine monophosphate; KCl=potassium chloride; KNO_3=potassium nitrate; NO_2^-=nitrite; NO_3^-=nitrate; NO_x=nitrite/nitrate).*

3.3.2.2 BP and HR

KNO_3 (24mmol) ingestion caused significant reductions in both SBP and DBP over 24 h, compared to KCl control (Figure 3.3 A-B). SBP decreased after 1 h following KNO_3 ingestion, with sustained, significant reductions in SBP and DBP apparent between 2.5-6 h after ingestion. The peak differences between the two limbs were 9.4±1.6 mmHg (at 6 h) and 6.0±1.1 mmHg (at 2.75 h) for SBP and DBP respectively. SBP was still significantly lower 24 h after KNO_3 ingestion compared to KCl control ingestion (change in SBP 6.1±1.2 mmHg) at 24 h. SBP and DBP were not significantly altered after ingestion of KCl control (Figure 3.3 A-B). There was no significant difference in the HR response between the two limbs (Figure 3.3 C).

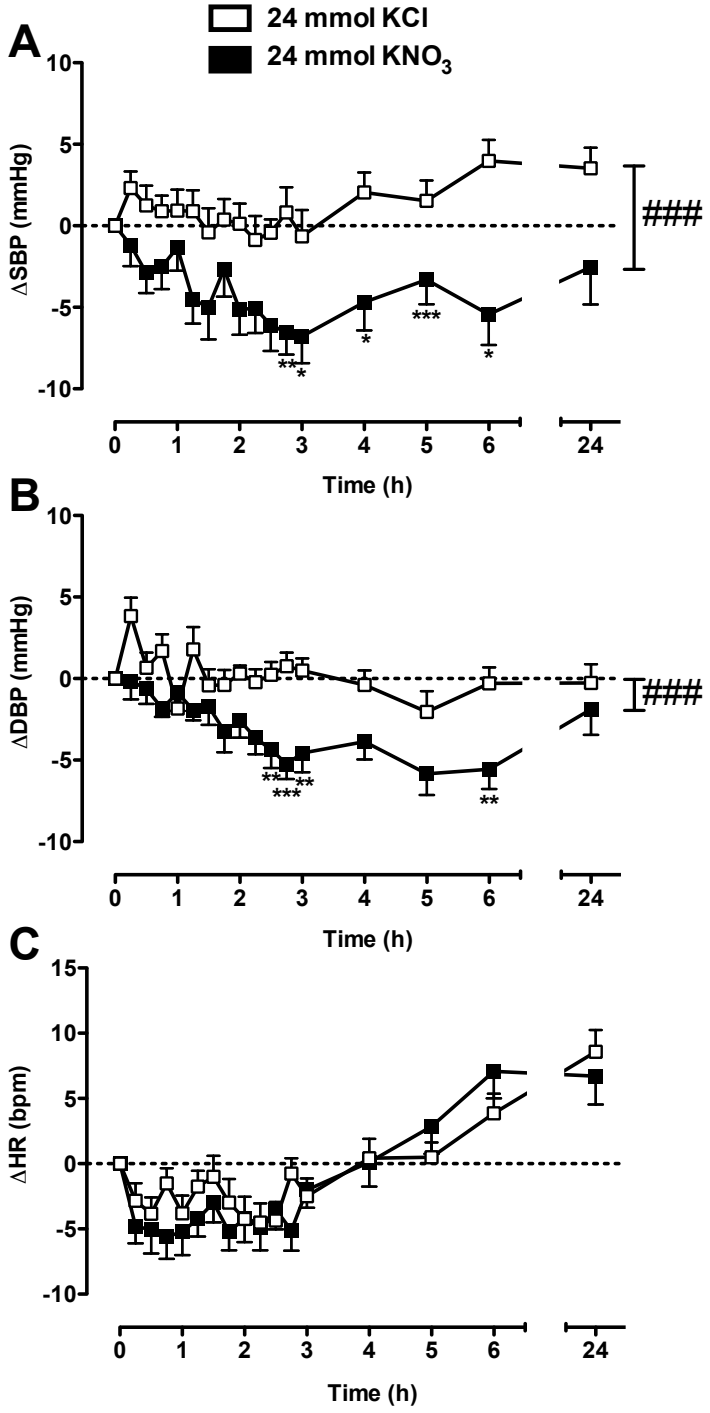


Figure 3.3 *Effect of inorganic NO_3^- supplementation on BP. The effects of 24 mmol KNO_3 and KCl (control) capsules on change from baseline in (A) SBP, (B) DBP and (C) HR (n=20). Data are expressed as mean±SEM. Significance shown for comparisons between groups as ###p<0.001 for 2-way ANOVA followed by *p<0.05, **p<0.01 and ***p<0.001 for Bonferroni post-hoc tests. (BP=blood pressure; DBP=diastolic blood pressure; HR=heart rate; KCl=potassium chloride; KNO_3=potassium nitrate; SBP=systolic blood pressure).*

3.3.2.3 Correlation of changes in BP to plasma NO_x levels and baseline BP

Decreases in SBP following 24 mmol KNO_3 ingestion were inversely correlated to changes in plasma NO_2^- levels (r^2=0.122, p<0.001) but not to changes in plasma NO_3^- levels (p=0.946) (Figure 3.4 A-B). In addition, post-hoc analyses revealed that the peak decreases in BP were also significantly correlated to baseline BP (SBP r^2=0.530, p<0.001; DBP r^2=0.431, p<0.01) (Figure 3.5 A-B). Finally, baseline BP was inversely correlated to baseline NO_2^- levels (r^2=0.139, p<0.05) but not NO_3^- levels (p=0.923) (Figure 3.6 A-B).

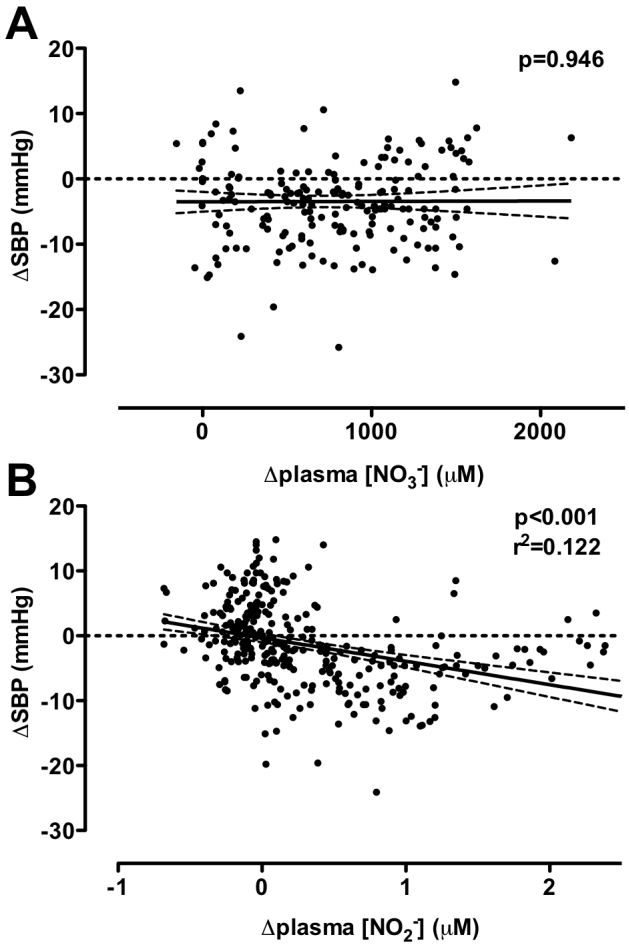


Figure 3.4 *Changes in plasma NO_2^- levels determine reductions in SBP. Correlation of changes in SBP from baseline following 24 mmol KNO_3 ingestion to plasma (A) NO_3^- and (B) NO_2^- levels. All graphs show Pearson's linear regression of best-fit±95% confidence intervals. (BP=blood pressure; KNO_3=potassium nitrate; NO_2^-=nitrite; NO_3^-=nitrate; SBP=systolic blood pressure).*

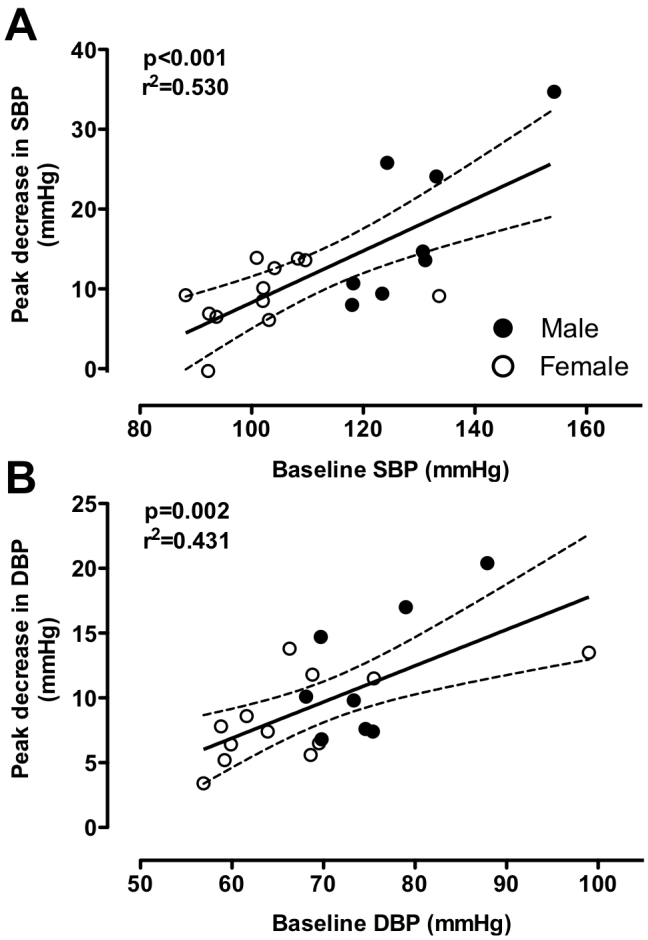


Figure 3.5 *Baseline BP determines magnitude of BP reduction. Correlation of peak changes in (A) SBP and (B) DBP following 24 mmol KNO_3 ingestion to baseline BP. All graphs show Pearson's linear regression of best-fit±95% confidence intervals. (BP=blood pressure; DBP=diastolic blood pressure; KNO_3=potassium nitrate; SBP=systolic blood pressure).*

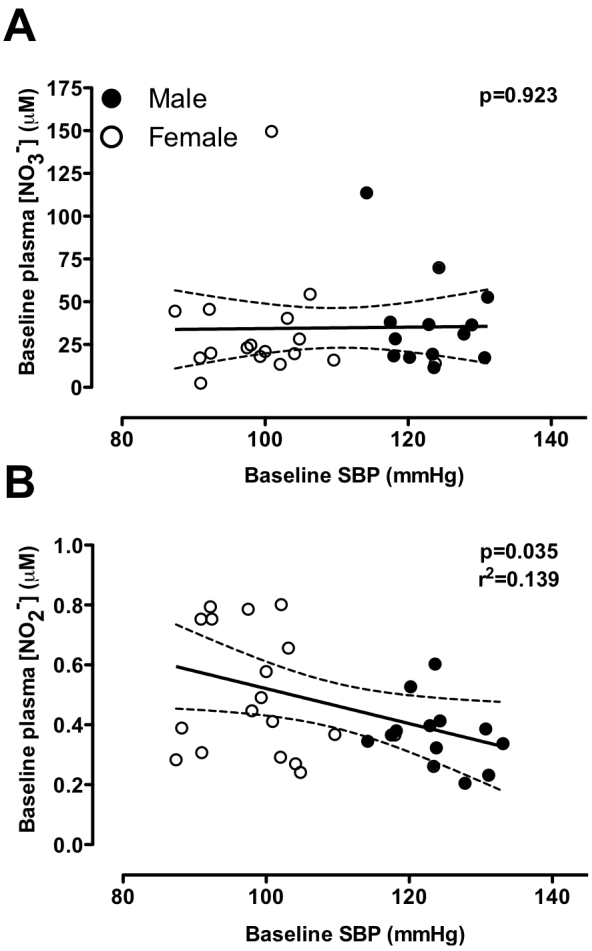


Figure 3.6 *Baseline NO_2^- levels inversely correlate to baseline BP. Correlation of baseline SBP to baseline plasma (A) NO_3^- and (B) NO_2^- levels. All graphs show Pearson's linear regression of best-fit±95% confidence intervals. (BP=blood pressure; KNO_3=potassium nitrate; NO_2^- =nitrite; NO_3^-=nitrate; SBP=systolic blood pressure).*

3.3.2.4 Sex differences in response to inorganic NO_3^- ingestion

Interestingly, the above post-hoc correlations exposed a prominent sex difference in the responses to NO_3^-. Separation of the 24 mmol KNO_3 vs. KCl study data by sex demonstrates that female subjects had significantly lower baseline SBP, DBP and BMI (Table 3.2) compared to the male subjects. In addition, whilst baseline plasma NO_3^- levels were similar between the sexes, plasma NO_2^- levels were significantly higher in the females (table 3.2).

Baseline Characteristics	Male (n=8)	Female (n=12)	Significance
Age (years)	23.0±1.4	22.3±1.2	p=0.70
BMI (kg/m^2)	24.2±1.0	21.4±0.7	p<0.05
SBP (mmHg)	126.4±2.5	101.5+2.3	p<0.001
DBP (mmHg)	73.3±1.4	66.7±2.2	p<0.05
Plasma [NO_3^-] µM	35.0±6.9	33.8±7.9	p=0.91
Plasma [NO_2^-] µM	0.362±0.03	0.536±0.05	p<0.01

Table 3.2 *Sex differences in demographics, baseline haemodynamic characteristics and baseline plasma NO_x levels for 24 mmol KNO_3 vs. KCl study. Significance values for unpaired Student t-test shown in last column. Data expressed as mean±SEM. (BMI=body mass index; DBP=diastolic blood pressure; KCl=potassium chloride; KNO_3=potassium nitrate; NO_2^- =nitrite; NO_3^-=nitrate; NOx=nitrite/nitrate; SBP=systolic blood pressure).*

Additionally, the rise in plasma NO_x levels in males following KNO_3 ingestion appeared significantly lower compared to females (Figure 3.7 A-B). However, the fold increases in plasma NO_2^- from baseline were similar (~3.3 and ~4.1-fold for males and females respectively). Conversely, KNO_3-induced reduction in SBP and DBP was substantially greater in males compared to females (Figure 3.8 A-B). There were no significant effects on HR (Figure 3.8 C). No sex differences in the response to KCl with respect to SBP, DBP or HR were found (Figure 3.9 A-C).

The dose/kg of NO_3^- administered to females was 0.45±0.02 mmol/kg and for males was 0.32±0.021 mmol/kg. After adjusting changes in plasma NO_x levels for body weight, there was no significant difference in changes to plasma NO_3^- levels (Figure 3.10 A). However, females had significantly higher adjusted plasma NO_2^- levels over the 24 h study compared to males and appeared to be able to sustain the elevation much longer than males, though there were no significant differences at any time-points by Bonferroni post-hoc tests (Figure 3.10 B).

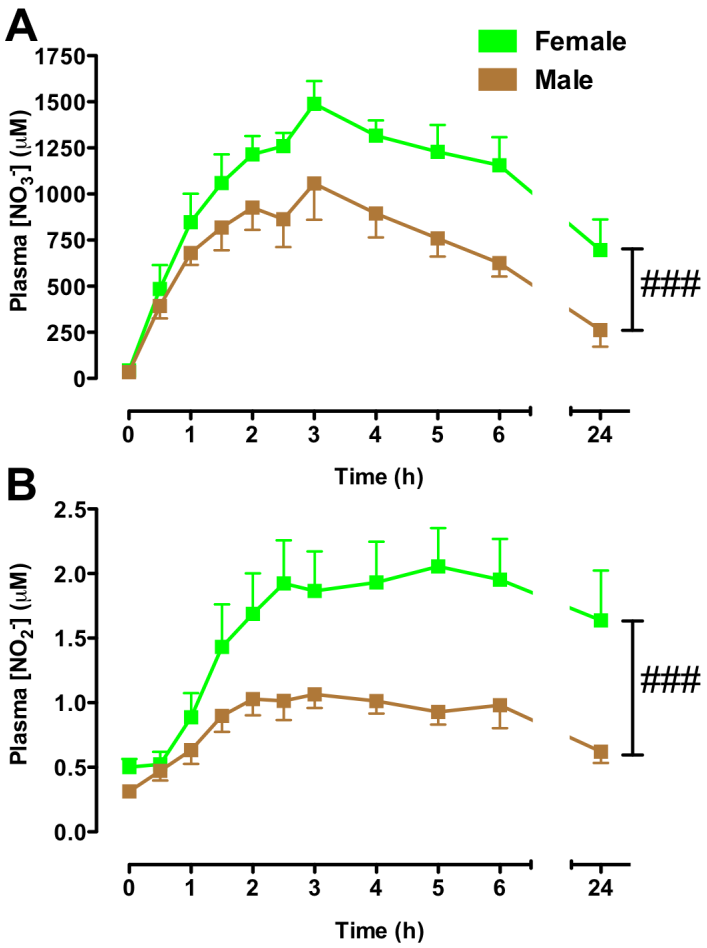


Figure 3.7 *Sex differences in plasma NO_x levels after inorganic NO_3^- supplementation. The effects of KNO_3 (24mmol) on plasma (A) NO_3^- and (B) NO_2^- levels in males and females. Data are expressed as mean±SEM (males n=8; females n=12). Significance shown for comparisons between groups as ###p<0.001 for 2-way ANOVA. (KNO_3=potassium nitrate; NO_2^-=nitrite; NO_3^-=nitrate; NO_x=nitrite/nitrate).*

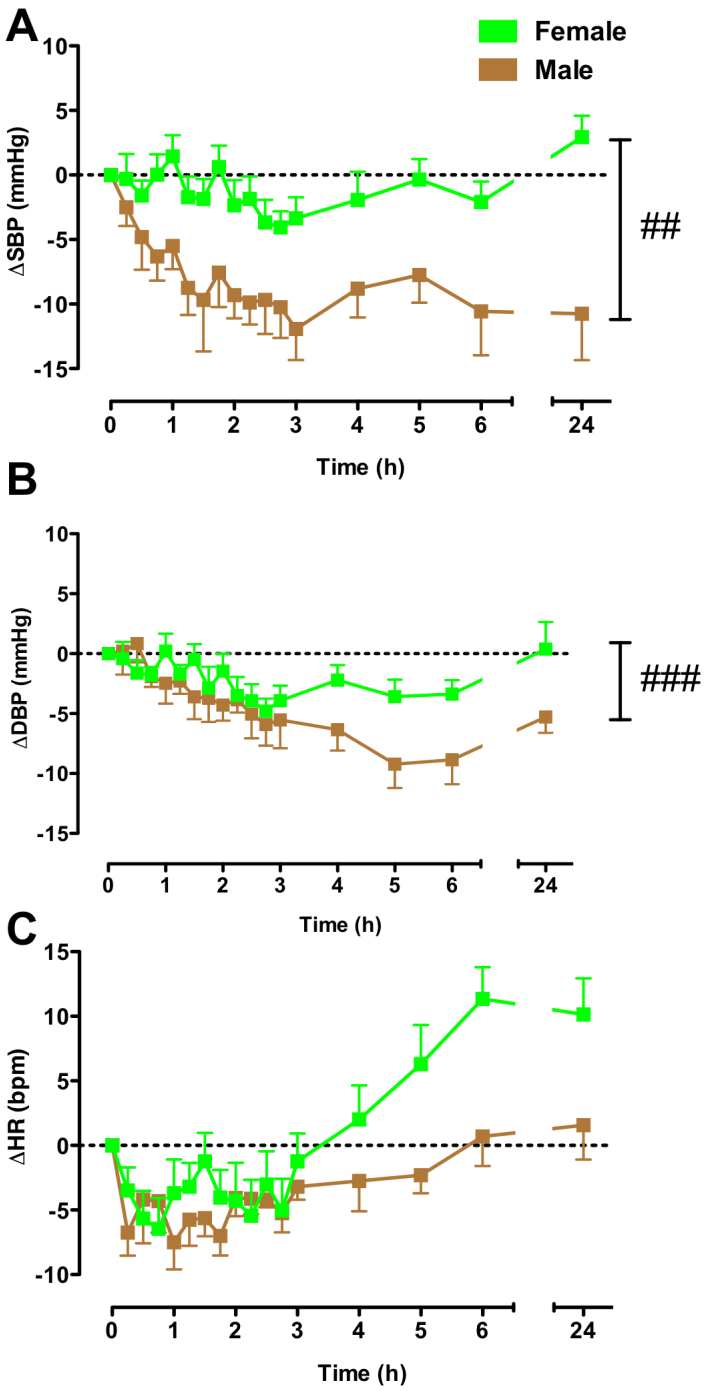


Figure 3.8 *Sex differences in BP after inorganic NO_3^- supplementation. The effects of KNO_3 (24mmol) on change from baseline in (A) SBP, (B) DBP and (C) HR in males and females. Data are expressed as mean±SEM (males n=8; females n=12). Significance shown for comparisons between groups as ##p<0.01, ###p<0.001 for 2-way ANOVA. (DBP=diastolic blood pressure; HR=heart rate; KNO_3=potassium nitrate; SBP=systolic blood pressure).*

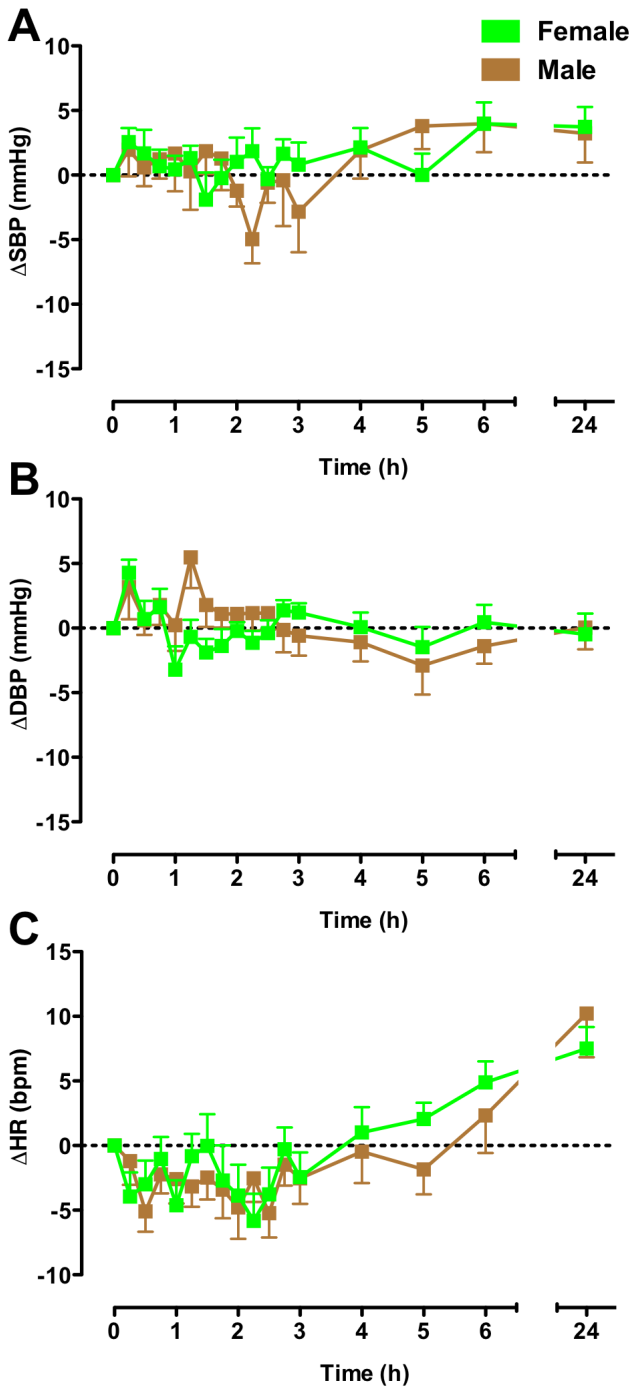


Figure 3.9 *Sex differences in BP after placebo. The effects of KCl control (24mmol) on change from baseline in (A) SBP, (B) DBP and (C) HR in males and females. Data are expressed as mean±SEM (males n=8; females n=12). (DBP=diastolic blood pressure; HR=heart rate; KCl=potassium chloride; SBP=systolic blood pressure).*

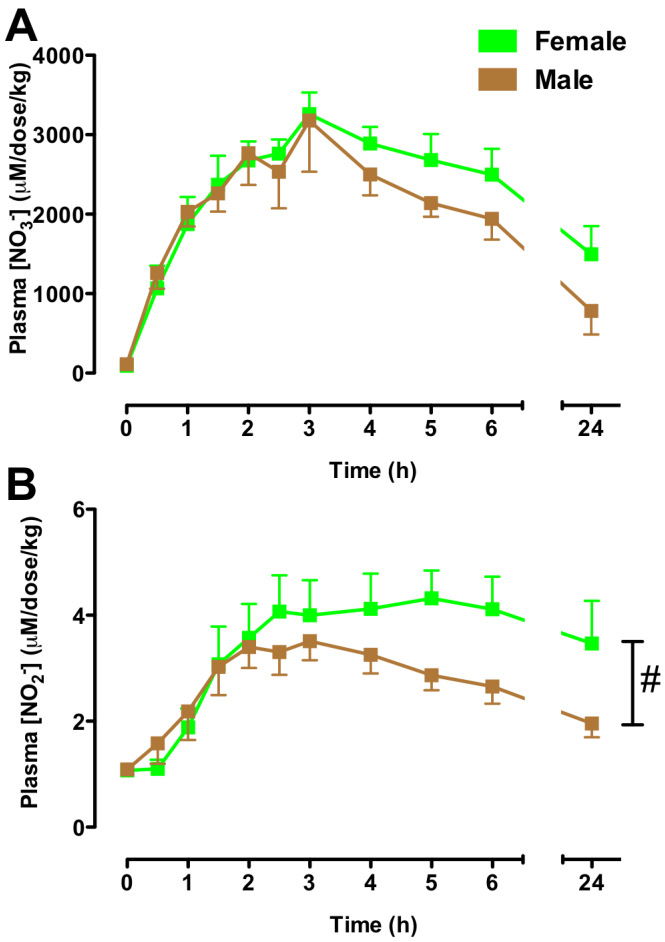


Figure 3.10 *Weight-normalized plasma NO_x levels by sex. Plasma (A) NO_3^- and (B) NO_2^- levels relative to dose/kg administered following 24mmol KNO_3 administration. Data are expressed as mean±SEM (males n=8; females n=12). Significance shown for comparisons between groups as #p<0.05 for 2-way ANOVA. (KNO_3=potassium nitrate; NO_2^-=nitrite; NO_3^-=nitrate; NO_x=nitrite/nitrate).*

3.3.3 Inorganic NO_3^- dose-response study

KNO_3 (4 and 12 mmol) capsules were well tolerated.

3.3.3.1 Plasma NO_x levels

The effects of KNO_3 were dose-dependent with plasma NO_3^- levels elevated above baseline by 7 and 27-fold after administration of 4 and 12 mmol of KNO_3 respectively (Figure 3.11 A). The rises in plasma NO_2^- levels also showed dose-dependency, albeit with a more moderate rise of 1.3 and 2.0–fold increase respectively (Figure 3.11 B).

3.3.3.2 Dose-dependent decreases in BP following inorganic NO_3^- ingestion

The effect of KNO_3 on BP was dose-dependent (Figure 3.12 A-B). Peak decreases in BP after 4mmol KNO_3 were ~2.6/4.6 mmHg, whilst there were greater decreases in BP after ingestion of 12 mmol KNO_3 of ~5.9/4.6 mmHg. 3 h after capsule consumption, changes from baseline in BP were 2.0/2.2 and 5.5/4.6 mmHg after 4 and 12 mmol KNO_3 respectively. There were no significant effects of either dose of KNO_3 on HR (Figure 3.12 C).

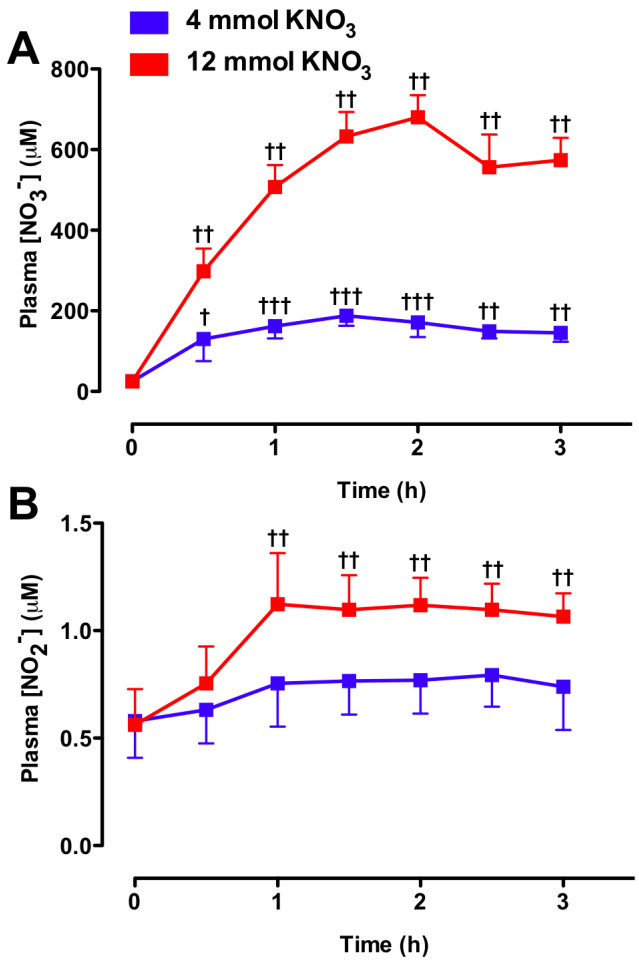


Figure 3.11 *Dose-dependent effect of inorganic NO_3^- on plasma NO_x levels. The effects of 4mmol and 12mmol of KNO_3 on plasma (A) NO_3^- and (B) NO_2^- levels (n=6). Data are expressed as mean±SEM. Significance shown for comparisons as †p<0.05, ††p<0.01 and †††p<0.001 for Dunnett's post-hoc test comparison to baseline (t= 0 h) following 1-way ANOVA (KNO_3=potassium nitrate; NO_2^-=nitrite; NO_3^-=nitrate; NO_x=nitrite/nitrate).*

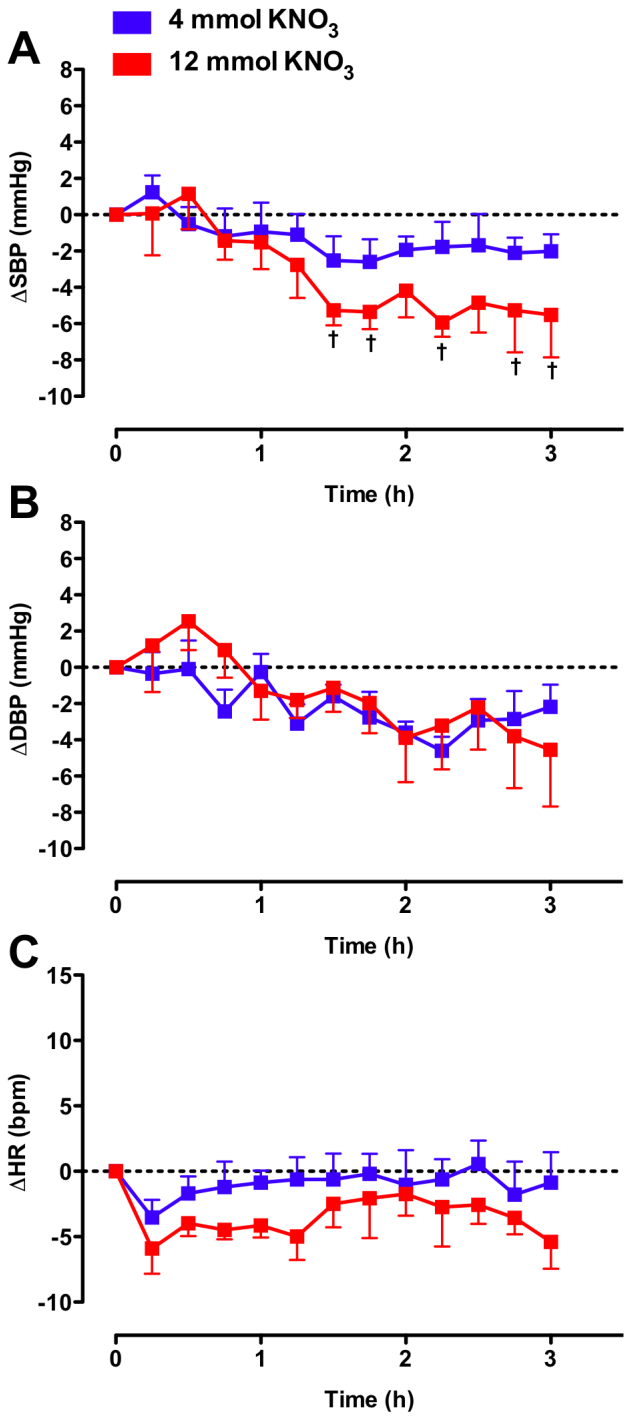


Figure 3.12 *Dose-dependent effect of inorganic NO_3^- on BP. The effects of 4mmol and 12mmol of KNO_3 on change from baseline in (A) SBP, (B) DBP and (C) HR (n=6). Data are expressed as mean±SEM. Significance shown for comparisons as †p<0.05 for Dunnett's post-hoc test comparison to baseline (t= 0 h) following 1-way ANOVA (DBP=diastolic blood pressure; HR=heart rate; KNO_3=potassium nitrate; NO_3^-=nitrate; SBP=systolic blood pressure).*

3.3.4 Dietary NO_3^- study

Dietary NO_3^- was generally well tolerated by the subjects. The [NO_3^-] in the dietary NO_3^- was 22.4±3.8 mM, whereas [NO_2^-] was <50nM (n=9).

3.3.4.1 Effects of reduced dose of dietary NO_3^- on plasma NO_x levels

Following dietary NO_3^- ingestion (~5.5 mmol NO_3^- dose) plasma NO_3^- levels rose rapidly, peaking at 1 h post-ingestion ~11-fold higher than basal plasma NO_3^- levels (Figure 3.13 A). Plasma NO_3^- levels remained elevated in a sustained manner over the entire 3 h time course, compared to water control (Figure 3.13 A). Plasma NO_2^- levels also increased after NO_3^- ingestion, peaking at 2.5 h with an ~1.6-fold rise above baseline levels and also remaining significantly elevated over the 3 h time course compared to water control (Figure 3.13 B). In addition, cGMP levels were elevated at 3 h compared to baseline after dietary NO_3^- ingestion compared to water control (Figure 3.13 C). There were no significant changes in plasma NO_x or cGMP levels after water control ingestion (Figure 3.13 A-C).

3.3.4.2 Effects of reduced dose of dietary NO_3^- on BP and HR

SBP decreased with a peak reduction in SBP of 5.4±1.5 mmHg compared to baseline and 7.2±1.8 mmHg compared to water control at 3 h post-ingestion (Figure 3.14 A). There were no significant changes in either DBP or HR after dietary NO_3^- or water control ingestion (Figure 3.14 B-C).

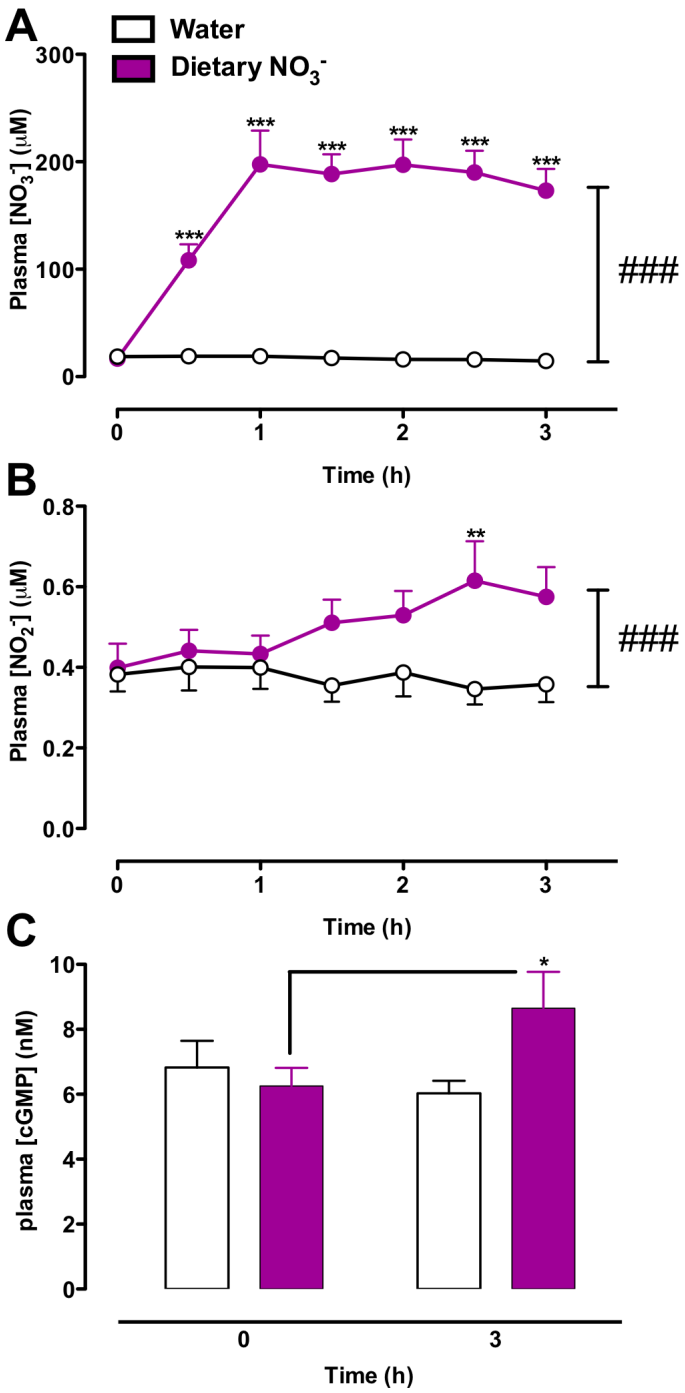


Figure 3.13 *Dietary NO_3^- supplementation raises plasma NO_x levels The effects of dietary NO_3^- (250 mL as beetroot juice; 5.5mmol NO_3^-) or water control on plasma (A) NO_3^-, (B) NO_2^- and (C) cGMP levels. Data are expressed as mean±SEM (n=9). Significance shown for comparisons between groups as ###p<0.001 for 2-way ANOVA, and by **p<0.01, ***p<0.001 for Bonferroni post-hoc tests following 1-way or 2-way ANOVA. (cGMP=cyclic guanosine monophosphate; NO_2^-=nitrite; NO_3^-=nitrate).*

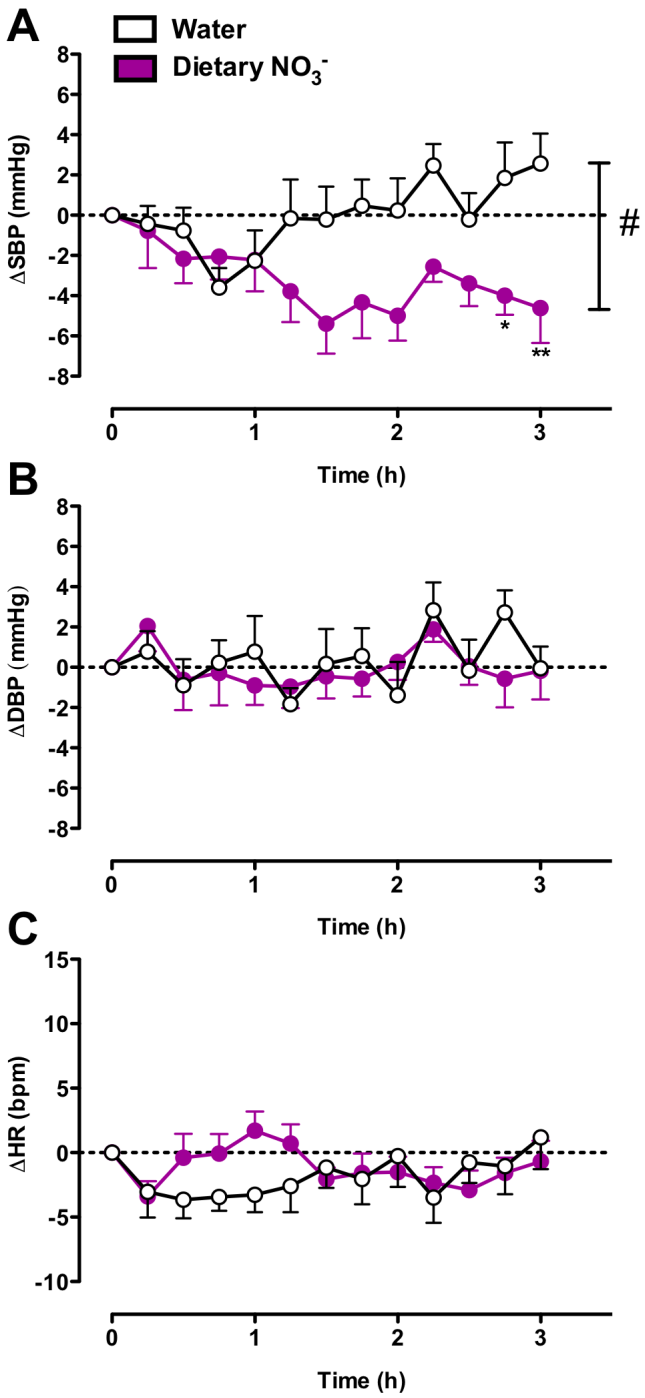


Figure 3.14 *Dietary NO_3^- lowers BP. The effects of dietary NO_3^- (250 mL as beetroot juice; 5.5mmol NO_3^-) or water control on plasma (A) SBP, (B) DBP and (C) HR. Data are expressed as mean±SEM (n=9). Significance shown for comparisons between groups as #p<0.05 for 2-way ANOVA followed by *p<0.05 and **p<0.01, for Bonferroni post-hoc tests. (BP=blood pressure; DBP=diastolic blood pressure; HR=heart rate; NO_3^-=nitrate; SBP=systolic blood pressure).*

3.4 Results from amalgamation of data

3.4.1 Baseline cGMP levels determine baseline SBP in healthy subjects

Amalgamating all the subjects across the two studies (n=29) in which cGMP levels were measured (24 mmol KNO_3 vs. KCl and dietary NO_3^- studies), baseline SBP inversely correlated with plasma cGMP levels at baseline ($p=0.024$, $r^2=0.203$ Figure 3.15 A) but baseline DBP was not significantly correlated to plasma cGMP levels at baseline ($p=0.053$, $r^2=0.153$, Figure 3.15 B) though there equally appeared to be a trend towards this.

3.4.2 BP-lowering effects of NO_3^- are dose-dependent

For increasing doses of inorganic NO_3^-, in the form of KNO_3 capsules (4, 12 and 24mmol) or from increasing doses of dietary NO_3^- as beetroot juice (250mL, ~5.5mmol NO_3^-, there is a graded reduction in SBP irrespective of the formulation of NO_3^- up to 3 h post-ingestion (Figure 3.16).

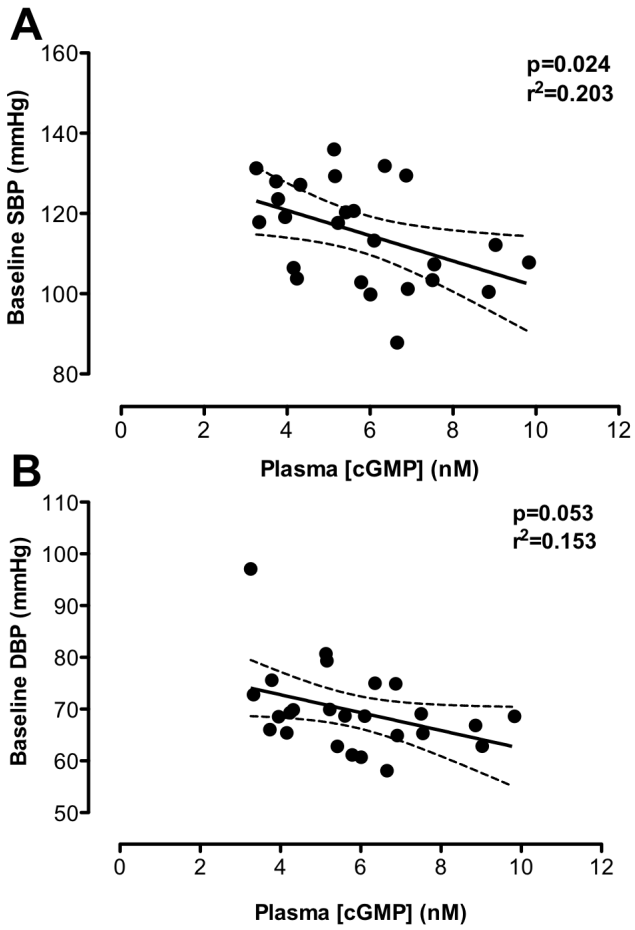


Figure 3.15 *Plasma cGMP levels inversely correlate with baseline BP. Correlation of baseline cGMP levels to (A) baseline SBP and (B) baseline DBP. All graphs show Pearson's linear regression of best-fit±95% confidence intervals (n=29 from 2 separate studies). (BP=blood pressure; cGMP=cyclic guanosine monophosphate; DBP=diastolic blood pressure; SBP=systolic blood pressure).*

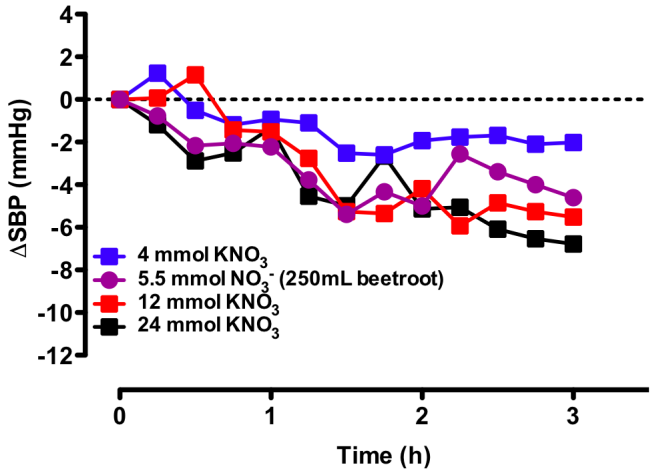


Figure 3.16 *Inorganic or dietary NO_3^- supplementation lowers SBP in a dose-dependent manner. The effects of KNO_3 (4, 12 or 24mmol) and dietary NO_3^- as beetroot juice (250 mL, ~5.5 mmol NO_3^-) on change in SBP from baseline over 3 h. (n=6-20). Data are expressed as mean only. (KNO_3=potassium nitrate; NO_3^-=nitrate; SBP=systolic blood pressure).*

3.5 Summary

1. Inorganic NO_3^- supplementation mimics the effects of dietary NO_3^-, causing
    a. Rapid elevations in plasma NO_3^- levels.
    b. Delayed, sustained elevations in plasma NO_2^- levels.
    c. Significant reductions in both SBP and DBP.
2. Inorganic NO_3^- supplementation is associated with increases in plasma cGMP levels.
3. Baseline BP and changes in BP are inversely correlated to NO_2^-, but not NO_3^-, levels.
4. Sex differences exist in plasma NO_2^- levels at baseline and after NO_3^- supplementation.
5. Inorganic NO_3^- supplementation causes dose-dependent increases in plasma NO_x levels, and dose-dependent decreases in BP.
6. A reduced dose of dietary NO_3^- causes elevations in plasma NO_x and cGMP levels and reductions in SBP.
7. Overall, NO_3^- whether in salt form or dietary form reduces BP in a dose-dependent manner.

CHAPTER 4

Investigation of the effect of interrupting the entero-salivary circulation on oral NO_3^- reductase activity, systemic NO_2^- levels and BP in healthy subjects

4.1 Introduction

There is now much evidence, including that presented in chapter 3, that inorganic (Larsen *et al.*, 2006) and dietary (Webb *et al.*, 2008a) NO_3^- lowers BP through the elevation of systemic NO_2^- levels in humans. Moreover, the importance of oral conversion to this process has been established in a few human studies that demonstrate that interruption of the entero-salivary circulation, by the avoidance of swallowing, prevents the elevations in systemic NO_2^- levels after NO_3^- ingestion (Lundberg and Govoni, 2004; Webb *et al.*, 2008a) and the BP-lowering effects thereof (Webb *et al.*, 2008a). However, up to ~1 mmol of NO_3^- is synthesized in humans on a daily basis (Green *et al.*, 1981) by the oxidation of NO produced by the action of NOS enzymes. Thus, under basal conditions without NO_3^- supplementation, this endogenously-derived NO_3^- should still enter the entero-salivary circulation to produce NO_2^- that may contribute not only to basal plasma NO_2^- levels but, since NO_2^- is bioactive in the circulation, may also regulate BP.

Thus, the aim of this study was to establish the basal rate of oral NO_3^- reductase activity at different salivary NO_3^- levels that may be encountered in normal physiological situations. In addition, the impact of disrupting the oral microflora responsible for oral NO_3^- reductase activity on systemic NO_2^- levels and BP. Lastly, the influence of sex on the above responses was also investigated.

4.2 Protocol

For this oral NO_3^- reductase activity study, the following *exclusion criteria* were used to those previously stated:

1. Self-reported use of mouthwash or tongue scrapes.
2. Recent or current antibiotic use (within 3 months).
3. History, or recent treatment of (within last 3 months) of any oral condition (excluding caries), including gingivitis, periodontitis and halitosis.

Design: To assess the contribution of the entero-salivary circulation of NO_3^- to NO_2^- (under basal conditions) to systemic NO_2^- levels, and to determine the impact of interruption of this pathway on the regulation of basal BP, an open-label, cross-over study of a chlorhexidine-based, antiseptic mouthwash was performed in healthy subjects. Subjects were instructed to instill 10mL 0.2% chlorhexidine-based mouthwash (Corsodyl™, purchased from GlaxoSmithKilne Company, Stevenage, UK) orally and hold in the oral cavity for 1 min, twice daily, 12 h apart for 7 days in the 2nd week of the oral NO_3^- reductase study to disrupt the activity of the oral microflora (Petersson *et al.*, 2009).

Power analysis: Previous data from the 24 mmol KNO_3 vs. KCl study gave a baseline value of plasma NO_2^- levels of 0.418 µM in 20 healthy subjects. Based upon this value, it was postulated that there would be a 30% reduction following repression of the bacterial NO_3^--reductase pathway, as according to published evidence it is thought that 30% of plasma NO_2^- levels is not derived from eNOS-derived oxidation of NO (Kleinbongard *et al.*, 2003). Therefore a change in plasma NO_2^- levels of 0.126 µM (SD=0.169) was expected. Based on these values, with an α=0.05 and 1-β=0.9 power calculations determined that n=22 is required. 2 extra subjects were

recruited to account for potential drop-outs, thus the final number recruited was n=24.

Measurements: On arrival, baseline clinic BP was measured (section 2.4.1). Following this, baseline blood, urine and whole saliva samples were collected (sections 2.6-2.8). Oral NO_3^- reductase capacity was ascertained (section 2.9) and then a 24 h ABP monitor attached (section 2.4.2), to be returned the following day. Subjects were then instructed to measure BP on a daily basis whilst at home (home BP) for 2 weeks (section 2.4.3). However, after 7 days, subjects were instructed to introduce rinsing of their oral cavities with 10 mL Corsodyl™ antiseptic mouthwash twice daily for the remaining 7 days. After this time subjects returned for a repeat assessment of clinic BP measurement, blood, urine and whole saliva sample collection and oral NO_3^- reductase capacity assessment. A further 24 h ABP monitor was attached and returned the following day (Figure 4.1). Subjects recorded daily nutritional intake during the 2 weeks using self-reported dietary diaries. The subjects were not aware of the hypothesis of the study.

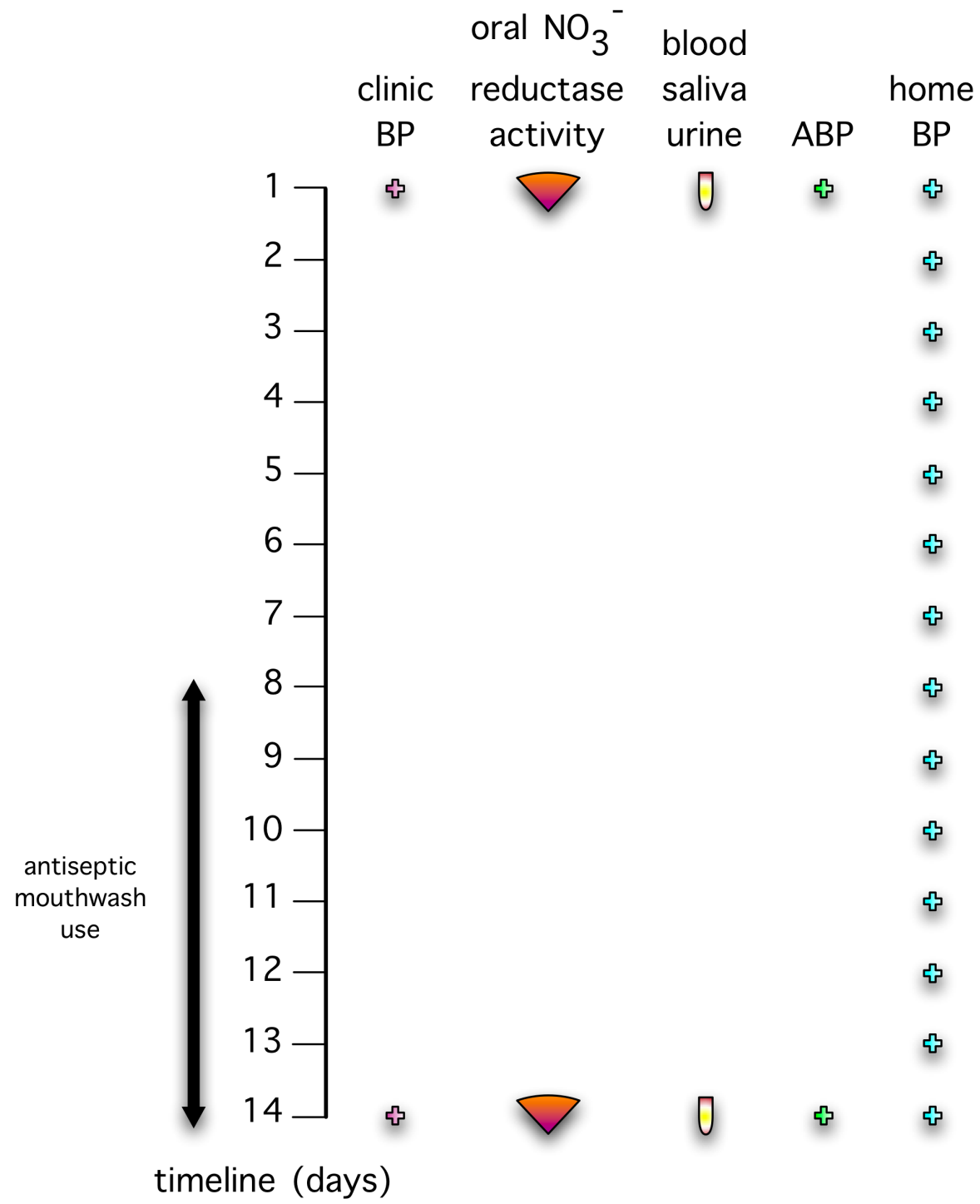


Figure 4.1 *Timeline for oral NO_3^- reductase activity study. (ABP=ambulatory blood pressure; BP=blood pressure; NO_3^-=nitrate).*

4.3 Results

4.3.1 General characteristics

24 healthy subjects were recruited; 5 dropped out during the course of the study (2 were unable to complete 2 weeks of home BP monitoring, 3 withdrew voluntarily), hence 19 subjects completed the protocol (see Table 4.1 for baseline demographics). Power analyses with this sample size estimated 1-β=0.86. BP measured by 24 h ABP was significantly greater than clinic or home SBP ($p<0.05$ and $p><0.001$ for Bonferroni post-hoc test following 1-way ANOVA, Table 4.1). Similarly, DBP measured by 24 h ABP was greater than clinic DBP ($p<0.01$) but not home BP (Table 4.1). Estimated daily portion intake of fruit, high-NO_3^- containing vegetables, low-NO_3^-containing vegetables (Hord *et al.*, 2009) and processed meat did not differ between the two study periods (Table 4.2).

Baseline characteristic			
Subjects (n)	19 (8 female)		
Age (years)	23.8±0.5		
BMI (kg/m^2)	23.0±0.6		
Clinic SBP (mmHg)	110.4±1.8	Plasma [NO_3^-] (µM)	26.7±2.4
Clinic DBP (mmHg)	66.2±1.6	Plasma [NO_2^-] (nM)	284.2±16.9
Home SBP (mmHg)	115.3±1.7	Salivary [NO_3^-] (µM)	441.8±68.6
Home DBP (mmHg)	67.3±1.1	Salivary [NO_2^-] (µM)	316.1±30.7
Ambulatory SBP (mmHg)	119.2±1.6	Urinary [NO_3^-] µM	1462.0±64.9
Ambulatory DBP (mmHg)	70.3±1.3	Urinary [NO_2^-] nM	203.1±8.8

Table 4.1 *Baseline characteristics in oral NO_3^- reductase activity study. Demographics, haemodynamics and baseline NO_x levels. Data are expressed as mean±SEM (n=19). (BMI=body mass index; DBP=diastolic blood pressure; NO_3^-=nitrate; NO_2^-=nitrite; NO_x=nitrite/nitrate; SBP=systolic blood pressure).*

Food group (mean portions/day)	**Baseline**	**Post-mouthwash**	**Significance (p)**
Fruit	0.5±0.2	0.5±0.2	0.205
Low-NO_3^- vegetables	0.6±0.1	0.6±0.1	0.928
High-NO_3^- vegetables	0.2±0.1	0.2±0.1	0.245
Processed meat	0.7±0.1	0.7±0.1	0.793

Table 4.2 *Self-reported daily food estimates in oral NO_3^- reductase activity study. Specific food groups mean portion daily intake in the 7 day study periods. Data are expressed as mean±SEM (n=19). Significance shown in last column for paired Student's t-test. (NO_3^- =nitrate).*

4.3.2 Antiseptic mouthwash use abrogates oral NO_3^- reductase activity

Prior to mouthwash treatment, oral NO_3^- reduction was found to be concentration dependent ($p<0.001$) with levels increasing in a linear fashion with respect to time the solutions were held in the oral cavity (Figure 4.2 A). The calculated rates of reduction increased with increasing NO_3^- concentration (Table 4.3). After antiseptic mouthwash use for 7 days, oral NO_3^- reduction was near abolished (~90% lower, $p<0.001$ for all solutions compared to baseline rates by 2-way ANOVA, Figure 4.2 B and Table 4.3 for calculated rates).

	Rate of oral NO_3^- reductase activity (nmol/min)	
Solution	**Baseline**	**Post-mouthwash**
NO_x-free water	19±3	2±1
0.8 mM [NO_3^-]	113±25	11±3
8 mM [NO_3^-]	212±31	28±7

Table 4.3 *Rates of oral NO_3^- reductase activity at baseline and after 7 days antiseptic mouthwash use. Data are expressed as mean±SEM (n=19). (NO_3^-=nitrate; NO_x=nitrite/nitrate).*

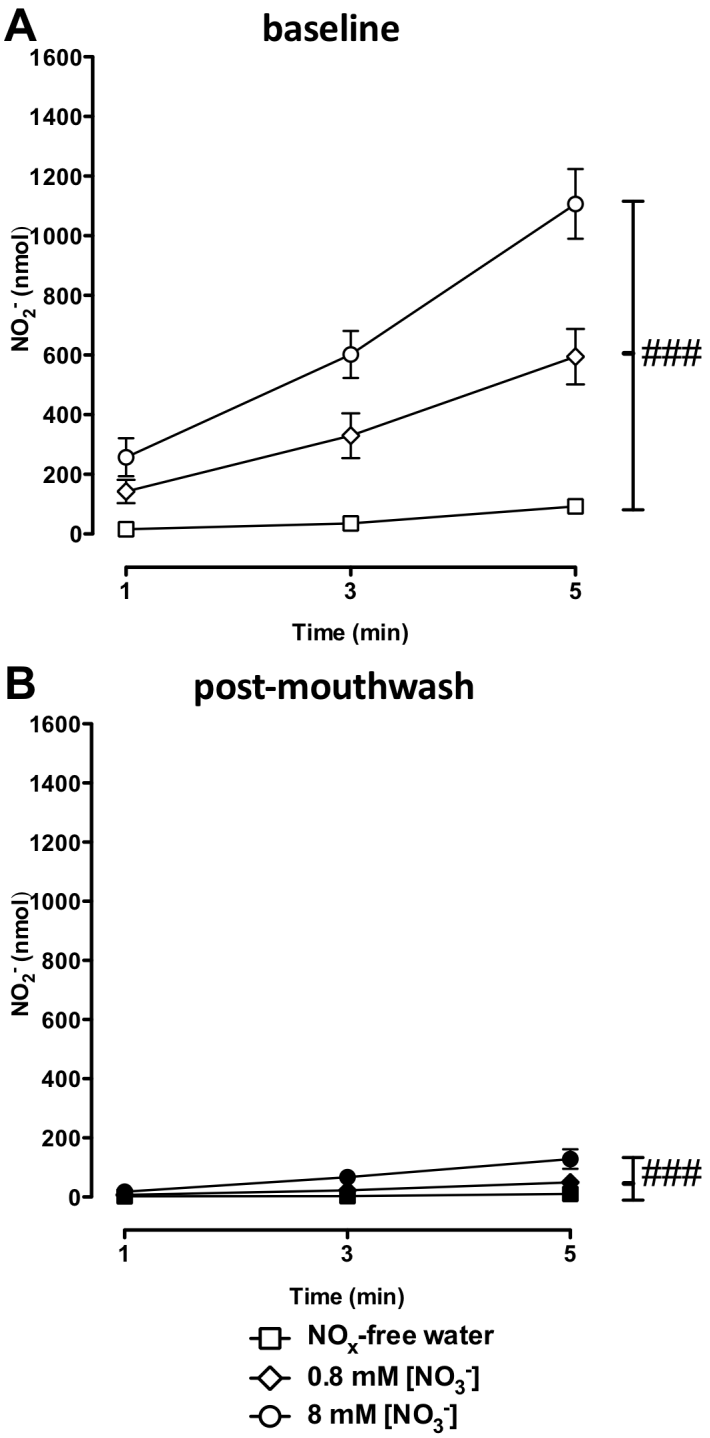


Figure 4.2. *Antiseptic mouthwash use abrogates oral NO_3^- reductase ability. Oral NO_3^- reductase activity after instillation of NO_3^--containing solutions for 1-5 min at (A) baseline and (B) after daily use of antiseptic mouthwash for 7 days. Data are expressed as mean±SEM (n=19). Significance shown as ###p<0.001 for 2-way ANOVA. (NO_3^-=nitrate NO_2^-=nitrite; NO_x=nitrite/nitrate).*

4.3.3 Antiseptic mouthwash use attenuates systemic NO_2^- levels

Following use of antiseptic mouthwash for 7 days, salivary, urinary and plasma NO_2^- levels were significantly attenuated (Figure 4.3 A-C). Salivary NO_2^- levels were reduced by ~90% (change in salivary NO_2^- levels: 282±35 μM, $p<0.001$). Plasma and urinary NO_2^- levels were similarly reduced by ~25% (change in plasma NO_2^- levels: 71±15 nM, $p<0.001$; and change in urinary NO_2^- levels: 51±12 nM, $p<0.001$ respectively). In contrast, salivary, plasma and urinary NO_3^- levels were significantly elevated after mouthwash treatment compared to baseline levels (Figure 4.4 A-C).

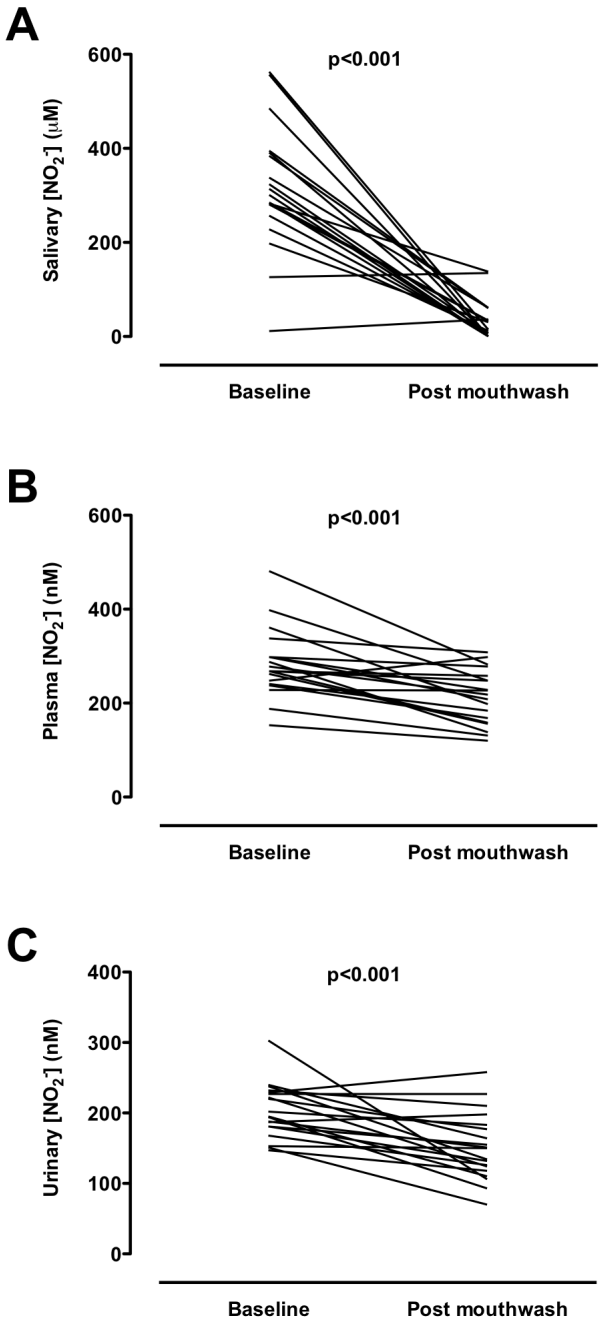


Figure 4.3 *Disruption of oral NO_3^- reductase activity reduces systemic NO_2^- levels. The effect of daily antiseptic mouthwash use for 7 days on (A) salivary (B) plasma and (C) urinary NO_2^- levels. Data are expressed as mean±SEM (n=19). Significance shown for paired Student's t-test. (NO_3^-=nitrate; NO_2^-=nitrite).*

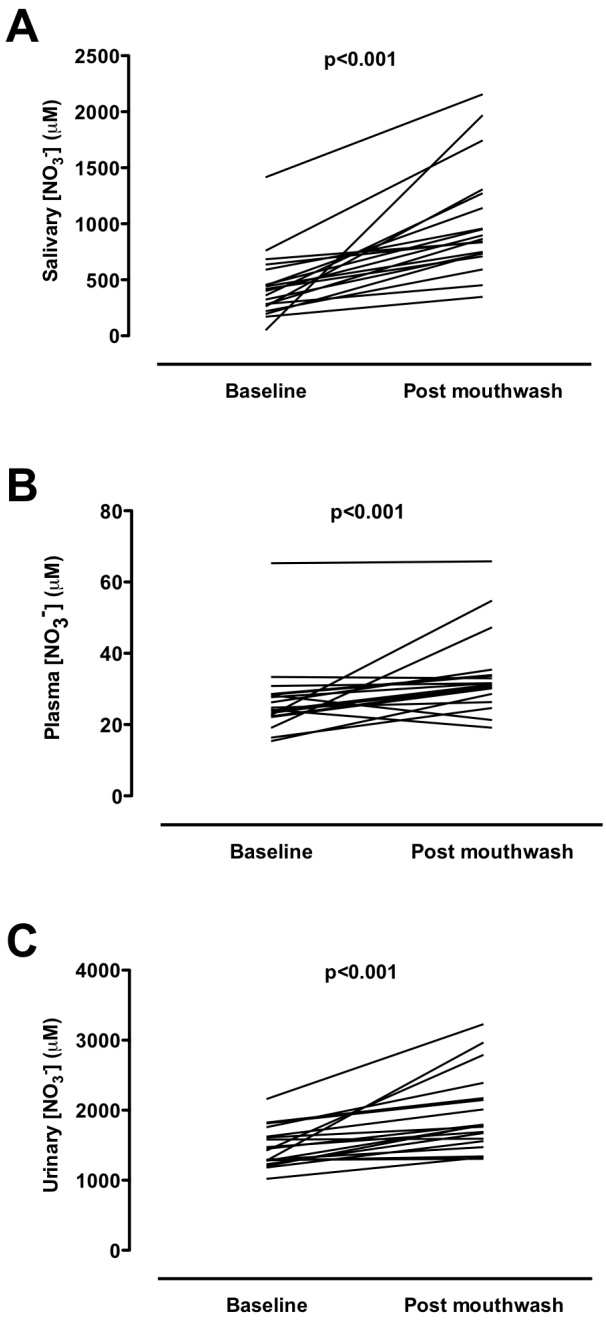


Figure 4.4 *Disruption of oral NO_3^- reductase activity increases systemic NO_3^- levels. The effect of daily antiseptic mouthwash use for 7 days on (A) salivary (B) plasma and (C) urinary NO_3^- levels. Data are expressed as mean±SEM (n=19). Significance shown for paired Student's t-test. (NO_3^-=nitrate).*

4.3.4 Disruption of the entero-salivary circulation elevates BP

Daily use of antiseptic mouthwash for 7 days was associated with significant increases in both SBP and DBP (Figure 4.5-4.7). These changes in BP were of similar magnitude, irrespective of the method of BP measurement (Table 4.4). There were no significant changes in HR following mouthwash use (Table 4.5).

	Method of BP measurement			
	Clinic	**Home**	**ABP**	**Significance (p)**
Change in SBP (mmHg)	3.5±1.1	2.9±0.4	2.4±0.9	0.542
Change in DBP (mmHg)	2.2±1.0	2.0±0.5	2.2±0.8	0.984

Table 4.4 *Change in BP following antiseptic mouthwash use for 7 days. Data are expressed as mean±SEM (n=19). Significance shown for 1-way ANOVA. (BP=blood pressure; DBP-diastolic blood pressure; SBP=systolic blood pressure).*

	Method of HR measurement		
	Clinic	**Home**	**ABP**
Change in HR (bpm)	2.0±1.7	1.3±1.0	2.4±0.9
Significance (p)	0.252	0.179	0.172

Table 4.5 *Change in HR following antiseptic mouthwash use for 7 days. Data are expressed as mean±SEM (n=19). Significance shown for paired Student's t-test, comparing HR at baseline to HR after antiseptic mouthwash use for 7 days. (bpm=beats per min; HR=heart rate).*

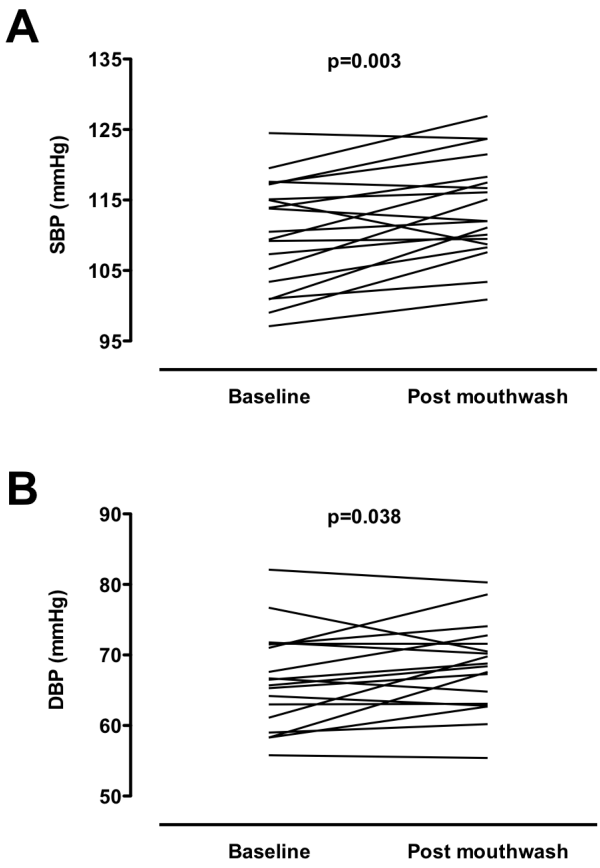


Figure 4.5 *Effects of antiseptic mouthwash use on clinic BP. Clinic (A) SBP and (B) DBP at baseline and following 7 days use of antiseptic mouthwash. Data are expressed as mean±SEM (n=19) and statistical significance determined using paired Student's t-test. (BP=blood pressure; DBP=diastolic blood pressure; SBP=systolic blood pressure).*

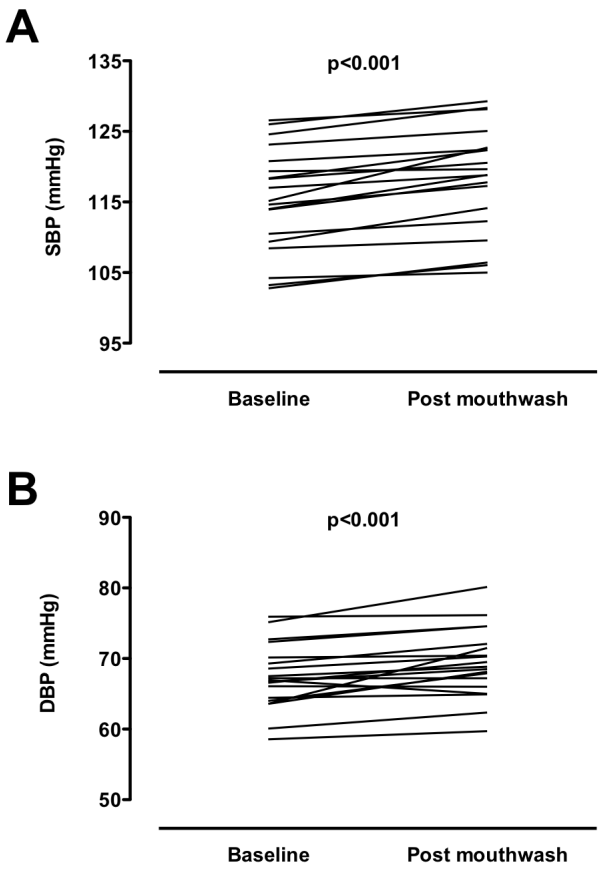


Figure 4.6 *Effects of antiseptic mouthwash use on home BP. Home (A) SBP and (B) DBP prior to and during 7 days use of antiseptic mouthwash. Data are expressed as mean±SEM (n=19) and statistical significance determined using paired Student's t-test. (BP=blood pressure; DBP=diastolic blood pressure; SBP=systolic blood pressure).*

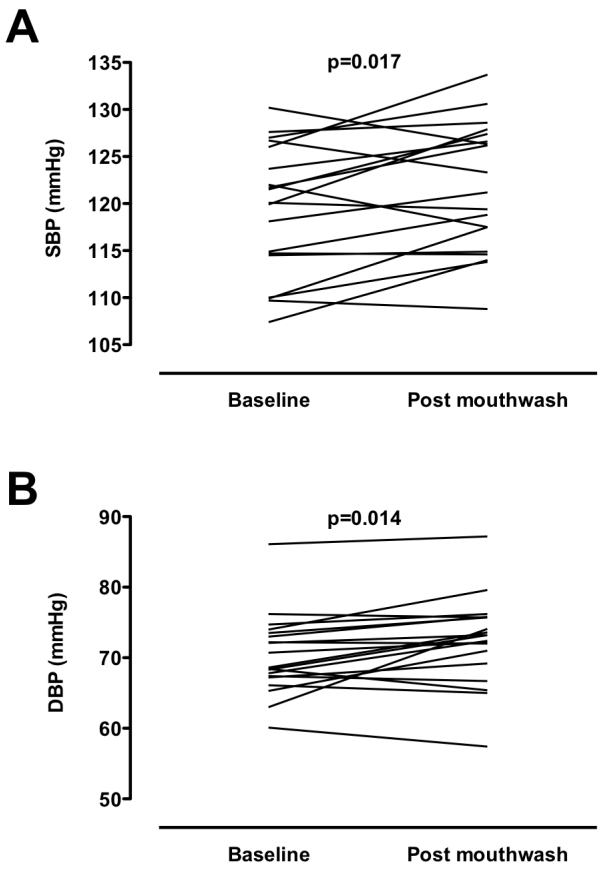


Figure 4.7 *Effects of antiseptic mouthwash use on 24 h ABP. (A) SBP and (B) DBP measured by ABP at baseline and following 7 days use of antiseptic mouthwash. Data are expressed as mean±SEM (n=19) and statistical significance determined using paired Student's t-test. (ABP=ambulatory blood pressure; BP=blood pressure; DBP=diastolic blood pressure; SBP=systolic blood pressure).*

Home SBP and DBP measurements over the course of the first 7 days (baseline) did not change significantly from day to day (Figure 4.8 A-B). However, following initiation of a single day's use of mouthwash, both home SBP and DBP were raised compared to prior to mouthwash use and remained elevated over the entire 7 day treatment period ($p<0.001$ for both SBP and DBP), with no apparent tachyphylaxis (Figure 4.8 A-B).

Separation of ABP data into daytime and nighttime means demonstrated that daytime ambulatory SBP and DBP were both increased post-mouthwash (Table 4.6), whilst daily use of antiseptic mouthwash was associated with increased nighttime SBP, but not DBP (Table 4.6).

	Daytime		**Nighttime**	
	SBP	**DBP**	**SBP**	**DBP**
Change in ABP (mmHg)	2.9±0.9	3.0±0.8	2.2±0.9	1.3±1.5
Significance (p)	0.004§§	0.002§§	0.022§	0.404

Table 4.6 *Effects of a 7 day intervention with antiseptic mouthwash on 24 h ABP, separated into daytime and nighttime periods. Data are expressed as mean±SEM (n=19) and statistical significance determined using paired Student's t-test and shown as §p<0.05 and §§p<0.01. (ABP=ambulatory blood pressure; DBP=diastolic blood pressure; SBP=systolic blood pressure).*

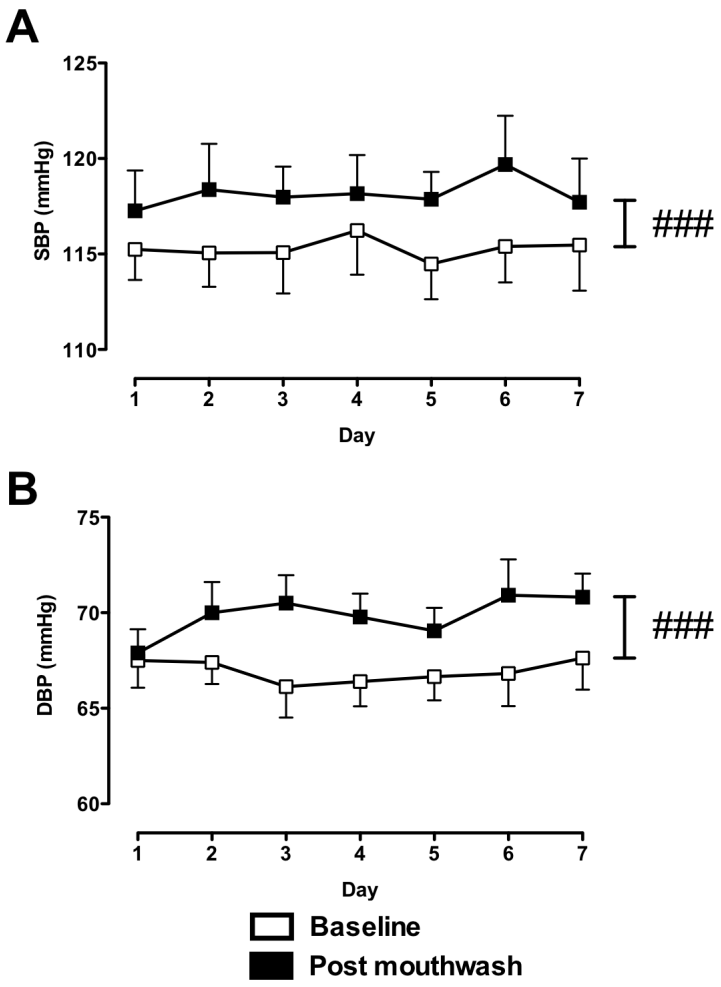


Figure 4.8 *Disruption of oral microflora elevates BP within 1 day. The daily profiles of (A) home SBP and (B) DBP during the 7 day study periods in healthy subjects. Data are expressed as mean±SEM (n=19). Statistical significance shown as ###p<0.001 for 2-way ANOVA. (BP=blood pressure; DBP=diastolic blood pressure; SBP=systolic blood pressure).*

4.3.5 Baseline BP and changes in plasma NO_2^- levels determine changes in BP

Daily use of antiseptic mouthwash for 7 days was associated with both significant attenuation of plasma NO_2^- levels (Figure 4.3 B) and elevations in SBP by all 3 methods that were employed to measure BP (Figure 4.5-4.7). Moreover, for each method of BP measurement, the change in plasma NO_2^- levels was inversely correlated to the changes in SBP (Figure 4.9 A-C). In addition, the magnitude of BP elevation was also inversely correlated to baseline BP, i.e. the lower the baseline BP, the greater the effect of antiseptic mouthwash use on BP (Figure 4.10 A-C).

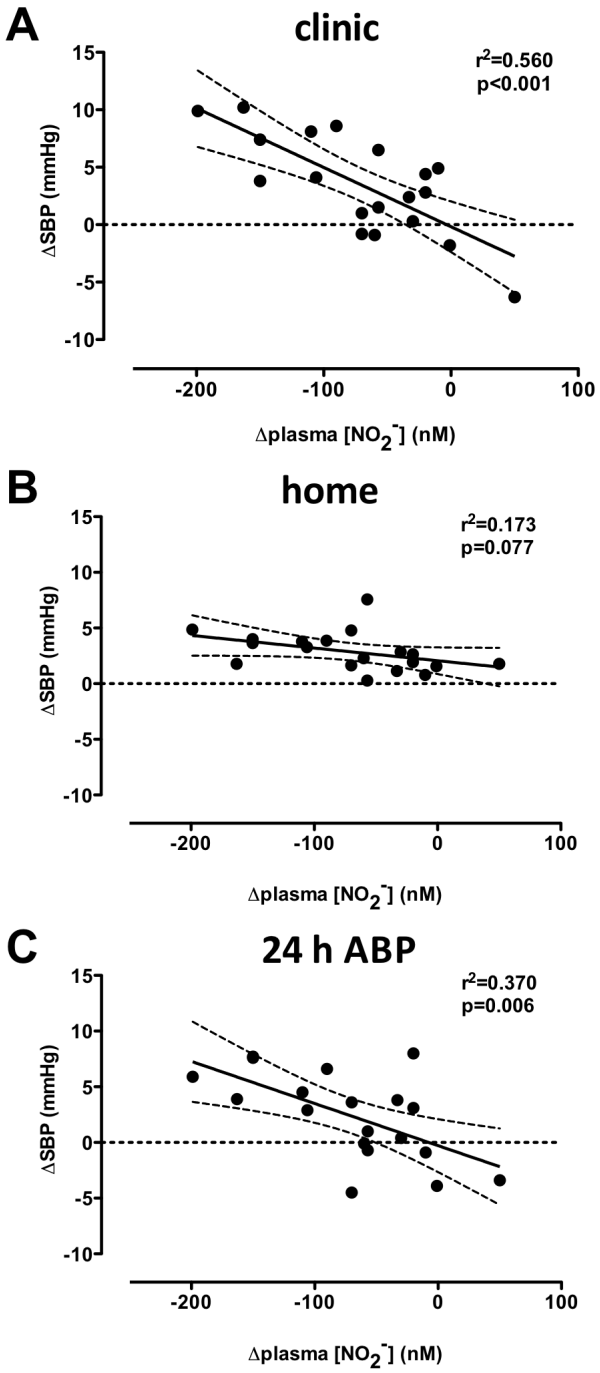


Figure 4.9 *Reduction in plasma NO_2^- levels determines elevation in BP. The relationship in change in plasma NO_2^- levels and changes in SBP measured in (A) clinic, (B) at home and by (C) 24h ABP after 7 days intervention with antiseptic mouthwash use. Significance shown for correlations determined using Pearson's linear regression of best-fit±95% confidence intervals. (ABP=ambulatory BP; NO_2^-=nitrite; SBP=systolic blood pressure).*

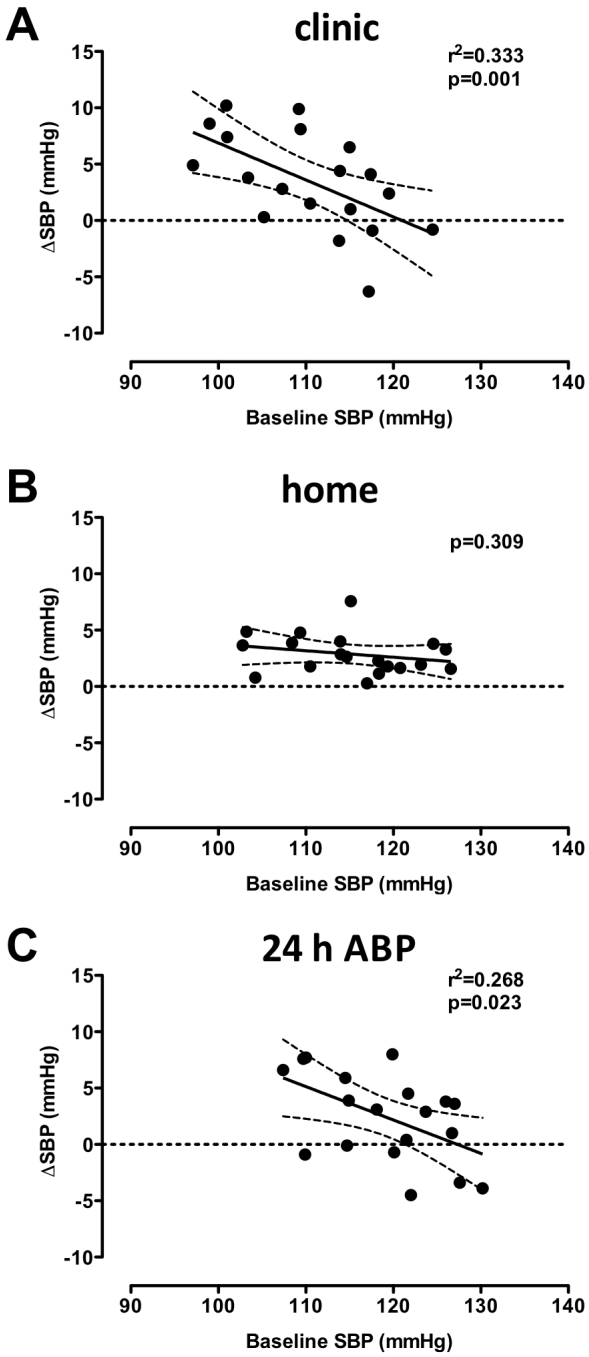


Figure 4.10 *Baseline BP determines elevation in BP. The relationship between baseline SBP and changes in SBP measured in (A) clinic, (B) at home and by (C) 24h ABP after 7 days intervention with antiseptic mouthwash use. Significance shown for correlations determined using Pearson's linear regression of best-fit±95% confidence intervals. (ABP=ambulatory BP; SBP=systolic blood pressure).*

4.3.6 Sex-specific differences after disruption of oral microflora

4.3.6.1 General characteristics

Pre-specified sub-group analyses of the data were conducted to stratify the results by sex. In the cohort that completed the study, there were 11 males and 8 females. Self-reported daily estimates of fruit, vegetable and processed meat intake were no different between sexes at baseline or after the treatment period (Table 4.7). There were some significant sex differences in baseline characteristics, with BMI and SBP (by all 3 methods) higher in males, and both plasma and salivary NO_2^- levels greater in females (Table 4.8).

Food group (mean portions/day)	**Baseline**		**Post-mouthwash**	
	Male	**Female**	**Male**	**Female**
Fruit	0.3±0.2	0.8±0.3	0.3±0.2	0.8±0.3
Low-NO_3^- vegetables	0.7±0.1	0.5±0.1	0.6±0.2	0.5±0.1
High-NO_3^- vegetables	0.3±0.1	0.2±0.1	0.3±0.2	0.3±0.1
Processed meat	0.7±0.2	0.7±0.1	0.8±0.2	0.6±0.1

Table 4.7 *Self-reported daily food estimates stratified by sex. Data are expressed as mean±SEM (n=11 males, n=8 females). No significant differences found after Bonferroni post-hoc tests following 2-way ANOVA. (NO_3^-=nitrate).*

Baseline characteristic	Male	Female	Significance (p)
Subjects (n)	11	8	
Age (years)	22.8±10.3	23.8±1.2	0.377
BMI (kg/m^2)	22.4±0.6	25.3±1.0	0.023§
Clinic SBP (mmHg)	113.9±1.9	105.5±2.4	0.012§
Clinic DBP (mmHg)	65.9±2.0	66.6±2.6	0.818
Home SBP (mmHg)	119.0±1.9	110.2±2.1	0.007§§
Home DBP (mmHg)	67.0±0.8	67.8±2.3	0.727
Ambulatory SBP (mmHg)	121.9±2.0	115.6±2.1	0.046§
Ambulatory DBP (mmHg)	70.2±1.2	70.4±2.7	0.930
Plasma [NO_3^-] (µM)	25.1±1.4	28.8±5.4	0.471
Plasma [NO_2^-] (nM)	255.9±9.7	323.1±34.3	0.045§
Salivary [NO_3^-] (µM)	392.4±47.5	509.8±252.2	0.413
Salivary [NO_2^-] (µM)	260.3±32.9	392.8±46.7	0.029§
Urinary [NO_3^-] (µM)	1392±66.1	1558±122.0	0.217
Urinary [NO_2^-] (nM)	202.5±7.6	203.8±19.0	0.948

Table 4.8 *Baseline characteristics stratified by sex. Demographics, haemodynamics and baseline NOx levels. Data are expressed as mean±SEM (n=11 males, n=8 females). Significance shown for unpaired Student's t-test in last column as §p<0.05 and §§p<0.01. (BMI=body mass index; DBP=diastolic blood pressure; NO_3^-=nitrate; NO_2^-=nitrite; NO_x=nitrite/nitrate; SBP=systolic blood pressure).*

4.3.6.2 Females exhibit greater oral NO_3^- reductase activity under basal conditions

There appeared to be little sex difference in oral NO_3^- reduction after instillation of NO_x-free water (Figure 4.11-4.12) but females had significantly higher oral NO_3^- reduction after instillation of KNO_3 solutions. After instillation of 0.8mM KNO_3 solution, oral NO_3^- reductase activity was ~2.5-fold higher (Table 4.9) in females compared to males ($p<0.01$ by 2-way ANOVA, Figure 4.11-4.12) (Table 4.9) and after 8mM KNO_3 solutions, ~1.4-fold higher (Table 4.9) compared to males ($p<0.05$ by 2-way ANOVA, Figure 4.11-4.12).

Solution	**Rate of oral NO_3^- reductase activity (nmol/min)**	
	Male	**Female**
NO_x-free water	20±4	17±5
0.8mM [NO_3^-]	69±16	173±43
8mM [NO_3^-]	183±29	252±51

Table 4.9 *Rates of oral NO_3^- reductase activity at baseline stratified by sex. Data expressed as mean±SEM (males, n=11; females n=8). (NO_3^-=nitrate; NO_x=nitrite/nitrate).*

Antiseptic mouthwash use diminished oral NO_3^- reductase activity similarly by ~90% in both sexes (all $p<0.001$ compared to baseline by 2-way ANOVA, Figure 4.11-12).

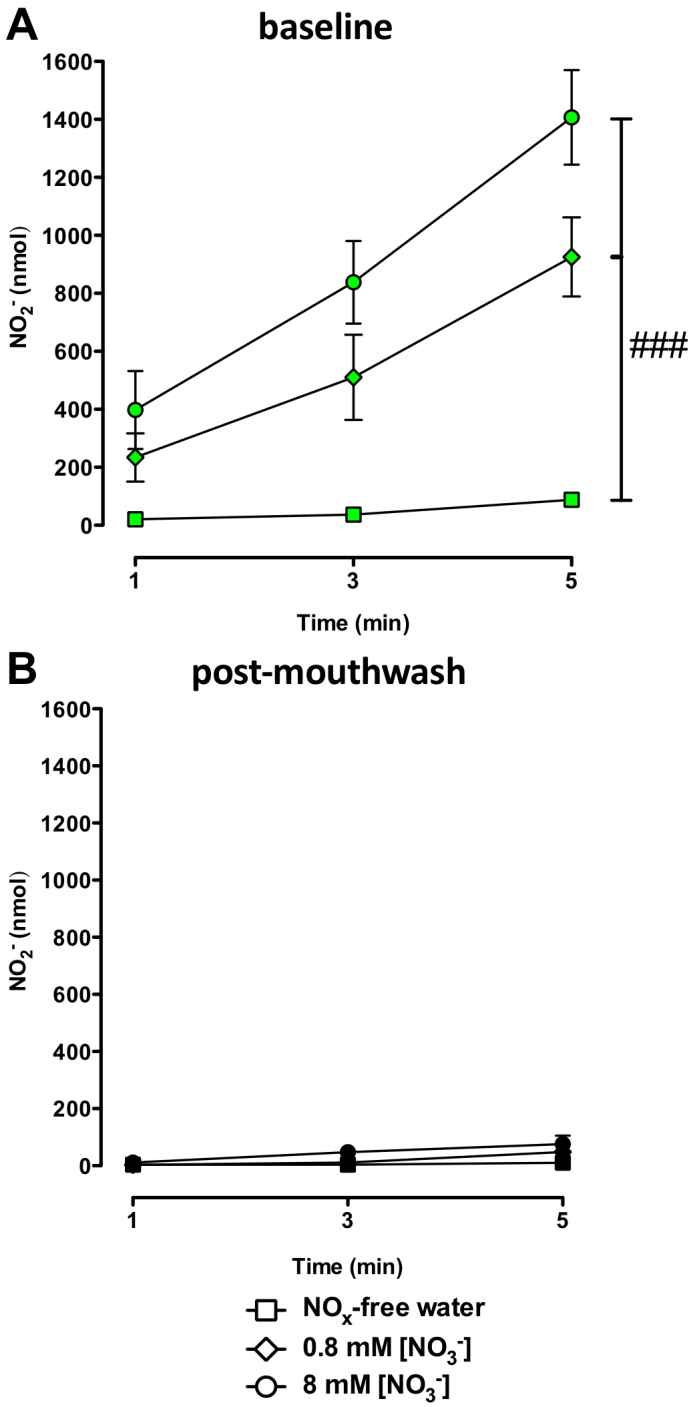


Figure 4.11 *Effects of antiseptic mouthwash use on oral NO_3^- reductase activity in females. Oral NO_3^- reductase activity after instillation of NO_3^--containing solutions for 1-5 min at (A) baseline and (B) after daily use of antiseptic mouthwash for 7 days. Significance shown as ###p<0.001 for 2-way ANOVA. Data are expressed as mean±SEM (n=8). (NO_3^-=nitrate NO_2^-=nitrite; NO_x=nitrite/nitrate).*

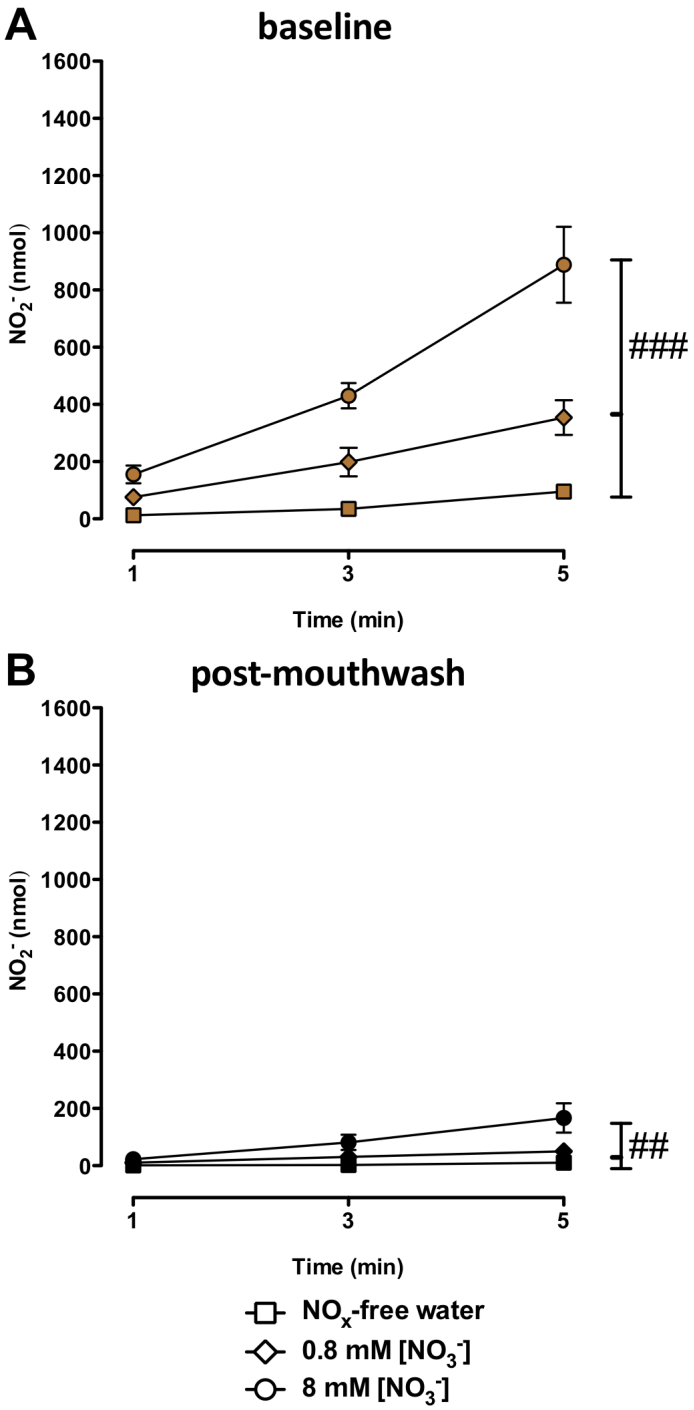


Figure 4.12 *Effects of antiseptic mouthwash use on oral NO_3^- reductase activity in males. Oral NO_3^- reductase activity after instillation of NO_3^--containing solutions for 1-5 min at (A) baseline and (B) after daily use of antiseptic mouthwash for 7 days. Data are expressed as mean±SEM (n=11). Statistical significance shown as ##p<0.01 and ###p<0.001 for 2-way ANOVA. (NO_3^-=nitrate; NO_2^-=nitrite; NO_x=nitrite/nitrate).*

4.3.6.3 Sex-specific effects of mouthwash use on systemic NO_x levels

Following 7 days continuous use of antiseptic mouthwash, salivary NO_2^- levels were reduced (Table 4.10) in relative terms by 85±10% and 87±6% in males and females respectively ($p<0.001$ for both). Plasma NO_2^- levels were reduced by 21±2% in males, but there was a greater relative reduction of 29±3% in females ($p<0.05$, unpaired Student t-test). The absolute decrease in plasma NO_2^- levels was greater in females compared to males (Table 4.10) with post-mouthwash plasma NO_2^- levels in males: 202.2±14.6 nM and in females: 228.8±24.6 nM. Similarly, there was a 20±5% reduction in urinary NO_2^- levels in males ($p<0.01$) and a corresponding 30±8% reduction in urinary NO_2^- levels in females ($p<0.05$), though the relative and absolute decreases in urinary NO_2^- levels in females were not greater than in males.

Salivary NO_3^- levels were elevated by ~2-fold in males and ~2.5-fold in females respectively after 7 days continuous use of antiseptic mouthwash (Table 4.10). There was no significant change in plasma NO_3^- levels in both sexes (Table 4.10). The change in urinary NO_3^- levels was significant in both males ($p<0.05$, by one-sample t-test) and females ($p<0.01$, by one-sample t-test).

Baseline characteristic	Female	Male
Subjects (n)	8	11
ΔPlasma [NO_3^-] (μM)	7.7±3.5	6.6±2.9
ΔPlasma [NO_2^-] (nM)	-94.4±15.8§	-53.7±9.3
ΔSalivary [NO_3^-] (μM)	765.2±180.1	432.1±92.1
ΔSalivary [NO_2^-] (μM)	-353.7±57.0	-231.5±39.7
ΔUrinary [NO_3^-] (μM)	599.6±152.9	386.2±138.2
ΔUrinary [NO_2^-] (nM)	-64.4±23.3	-41.6±12.5

Table 4.10 *Sex-specific changes in systemic NO_x levels following antiseptic mouthwash use. Data are expressed as mean±SEM (n=11 male, n=8 female). Significance shown as §p<0.05 for unpaired Student's t-test comparing males to females. (NO_3^-=nitrate; NO_2^-=nitrite; NO_x=nitrite/nitrate).*

4.3.6.4 Sex-specific effects of mouthwash use on BP

Home SBP and DBP increased in both sexes after mouthwash use (Table 4.11). However, the increases in clinic BP and ABP were only apparent in females (Table 4.11).

Characteristic	Female	Male
Subjects (n)	8	11
ΔClinic SBP (mmHg)	5.2±1.2§§	2.2±1.4
ΔClinic DBP (mmHg)	4.3±1.4§	0.7±1.2
ΔHome SBP (mmHg)	3.1±0.5§§§	2.7±0.6§§
ΔHome DBP (mmHg)	2.0±0.8§	2.0±0.7§
ΔAmbulatory SBP (mmHg)	4.4±0.9§§	1.3±1.3
ΔAmbulatory DBP (mmHg)	3.7±1.4§	1.1±0.8

Table 4.11 *Changes in BP after antiseptic mouthwash use stratified by sex. Changes in BP after 7 days use of antiseptic mouthwash measured in the clinic, at home and by 24 h ABP; Data are expressed as mean±SEM (n=11 male, n=8 female). Significance shown as §p<0.05, §§p<0.01 and §§§p<0.001 for one-sample Student t-test. (ABP=ambulatory blood pressure; DBP=diastolic blood pressure; SBP=systolic blood pressure).*

4.4 Summary

1. Reduction of NO_3^- to NO_2^- in the oral cavity can be abrogated by twice-daily use of antiseptic mouthwash in healthy subjects.
2. Interruption of the entero-salivary circulation by antiseptic mouthwash use attenuates plasma NO_2^- levels by ~25%.
3. These changes in plasma NO_2^- levels are significantly inversely correlated with small, significant elevations in BP of 2-3.5 mmHg in magnitude.
4. Females have higher baseline plasma NO_2^- levels and oral NO_3^- reductase activity than males.
5. Interruption of the entero-salivary circulation has a greater effect on lowering plasma NO_2^- levels in females than in males.
6. Changes in clinic BP and ABP after antiseptic mouthwash were only apparent in females, not males.

CHAPTER 5

Investigation of the effect of dietary NO_3^- on systemic NO_2^- levels and BP in hypertensive subjects

5.1 Introduction

Elevations of plasma NO_2^- levels, via inorganic or dietary NO_3^- supplementation, have been demonstrated to produce robust, dose-dependent BP reductions in healthy subjects over 3-24 h in the studies described in chapter 3, confirming earlier reports of the BP-lowering effects of NO_3^- ingestion. (Larsen *et al.*, 2006; Webb *et al.*, 2008a).

Although BP represents a continuous variable, with increasing risk of CVDs related to increasing levels of BP in a strong linear relationship down to a nadir of ~115/75 mmHg (Lewington *et al.*, 2002), national (Williams *et al.*, 2004) and international (Mancia *et al.*, 2007) guidelines do not suggest treatment of BP until levels reach a threshold, most commonly determined to be >140/90 mmHg. Thus it is important to establish the efficacy of inorganic NO_3^- supplementation in lowering BP in a hypertensive cohort, as this is the cohort most likely to benefit from such a therapeutic strategy. Thus the aim of this study was to determine whether dietary NO_3^- ingestion has similar effects on plasma NO_x levels and BP in hypertensive subjects as in healthy subjects.

5.2 Protocol

Design: To investigate the effects of inorganic NO_3^- supplementation in a hypertensive cohort, a randomized, open label, cross-over study was performed with subjects receiving 250 mL of dietary NO_3^- (beetroot juice; purchased from James White Drinks, Ipswich, UK) or an equal volume of low-NO_x containing water

(placebo, $[NO_3^-]$= 70.9±10.0 µM; $[NO_2^-]$ <50 nM), with at least 7 days between each limb of the study.

Power analysis: Given the higher pre-treatment BP in hypertensives, it was hypothesized that a similar BP reduction would be found as in the 24 mmol KNO_3 vs. KCl study. Observed peak decrease in SBP occurred at 2.5-3 h, post-KNO_3 ingestion with peak ΔSBP 7.4 mmHg (SD 7.2). Based on these values and an α=0.05 and 1-β=0.90, power calculations determined n=13 is required. An additional 2 subjects were recruited to a final n=15 to account for potential dropouts.

Measurements: Following arrival in the clinic, clinic BP and HR were measured for 1 h to establish baseline values (section 2.4.1). Following this, subjects ingested the randomized intervention with a light breakfast. BP, HR and arterial stiffness (section 2.5) were measured, and blood samples for plasma NO_x and cGMP analysis collected (section 2.6) at specific time-points over the following 6 h. After this time, the subjects left and then returned the following morning for 24 h measurements (see Figure 5.1).

This study is registered with clinicaltrials.gov (NCT01236872).

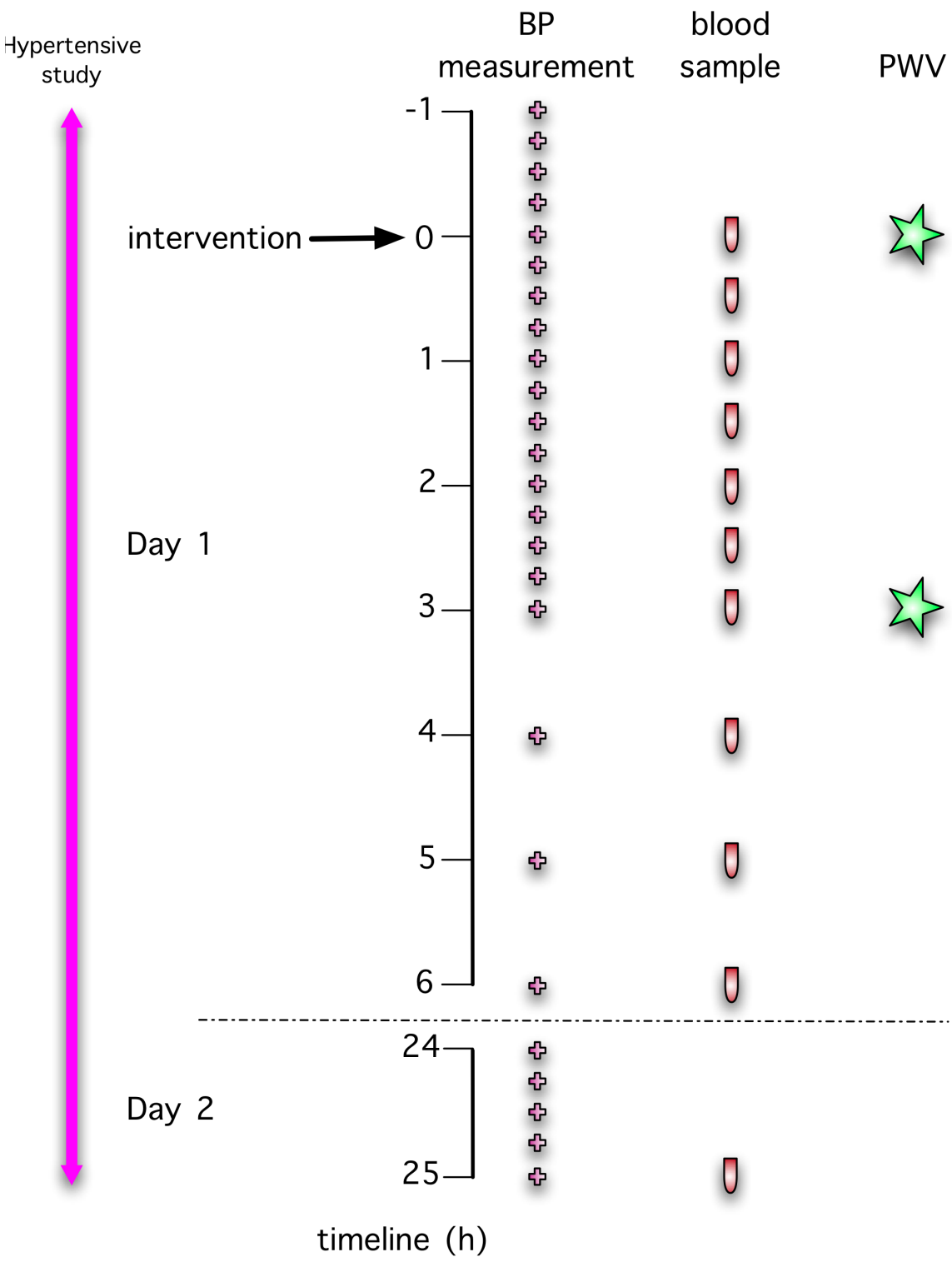


Figure 5.1 *Timeline for hypertensive study. (BP=blood pressure; PWV=pulse wave velocity).*

5.3 Results

5.3.1 General characteristics

All individuals recruited were classified on the screening visit as having grade 1 hypertension assessed according to the BHS guidelines (Williams *et al.*, 2004) (Table 5.1). There were no significant differences in the general characteristics of the individuals recruited between the separate limbs of the study (Table 5.2). Dietary NO_3^-, as beetroot juice, was well tolerated by the subjects. The [NO_3^-] of the beetroot juice was 13.2±0.94 mM, [NO_2^-] was <50 nM (n=15).

Characteristic		
Subjects (n)		15 (7 female)
ABP (daytime mean) (mmHg)	SBP	151.5±1.8
	DBP	89.7±2.2
estimated GFR (MDRD) (mL/min)		77.3±3.3
total serum [cholesterol] (fasting) (mM)		5.7±0.3
serum [LDL-cholesterol] (fasting) (mM)		3.3±0.3
plasma [glucose] (fasting) (mM)		5.5±0.3

Table 5.1 *Haemodynamic/biochemical parameters of hypertensive subjects at screening. Data are shown as mean±SEM. ABP values adjusted as per British Hypertension Society guidelines* (Williams *et al.*, 2004)*. (ABP=ambulatory BP; BP=blood pressure; DBP=diastolic blood pressure; GFR=glomerular filtration rate; LDL=low-density lipoprotein; MDRD=Modification of Diet in Renal Disease formula; SBP=systolic blood pressure).*

Characteristic	Placebo limb	Dietary NO_3^- limb	Significance (p)
Subjects (n)	15		
Age (years)	52.9±3.7		
BMI (kg/m^2)	26.2±0.9		
Serum [creatinine] (μM)	89.4±3.8		
Baseline clinic SBP (mmHg)	140.2±3.6	139.9±3.9	0.90
Baseline clinic DBP (mmHg)	86.2±2.7	86.5±2.6	0.80
Plasma [NO_3^-] (μM)	33.7±4.6	46.8±10.3	0.15
Plasma [NO_2^-] (μM)	0.41±0.05	0.43±0.04	0.68

Table 5.2 *Demographic characteristics and baseline (pre-treatment) haemodynamic / biochemical parameters in hypertensive subject study. Data are shown as mean±SEM. Significance values for paired Student t-test shown in last column. (BMI=body mass index; DBP=diastolic blood pressure; NO_3^-=nitrate; NO_2^-=nitrite; SBP=systolic blood pressure).*

5.3.2 Dietary NO_3^- elevates plasma NO_x levels

Following dietary NO_3^- ingestion (~3.3 mmol), plasma NO_3^- levels rapidly increased with significant elevations above baseline evident at 30 min ($p<0.05$). Increases in plasma NO_3^- levels peaked at 2 h (peak ~5.5-fold increase from baseline: 156.8 ± 22.9 µM; $p<0.001$) and remained elevated for the first 6 h of measurement, returning to baseline at 24 h (Figure 5.2 A). Plasma NO_2^- levels had a slower rise following dietary NO_3^- ingestion, becoming significantly elevated compared to water control at 4 h (peak ~1.5-fold increase from baseline: 0.24±0.06 µM, $p<0.05$; Figure 5.2 B), with NO_2^- levels approaching baseline levels by 24 h. In contrast there were no changes in plasma NO_x levels at any time-point in the control limb (Figure 5.2 A-B).

5.3.3 Dietary NO_3^- elevates plasma cGMP levels

Consumption of NO_3^-, with subsequent formation of NO_2^-, was associated with NO-like bioactivity as evidenced by rises in plasma cGMP levels measured at 24 h post-NO_3^- ($p<0.05$ versus water control, Figure 5.2 C).

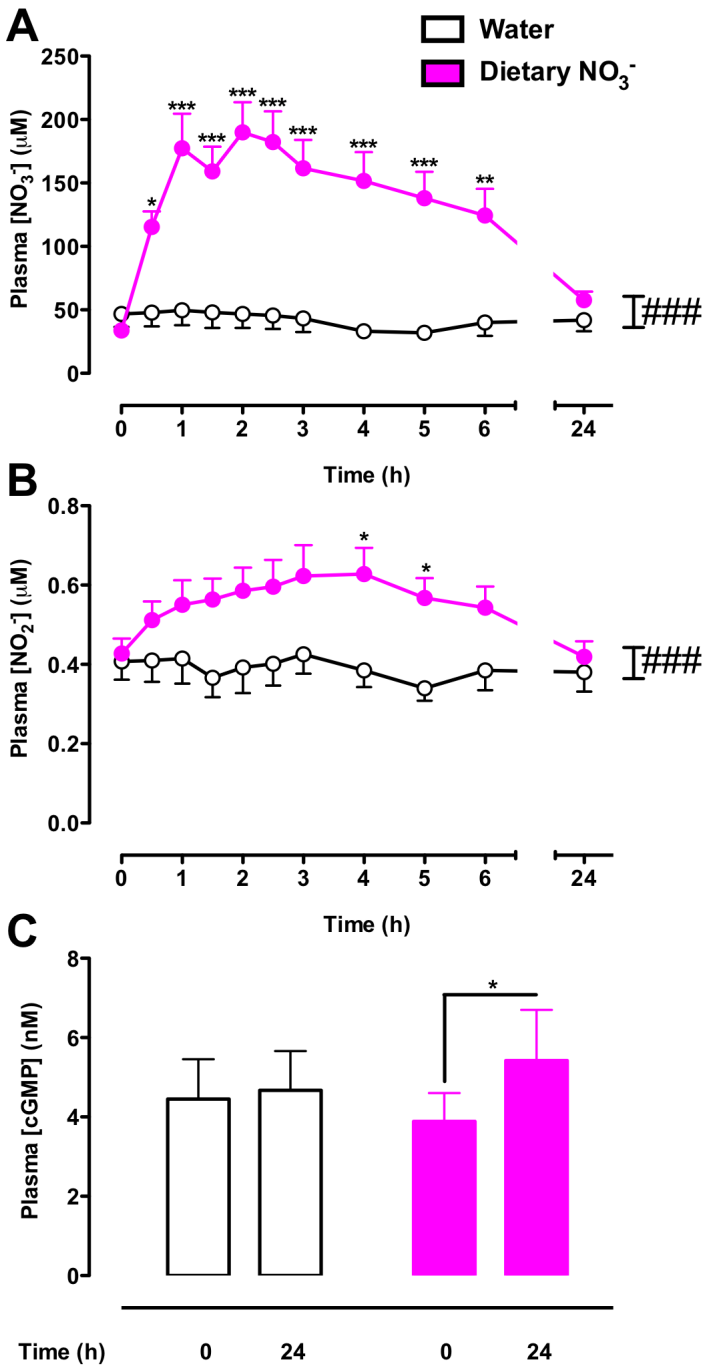


Figure 5.2 *Dietary NO_3^- supplementation elevates plasma NO_x and cGMP levels in grade 1 hypertension The effects of dietary NO_3^- ingestion (beetroot juice 250 mL) or placebo (water 250 mL) on plasma (A) NO_3^-, (B) NO_2^- and (C) cGMP levels. Data are expressed as mean±SEM (n=15). Significance shown for comparisons between groups as ###p<0.001 for 2-way ANOVA; and *p<0.05, **p<0.01, and ***p<0.001 for Bonferroni post-hoc tests following 1-way or 2-way ANOVA. (cGMP=cyclic guanosine monophosphate; NO_3^-=nitrate; NO_2^-=nitrite; NO_x=nitrite/nitrate).*

5.3.4 Dietary NO_3^- lowers BP in grade 1 hypertensive subjects

Consumption of dietary NO_3^- caused a significant decrease in SBP and DBP ($p<0.001$ vs. water control, Figure 5.3 A-B). SBP started to fall after 1.5 h, with the greatest reductions in SBP occurred between 3 and 6 h post-NO_3^- ingestion. The peak decrease was 11.2±2.6 mmHg versus 0.7±1.9 mmHg in the water control. At 24 h, SBP was still significantly lower than the water control (difference NO_3^- versus water of 8.5±1.3 mmHg, $p<0.01$, Figure 5.3 A) and significantly reduced from baseline (7.2±2.1 mmHg, $p<0.05$ Dunnett's post-hoc test of comparison to baseline (t= 0 h) following 1-way ANOVA). The greatest change in DBP occurred at 4 h post-NO_3^- ingestion with a drop of 9.6±1.2 mmHg versus 2.9±1.4 mmHg in the water control. DBP remained lower in the NO_3^--treated limb up to 6 h and returned to the pre-treatment pressure after 24 h (Figure 5.3 B). No significant differences in HR were observed after ingestion of dietary NO_3^- or water control (Figure 5.3 C).

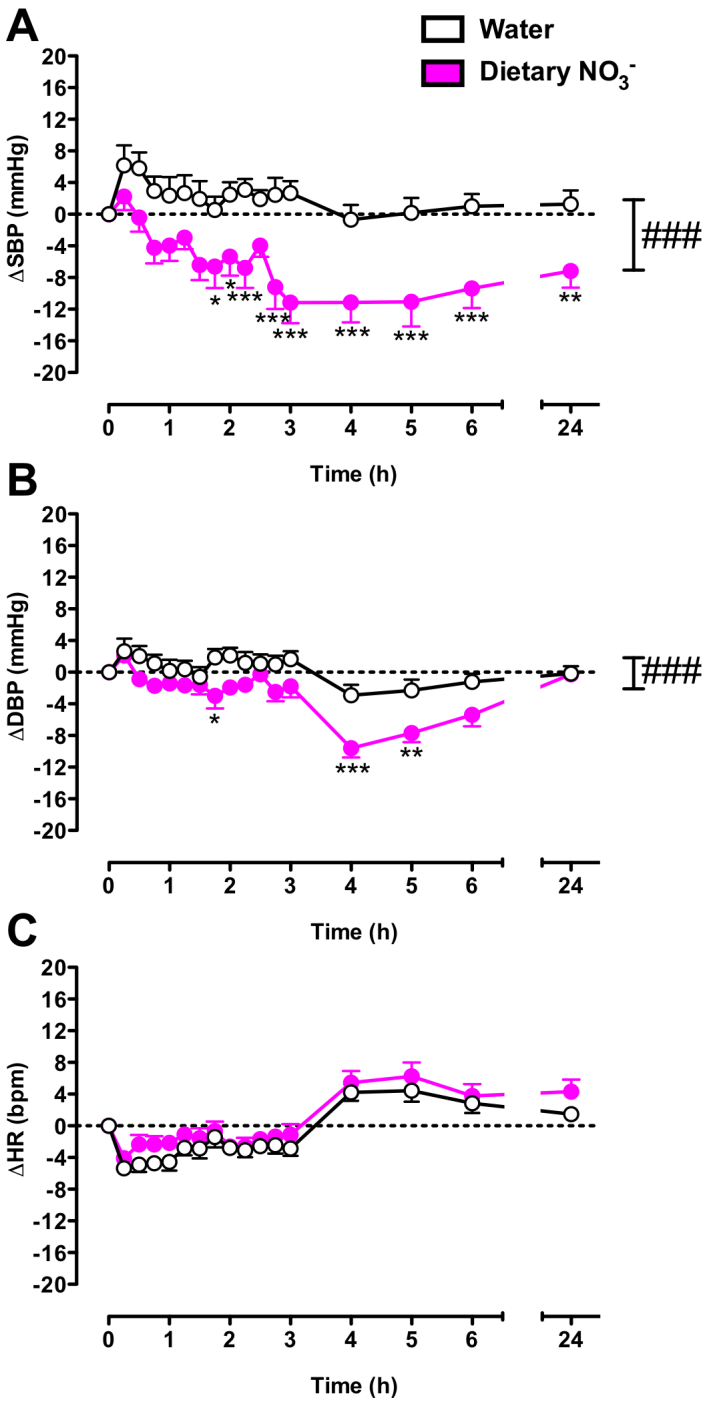


Figure 5.3 *Dietary NO_3^- supplementation reduces BP in grade 1 hypertension. The effects of dietary NO_3^- ingestion (beetroot juice 250 mL) or placebo (water 250 mL) on changes from baseline in (A) SBP, (B) DBP and (C) HR. Data are expressed as mean±SEM (n=15). Significance shown for comparisons between groups as ###p<0.001 for 2-way ANOVA; and as *p<0.05, **p<0.01 and ***p<0.001 for Bonferroni post-hoc tests. (BP=blood pressure; DBP=diastolic blood pressure; HR=heart rate; NO_3^-=nitrate; SBP=systolic blood pressure).*

5.3.5 Dietary NO_3^- ingestion lowers PWV

Aortic stiffness, measured by PWV, was significantly decreased at 3 h following dietary NO_3^- ingestion (change in PWV: 0.83±0.31 ms^{-1}); there were no changes after consumption of water and importantly, there were no differences in PWV between the two groups at baseline (Figure 5.4).

5.3.6 Electrolyte and renal indices following dietary NO_3^- ingestion

In these subjects, both serum K^+ and Cl^- levels were greater after dietary NO_3^- ingestion that after placebo ingestion. (Figure 5.5 A-B). No significant differences in serum HCO_3^-, urea or creatinine levels were observed between the two limbs over the entire 24 h period (Figure 5.6 A-C).

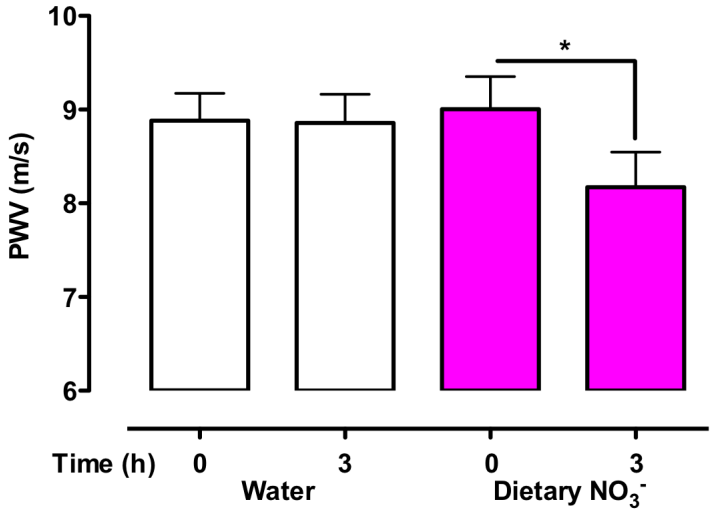


Figure 5.4 *Dietary NO_3^- supplementation reduces arterial stiffness in hypertensive subjects. The effects of dietary NO_3^- ingestion (beetroot juice 250 mL) or placebo (water 250 mL) on aortic PWV. Data are expressed as mean±SEM (n=15). Significance shown for *p<0.05 for Bonferroni post-hoc tests following 1-way ANOVA. (NO_3^-=nitrate; PWV=pulse wave velocity).*

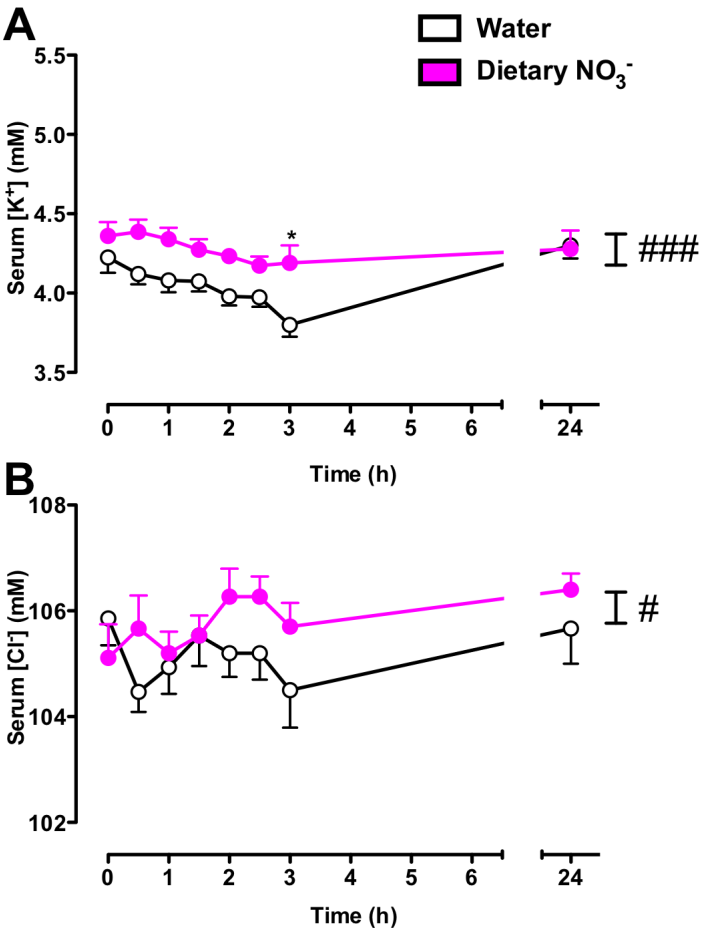


Figure 5.5 *Elevated serum K^+ and Cl^- levels following dietary NO_3^- ingestion. Changes in serum (A) K^+ and (B) Cl^- levels after dietary NO_3^- or water control ingestion over 24 h. Data are expressed as mean±SEM (n=15). Significance shown for comparisons between groups #p<0.05 and ###p<0.001 for 2-way ANOVA; followed by *p<0.05 for Bonferroni post-hoc tests. (Cl^-=chloride; K^+=potassium; NO_3^-=nitrate).*

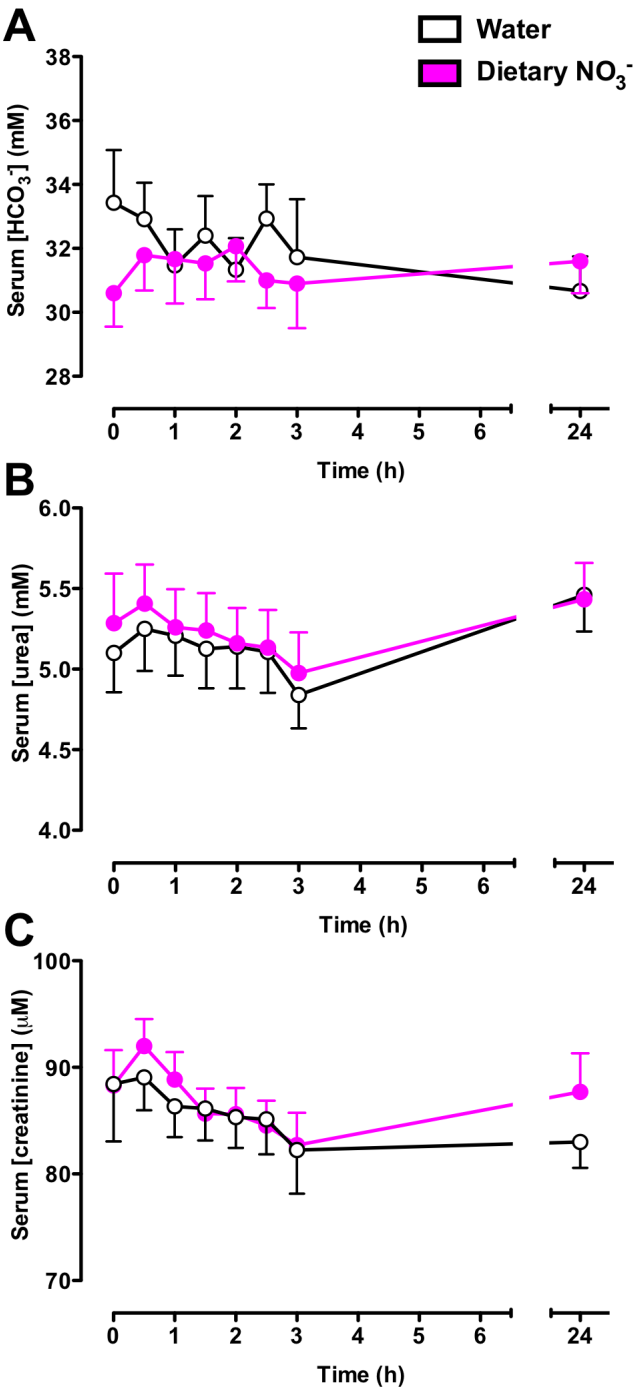


Figure 5.6 *Renal indices do not change following dietary NO_3^- ingestion. Changes in serum (A) HCO_3^-, (B) urea and (C) creatinine after dietary NO_3^- or water control ingestion over 24 h. Data are expressed as mean±SEM (n=15). Significance shown for comparisons between groups #p<0.05 and ###p<0.001 for 2-way ANOVA; followed by *p<0.05 for Bonferroni post-hoc tests. (HCO_3^-=bicarbonate; NO_3^-=nitrate).*

5.3.7 Changes in BP are correlated with plasma NO_2^- levels

Despite higher serum K^+ and plasma NO_3^- levels after dietary NO_3^- ingestion, there were no correlations between changes in SBP with either changes in serum K^+ ($p=0.396$) or plasma NO_3^- ($p=0.120$) levels observed (Figure 5.7 A-B). However, linear regression analysis showed a significant inverse correlation between changes in plasma NO_2^- levels and changes in SBP ($p<0.001$, $r^2=0.078$; Figure 5.7 C). In addition, the peak decreases in SBP and DBP coincided with the peak increase in plasma NO_2^-, not NO_3^-, levels (Figures 5.2 A-B; 5.3 A-B). The peak decrease in SBP was correlated to baseline SBP ($p=0.029$, $r^2=0.315$; Figure 5.8 A), although such a correlation was not evident for DBP ($p=0.890$, Figure 5.8 B). Finally, baseline SBP inversely correlated to baseline plasma NO_2^- levels ($p=0.043$, $r^2=0.148$; Figure 5.8 C).

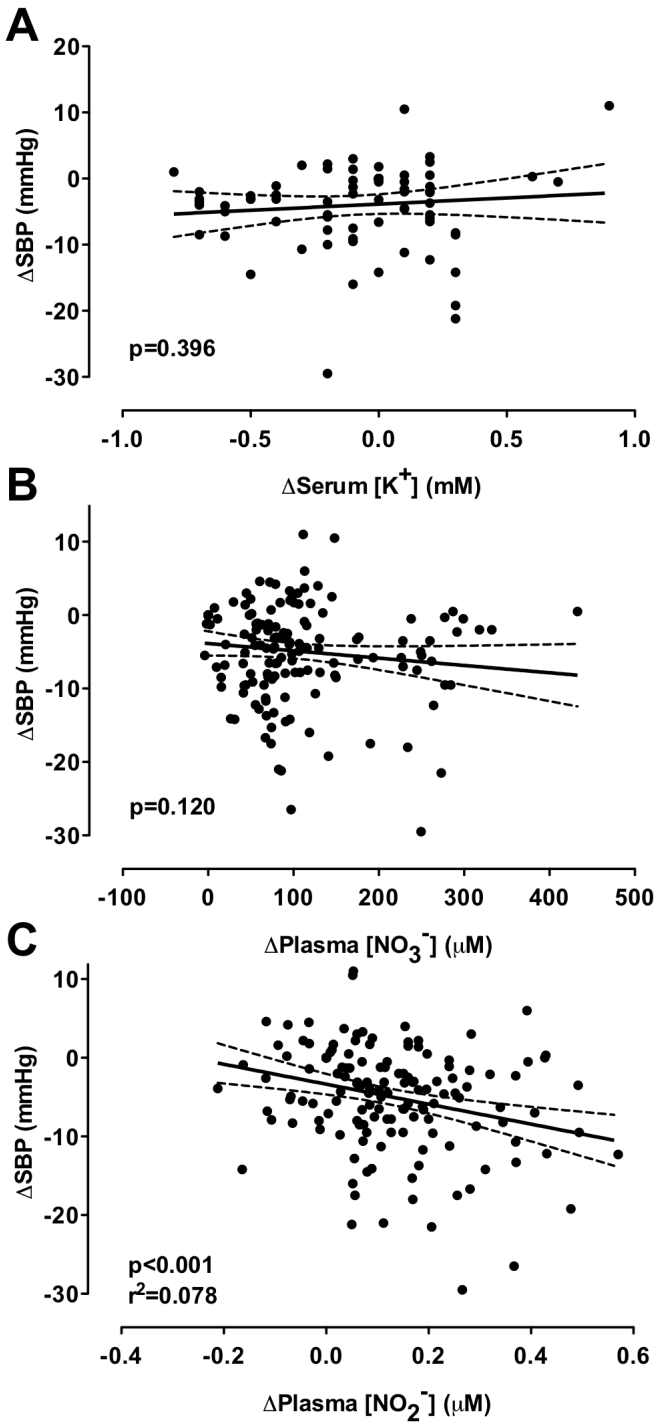


Figure 5.7 *Changes in plasma NO_2^- levels determine change in BP in hypertensive subjects. Correlation of changes in (A) K^+, (B) NO_3^- and (C) NO_2^- levels to changes in SBP from baseline after dietary NO_3^- ingestion. All of the graphs show Pearson's linear regression of best-fit±95% confidence intervals. (BP=blood pressure; K^+=potassium; NO_3^-=nitrate; NO_2^-=nitrite; SBP=systolic blood pressure).*

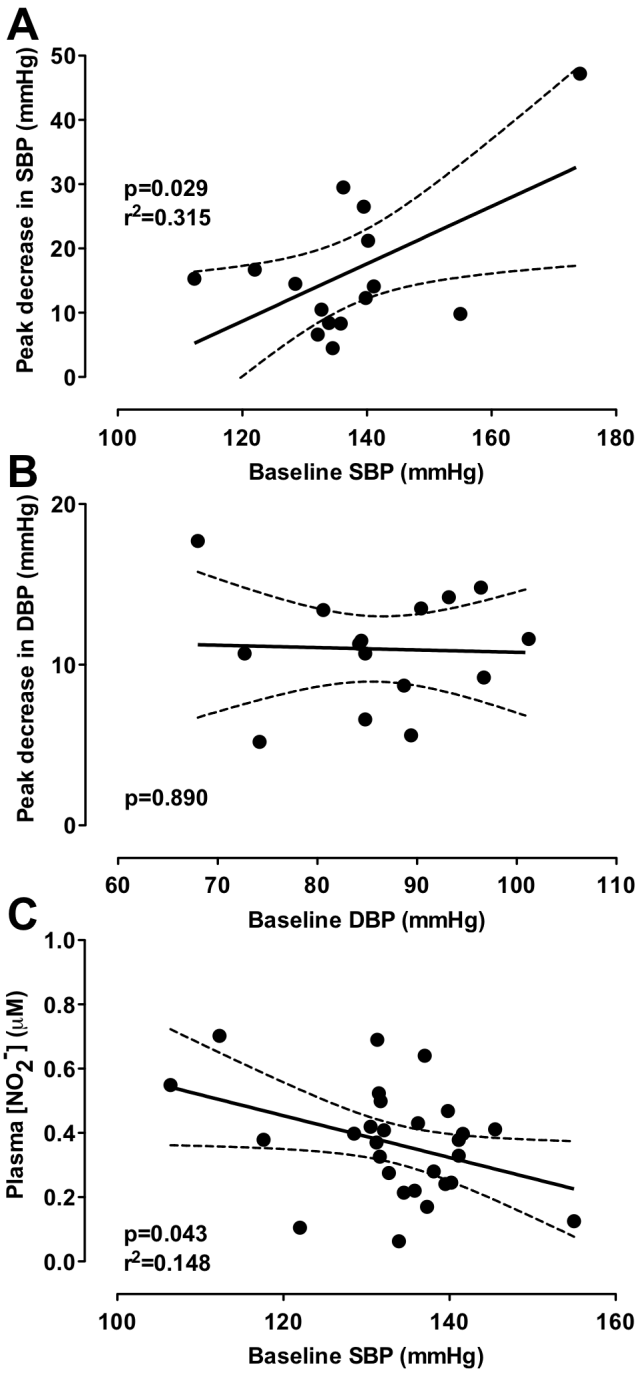


Figure 5.8 *Baseline BP determines response to dietary NO_3^- and is related to baseline plasma NO_2^- levels. Correlation of peak changes from baseline in (A) SBP and (B) DBP after dietary NO_3^- ingestion against baseline BP; and (C) baseline plasma NO_2^- levels against baseline SBP. All of the graphs show Pearson's linear regression of best-fit±95% confidence intervals. (BP=blood pressure; DBP=diastolic blood pressure; NO_3^-=nitrate; NO_2^-=nitrite; SBP=systolic blood pressure).*

5.4 Summary

1. Dietary inorganic NO_3^- elevates plasma NO_x levels in hypertensive subjects in a similar manner to normotensive subjects.
2. Elevations of plasma NO_2^- levels coincide with significant reductions in SBP and DBP in hypertensive individuals 3-4 h post-ingestion of dietary NO_3^-, the magnitude of which are related to baseline BP levels, and therefore greater than in normotensive individuals.
3. SBP was still significantly reduced 24 h post-dietary NO_3^- ingestion and was associated with significant increases in plasma cGMP levels.
4. Additionally, 3 h post-dietary NO_3^- ingestion, arterial stiffness was significantly reduced compared to baseline values.
5. Although ingestion of dietary NO_3^- additionally results in significant differences in serum K^+ levels, elevations in serum levels of this cation do not appear to be responsible for the BP-lowering effects of dietary NO_3^-.
6. Changes in plasma NO_2^- levels inversely correlate to changes in SBP whilst the same is not true for changes in plasma NO_3^- levels.
7. Similar to normotensive individuals, baseline plasma NO_2^- is inversely correlated to baseline SBP.

CHAPTER 6

Discussion

6.1 Introductory remarks

The elucidation of the identity of EDRF as NO and its protean roles in human physiology and pathophysiology has been one of the seminal discoveries in biology in the late 20th century. It was designated 'molecule of the year' by *Science* in 1992 (Koshland, 1992), and its 'discoverers' were awarded the Nobel Prize in 1998 (section 1.3). Over the past 25 years, there has been a remarkable escalation in the volume of NO research, especially in the realm of cardiovascular health and disease (Moncada and Higgs, 2006). A simple PubMed search with the search term '*Nitric Oxide'* (http://www.ncbi.nlm.nih.gov/pubmed?term=nitric%20oxide) reveals 6063 citations, from the beginning of its records, up to and including 1993; in 2011 alone, there were 6762 citations.

Until recently, a catholic view of mammalian NO biology would include the production of NO uniquely from the 5 e^- *oxidation* of the amino acid, L-arginine by NOS enzymes (Stuehr, 1999). In recent years, there have been increasing evidences of a complementary system for NO production from the 1 e^- *reduction* of NO_2^- (and, with the involvement of symbiotic, facultative, anaerobic, oral bacteria, from NO_3^-) that is especially prominent in situations of hypoxia and acidosis when the traditional NOS enzymes are dysfunctional. As such, the NO_3^--NO_3^--NO pathway can be considered to be a *back-up* system for NO generation (Lundberg *et al.*, 2008). Prior to the appreciation of this alternative pathway, NO metabolism ended in a one-way, linear termination of activity by the oxidation to both NO_2^- and NO_3^-. However, this novel paradigm reveals the 2 species to be in a 'NO cycle' (Reutov and Sorokina, 1998) (Figure 1.6).

Over the past 25 years there have been many attempts to manipulate the L-arginine:NO pathway, through provision of substrate or co-factors to the NOS system, to facilitate greater NO production (reviewed in Zhang *et al.*, 2011). However, the discovery of authentic NO production from NO_2^- reduction (Benjamin *et al.*, 1994; Lundberg *et al.*, 1994; Zweier *et al.*, 1995) has provided a further avenue within which to explore NO-based therapeutics that was explored in this thesis.

The data presented in this thesis reveal robust, dose-dependent decreases in BP in healthy subjects after ingestion of dietary NO_3^- or supplementation with inorganic NO_3^- salts and, for the first time, BP-lowering effects of dietary NO_3^- in hypertensive subjects. These effects are dependent on elevation of plasma NO_2^- levels, via the entero-salivary circulation, and conversion of this NO_2^- to bioactive NO in the circulation. Moreover, I have shown that interruption of the entero-salivary circulation of NO_3^- to NO_2^- under basal conditions has important, detrimental consequences on BP in healthy subjects.

This thesis suggests that dietary or endogenously-derived NO_3^-, and its oral conversion to NO_2^- and subsequent appearance in the circulation and thence reduction to NO, via the alternative pathways described (see section 1.8), plays an important part in regulating BP and potentially, vascular health. This thesis also proposes a physiologically balanced reasoning for the hitherto unexplained actions of the salivary glands selectively concentrating NO_3^- from the circulation and secreting NO_3^--rich saliva into the oral cavity.

6.2 NO_3^- supplementation to elevate plasma NO_2^- levels and produce bioactive NO

6.2.1 Dose-dependent elevations of plasma NO_2^- levels in healthy subjects after NO_3^- supplementation

In chapter 3, I demonstrated that ingestion of KNO_3 capsules caused rises in plasma NO_3^- levels and thence NO_2^- levels that were dose and time-dependent in healthy subjects. After ingestion of 24 mmol KNO_3, significant elevations in plasma NO_3^- levels were evident after 30 min, whilst it took 1.5 h for plasma NO_2^- levels to rise significantly. This lag with respect to time for elevation in plasma NO_2^- levels is consistent with previous reports in healthy subjects describing the effects of inorganic or dietary NO_3^- ingestion on plasma NO_2^- levels (Lundberg and Govoni, 2004; Webb *et al.*, 2008a) and reflects the time required for circulation through the entero-salivary pathway and reduction of NO_3^- to NO_2^- by oral microflora. Plasma NO_3^- levels peaked at ~35-fold higher than basal levels, whilst plasma NO_2^- levels peaked at ~4-fold higher than basal levels. This difference also reflects passage through the entero-salivary circulation and demonstrates that not all plasma NO_3^- is eventually converted to plasma NO_2^- through the entero-salivary circulation. It has been estimated that the bioavailability of inorganic NO_3^- is 100% after an oral load (van Velzen *et al.*, 2008) and that ~75% of an oral dose of inorganic NO_3^- is excreted in the urine (Packer *et al.*, 1989; Wennmalm *et al.*, 1993; Pannala *et al.*, 2003). The remaining 25% is concentrated by the salivary glands and is present in NO_3^--rich saliva as substrate for the oral microflora to reduce to NO_2^- in the oral cavity (Spiegelhalder *et al.*, 1976; Kortboyer *et al.*, 1994). Approximately ⅓ of salivary NO_3^- is converted to NO_2^- in the mouth (Lundberg and Govoni, 2004; Webb *et al.*, 2008a). Indeed it has been estimated that ~5-8% of an oral, inorganic NO_3^- dose is

converted finally to plasma NO_2^- (Spiegelhalder *et al.*, 1976; Eisenbrand *et al.*, 1980), which explains the reduced fold increases in plasma NO_2^- compared to plasma NO_3^- levels after an oral inorganic NO_3^- load as noted above and previously (Lundberg and Govoni, 2004; Webb *et al.*, 2008a).

The time-course for the changes in plasma NO_x levels were similar with lower doses of NO_3^- provided by either KNO_3 capsule (4 and 12 mmol) or in dietary form as beetroot juice (~5.5 mmol). Peak plasma NO_3^- levels were evident within 1 h and peak plasma NO_2^- levels were evident between 2-3 h post-NO_3^- ingestion. In addition, the changes in plasma NO_x levels were also dose-dependent. Indeed, the fold increase from basal levels in plasma NO_3^- after ingestion of 4 (inorganic salt), 5.5 (dietary form as beetroot juice) and 12 (inorganic salt) mmol NO_3^- were ~7, ~11 and ~27-fold respectively. These findings indicate that irrespective of source of NO_3^- i.e. in salt or in dietary form, the pharmacokinetics of NO_3^- following an oral NO_3^- load are similar. There were also dose-dependent increases in plasma NO_2^- levels irrespective of formulation. The fold increase in plasma NO_2^- levels from basal levels after ingestion of 4 (inorganic salt), 5.5 (dietary form as beetroot juice) and 12 (inorganic salt) mmol NO_3^- were ~1.3, ~1.6 and ~2-fold respectively.

6.2.2 NO_3^- supplementation elevates plasma NO_2^- levels in hypertension

In chapter 5, I showed that a similar profile in the pharmacokinetics of NO_3^- was also evident in hypertensive subjects. Following dietary NO_3^- ingestion, plasma NO_x levels were raised with respect to basal levels. Similar to healthy subjects, the rise in plasma NO_2^- levels was delayed relative to plasma NO_3^- levels, with the peak rise in

plasma NO_2^- levels occurring 4 h post-NO_3^- consumption. This time-lag relative to the very rapid (30 min) appearance of NO_3^- again reflects the dependence upon the entero-salivary circuit for the reduction of NO_3^- to NO_2^- in humans. Comparison of these present findings to healthy subjects suggests that the time-course of travel and appearance within the circulation of the two distinct anions is similar between normotensive, healthy subjects and grade 1, drug-naïve hypertensive subjects.

The relative rise in plasma NO_3^- levels between normotensives and hypertensives also followed a similar pattern. After ingestion of a ~3.3 mmol dietary NO_3^- load in hypertensive subjects, plasma NO_3^- levels were elevated above baseline by ~5.5-fold, which fits well with the data obtained from healthy subjects, in whom plasma NO_3^- levels increased ~7, ~11 and ~27-fold after ingestion of 4, 5.5 and 12 mmol of NO_3^- as discussed previously.

Perhaps more importantly, the relative rises in plasma NO_2^- levels after NO_3^- ingestion in hypertensive subjects fitted well with the profiles in healthy subjects. In normotensive subjects, a 4 mmol dose of KNO_3 caused a ~1.3-fold peak increase from baseline in plasma NO_2^- levels. The hypertensive subjects in this study received a relatively smaller dose of ~3.3 mmol NO_3^- with a peak fold increase of plasma NO_2^- levels of ~1.5-fold rise suggesting that if anything the processing of NO_3^- to NO_2^- is improved in hypertensives, although a prospective study comparing age-matched normotensives and hypertensives would be required to confirm this, as the

pharmacokinetics of drugs may be altered by the many physiological changes that occur with normal ageing (Mangoni and Jackson, 2004) and the mean ages in the cohorts studied were appreciably different (mean age: 52.9±3.7 years (hypertensive) vs. 28.8±1.7 years (4 mmol KNO_3 normotensive cohort). These similarities indicate that hypertension *per se* does not result in *substantial* changes in the bioconversion rate and extent of NO_3^- processing via the entero-salivary circuit. However, it is important to note that the relative importance of renal handling of these products was beyond the scope of these studies and deserves its own investigation. The pathways for renal tubular transport of NO_x are poorly understood and clearly may be important when discussing similarities and differences in plasma levels of these anions after NO_3^- ingestion or at baseline in different cohorts.

6.2.3 NO_3^- supplementation results in bioactive NO generation in the circulation

Interpretation of plasma NO_x levels is challenging due to the fact that multiple pathways for the generation and destruction of NO_x and NO exist (Lundberg *et al.*, 2008). Indeed, changes in plasma NO_2^- levels may reflect eNOS activity (Lauer *et al.*, 2001; Kleinbongard *et al.*, 2003, 2006b) NO oxidation (Ignarro *et al.*, 1993), NO_3^- reduction (Lundberg and Govoni, 2004; Webb *et al.*, 2008a) and, most likely, all three at once.

Whilst it is largely accepted that NO underlies the bioactivity of NO_2^- this has not been clearly demonstrated in humans *in vivo* previously. In this current work I have

demonstrated a temporal relationship between the rise in plasma NO_2^- levels with a rise in cGMP levels, both after dietary NO_3^- and inorganic NO_3^- supplementation in both normotensive and hypertensive subjects. These observations confirm the formation of bioactive NO within the human circulation. cGMP is an exquisitely sensitive indicator of NO production, via activation of NO-sensitive sGC (Batchelor *et al.*, 2010). Thus detection of elevated levels of cGMP provides strong evidence of the generation of bioactive NO (Hobbs, 1997; Hobbs and Stasch, 2010) from both formulations of NO_3^- in healthy and hypertensive subjects. However, there is some evidence suggesting that NO_2^- may exert direct effects on sGC independent of NO formation (Bryan *et al.*, 2005), a possibility that cannot be excluded by the detection of elevated cGMP levels in these studies.

What cannot be deduced from the studies in this thesis is the exact location of NO_2^- reduction to NO. There are lines of evidence that purport to demonstrate NO_2^- reduction inside erythrocytes (Cosby *et al.*, 2003), at the level of erythrocyte membranes (Webb *et al.*, 2008b) and within the vascular wall (Liu *et al.*, 2007; Alzawahra *et al.*, 2008; Feelisch *et al.*, 2008), but these studies have used either isolated cell or tissue preparations and have not addressed NO_2^- reduction *in vivo*. This is challenging because of the numerous, putative NO_2^- reductases described earlier in section 1.8 and difficulty in measuring both NO_x and cGMP in specific acellular, cellular and sub-cellular fractions.

As well as uncertainty regarding cellular location of NO_2^- reduction, there is also uncertainty regarding the identity of the NO_2^- reductase that might mediate NO

formation from NO_2^-. Whilst a number of NO_2^- reductases have been described within the circulating elements and within tissues in pre-clinical models (section 1.8), confirmation of many of these pathways in humans is limited, except for deoxyHb (Cosby *et al.*, 2003) and XOR (Webb *et al.*, 2008b). It has been previously shown that in deoxygenated human erythrocytes, addition of NO_2^- (100-200 μM) was associated with a liberation of NO detectable by gas-phase ozone chemiluminescence (Cosby *et al.*, 2003). In human internal mammary artery homogenates, prepared from arteries harvested at the time of coronary artery bypass surgery, and also in washed erythrocytes from healthy subjects, the liberation of NO after the addition of NO_2^- (100 μM) was attenuated by pre-treatment with the XOR inhibitor, allopurinol (100 μM) (Webb *et al.*, 2008b).

It is also possible that the effects seen may relate to the activity of newly formed S-nitrosothiols in blood and tissue; compounds known to be potent transducers of NO bioactivity and of particular relevance to vasodilation (Stamler *et al.*, 1997). Indeed, interactions between haem-containing proteins, such as Hb, and NO_2^- have been shown to promote the formation of S-nitrosothiols (Angelo *et al.*, 2006). In the studies presented in this thesis, no other NO-species besides NO_2^- and NO_3^- were measured. Indeed, all plasma samples were subject to low molecular weight cut-off filtration to enable specific estimation of plasma NO_2^- levels. This method, therefore, excluded the possibility of measurement of major NO-adducts found within the circulation that others have proposed might underlie the effects of NO_2^- such as S-nitrosothiols (particularly S-nitroso-albumin which is the predominant S-nitrosothiol in the plasma (Stamler *et al.*, 1992)) or NO-haem compounds. Although,

pertinent to this possibility of a role for S-nitrosothiols, a study in healthy subjects supplemented with oral NO_3^- has shown that whilst plasma NO_2^- levels were elevated 4-5 fold there were no measureable changes in S-nitrosothiol levels (Lundberg and Govoni, 2004).

The potential clinical importance of these findings demonstrating the sequential reduction of NO_3^- to NO_2^- and thence to NO in humans is considerable. The main source of endogenous NO within the cardiovascular system is eNOS-derived NO. However, an important feature of hypertension, as well as most CVDs for which hypertension is a risk factor, is 'endothelial dysfunction'. This is a phenomenon characterized by decreased bioavailability of eNOS-derived NO (for review see Brunner *et al.*, 2005). In hypertensive subjects, total NO_x levels in the circulation and urine have been used as an index to reflect endothelial dysfunction and reduced eNOS-derived NO synthesis (Forte *et al.*, 1997). Observations described in this thesis with dietary and inorganic NO_3^-, suggest that NO synthesis via the *alternative* NO_3^--NO_2^--NO generating pathway remains intact in hypertension and, as such, may be a useful strategy that could be employed to elevate systemic NO generation. Indeed, a recent *meta* analysis of 11 RCTs of oral L-arginine supplementation (4-24 g/day, for 2-24 weeks (median 9 g/day, 4 weeks)) in hypertensive subjects demonstrated BP reduction of ~5.4/2.7 mmHg, suggesting that a strategy based on increasing endogenous NO synthesis may be useful in hypertension (Dong *et al.*, 2011).

6.3 BP-lowering effects of NO_3^- supplementation

In this thesis, I have shown that inorganic NO_3^- capsules or a dietary NO_3^- load, in the form of beetroot juice, results in dose-dependent increases in plasma NO_2^- levels via bioconversion *in vivo* and my findings confirm that this bioactive NO_2^-, following reduction to NO, causes dose-dependent decreases in BP in healthy subjects and robust BP decreases in drug-naïve hypertensive subjects as well.

6.3.1 BP-lowering effects of NO_3^- are dose-dependent

KNO_3 capsule ingestion (24 mmol) substantially lowered SBP and DBP over 24 h, whilst a similar dose of KCl did not alter BP over the same time period. A dose of 24 mmol was chosen in this sub-study to closely approximate the NO_3^- dose given in dietary form (~22.5 mmol NO_3^- as beetroot juice) previously (Webb *et al.*, 2008a). The effects of inorganic NO_3^- supplementation at a similar dose to dietary NO_3^- supplementation elicited similar changes in BP, with peak changes in BP of ~9.4/6mmHg (after 24 mmol inorganic NO_3^- ingestion) and ~10.4/8mmHg (after ~22.5 mmol) (Webb *et al.*, 2008a).

Previous studies have demonstrated that NO_2^- vasodilates both the arterial and venous sides of the forearm circulation of humans (Cosby *et al.*, 2003; Maher *et al.*, 2008) and systemic NO_2^- application decreases BP in both primates (Dejam *et al.*, 2007) and humans (Cosby *et al.*, 2003; Dejam *et al.*, 2007; Pluta *et al.*, 2011). The studies described in chapter 3 further contribute to the evidence previously accumulated (Larsen *et al.*, 2006; Webb *et al.*, 2008a) that inorganic NO_3^-

supplementation can elevate plasma NO_2^- levels sufficiently to elicit robust BP decreases over 3-24 h in healthy subjects.

The BP-lowering effects of inorganic NO_3 were found to be dose-dependent as reflected by the decreasing magnitude of response in SBP to a 24, 12, 5.5 and 4 mmol dose. Importantly, as with plasma NO_x levels, this dose-dependency appeared to hold irrespective of whether the inorganic NO_3^- load was administered by KNO_3 capsules or in dietary NO_3^- form, as beetroot juice. The ability to replicate the effects of beetroot juice on the cardiovascular system with inorganic NO_3^- and to show dose-dependency by either a dietary or inorganic formulation provides further support for the proposal that inorganic NO_3^- underlies the beneficial effects of beetroot juice on the cardiovascular system.

6.3.2 BP-lowering effects of NO_3^- are dependent upon NO_2^-

Post-hoc analyses of the 24 mmol KNO_3 vs. KCl study data in healthy subjects revealed that the changes in BP from baseline (following supplementation with 24 mmol KNO_3) inversely correlated only to changes in plasma NO_2^- levels, but not NO_3^- levels, revealing that NO_2^- is the bioactive moiety, not NO_3^-. These data fit with previous observations after dietary NO_3^- ingestion in healthy subjects (Webb *et al.*, 2008a) and also fit with the prevailing dogma that humans are unable to metabolize NO_3^- directly (Lundberg *et al.*, 2004). These results suggest that the measure of plasma NO_2^- levels do reflect, at least in part, NO_2^- bioactivity, via bioconversion to NO *in vivo*.

Interestingly, in this cohort, baseline BP was closely inversely correlated to baseline plasma NO_2^- but not NO_3^- levels. Baseline plasma NO_2^- levels have been proposed to be an accurate reflection of endogenous NO generation via eNOS-dependent conversion of L-arginine to NO (Lauer *et al.*, 2001; Kleinbongard *et al.*, 2003, 2006b) and these findings may simply be highlighting the known relationship between classical eNOS-derived NO and BP. However, with the appreciation that NO_2^- is a bioactive molecule, these findings also support the possibility that the correlation of baseline plasma NO_2^- with BP is actually a reflection of the functional activity of physiological NO_2^- reduction as first proposed in 2000 by Cannon and colleagues (Gladwin *et al.*, 2000). This, in turn, raises the possibility that intrinsic plasma NO_2^- levels may be involved in 'setting' the BP of healthy subjects. These results suggest that the measure of plasma NO_2^- levels do reflect, at least in part, NO_2^- bioactivity, via bioconversion to NO *in vivo*. These data also appear to suggest some clustering within healthy subjects of responsiveness to NO_2^- into two groups i.e. that whilst small changes in NO_2^- (~<1 μM) effected apparently substantial changes in BP that where the changes in NO_2^- were >1μM little effect on BP was evident. Further analyses of these healthy subject data suggested that indeed, two distinct groups of individuals that could be separated by sex did exist within the cohort as will be discussed in section 6.5.

Further post-hoc analyses of the 24 mmol KNO_3 vs. KCl data in healthy subjects demonstrate that the magnitude of the BP response is directly related to baseline BP (i.e. the higher the baseline BP the greater the peak BP reduction achieved). This

relationship is consistent with the observation that the effect of BP-lowering drugs is proportional to resting BP (Law *et al.*, 2003).

6.3.3 NO_3^- is more potent in hypertension

Hypertensive subjects exhibited greater BP-lowering in response to elevations in NO_2^- levels compared to the healthy subjects studied herein and published previously (Larsen *et al.*, 2006; Webb *et al.*, 2008a). The peak BP drop following dietary NO_3^- ingestion (~3.3 mmol NO_3^-) in the hypertensive study was 11.2/9.6 mmHg. This compares to peak BP reductions in healthy subjects of only 1.7/4.6 mmHg in response to a comparable 4 mmol dose of NO_3^- (KNO_3 capsules). Indeed, similar BP decreases (~9.4/6mmHg) in healthy subjects, with inorganic NO_3^- supplementation, were achieved with ~7x higher dose of KNO_3 (24 mmol), or, previously, as dietary NO_3^- (~10.4/8mmHg after ~22.5 mmol, Webb *et al.*, 2008a). The BP-lowering effects of NO_3^-, in inorganic salt or dietary form (as beetroot juice) in healthy subjects demonstrated dose-dependency with doses ranging between 4-24 mmol NO_3^- and fitted well with previous observations in healthy subjects given ~22.5 mmol NO_3^- (500 mL beetroot juice, Webb *et al.*, 2008a) (Figure 6.1). However, the BP-lowering after dietary NO_3^- in hypertensive subjects does not fit into the same dose-dependent pattern (Figure 6.1).

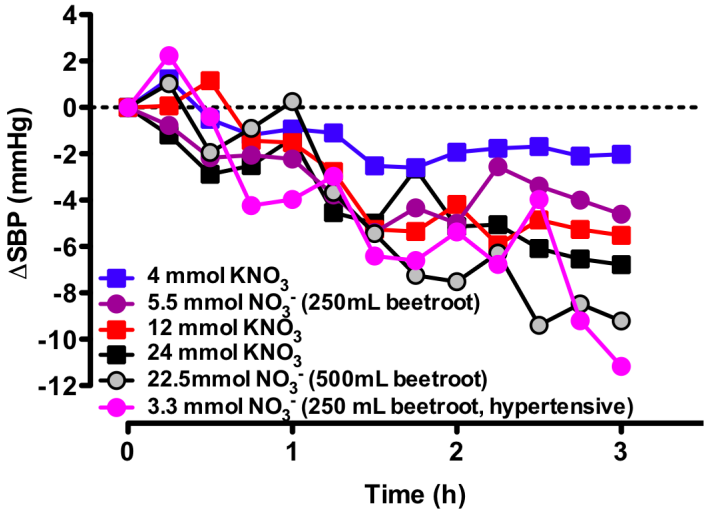


Figure 6.1 *BP-lowering effects of inorganic or dietary NO_3^- in healthy and hypertensive subjects. The effects of KNO_3 (4, 12 or 24mmol) in healthy subjects and dietary NO_3^- as beetroot juice (250 mL, ~5.5 mmol NO_3^-; 500 mL, ~22.5 mmol) in healthy subjects; and dietary NO_3^- as beetroot juice (250 mL, ~3.3 mmol) in hypertensive subjects) on SBP over 3 h. (n=6-20). Data are expressed as mean only. (Data for effects of ~22.5 mmol dietary NO_3^- adapted with permission from Webb et al., 2008a) (KNO_3=potassium nitrate; NO_3^-=nitrate; SBP=systolic blood pressure).*

As with healthy subjects, baseline BP inversely correlated only to baseline plasma NO_2^- levels, and the change in BP inversely correlated only to changes in plasma NO_2^- levels, but not NO_3^- levels, affirming the importance of the NO_2^- anion as the mediator of the BP-lowering effects of NO_3^- ingestion and suggesting that baseline plasma NO_2^- levels may be involved in BP regulation.

This enhanced sensitivity of BP responses in hypertensive subjects could simply reflect the higher baseline BP evident in the hypertensive cohort, a view consistent with the observation from *meta* analyses that the effect of BP-lowering drugs in patients is proportional to pre-treatment BP (Law *et al.*, 2003) and the peak BP decrease was correlated to baseline BP, as was apparent in healthy subjects.

An additional explanation for the greater BP reductions in hypertensive subjects is sensitization of downstream pathways in the classical NO-signalling paradigm. For instance, it has been demonstrated that there is an enhanced vasorelaxant response to NO donors at the level of soluble guanylyl cyclase in wild-type mice treated with NOS inhibitors (Moncada *et al.*; 1991) and in eNOS knockout mice (Brandes *et al.*, 2000). However, whether this is similar in human hypertension, a situation in which eNOS activity is similarly reduced (Linder *et al.*, 1990; Panza *et al.*, 1993), has not been explored.

In hypertensive subjects, SBP was still appreciably reduced at 24 h by ~8.5 mmHg compared to water control, which is very close to the expected BP-lowering effects

of any anti-hypertensive drug at standard dose in grade 1 hypertension (9.1 mmHg) (Law *et al.*, 2009) This observation suggests that a dietary NO_3^- intervention could be considered as a once daily, effective anti-hypertensive agent, especially as the peak:trough ratio of BP-lowering effect is ~60% for dietary NO_3^- in this study which is consistent with an effect size necessary for once daily dosage of anti-hypertensive medications (Lipicky, 1994).

6.3.4 NO_3^- reduces arterial stiffness

It is also very encouraging, if at first somewhat surprising, that dietary NO_3^- ingestion reduced aortic PWV, considered to be the *gold*-standard approach to assessing arterial stiffness (Laurent *et al.*, 2006). Arterial stiffness is considered to be an important, independent predictive marker of cardiovascular risk and is best measured by aortic PWV (Laurent *et al.*, 2006). Previous studies has shown that acute BP reduction (using *organic* nitrovasodilators) did not alter PWV in *treated* hypertensive subjects, though pharmacological manipulation of BP did alter PWV in drug-naïve normotensive controls (Stewart *et al.*, 2006). These findings suggest that, although raised BP contributes to increased functional arterial stiffness through increased distending pressure, it may also be related to structural changes in the arterial wall after prolonged increases in arterial tension. However, contrasting data suggest that acute inhibition of endogenously generated NO does modulate elastic artery distensibility and hence stiffness *in vivo* (Wilkinson *et al.*, 2002; Schmitt *et al.*, 2005; Bellien *et al.*, 2010). Therefore, a stratagem to increase bioavailable NO, through increases in systemic NO_2^- levels, in the situation of

endothelial dysfunction and reduced endogenous NO production, could be expected to have a favorable effect. Other studies have shown acute reductions in PWV (within 4 h) after the administration of vasoactive compounds, more than would be expected from BP-lowering on its own, such as with endothelin receptor antagonism (Dhaun *et al.*, 2009). This suggests that, although the reduction in PWV is likely to be due to reduced BP, further studies to elucidate the mechanism and potentially using other BP-lowering drugs to achieve equivalent BP reductions, are needed.

Indeed, since the inception of these studies described herein, further work in healthy subjects that did not form part of this thesis, demonstrates that inorganic NO_3^- supplementation reduces arterial stiffness, as measured by PWV, in healthy subjects as well (Bahra *et al.*, 2012). In this particular study, healthy subjects were supplemented with 8 mmol of KNO_3 (and matched KCl control in a randomized, double-blind, cross-over design). PWV was measured at baseline and 3 h post-intervention, when plasma NO_2^- levels peak post-NO_3^- ingestion (see section 6.2). Although there was no effect of the placebo, NO_3^- supplementation resulted in significant elevations in plasma NO_2^- and cGMP levels, reductions in BP and reductions in PWV (Bahra *et al.*, 2012). These results suggest that the effects of NO_3^- supplementation observed in our hypertensive cohort are not simply a reflection of elevated arterial tone and may indeed reflect modulation of elastic distensibility of the arterial tree by NO_2^--derived NO.

6.3.5 Implications of the lack of effect of placebo

Finally, in all of the measures of bioactivity, no significant changes were observed in the control limbs using KCl capsules or water. The significance of this finding is two-fold. Firstly, this suggests that the effects on BP were due specifically to the activity of NO_3^-. Beetroot juice contains other potentially vasoactive components, most important amongst these, as with many other vegetables, significant levels of K^+ (up to 93 mM (Webb *et al.*, 2008a)), that have previously been suggested as a putative mechanism of the BP-lowering effects of a vegetable-rich diet (Adrogué and Madias, 2007). Thus, by using a matched KCl dose as a control, the K^+ content of dietary forms of NO_3^- has been accounted for. Thus, these findings suggest that the BP changes after inorganic, or dietary, NO_3^- ingestion were not due to the K^+ content, as there was no decrease in BP after KCl ingestion. Moreover, the lack of correlation between change in plasma K^+ levels and change in SBP after dietary NO_3^- ingestion in the hypertensive study also supports the view that the BP-lowering effects of beetroot juice are not due to the activity of this particular cation, as had been previously suggested for the DASH diet (Appel *et al.*, 1997). In addition, matched KNO_3 supplementation closely replicated the BP-lowering effects of dietary NO_3^- (Webb *et al.*, 2008a) Hence, the BP-lowering mechanism of NO_3^- supplementation is likely to be due to the endogenous conversion of NO_3^- to NO_2^-, and thence to NO, especially since the changes in plasma NO_2^- levels correlated closely with reductions in BP.

In the hypertensive study, water-placebo produced a short-lived *pressor* response after ingestion. It is well recognized that water can elevate SBP in healthy middle-

aged subjects of up to ~12mmHg within 1 h (Jordan *et al.*, 2000), and it is interesting to note that there was no apparent *pressor* response to volume ingestion after dietary NO_3^-. Nevertheless, dietary NO_3^- ingestion significantly reduced BP compared to baseline in itself.

6.3.6 NO_3^- supplementation does not alter HR

There were no significant elevations in HR with the BP-lowering effects of dietary NO_3^- in healthy or hypertensive subjects, similar to the effects on HR in healthy subject in previous investigations (Webb *et al.*, 2008a). This is surprising, as it would be expected that a vasodilator, through reductions in BP, would cause a reflex increase in HR. It may be that NO_3^- or NO_2^- have negatively chronotropic cardiac or sympatholytic effects; a possibility that deserves further, separate investigations. It may be that the slow-developing, smooth reduction in BP observed after inorganic or dietary NO_3^- ingestion mitigates against the reflex increases in HR normally observed with vasodilator therapy.

6.4 NO_3^- reduction by oral bacteria contributes to BP regulation

The colonization by micro-organisms of most surfaces of the human body, including the GI tract, provides us with a number of beneficial functions that we do not possess innately. For example the symbiotic relationship that humans have with GI microflora endows the host with a number of qualities not least the capacity to digest components of plants, host defense and the development of tolerance to a number of pathogens (Bäckhed *et al.*, 2005). Whilst a symbiotic relationship has been demonstrated for a number of host-microbial interactions in the lower GI tract, the oral microflora, however, remains virtually unexplored in this aspect. In fact, the general view is of an overall detrimental role for this microflora, traditionally associated with negative health effects ranging from halitosis and caries (Ratcliff and Johnson, 1999) to CVDs (Desvarieux *et al.*, 2003, 2005, 2010). This negative view is clearly reflected in the widespread usage (e.g. 30-45% in the USA and UK) of antiseptic mouthwashes in the general population (Elmore and Horwitz, 1995; Fedorowicz *et al.*, 2008; Chadwick *et al.*, 2011).

The conversion of dietary-derived NO_3^- to NO_2^- by oral bacteria is a classical example of a proposed harmful process since salivary NO_2^-, when swallowed, can give rise to N-nitrosamines (Harada *et al.*, 1974; Tannenbaum *et al.*, 1974; Ishiwata *et al.*, 1975a, b, c, d; Ishiwata, 1976a, b; Spiegelhalder *et al.*, 1976; Tannenbaum *et al.*, 1976; Eisenbrand *et al.*, 1980; Tannenbaum and Correa, 1985), with potentially carcinogenic effects (Bogovski and Bogovski, 1981). This has led to strict regulations of permissible levels of NO_3^- in food and drinking water. In contrast, the results presented herein support the notion that oral NO_3^--reducing bacteria are beneficial

to the host and participate in control of cardiovascular NO homeostasis to modulate BP.

As discussed in section 1.6.4, oral bacteria are crucial in the initial step of a NO_3^--NO_2^--NO pathway. Thus, it was important to clarify the role of oral bacteria in physiological processes related to NO bioactivity. The oral NO_3^- reductase study presented in chapter 4 investigated whether reduction of endogenously-generated NO_3^- to NO_2^- by oral bacteria under basal conditions (i.e. without exogenous provision of inorganic NO_3^-), contributes to plasma NO_2^- levels and BP regulation in healthy subjects. For this, an intervention with antiseptic mouthwash for 7 days to disrupt oral NO_3^- reduction was provided and thence plasma NO_2^- levels and BP were measured. Twice-daily use of an antiseptic mouthwash for 7 days near abolished oral conversion of NO_3^- to NO_2^-. More importantly, this was accompanied by a decrease in plasma NO_2^- levels (~25%) and a concomitant increase in BP. Furthermore, as evidenced by the home BP measurements, the persistence of the effect of mouthwash on BP from the first day of instigation suggests that there is no immediate tachyphylaxis, at least over a 7 day period, in response to the mouthwash. The hypothesis is that the mechanism underlying the observed increase in BP is a decrease in the amounts of NO or other bioactive nitrogen oxides generated from NO_2^- via recently described reductive pathways (as discussed in section 1.8). Support for this hypothesis emanates from the robust correlation evident between changes in plasma NO_2^- and changes in BP that were apparent after 7 days used of the antiseptic mouthwash intervention. It is unlikely that these effects relate to other mechanisms, such as increased stress due to instillation of

mouthwash, since the effects on SBP were present on every occasion throughout the 7 day home BP measurement protocol and also evident in the averaged nighttime BP mean.

All subjects were fasted on clinic visits, therefore the majority of the circulating NO_3^- measured in their plasma likely originates from the endogenous source of the NOS pathway. The NO_3^- levels that were apparent in the subjects at baseline are in good agreement with previous estimates of the relative contributions of exogenous vs. endogenous NO_3^- (Packer *et al.*, 1989). In light of this, the present data suggest the existence of a salvage pathway in which NO_3^-, derived from oxidized NO, is partly recycled back to NO with the help of oral NO_3^--reducing bacteria. This represents the first demonstration of the functional activity of endogenously generated NO_3^- in humans.

NO_3^- is a very stable molecule and, unlike bacteria, human cells cannot efficiently metabolize this anion. This fact together with these observations suggests that the process we describe herein fulfill the criteria for a true symbiotic relationship whereby the host provides the oral bacteria with NO_3^- via active secretion to the site where they reside. The bacteria then use this NO_3^- as a terminal e^- acceptor to allow respiration in the absence of O_2. In return, the bacteria provide the host with NO_2^-, a substrate used for further generation of the biological messenger NO (Moreno-Vivian *et al.*, 1999; Lundberg *et al.*, 2004). That NO is the likely mediator of the effects seen is supported by observations demonstrating that the BP-reducing effects of exogenously administered inorganic or dietary NO_3^- are associated with

levels of the signalling nucleotide cGMP in healthy and hypertensive subjects, measurement of which has been recently shown to provide a sensitive indicator of NO bioactivity and to underlie many of the beneficial effects of NO (Hobbs and Stasch, 2010).

It has been estimated that 30-45% of the adult population in the US and UK (Elmore and Horwitz, 1995; Fedorowicz *et al.*, 2008; Chadwick *et al.*, 2011) use some form of antiseptic mouthwash on a regular basis. In the present study the mouthwash contained chlorhexidine as the active ingredient. It remains to be determined whether other antiseptic mouthwash products would affect NO_2^- and NO homeostasis in a similar manner. Nevertheless, the present results clearly demonstrate effects on BP that could be of clinical significance if sustained over prolonged periods of time beyond the 7 days measured in the present study. The increase in BP was reflected in all of the 3 methods employed to accurately measure BP. Indeed, home BP and 24h ABP are being increasingly recognized as important BP measures that are independently predictive of cardiovascular mortality (Mancia *et al.*, 2007).

Although the increases in BP may seem small (~ΔSBP 2-3.5mmHg), on a population scale if these increases were applied to a pre-hypertensive or hypertensive cohort, then it could reasonably be estimated from previous analyses that oral NO_3^- reduction likely plays an important role in decreasing both stroke and cardiovascular morbidity and mortality, since every 2 mmHg increase in SBP results in a 7% increase in the risk of mortality due to ischaemic heart disease and a 10%

increase in the risk of mortality due to stroke (Lewington *et al.*, 2002). Whether a similar pathway operates in individuals with hypertension or in individuals with increased risk of developing clinical hypertension (i.e. pre-hypertension), or indeed whether dysfunction of this pathway might contribute to CVD progression is unknown, although recent evidence clearly demonstrates the presence of NO_3^--reducing bacterial species, including *Veilonella spp.* and *Pseudomonas luteola* (Koren *et al.*, 2011) in the oral cavity of individuals with atherosclerotic disease. However, this possibility has been addressed, at least in part in chapter 5, as inorganic dietary NO_3^- supplementation in hypertensive subjects elevated plasma NO_2^- levels and lowered BP in a similar vein to that demonstrated to occur in healthy individuals, demonstrating effective oral NO_3^- reduction after dietary NO_3^- supplementation.

In conclusion, these studies provide evidence of a role for oral NO_3^--reducing bacteria in modulation of vascular NO_2^- and NO homeostasis and thereby, a physiological role of NO_2^-, derived from the oral reduction of endogenously generated NO_3^-, in BP regulation. These data further suggest that disturbances in NO_2^- homeostasis (herein achieved via interruption of NO_3^--reduction in the oral cavity by commercial mouthwash use) have small, yet potentially important implications for cardiovascular health.

6.5 Sex differences in handling and response to inorganic NO_3^- supplementation

The post-hoc analyses of the 24 mmol KNO_3 vs. KCl study exposed apparent sex differences in the response to inorganic NO_3^- supplement. Significant differences in baseline plasma NO_2^-, but not plasma NO_3^-, levels associated with lower baseline BP were evident in the female subjects compared to the male subjects. This finding is supportive of the view that a close relationship between NO_2^- levels and BP exists in humans. This correlation has been demonstrated previously, but attributed to differences in vascular eNOS expression and activity (Forte *et al.*, 1998), an effect that, in addition to endothelium-derived hyperpolarising factor (Scotland *et al.*, 2005), has been proposed to mediate the prevalence of lower BP in premenopausal women compared to age-matched men (Lerner and Kannel, 1986). These data, herein, also raises the further possibility that the association of plasma NO_2^- levels with lower BP evident in pre-menopausal women may relate, in part, to the bioactivity of the elevated NO_2^- levels. This difference in basal NO_2^- levels may underlie the apparent decreased sensitivity to further elevations in plasma NO_2^- levels in females due to a possible *saturation* of the vasodilating effect of NO_2^-, as proposed by Gladwin and colleagues (Dejam *et al.*, 2007). In this study, it was demonstrated that increases in plasma NO_2^- levels (low µM) produced substantial increases in FBF but that a saturation of the vasodilatory effect was observed with higher µM plasma NO_2^- levels (Dejam *et al.*, 2007).

In addition, these analyses intimate sex-differences in the endogenous handling of NO_3^-. Indeed, an identical inorganic NO_3^- load (24 mmol KNO_3) whilst resulting in only subtle, but significant, differences in NO_3^- levels between the sexes, caused ~2-

fold higher plasma NO_2^- levels in females compared to males. It is possible that the differences in plasma NO_2^- and NO_3^- levels after ingestion of 24 mmol KNO_3 reflects the differences in body weight between the 2 sexes, which was greater in the females compared to the males. However, normalization of plasma NO_x levels to body weight did not alter the shape of the profiles seen and significant differences in plasma NO_2^- levels still persisted.

Whilst differences in absorption and excretion of NO_x may underlie some of these differences, it is apparent that the differences in plasma NO_2^- levels reflect, in part, sex-differences in bacterial conversion of NO_3^- to NO_2^- in the oral cavity. There were significantly higher rates of oral NO_3^- reduction in the oral cavity in females in response to physiological, basal salivary NO_3^- concentrations (~0.8 mM) and also in response to NO_3^- levels that are apparent in the mouth after ingestion of 8-10 mmol inorganic NO_3^- (Lundberg and Govoni, 2004; Bahra *et al.*, 2012) that may be found in ~300 g meal of spinach or lettuce (Hord *et al.*, 2009).

There are several, potential explanations as to how there could be differences in this part of the entero-salivary circulation. There could be a difference in bacterial load between the sexes. Additionally, there could be differences in the prevalent species of bacteria, and there is evidence within the oral cavity that sex hormones can alter the prevailing microbial flora (Klinger *et al.*, 1998). It is well recognized that for different bacterial species, the NO_3^--reductase enzymes have significantly different K_m (Betlach and Tiedje, 1981) and different bacterial species also may contain vastly different copy number (Smith *et al.*, 2007) of the 3 distinct

prokaryotic NO_3^--reductase genes (Moreno-Vivian *et al.*, 1999; Lundberg *et al.*, 2004). Hence, there is a potential for wide variation in NO_2^- production with differences in either load or species of symbiotic bacteria, which may be altered by sex hormones.

Currently, it is thought that the predominant lingual bacteria responsible for NO_3^- reduction in the oral cavity are gram-negative *Veillonella spp.* and gram-positive *Actinomyces spp.* (Duncan *et al.*, 1995; Doel *et al.*, 2005). However, whether sex differences exist in the colonization of the tongue or NO_3^- reductase activities of these bacteria in humans is currently unknown.

However, these studies cannot rule out sex-differences in excretion as another important factor. Urinary NO_2^- levels was similar in males and females, despite significantly higher plasma NO_2^- levels in females, suggesting that there may be more efficient reabsorption or different renal threshold for NO_2^- reabsorption in females.

One of the problems in investigating these possibilities is that the specific pathways for NO_x movement across renal tubular cell membranes have not been fully elucidated. In erythrocytes, there may be a role for AE-1 (Vitturi *et al.*, 2009; Jensen and Rohde, 2010) and the sodium-dependent phosphate transporter (May *et al.*, 2000) but only recently have renal carbonic anhydrases been implicated in renal NO_2^- handling in humans (Chobanyan-Jürgens *et al.*, 2012). Very recently, a pathway for NO_3^- transport that may be important in the salivary glands has been identified

as the sialic acid transporter, sialin, which can additionally facilitate intracellular movement of NO_3^- in a $2NO_3^-/H^+$ electrogenic manner (Qin *et al.*, 2012). Sialin is also present in renal tissue (Reimer and Edwards, 2004) and therefore may be important for renal NO_3^- transport as well.

These differences in oral NO_3^- reduction between females and males was reflected in not only higher baseline plasma NO_2^- levels despite similar plasma NO_3^- levels, but also disruption of the entero-salivary circulation by use of antiseptic mouthwash reduced plasma NO_2^- levels to a greater extent in females. Following antiseptic mouthwash use, plasma NO_2^- levels were similar in both sexes. These results possibly suggest that bacterial NO_3^- reduction may be an important determinant in the sex-differences in plasma NO_2^- levels seen. In addition, reduction in plasma NO_2^- levels was associated with significant elevation in clinic BP and 24 h ABP only in females, though the change in home BP was significant in both sexes.

Taking all of the analyses together the data suggest that these apparent differences in processing of NO_3^- may contribute to the prevalence of lower baseline BP in women compared to men (Lerner and Kannel, 1986).

6.6 Nutritional and toxicological aspects

Determining how vegetables confer protection against CVD and exploiting this to therapeutic advantage is likely to have considerable health and economic implications. Recently, it has been suggested that dietary NO_3^- found in high levels in vegetables might underlie some of the beneficial effects of vegetable-rich diets (Lundberg *et al.*, 2006; Webb *et al.*, 2008a; Ralt, 2009). It is ironic that NO_3^-, a natural component of vegetables with a proposed harmful effect (via proposed carcinogenic properties as discussed earlier), is now emerging as a possible mediator of cardiovascular benefits. Indeed, it would be strange, in evolutionary terms, if NO_3^- would be purposefully concentrated by the salivary glands and then secreted into the oral cavity, followed by entero-salivary recycling, if this was actually harmful. It would make biological sense that such a sophisticated, precise mechanism has evolved for a net beneficial reason. These studies described herein suggest that the recycling of NO_3^- to NO_2^- may play an important role in vascular homeostasis.

6.6.1 Pharmacodynamic advantages of NO_3^--based therapeutics

As has been alluded throughout, NO_3^- can be thought of as a 'pro-drug' for bioactive NO_2^-, formed by the action of bacterial NO_3^- reductases. However, there are some advantages to this dietary NO_3^- approach over NO_2^-. Inorganic and dietary NO_3^- has a much longer $t_{1/2}$ in human plasma (~6 h) (van Velzen *et al.*, 2008) compared to NO_2^- given either by oral or *i.v.* routes (~15-45 min) (Dejam *et al.*, 2007; Hunault *et al.*, 2009) and, therefore, could be given as an once-daily dosing regime. The peak:trough ratio of the pharmacodynamic effects of dietary NO_3^- in hypertensive

subject described above also suggest that this is a viable dosing strategy. Importantly, the rise in plasma NO_2^- levels after NO_3^- ingestion is slow and sustained to 6 h and remaining slightly elevated at 24 h after a single dose. One could speculate that such a once-a-day approach is one that has the potential for improved compliance in the clinical setting, although this warrants further exploration. There is, of course, the added attraction that dietary approaches to chronic disease may be more attractive since they offer a 'natural' and potentially low cost approach to CVD. However, there are some important aspects of dietary NO_3^- and NO_2^- that require consideration before such an approach can be taken.

6.6.2 Agricultural issues with a NO_3^--based therapeutic approach

An important limitation of the dietary approach to NO_3^- supplementation is the range of NO_3^- concentrations found naturally in batches of plants. Plants accumulate NO_3^- through the roots and use it for novel synthesis of amino acids and proteins (Vogtmann and Biedermann, 1985). If the plant does not use the NO_3^- immediately, it is stored in vacuoles and it remains in the vacuoles if the plants are supplied with more NO_3^- than they can use (Martinoia *et al.*, 2007). NO_3^- will therefore be stored more readily in vegetables with low rate of photosynthesis (Martinoia *et al.*, 2007). Hence, vegetables harvested in winter have higher NO_3^- content than those harvested in the summer (Food Standards Agency, 2004). This produces a problem when trying to give a fixed dose of NO_3^- in the form of a vegetable or vegetable-based drink. In the studies reported herein, the [NO_3^-] of beetroot juice used was 13.2±0.94 mM (n=15) and 22.4±3.8 mM (n=9), whilst previous research from Prof Ahluwalia's group used a beetroot juice with [NO_3^-] of

45.0±2.6 mM (Webb *et al.*, 2008a). It is impractical to alter the volume ingested on a batch-by-batch basis. In this regard, a commercial company has recently produced a beetroot juice concentrate with a fixed amount of NO_3^- (~5mmol, http://www.beet-it.com/organic) that is currently aimed at use in elite athletes and this approach to provide a fixed dose could be used, as clearly, could a inorganic NO_3^- capsule formulation.

6.6.3 NO_3^- supplementation is unlikely to cause significant methaemoglobinaemia

The fruit and vegetable-rich DASH diet (Appel *et al.*, 1997) that lowers BP is estimated to contain up to ~20 mmol of NO_3^- (Hord *et al.*, 2009), exceeding the recommended ADI limit for NO_3^-, which currently is set at 3.7mg/kg daily (Speijers and van den Brandt, 2003) which would be ~4.2 mmol in a 70 kg person. However, most of the doses of NO_3^- given that show beneficial effects clearly exceed this advised limit.

The levels of NO_3^- consumption advised and concentration in drinking water is strictly controlled in many countries and is based upon two main concerns. Firstly, NO_3^- in drinking water has long been thought to cause methaemoglobinaemia and on the basis of observational data (Comly, 1945; Walton, 1951) a maximum limit of 50 mg/L (=0.8 mM [NO_3^-]) has been implemented in Europe and USA. Methaemoglobinaemia caused by NO_3^- is a consequence of the interaction of NO_2^-, formed as an intermediate, with erythrocytes, resulting in the oxidation of the Fe^{2+} (ferrous state) in Hb to the Fe^{3+} (ferrous) state, forming stable metHb (Doyle *et al.*, 1981), which is incapable of O_2 transport.

However more recently, there has been a reappraisal of these concerns and a reevaluation of those early data (Fewtrell, 2004). Most of the cases of methaemoglobinaemia reported in the 1940's were associated with high-NO_3^- containing well water in infants who developed co-existent diarrhoea from presumed infectious gastroenteritis (Comly, 1945; Walton, 1951). It has since been suggested that bacterial contamination of the well-water (Avery, 1999), or bacterial NO_3^- reduction *in vivo* during intestinal infection (Hanukoglu and Danon, 1996) may be responsible for apparent NO_3^--induced infantile methaemoglobinaemia, especially as recent reports have demonstrated no consistent association between either metHb levels or the risk of developing clinical methaemoglobinaemia with drinking water NO_3^- levels (reviewed in Ward *et al.*, 2005).

Interestingly, in the measurements of commercially available beetroot juice, the NO_3^- concentration ranged from 13-45 mM, clearly above the set limits for drinking water. However, post-ingestion of dietary NO_3^-, maximal plasma NO_2^- levels remained on average below 1 µM (a level within the normal physiological realm). It is also noteworthy that after intra-arterial infusion of NO_2^- in healthy subjects, systemic NO_2^- levels of 16 µM were achieved (far higher levels than those needed to see BP lowering) but with no evidence of substantial methaemoglobinaemia with levels being measured at ~1% (Cosby *et al.*, 2003). More recently, a pharmacokinetic study was performed to determine dose-limiting toxicity in healthy subjects (Pluta *et al.*, 2011). Doses of $NaNO_2$ between 6.4-7.7 µmol/kg/h *i.v.* for 3-9 h produced large, symptomatic reductions in BP (~20 mmHg decrease in MAP) but only clinically insignificant, asymptomatic metHb levels of between 2-5% with

plasma NO_2^- levels between 1-5 µM (Pluta *et al.*, 2011); methaemoglobinaemia becoming symptomatic when levels >8% (Ash-Bernal *et al.*, 2004). Importantly, there have been no cases of vegetable-induced methaemoglobinaemia in adults reported in the literature and cases in children have been related to improper storage of cooked or salted vegetables that allowed bacterial contamination and elevated NO_2^- levels to accumulate pre-ingestion (for review of this area see Chan, 2011).

6.6.4 NO_3^- is unlikely to increase risk of carcinogenesis

There has also been a concern for many years about the formation of N-nitrosamines after ingestion of both NO_2^- and NO_3^- and therefore, potential carcinogenesis (Harada *et al.*, 1974; Tannenbaum *et al.*, 1974; Ishiwata *et al.*, 1975a, b, c, d; Ishiwata, 1976a, b; Spiegelhalder *et al.*, 1976; Tannenbaum *et al.*, 1976; Eisenbrand *et al.*, 1980; Tannenbaum and Correa, 1985), especially in the upper GI tract (Iijima *et al.*, 2003). However, a comprehensive review of the data in 2003 by the World Health Organization Expert Committee on Food Additives concluded that there was no evidence that NO_3^- was carcinogenic to humans (Speijers and van den Brandt, 2003). Reassuringly and importantly, in large cohorts (>100,000 subjects) followed for many years, a fruit and vegetable-rich diet, where the ADI is exceeded several fold, is not associated with any increase in cancer or mortality (Hung *et al.*, 2004), indeed the contrary is true (World Cancer Research Fund/American Institute for Cancer Research, 2007; Boffetta *et al.*, 2010).

6.6.5 Oral hygiene treatments may have adverse cardiovascular effects

The studies in this thesis also intimate possible adverse cardiovascular effects of excessive antiseptic mouthwash use in healthy individuals. This view is in contrast with the clear association of oral bacterial periodontal infection with hypertension and vascular dysfunction (Tonetti *et al.*, 2007; Desvarieux *et al.*, 2010) but this difference may reflect health status, possible translocation of bacteria and bacterial species prevalent. However, considering the wide-scale use of over-the-counter mouthwash within the general population (estimates suggest 30-45% of the US population use daily mouthwash (Elmore and Horwitz, 1995; Fedorowicz *et al.*, 2008; Chadwick *et al.*, 2011) the potential impact of these findings are substantial.

6.7 Limitations

6.7.1 Sample size

These studies in healthy and hypertensive subjects represent phase I/IIa-type studies that would be performed if dietary or inorganic NO_3^- were novel small chemical entities. Although the sample sizes were not large in all studies (n=6-20 completed), they were adequately powered by prospective power analyses to detect differences in total cohorts for changes in NO_2^- levels and BP. The oral NO_3^- reductase study, described in chapter 4, recruited n=19 subjects to completion, rather than the prescribed n=21 by the initial power calculation. However, even with this *n* number, the achieved 1-β of the study was 0.86. This power level is above the conventionally accepted level of 0.80 that is expected for medical research (Bacchetti, 2010; Norman *et al.*, 2012). Post-hoc analyses in healthy subjects and stratification of results by sex (the latter due to dropouts) were underpowered to robustly detect statistical sex-differences in BP. However, sex-differences in baseline plasma NO_2^- levels were detected in both healthy subject cohorts and the large sex-dependent differences in oral NO_3^- reduction are likely to be significant. An important limitation of the findings is that the studies were not controlled for the stage of the menstrual cycle in female subjects and this may have some relevance since resting BP is different throughout the menstrual cycle (Dunne *et al.*, 1991). However, this is likely to have increased the heterogeneity of the female results and reduced the likelihood of determining significant differences.

6.7.2 Study design

The *gold*-standard design for clinical intervention studies is the randomized, double-blind, placebo-controlled study. However the taste and colour of the dietary NO_3^- used (beetroot juice) is hard to blind and so water was used in an open-label design. Other researchers have used fruit juice cordials as a placebo (Bailey *et al.*, 2009; Vanhatalo *et al.*, 2010), though this design introduces other chemicals in the placebo that may have vascular effects. More recently, Prof N Benjamin (University of Exeter) has developed a beetroot juice 'placebo' made by passing beetroot juice through a commercially available anion-exchange resin, Purolite a520E, (Purolite, Ltd., Bala Cynwyd, USA) as used to control NO_3^- in water supplies. This has recently been used as placebo in ethically-approved research studies (Lansley *et al.*, 2011a, b; Vanhatalo *et al.*, 2011) and contains only 0.008 μM [NO_3^-] and is indistinguishable by taste or appearance from NO_3^--rich beetroot juice (Vanhatalo *et al.*, 2011).

Similarly, there was no adequate placebo for the oral mouthwash. All commercially-available mouthwashes contain antiseptic ingredients and the taste of the mouthwash precludes blinding with water or saline. When this study was designed, it was not clear how long the effects of antiseptic mouthwash would last for after termination of use (it has recently been demonstrated that oral NO_3^- reductase activity returns to baseline levels 48 h following cessation of antiseptic mouthwash use (Kanady *et al.*, 2012)). Therefore, it was not possible to conduct a cross-over design in the oral NO_3^- reduction study and there is a potential problem with order effect. These problems could have been solved using a parallel design if a useful placebo could have been found. However, the required sample size would have

been much larger and there were ongoing problems throughout these studies with adequate recruitment. All participants in the oral NO_3^- reduction study had a control period followed by an intervention period, so treatment effects could be confounded with a change over time. However, efforts were made to minimize this possibility by using 3 different measures of BP. In particular, home BP was measured on a daily basis for 7 days prior to the intervention. This data demonstrates the reproducibility of the BP measure on a day-to-day basis and clearly demonstrates no change from one day to the next supporting the view that instigation of the intervention did indeed affect BP. In spite of the lack of a placebo mouthwash, the dramatic drop in salivary NO_2^- levels together with the strong correlation between the change in plasma NO_2^- levels and change in BP, strongly supports the view that disruption of oral bacterial conversion of NO_3^- to NO_2^- is the cause of the observed effects. Saliently, none of the participants in the dietary NO_3^- or oral NO_3^- reduction studies were aware of the hypotheses and statistical analyses were performed blind to the treatment groups, making bias from the open-label designs less likely.

6.7.3 Time-course

The studies that involved NO_3^- supplementation were conducted over a range of times between 3-24 h. The initial study described in this thesis (24 mmol KNO_3 vs. KCl study) was so designed to investigate whether inorganic NO_3^- supplementation would replicate the effects of dietary NO_3^- ingestion, thus providing evidence that it was the NO_3^- content of the beetroot juice (as a source of dietary NO_3^-) that was responsible for the BP-lowering effects noted previously (Webb *et al.*, 2008a) and

thus the study design was replicated. In addition, this study design over 24 h was used in hypertensive subjects, as the time-course of changes in plasma NO_x levels and BP response following NO_3^- ingestion in hypertensives were not known. It was elucidated from these healthy subject studies that the time to peak plasma NO_2^- levels and BP-lowering after NO_3^- ingestion was consistent at ~3 h. Thus, in the further dose-response and dietary NO_3^- studies in healthy subjects, it was decided that the design would last only up to 3 h post-NO_3^- ingestion to maximize the recruitment for the studies. Throughout these studies, recruitment was a significant hurdle and recruitment was improved by limiting the length of the study visits.

6.7.4 Interpretation of oral NO_3^- reductase activity

The biological nitrogen cycle in bacteria is complex and can take inorganic NO_3^- (+5 oxidation) all the way to ammonia (-3 oxidation) (Schreiber *et al.*, 2010). NO_3^- reduction is only the first of many steps and several enzymes are involved in denitrification (Lundberg *et al.*, 2004). Many of these enzyme systems are co-localized within bacterial membranes and many bacteria have the complete denitrification pathway taking metabolites produced from one enzyme and reducing it in a step-wise fashion. Therefore, measuring NO_2^- accumulation may not be precise enough to adequately determine and quantify oral NO_3^- reductase activity. Any NO_2^- that may be produced may be further reduced to NO or all the way down to ammonia. NO_3^- disappearance from solution is an alternative indicator of NO_3^- reduction, but does not always mean that it ends up as NO_2^- and in these studies the primary interest was the contribution of the entero-salivary circulation to salivary and plasma NO_2^- levels as this is the anion with reported bioactivity.

6.8 Future perspectives

Despite the substantial advances made in anti-hypertensive pharmacotherapy, it is estimated that by 2025 the world will have 1.5 billion hypertensive patients (Kearney *et al.*, 2005). Indeed, over the past 3 decades the numbers of patients with uncontrolled essential hypertension has continued to rise year on year (Egan *et al.*, 2011). These are worrying statistics since raised BP is thought to underlie approximately 50% of all coronary events and greater than 60% of all strokes (Rodgers *et al.*, 2002); an observation supported by recent a *meta* analysis (Law *et al.*, 2009). Such statistics clearly support the rationale for identification of novel therapeutic approaches for hypertension.

In addition to identification of novel pharmacological and surgical approaches to treat hypertension (Paulis and Unger, 2010), there has been the implementation of large, relatively 'low cost' public health initiatives to improve a number of modifiable lifestyle factors, such as increasing vegetable consumption (Department of Health, 2003; Lichtenstein *et al.*, 2006). Whilst the benefits of vegetable-rich diet in populations at risk of CVD are well recognized (Appel *et al.*, 1997), the constituent elements of such diets have not replicated the same beneficial outcomes. For the first time it has been demonstrated in hypertensive subjects that a dietary NO_3^- load (in the form of beetroot juice) significantly elevates plasma NO_2^- levels with resulting reductions in BP. For any therapy to become part of the established armamentarium for CVD typically requires large-scale outcome trials. The results presented in this thesis offer support for advocating a fruit and vegetable-rich dietary approach to tackling the growing global burden of

hypertension and associated CVD, that is cheap, robust and a palatable alternative to medications that could be form part of both public health initiatives as well as treatment.

Given that ~50% of treated hypertensive subjects fail to achieve their target BP (Egan *et al.*, 2010, 2011), an additional strategy, based on intake of NO_3^--rich vegetables, may prove to be both cost-effective and favourable for public health. Hence, determining the long term therapeutic potential of inorganic and dietary NO_3^- is warranted and is currently underway and will inform as to whether a long-term elevation of dietary NO_3^- intake can provide sustained BP benefits. To date, >50% of patients have been recruited to a double-blind, randomized, parallel, placebo-controlled 4-week intervention in hypertensive subjects with dietary NO_3^- as beetroot juice (250mL daily) or a matched NO_3^--depleted beetroot juice (clinicaltrials.gov: NCT01405898). In this study, it will be determined whether dietary NO_3^- lowers BP and, in addition, whether dietary NO_3^- can improve endothelial function and arterial stiffness in hypertensive subjects. Some recently published results that do not appear in my thesis have shown that inorganic NO_3^- supplementation (8 mmol KNO_3) did not improve endothelial function *per se* in healthy subjects with normal endothelial function (Bahra *et al.*, 2012). However, our group has previously demonstrated that ingestion of dietary NO_3^- protected against IR-induced endothelial dysfunction of conduit vessels in healthy subjects (Webb *et al.*, 2008a). In 10 healthy subjects, IR caused reductions in brachial artery FMD that could be prevented by ingestion of a single dose of dietary NO_3^- (22.5 mmol dose) 2 h prior to the IR injury (Webb *et al.*, 2008a), reflecting the time taken for systemic

NO_2^- levels to elevate due to entero-salivary circulation of NO_3^- to NO_2^- (Lundberg and Govoni, 2004; Webb *et al.*, 2008a). Hypertensive subjects have endothelial dysfunction (Linder *et al.*, 1990; Panza *et al.*, 1993) and it may be that elevation of NO_2^- levels may improve endothelial function in this setting and this hypothesis is currently being tested (clinicaltrials.gov: NCT01405898).

In this regard, it has recently been suggested that provision of NO via the alternative reductive pathway (i.e. from NO_2^- or NO_3^-) might also have beneficial effects in the pathogenic processes associated with endothelial dysfunction (Stokes *et al.*, 2009). Tsuchiya and colleagues demonstrated that in a L-NAME induced model of hypertension, co-administration of dietary NO_2^- (~20-200 μM in drinking water) for 8 weeks prevented NOS-inhibition induced hypertension (Tsuchiya *et al.*, 2005), elevated systemic NO production (as measured by circulating HbNO) (Tsuchiya *et al.*, 2005; Kanematsu *et al.*, 2008) and prevented associated renal damage assessed histologically and by urinary proteinuria (Kanematsu *et al.*, 2008) suggesting that dietary NO_2^- can play a role in compensating for the depletion of eNOS-derived NO in situations such as endothelial dysfunction (Tsuchiya *et al.*, 2010).

More recently, eNOS knockout mice were fed 1 mM NO_3^- water or placebo water for 8-10 weeks. Surprisingly, weight in the obese mice was significantly reduced at the end of the supplementation period (~4.3g different to control population) with no significant difference in food or water intake (Carlström *et al.*, 2010). Triglycerides, visceral fat, blood glucose homeostasis after oral glucose tolerance

testing and glycated Hb were all also significantly improved in the active group (Carlström *et al.*, 2010), suggesting that dietary NO_3^- could compensate for some of the metabolic consequences of disturbances in eNOS-derived NO production.

Interestingly, raising plasma NO_2^- levels in a sustained manner by dietary supplementation with either NO_2^- or NO_3^- in the drinking water also produced similar protection against myocardial IR injury. Lefer and colleagues demonstrated that pre-treatment with dietary NO_2^- (~0.7 μM) or NO_3^- (~12 mM) in mice for 7 days increased plasma and heart NO_2^- levels by ~2-3 fold for both treatments (Bryan *et al.*, 2007). These elevations in plasma and tissue NO_2^- levels were associated with reduced infarct size in response to a myocardial injury (30 min left coronary ligation and then reperfusion) (Bryan *et al.*, 2007). There was a 48% reduction in infarct size after dietary NO_2^- supplementation and 33% reduction in infarct size after NO_3^- supplementation in drinking water (Bryan *et al.*, 2007). In addition, depletion of basal plasma and myocardial NO_2^- stores, by putting mice on a low-NO_2^- diet for 7 days, caused larger infarct size than mice fed normal chow diet alone, and this worsening of outcome was entirely ameliorated by provision of NO_2^- in the drinking water, restoring plasma and tissue NO_2^- levels to normal physiological levels (Bryan *et al.*, 2007). These findings support the view that elevation of plasma NO_2^- levels may be a useful preventative strategy for at risk individuals and that basal, systemic NO_2^- levels afforded by the entero-salivary circulation have important effects on cardiovascular homeostasis (Bryan *et al.*, 2007).

Further translation of these results in a canine model of myocardial infarction (MI) demonstrated that infusion of NO_2^- (0.2 μmol/min) in just the last 5 min of a 2 h period of ischaemia reduced infarct size by ~50% compared to control (Gonzalez *et al.*, 2008). Such observations suggest that NO_2^- may have use in the treatment of acute MI in humans, in which NO_2^- could be delivered at the time of primary coronary intervention. Indeed, our group is translating this to the clinical setting currently, where $NaNO_2$ has entered phase II trials to determine whether intracoronary NO_2^- infusion can reduce infarct size and improve left ventricular function following acute MI (clinicaltrials.gov: NCT01584453).

Another intriguing aspect of the findings in this thesis is the role of the oral microflora in regulating NO-dependent mechanisms, such as BP in these studies. Apart from vasodilator actions, NO_2^--derived NO also has a number of other potentially beneficial effects in humans, including cytoprotection after IR injury (Lang *et al.*, 2007), inhibition of platelet aggregation (Richardson *et al.*, 2002; Webb *et al.*, 2008a; Srihirun *et al.*, 2012) and improvement of mitochondrial efficiency (Larsen *et al.*, 2011). Future studies will reveal if the disturbances in circulating NO_2^- homeostasis following the use of an antiseptic mouthwash or other similar interventions, might affect these processes too.

It is already apparent that the entero-salivary circulation additionally has important effects on gastric physiology, including protecting against stress and NSAID-induced ulcers and that disruption of this pathway is detrimental (Björne H *et al.*, 2004; Jansson *et al.*, 2007; Petersson *et al.*, 2007, 2009; Jädert *et al.*, 2012). The role and

importance of this pathway in the prevention and treatment of disease in which NO plays an important part including osteoporosis (Wimalawansa, 2010) and pre-eclampsia (Savvidou *et al.*, 2003) are clearly warranted. Similarly, further investigation into the oral microflora may elucidate species differences between the sexes that explain differences in oral NO_3^- reduction that appear to have functionally important cardiovascular effects.

Depending on the appropriate validation of these results in larger populations of healthy subjects and patient populations, with hard end-point data such as major cardiovascular event and mortality outcomes and with the caveats listed above in mind, finally I wish to speculate on how this research could possibly change the medical and therapeutics landscape.

There should be a further public health push to increase the intake of specifically dietary NO_3^--rich green leafy vegetables, led by governments and inter-governmental agencies. There is the possibility that this focused approach may be more successful in persuading the general public to increase vegetable intake, with the backing of a definitive, scientifically-robust mechanism behind the calls, with a concurrent change in the ADI for NO_3^- to reflect this.

One could envisage a situation where dietary NO_3^-, in the form of a vegetable drink, could be recommended, or prescribed, long-term to patients with high-normal BP to retard the progression to a clinical diagnosis of hypertension. In the same vein, the same approach could be used as first-line treatment, and in conjunction with

established therapeutics, for hypertension and possibly other cardiovascular risk factors and CVDs.

In addition, there may an imperative to develop antibiotics, for common infections and infectious agents that require systemic antibiotics, which do not target the important oral-NO_3^- reducing microflora. Similarly, over-the-counter antiseptic mouthwashes should be developed that only target the pathogenic bacteria behind medical problems such as halitosis and periodontitis, without targeting NO_3^--reducing bacteria that inhabit the posterior, dorsal lingual surface.

Lastly, for patients that do require systemic antibiotics that destroy the NO_3^--reducing microflora in the oral cavity, one could envisage an oral suspension of the important species that could be instilled after the end of the antibiotics course to actively repopulate the oral cavity, in the same fashion as bio-cultures currently commercially available to replenish GI microflora. Other patients that have disrupted entero-salivary circulations, such as those intubated and ventilated on intensive therapy units (Weitzberg *et al.*, 2010) or with salivary gland dysfunction (Chen *et al.*, 2010a) may require systemic supplementation with NO_2^- itself to elevate circulating and tissue NO_2^- levels to avoid the detrimental effect of decreased NO_2^- levels.

6.9 Conclusions

In summary, inorganic NO_3^- administration either via the diet, in the form of beetroot juice, or supplementation exerts dose-dependent decreases in BP that are due to its entero-salivary processing to NO_2^- and then to NO in healthy and hypertensive subjects. These findings suggest a role for NO_3^- in preventing and treating hypertension, and supplementation either in water or by diet may provide a cheap and effective health strategy to combat the prevalence of CVD.

These observations also support the notion that oral NO_3^--reducing bacteria participate in physiological control of cardiovascular NO_2^- and NO homeostasis to modulate BP and suggest a potentially novel adverse cardiovascular effect of antiseptic mouthwash in healthy individuals in terms of BP regulation.

To conclude, this thesis challenges the current dogma that NO_3^- intake is only detrimental, and on the contrary suggests that dietary NO_3^- is important for cardiovascular health. It may be that sufficient supply of NO_3^- through the diet together with the functioning, oral microflora is essential for normal cardiovascular homeostasis.

These concepts resurrect the prescience of the early pioneers in the NO_2^- field from more than 80 years ago (Stieglitz and Palmer, 1937):

"Because of the rapid disappearance of nitrite from blood and the relative constancy of the level found in freshly drawn blood, one may tentatively assume that there is a constant production and destruction of the nitrite going on in the body.

"The source of the nitrite of the blood may be from administration by any route, absorption of nitrites from the bacterial reduction of food or drug nitrates in the lower portion of the bowel or absorption of nitrates and a subsequent reduction in the blood stream itself or a reduction of nitrates in the tissues. Any of these sources may be foci of a more or less constant production of nitrite…

"Nitrite… has profound effects in very small amounts on a great many functions of the body directly by its action of relaxing smooth muscle, especially arteriolar muscle, and indirectly by its effects on the blood flow in secretory organs.

The exact physiological significance of the blood nitrite is uncertain, but it may be that normally it aids in maintaining those functions which are stimulated by the administration of therapeutic doses. Clinical application of nitrite analysis of the blood may reveal some correlation between a disturbed nitrite metabolism and abnormalities of the arterial tension."

CHAPTER 7

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