

Dietary nitrate, nitric oxide and cardiovascular health

Catherine P Bondonno PhD^a, Kevin D Croft PhD^a, Jonathan M Hodgson PhD^a

^a School of Medicine and Pharmacology, University of Western Australia, Perth, WA 6000, Australia

Address for correspondence and requests for reprints:

Catherine P Bondonno

School of Medicine and Pharmacology

Level 3, Medical Research Foundation

Rear 50 Murray St, Perth Western Australia, Australia WA6000

GPO Box X2213 Perth Western Australia, Australia WA6847

Tel: 618 9224 0342

Fax: 618 9224 0246

Email: cbondonno@meddent.uwa.edu.au

ABSTRACT

Emerging evidence strongly suggests that dietary nitrate, derived in the diet primarily from vegetables, could contribute to cardiovascular health via effects on nitric oxide (NO) status. NO plays an essential role in cardiovascular health. It is produced via the classical L-arginine-NO-synthase pathway and the recently discovered enterosalivary nitrate-nitrite-NO pathway. The discovery of this alternate pathway has highlighted dietary nitrate as a candidate for the

cardioprotective effect of a diet rich in fruit and vegetables. Clinical trials with dietary nitrate have observed improvements in blood pressure, endothelial function, ischaemia-reperfusion injury, arterial stiffness, platelet function, and exercise performance with a concomitant augmentation of markers of NO status. While these results are indicative of cardiovascular benefits with dietary nitrate intake, there is still a lingering concern about nitrate in relation to methaemoglobinaemia, cancer and cardiovascular disease. It is the purpose of this review to present an overview of NO and its critical role in cardiovascular health; to detail the observed vascular benefits of dietary nitrate intake through effects on NO status as well as to discuss the controversy surrounding the possible toxic effects of nitrate.

Keywords: vegetables, nutrition, nutrients, L-arginine-NO-synthase pathway, nitrate-nitrite-NO pathway

INTRODUCTION

“We are what we eat”, Hippocrate’s famous maxim, is now more pertinent than ever. Evidence is mounting that diet is a critical determinant of health status. Epidemiological studies (Bazzano et al., 2003; Ness and Powles, 1997; van't Veer et al., 2000) indicate that increased fruit and vegetable intake reduces the incidence of cardiovascular disease. With cardiovascular disease being the leading cause of death in industrialized countries, there is considerable interest in identifying optimal diets and their exact components that can prevent or reduce its severity. Since the discovery of nitric oxide (NO) and the fundamental role it plays in endothelial function and cardiovascular health, components of a cardioprotective diet that could mediate their effect through NO pathways have come under the spotlight. One such candidate is dietary nitrate. It is the aim of this review to provide an overview of NO, focusing on the vasculature, and to review the controversial role that dietary nitrate, through effects on NO status, may play in cardiovascular health. We address the evidence that dietary nitrate provides an important contribution to the health benefits of a vegetable-rich diet.

NITRIC OXIDE

Historical Perspective: From *NO*xious to *NO*table

Since its discovery in 1772 as “nitrous air”, the understanding of the importance of NO has undergone a radical shift. What was once considered a noxious environmental pollutant is now regarded a key molecule in biological processes. Science magazine awarded NO the title of “Molecule of the year” in 1992 (Koshland, 1992) and the Nobel Prize in Physiology or Medicine

in 1998 was awarded to the researchers involved in its discovery: Furchgott, Ignarro and Murad. From its non-illustrious beginnings it is now recognised that NO plays a critical role in the biological processes of the heart and blood vessels, neurotransmission and non-specific host defence. Indeed, there is hardly a disease where altered NO homeostasis is not implicated in some way.

Isoforms of the nitric oxide synthases (NOS)

NO is produced from the conversion of the amino acid L-arginine to L-citrulline by one of a family of enzymes, the nitric oxide synthases (NOS)(Palmer et al., 1988). Three distinct NOS isoforms have been identified (Alderton et al., 2001) and are officially termed according to the order of their isolation and characterisation. NOS I is more commonly known as neuronal NOS (nNOS), NOS II as inducible NOS (iNOS) and NOS III as endothelial NOS (eNOS). These isoforms have distinct characteristics determined by their site of synthesis, pattern of expression and Ca^{2+} dependency (**Table 1**). They share between 50 and 60% sequence homology (Lamas et al., 1992), have similar enzymatic mechanisms which require five cofactors and are all calmodulin dependent (Bredt, 1999). The action of the NO generated by these isoforms is dependent on both its location and its concentration.

Two pathways to NO

L-arginine NO synthase pathway

The L-arginine NO synthase pathway is well defined and has been extensively reviewed (Alderton et al., 2001; Bredt, 1999, 2003; Jin and Loscalzo, 2010; Liu and Huang, 2008). In the

endothelium, eNOS (NOS III) synthesis is increased in response to biochemical stimuli (thrombin, adenosine diphosphate (ADP), serotonin, acetylcholine and bradykinin) as well as mechanical stimuli such as shear stress and cyclic strain (**Figure 1**). These stimuli cause the transient release of Ca^{2+} from intracellular stores. Ca^{2+} binds to calmodulin to form a Ca^{2+} -calmodulin complex which activates eNOS (Jin and Loscalzo, 2010). The enzymatic action of eNOS is highly regulated by cofactors such as flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin (BH_4), calmodulin and heme (Marletta, 1994). eNOS catalyses the synthesis of NO by the 5-electron oxidation of the terminal guanidine nitrogen atom of L-arginine with L-citrulline produced as a by-product. Nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen are cosubstrates. Once synthesised NO, which has a very short half-life, rapidly diffuses across the endothelial cell membrane where it activates guanylate cyclase in vascular smooth muscle cells by binding to its heme moiety, causing a rise in cGMP concentrations. cGMP acts as a second messenger for many important cellular processes, including smooth muscle relaxation. Indeed, cGMP, through its principal mediator PKG, is responsible for many of the biological effects of NO (Munzel et al., 2005). From experiments with L-N monomethyl arginine (LNMMA), an inhibitor of NOS, it is now clear that NO is continually being synthesized in the endothelium (Vallance et al., 1989).

Nitrate-nitrite-NO pathway

Since the discovery of the L-arginine NO synthase pathway, an alternate pathway for NO production, involving nitrate and nitrite, has been described (**Figure 2**) (Govoni et al., 2008; Petersson et al., 2009; Webb et al., 2008). Nitrate and nitrite have an interesting history in the

literature. They were initially considered environmental pollutants, potential carcinogens and endogenous inert end products of nitric oxide metabolism; however it is now apparent that nitrate and nitrite are important molecules in cardiovascular health. Through the endogenous nitrate-nitrite-NO pathway they have the potential to be converted into NO and form a large and abundant storage pool for this molecule in blood and tissues (Larsen et al., 2006; Lundberg and Govoni, 2004).

There are two major sources of nitrate and nitrite in the body: end products of the L-arginine-NO synthase pathway and the diet. Vegetable consumption accounts for 80% of dietary nitrate (Hord et al., 2009) and while there is some nitrite intake from the diet, the majority is derived from endogenous biochemical pathways. Endogenously nitrate could be reduced to nitrite via the entero-salivary circulation of nitrate (Govoni et al., 2008; Petersen and Stoltze, 1999; Webb et al., 2008) or, to a lesser extent, by the action of mammalian nitrate reductases such as xanthine oxidase (Jansson et al., 2008). The entero-salivary circulation of nitrate involves the active uptake of nitrate from blood, about 25% of plasma nitrate, by salivary glands. Nitrate is concentrated and secreted in the saliva where a portion of it is reduced to nitrite by oral facultative anaerobic nitrate reducing bacteria found in the deep clefts on the dorsal surface of the tongue (Gladwin et al., 2005). These bacteria use nitrate as an alternative terminal electron receptor to oxygen, producing nitrite as a by-product. This step is inhibited by use of antibacterial mouthwash (Webb et al., 2008) but not antibacterial toothpaste (Bondonno et al., 2012a). Once swallowed, nitrite can be converted to NO in the acidic environment of the stomach where it reduces gastrointestinal infection (Dyckhuizen et al., 1996), increases mucous barrier thickness (Bjorne et al., 2004) and stimulates gastric blood flow (Pettersson et al., 2007).

The remaining nitrite is absorbed into the blood stream (Lundberg and Govoni, 2004). Together with the nitrite produced as an end product of the L-arginine-NO synthase pathway, or by action of the mammalian nitrate reductases (Jansson et al., 2008), this nitrite is rapidly distributed throughout the body becoming a source of vasodilatory NO or acting as a signalling molecule in its own right (Bryan et al., 2005).

The exact mechanism by which nitrite is converted to NO is still uncertain. It could occur through the intermediate formation of *S*-nitrosothiols and other nitrogen oxides (Bryan et al., 2004; Gladwin et al., 2000) or by a number of substances and enzymes identified to have nitrite reducing potential such as xanthine oxidase (Zhang et al., 1997), haemoglobin (Cosby et al., 2003), myoglobin (Shiva et al., 2007a), neuroglobin (Petersen et al., 2008), respiratory chain enzymes (Kozlov et al., 1999), cytochrome P 450 (Kozlov et al., 2003), aldehyde oxidase (Li et al., 2008), carbonic anhydrase (Aamand et al., 2009) and NO synthase (Vanin et al., 2007). We recently observed a dose-related increase in nitrate reduction in the mouth and NO status with increasing nitrate intake (Bondonno et al., 2012a). Although the exact mechanisms still need to be clarified it is clear that through the nitrate-nitrite-NO pathway, dietary nitrate and nitrite could be a source of NO, supplementing NO generation from L-arginine by the nitric oxide synthase (NOS) enzymes. This is particularly evident when oxygen tension falls rendering the L-arginine-NOS pathway inactive (Feelisch et al., 2008). The nitrate-nitrite-NO pathway is not oxygen dependent and could therefore be a backup system when oxygen is depleted. The discovery of this alternate pathway for NO production could account for the observation in epidemiological studies that green leafy vegetables (high in nitrate) protect against cardiovascular disease (Hu

and Willett, 2002; Hung et al., 2004; Joshipura et al., 1999). The sources, half-lives and basal plasma concentrations of nitrate, nitrite and NO are summarised in **Table 2**.

Metabolic fate of NO

Once generated in the endothelium, NO has a number of potential effects which are largely determined by the availability and concentration of surrounding bioreactants (**Table 3**). These effects could be direct or indirect depending on whether NO itself, reacts with target molecules (direct) or whether, by its conversion to nitrite or the *S*-nitrosothiol (RNOS) derivatives of plasma proteins, its effects are directed at distant targets (indirect) (Wink et al., 1996).

NO has a half-life of only a few milliseconds when in close contact with other bioreactants (Thomas et al., 2001). However, in the red blood cell free zone close to the endothelial surface of a blood vessel, the biochemical lifetime of NO could be as much as 100-500 s (Ford et al., 1993). NO is uncharged and lipophilic allowing it to diffuse freely across biological membranes. The majority of NO that does not diffuse across biological membranes to activate cGMP will react with both oxy- and deoxyhaemoglobin (Liu et al., 1998). NO is oxidised by oxyhaemoglobin to form nitrate and methaemoglobin. NO's reaction with deoxyhaemoglobin results in the formation of iron-nitrosyl haemoglobin. NO also reacts rapidly with superoxide to form peroxynitrite, a reaction thought to be the reason for the pathological effects associated with NO (Beckman, 2009). The small amount of NO that escapes inactivation by molecules such as haemoglobin and superoxide, approximately 20%, is either oxidised to nitrite, reacts with thiol groups on proteins to form RSNOs or reacts with lipids to form nitrated lipids (MacArthur et al., 2007b).

It is now recognised that nitrate, nitrite and RSNOs are not only end products of NO metabolism but are important in preserving NO from local inactivation, in the long-distance transport of NO and in forming a storage pool of NO. These molecules have the potential to be converted back to NO when required and may explain some of the systemic effects of NO that occur after its administration by inhalation or infusion (Barbotin-Larrieu et al., 1996; Cannon et al., 2001; Fox-Robichaud et al., 1998; Takahashi et al., 1998; Troncy et al., 1997). An example of this was presented by Rassaf et al (Rassaf et al., 2002) who demonstrated that when NO is administered intravenously, the systemic effects observed are mediated by the conversion of NO to RSNOs.

Measurement of NO status

With the understanding that NO plays a critical role in health and that its levels are disturbed in disease states, has come the need to accurately detect and quantify this extremely labile molecule. NO's short half-life, gaseous nature and free radical structure makes direct measurement of NO an analytical challenge (Hausladen et al., 2007; Rogers et al., 2005; Wang et al., 2006). Since nitrate, nitrite and RSNOs are end products of NO metabolism and act as a storage pool for NO, the focus has been on quantifying these molecules as indicators of NO status. However this is not without difficulty and controversy (Giustarini et al., 2007; Gow et al., 2007; MacArthur et al., 2007a). These molecules are found in extremely low concentrations in complex biological matrices (Bryan and Grisham, 2007; Tsikas, 2000). Variations in sample collection and preservation procedures, the large number of different analytical approaches used as well as nitrite contamination has resulted in big disparities in reported values. For example,

the range of basal RSNO has been reported from undetectable up to micromolar values which is over 3 orders of magnitude (MacArthur et al., 2007b).

A wide variety of techniques have been used to measure NO status including photolysis chemiluminescence (Hausladen et al., 2007; Wang et al., 2006), spectrophotometry (Grisham et al., 1996), HPLC (Bryan and Grisham, 2007), GC-MS (Tsikas, 2000), electron paramagnetic resonance spectroscopy (Rocks et al., 2005), electrochemistry (Rafikova et al., 2004), immunological (Jaffrey et al., 2001) and fluorescent based methods (Bryan and Grisham, 2007). Of these techniques, gas-phase ozone based chemiluminescence has emerged as one of the most reliable assays for NO metabolites (Wang et al., 2006). The basal RSNO levels reported using this method are typically at a nanomolar level and therefore lower than those reported using other methods. Our lab has since validated a procedure that measures RSNO, nitrite and nitrate in biological matrixes and we report values that are consistent with the low levels reported using ozone based chemiluminescence (Yang et al., 2013).

Nitrate levels in the plasma are now considered to be an unreliable marker of eNOS activity. Nitrate levels are influenced by dietary nitrate intake, bacterial synthesis of nitrate within the gastrointestinal tract, denitrifying liver enzymes, saliva formation and renal function. In addition, nitrate is present in plasma in the micromolar range and is produced in the nanomolar amounts by the L-arginine NOS pathway. This makes measurement of any changes due to eNOS activity difficult (Kelm, 1999).

CARDIOVASCULAR HEALTH AND VASCULAR FUNCTION

Overview and importance

Cardiovascular disease is one of the leading causes of death and morbidity as well as imposing a huge economic burden at both country and household level (Fuster et al., 2011; Lloyd-Jones et al., 2010). A healthy endothelium plays a pivotal role in cardiovascular health and indeed endothelial dysfunction is associated with the development of atherosclerosis, hypertension and heart failure (Vallance and Chan, 2001).

Endothelial structure and function

The healthy endothelium is a monolayer of cells that is strategically placed between the lumen and vascular smooth muscle cells. Originally viewed as a simple semi permeable barrier between blood and interstitium, it is now recognised as a multifunctional key regulator of vascular homeostasis (Widlansky et al., 2003). Endothelial cells respond to both physical and chemical stimuli by synthesis or release of a wide range of molecules that regulate vascular tone (influencing arterial stiffness and blood pressure), inflammation, permeability and growth as well as blood fluidity and coagulation (Widlansky et al., 2003). An alteration in endothelial function, endothelial dysfunction, is considered to be the initial (and reversible) stage in the development of cardiovascular disease (Endemann and Schiffrin, 2004; Vanhoutte, 2009). Endothelial dysfunction is an impairment of endothelium-dependent relaxation, with a tendency towards a proinflammatory and prothrombotic state (Herrmann and Lerman, 2008). It is strongly associated with cardiovascular risk factors including obesity, smoking, aging, hypercholesterolaemia, hypertension, hyperglycaemia, systemic infection and a family history of early atherosclerotic disease (Widlansky et al., 2003). These risk factors are also associated with arterial stiffness

which is an independent predictor of cardiovascular risk (Benetos et al., 1993; Mahmud and Feely, 2003; O'Rourke and Hashimoto, 2007; Tanaka et al., 1998; Wilkinson et al., 2002a). While the pathophysiology of endothelial dysfunction is complex, it is now recognised that the central element is an attenuation of NO production and/or bioavailability.

Vascular effects of NO

NO is a key regulator of vascular homeostasis and integrity. Decreased production and/or bioavailability of NO and impairment of endothelial function is implicated in a number of cardiovascular disorders including hypertension, atherosclerosis and ischaemic disease. Whether reduced NO bioavailability is the cause or result of endothelial dysfunction is not yet understood. However, the importance of NO in maintaining vascular tone, vasodilation, as well as its antithrombotic, antiatherogenic and antiproliferative properties are now accepted (Jin and Loscalzo, 2010). This has been demonstrated experimentally by inhibiting its synthesis. In addition, mice lacking eNOS have endothelial dysfunction, are hypertensive and show a more severe outcome in response to vascular injury, stroke, cerebral ischemia and diet induced atherosclerosis (Liu and Huang, 2008).

Vascular tone and vasodilation

NO is important in vascular homeostasis playing a key role in maintaining basal vascular tone through its continual low level release from endothelial cells (Vallance et al., 1989). These low levels maintain vasorelaxation through the cGMP mediated relaxation of vascular smooth muscle cells described above. The NO produced is in balance with endothelial derived vasoconstrictors

and the sympathetic nervous system (Vallance and Chan, 2001). NO and other vasoactive mediators have functional effects on endothelial cells and smooth muscle cells. Both functional and structural characteristics of the arterial wall play an important role in arterial stiffness. Indeed, inhibition of NO synthesis results in an increase in local arterial stiffness (Schmitt et al., 2005; Wilkinson et al., 2002b). Endothelial derived NO is a potent vasodilator. Its synthesis is increased in response to both biochemical stimuli (thrombin, ADP, serotonin, acetylcholine and bradykinin) as well as mechanical stimuli (shear stress and cyclic strain) (Jin and Loscalzo, 2010).

Antithrombotic, antiatherogenic and antiproliferative properties

NO maintains vascular integrity by exerting antithrombotic, antiatherogenic and antiproliferative properties. Platelets play an important role in wound healing and hemostatic plug formation. However, modulation of their activity is required to prevent vascular thrombosis and its clinical vascular outcomes (Loscalzo, 2001). NO is one of 3 biochemical systems that suppress platelet activity. NO exerts this effect principally via cGMP production with a resultant decrease in intracellular platelet Ca^{2+} levels. Increases in Ca^{2+} concentration within platelets causes a number of structural changes which leads to platelet aggregation (Jin and Loscalzo, 2010). NO also plays an important role in the vasculature by suppressing leucocyte migration and cellular adhesion to the endothelium, as well as preventing smooth muscle cell proliferation and migration (Vallance and Chan, 2001).

DIETARY NITRATE AND VASCULAR FUNCTION

Dietary nitrate was highlighted as a potential candidate for the cardioprotective effect of a fruit and vegetable - rich diet (Lundberg et al., 2006) with a landmark population study by Joshipura et al. This study provided evidence that vegetables generally and green leafy vegetables specifically afforded the greatest protection from cardiovascular disease (Joshipura et al., 1999; Joshipura et al., 2001). Whether this effect can be attributed to the high nitrate content of green leafy vegetables has not been determined from epidemiological studies due to inherent study limitations. Vegetables contain several factors which could contribute to cardiovascular health. These include vitamins such as vitamin C, minerals such as potassium, dietary fibre, polyphenols and carotenoids.

The cardiovascular benefits of nitrate intake have been observed in clinical studies. Studies have demonstrated lowering of blood pressure, improvement of endothelial function, protection against ischaemia reperfusion injury, reduction in platelet aggregation and improvement of exercise performance with nitrate intake. Research indicates that these effects could occur as a result of enhanced NO production through the nitrate- nitrite-NO pathway, as described above. This pathway could be an important back-up for the L-arginine NO pathway.

Dietary sources

The two main sources of nitrate in the diet are vegetables and drinking water. Since nitrate is absorbed effectively with a bioavailability of 100% (van Velzen et al., 2008), total nitrate consumption is largely dependent on the nitrate content of vegetables, quantity of vegetable intake as well as the level of nitrate in local drinking water (Jean A.T, 1998). Certain vegetables, especially beetroot, lettuce, rocket and spinach (**Figure 3**) are naturally rich in nitrate while other

vegetables, such as peas, potato and tomato, contain nitrate at lower concentrations (Santamaria, 2006). The level of nitrate in vegetables also varies according to soil conditions, time of year, nitrate content of fertilizers, growing conditions (e.g. plants grown in low light have higher nitrate concentrations), the storage and transport environment as well as cooking procedures (Anjana and Abrol, 2007; Pennington, 1998; Petersen and Stoltze, 1999; Ysart et al., 1999). The nitrate content of drinking water, which is regulated in most countries due to health concerns, fluctuates and is dependent on bacterial nitrogen fixation, decay of organic matter in the soils, manure from large-scale livestock production and fertilizer use (Addiscott, 2005).

Nitrate intake varies greatly between individuals and populations. Mean daily intakes are estimated to be between 0.4 to 2.6 mg/kg or 31 to 185 mg (Gangolli et al., 1994), and actual individual daily nitrate intakes ranging from less than 20 mg to greater than 400 mg (Petersen and Stoltze, 1999; Ysart et al., 1999). Indeed, individuals who follow the Dietary Approaches to Stop Hypertension (DASH) diet may consume as much as 1000 mg/d. The European Food Safety Authority has set the Acceptable Daily Intake (ADI) for nitrate at 3.7 mg/kg (approximately 260 mg for a 70 kg adult). The consumption of a diet rich in high-nitrate vegetables could markedly exceed the ADI. It seems unlikely that this would be related to detrimental health effects. Although the consumption of green leafy vegetables is widely promoted, the diets of many populations are low in these vegetables, and as a result low in nitrate with intakes often less than 100 mg/d. There is, however, concern among some researchers about the toxicity associated with intake of nitrate and nitrite, particularly in relation to methaemoglobinaemia, colorectal cancer and cardiovascular disease. Nevertheless, due to the health benefits observed and the uncertainty surrounding the toxic effects, a number of

researchers are calling for a re-evaluation of the guidelines for the acceptable levels of nitrate in water and intake from food (Hord et al., 2009; Kapil et al., 2010b).

Beneficial effects on vascular health

Epidemiological evidence and biomarkers of nitrate intake

The Seven Countries Study was the first to identify an association between the Mediterranean Diet and a very low incidence of cardiovascular disease. People eating a Mediterranean Diet have a high intake of fruit and vegetables, eat low levels of red meat, consume a large amount of fish and white meat as well as have a moderate intake of red wine (Keys, 1995). There is current speculation that the cardioprotective effect of the Mediterranean Diet is partially due to the high nitrate/nitrite content (Hord et al., 2009; Lundberg et al., 2006). Indeed, a Mediterranean Diet may contain as much as 20 times the nitrate/ nitrite present in a typical Western Diet (Bryan and Hord, 2010). While clinical trials with nitrate provide support for this hypothesis, no epidemiological study has investigated nitrate consumption and cardiovascular disease. This is possibly due to a number of difficulties linking nitrate intake specifically with cardiovascular disease risk. Nitrate intake is closely linked to vegetable intake and as such is highly correlated with other nutrients particularly fibre, carotenoids and folate (Lajous and Willett, 2011). Additionally, accurate estimates of nitrate exposure are complicated due to large within-foods variations as well as geographical differences in drinking water nitrate concentration. Another difficulty is the lack of a good biomarker of nitrate intake. Plasma and urinary nitrate/nitrite

levels may not be a good indication of long-term nitrate and nitrite exposure as they are affected by both dietary nitrate intake and the L-arginine-NO synthase pathway. Removal of nitrate from the diet results in reduced tissue levels of nitrite but little changes in plasma nitrite (Feelisch et al., 2008; Lundberg et al., 2009). Other considerations are the short half-lives of nitrate (5-8 h) and nitrite (20-45 min) and their variability related to other factors such as exercise (Rassaf et al., 2007).

Blood pressure

Recent studies have shown that a diet rich in fruit and vegetables lowers blood pressure (Appel et al., 1997; Rouse et al., 1983) with the greatest protective effect from consumption of green leafy and cruciferous vegetables with high nitrate content (Joshiyura et al., 1999; Joshiyura et al., 2001). While the exact compounds responsible for this blood pressure lowering effect are not known, the possibility that nitrate is a key molecule was highlighted with the discovery of the enterosalivary pathway of nitrate (Benjamin et al., 1994; Lundberg et al., 1994; Zweier et al., 1995) and the evidence of decreased NO production in hypertension (Giansante and Fiotti, 2006). Indeed, this has been translated into the clinical setting with a number of studies showing a reduction in blood pressure after an acute or chronic dose of nitrate whether in the form of beetroot juice, high green leafy vegetable diet, or the nitrate salts (**Table 4**). These effects were observed with a concomitant rise in plasma nitrate and nitrite concentrations (Bailey et al., 2009; Kapil et al., 2010a; Larsen et al., 2006; Larsen et al., 2007; Webb et al., 2008). Govoni et al demonstrated the rise in plasma nitrite and reduction in blood pressure after a nitrate dose can be

prevented by interrupting the enterosalivary circulation of nitrate by use of antibacterial mouthwash (Govoni et al., 2008).

The effects on blood pressure are not consistent, however, with some studies showing a reduction in both systolic and diastolic blood pressure while others show a decrease in either systolic or diastolic blood pressure. In a recent study by Kapil et al, suppression of the oral microflora by a chlorhexidine based mouthwash for 7 days resulted in a 25% decrease in plasma nitrite and a concomitant increase in both systolic and diastolic blood pressure (Kapil et al., 2013). This raises the possibility that the enterosalivary nitrate-nitrite-NO pathway plays an important role in maintaining plasma nitrite levels with follow on effects on blood pressure. Additionally, this study demonstrates that, even in the absence of dietary nitrate intake, endogenous nitrate derived from the L-arginine NOS pathway contributes to physiological modulation of blood flow. The blood pressure lowering effect of sunlight has been attributed to the conversion of nitrate in sweat to nitrite by commensal bacteria on the surface of the skin (Gilchrist et al., 2011).

Endothelial function

Endothelial dysfunction with attenuated NO production is central to the pathogenesis of cardiovascular disorders. Dietary nitrate could improve endothelial function by serving as an alternate source of vasoactive NO through the nitrate-nitrite-NO pathway. Flow-mediated vasodilation (FMD) of the brachial artery is currently the gold standard for non-invasive measurement of conduit artery endothelial function (Celermajer et al., 1992). There is evidence to suggest the size of FMD response is prognostic not only for the occurrence of a cardiovascular

event in high and low risk individuals (Modena et al., 2002; Shimbo et al., 2007; Witte et al., 2005) but also the severity of the disease (Neunteufl et al., 1997). Brachial artery diameter is assessed by high resolution ultrasound before and after a period of transient (5 min) ischaemia induced by inflation of a blood pressure cuff placed on the distal or proximal part of the arm. The increase in blood flow after cuff deflation generates a sheer stress stimulus which results in vasodilation (**Figure 4**). FMD, when performed according to published protocols provides a measure of in vivo endothelium-derived NO bioavailability (Green, 2005). We recently observed a small but significant improvement in FMD in healthy volunteers following ingestion of 200 mg spinach (182 mg nitrate) (Bondonno et al., 2012b). Heiss et al also demonstrated a significant improvement in FMD post 12.7 mg/kg (approximately 1g) sodium nitrate (Heiss et al., 2012). Bahra et al (Bahra et al., 2012), however, found no significant effect on FMD in healthy volunteers post 8 mmol (500 mg) potassium nitrate intake.

Ischemia reperfusion injury

Ischaemia reperfusion injury is the tissue damage that occurs on restoration of circulation after a period of ischaemia or lack of oxygen. The consequences of ischaemia reperfusion injury can be mild, resulting in reversible cell dysfunction, or severe, with multiple organ failure and death. Particularly in the heart and brain, ischaemia reperfusion injury is a major cause of death and morbidity (Murray and Lopez, 1997). Currently, it is the subject of intense research with several approaches and procedures being studied or trialled in order to prevent or limit the damage.

One approach receiving attention is the protection mediated by nitrite through its conversion to NO. This is enhanced in hypoxia when oxygen tension and pH falls rendering the L-arginine-

NOS pathway inactive. In animal models administration of nitrite protects against ischemia-reperfusion injury in liver (Duranski et al., 2005; Lu et al., 2005), heart (Baker et al., 2007; Duranski et al., 2005; Webb et al., 2004), brain (Jung et al., 2006), kidney (Tripatara et al., 2007) and chronic hind-limb ischaemia (Kumar et al., 2008). This protection was not observed in renal ischaemia reperfusion injury (Basireddy et al., 2006) indicating that nitrite protection may be tissue specific. Plasma nitrite levels decrease with increasing cardiovascular risk (Kleinbongard et al., 2006) and this may be reflected in tissues such as the heart (Bryan et al., 2007). Increasing plasma and tissue nitrite levels, therefore, would enlarge the storage pool of NO and could prevent or limit damage caused by ischaemia-reperfusion injury. This could possibly be of therapeutic value in human diseases such as myocardial infarction, stroke, organ transplantation and cardiopulmonary arrest.

The mechanism of action of nitrite, in exerting its protective effect, has not been fully elucidated. However, nitrite is equally effective when it is delivered by oral or systemic administration either before, during or after ischaemia (Shiva et al., 2007b). While most of these studies have investigated the administration of nitrite, it is assumed that nitrate, through the nitrate-nitrite-NO pathway, would have a similar effect. Indeed, Webb et al (Webb et al., 2008) demonstrated a prevention of ischemia-induced endothelial dysfunction, as measured by flow mediated dilatation (FMD) of the brachial artery after 20 min occlusion of blood flow, 2 hours after healthy volunteers ingested 1400 mg nitrate in the form of beetroot juice. This improvement coincided with an increase in plasma nitrite levels. Confirmation of this finding was provided by Kapil et al (Kapil et al., 2010a) in a study with healthy volunteers after ingestion of beetroot juice (350 mg nitrate) or potassium nitrate (1488 mg nitrate). Bryan et al (Bryan et al., 2007)

demonstrated that 7 days pretreatment with dietary nitrite or nitrate decreased infarct size in mice after myocardial ischaemia reperfusion injury. Mice fed on a low nitrite diet for 7 days had a reduced plasma and myocardial nitrite level and a subsequent larger infarct size after ischaemia reperfusion injury compared to mice fed a normal diet. This difference was not observed with provision of nitrite in the drinking water. Recently, Hendgen-Cotta et al (Hendgen-Cotta et al., 2012) observed an improvement in ischaemia reperfusion recovery and revascularization in nitrate fed mice compared to controls. This effect was not observed in nitrate fed mice whose nitrate-reducing bacteria on the oral cavity had been eradicated with antiseptic mouthwash.

Arterial stiffness

Arterial stiffness, a decreased capacity of expansion and contraction in response to change in pressure, is increasingly recognised as an important assessment of cardiovascular risk. NO influences vascular tone, and therefore arterial stiffness, through effects on smooth muscle relaxation. A number of non-invasive methods for measuring arterial stiffness have been developed which are generally derived from pulse wave velocity (PWV), peripheral arterial waveforms or arterial distensibility measures. PWV is considered the gold-standard measurement of arterial stiffness and is independently predictive of cardiovascular events (Willum Hansen et al., 2006). PWV is derived from the time taken for a pulse pressure to travel between two points (generally the carotid and femoral artery) a measured distance apart. Inhibition of NO alters PWV through effects on mean arterial pressure (Stewart et al., 2003). Improving NO status via the enterosalivary nitrate-nitrite-NO pathway could potentially improve arterial stiffness. Bahra et al (Bahra et al., 2012) found a significant decrease in both aortic PWV

and systolic blood pressure 3 hours after ingestion of 500 mg nitrate although no change in FMD was observed. It is possible that the decrease in PWV is related to the decrease in blood pressure observed. Additional studies are required to assess the effect of dietary nitrate intake on measures of arterial stiffness.

Platelet function

Platelet aggregation is a key event in the pathogenesis of atherosclerosis and the development of acute thrombotic events, including myocardial infarction and unstable angina (Davi and Patrono, 2007). Anti-platelet therapy, such as aspirin, highlights the importance of platelet aggregation in these disorders by reducing cardiovascular risk (Awtry and Loscalzo, 2000). The aggregation and adherence of platelets to the endothelium is normally prevented by the production of endogenous antiplatelet agents, including NO (Alheid et al., 1987; Radomski et al., 1987). This NO is produced by the vascular endothelium or within the platelet itself (Radomski et al., 1990). An effect of nitrate, through the nitrate-nitrite-NO pathway, to reduce platelet activity could have a large impact on cardiovascular disease. Indeed, this has been demonstrated after dietary supplementation with beetroot juice and potassium nitrate. Webb et al demonstrated an inhibition of *ex vivo* platelet aggregation, in response to collagen and ADP, 2.5 hours after healthy volunteers ingested beetroot juice containing 1400 mg nitrate (Webb et al., 2008). Intake of potassium nitrate also had a significant effect on platelet aggregation in response to collagen up to 2 hours post ingestion (Richardson et al., 2002).

Improvement of exercise performance

Exercise is cardioprotective with a number of cardiovascular disease risk factors reduced with regular exercise (Cornelissen and Fagard, 2005). In addition, exercise confers protection following a heart attack in humans (Hull Jr et al., 1994). While the exact mechanisms behind the cardioprotective effects of exercise are unknown, enhanced eNOS expression and elevated NO in response to shear stress during exercise could play a role (Hambrecht et al., 2003; Sessa et al., 1994).

NO plays an important role in the physiological adaptation to exercise by enhancing muscle blood flow, altering glucose uptake and metabolism, calcium homeostasis and modulating muscle contraction (Stamler and Meissner, 2001). In addition it controls cellular respiration through its interactions with mitochondrial respiratory chain enzymes (Moncada and Erusalimsky, 2002). Because NO production is increased in the vascular endothelium by exercise, it has been suggested that plasma nitrite levels post exercise are a good predictor of exercise capacity (Rassaf et al., 2007). Whether the increase in systemic nitrite after administration of dietary or inorganic nitrite would have an effect on oxygen consumption during exercise was recently investigated in humans. Bailey et al (Bailey et al., 2010; Bailey et al., 2009) found that 4-6 days of dietary nitrate supplementation in the form of beetroot juice not only reduced blood pressure but also significantly reduced the O₂ cost of low and moderate intensity exercise while the time to exhaustion during high intensity exercise was significantly increased. These results were not expected. It was previously thought that the O₂ cost of submaximal exercise was fixed. Larson et al found that 3 days supplementation with sodium nitrate resulted in a reduction in oxygen consumption during submaximal and maximal exercise

(Larsen et al., 2007; Larsen et al., 2010). This occurred with no concomitant increase in plasma lactate suggesting that energy production was more efficient.

The exact mechanisms involved still need to be determined but there is some evidence to suggest that it involves the mitochondrion (Clerc et al., 2007; Larsen et al., 2011; Lundberg et al., 2011) and/or an increase in blood flow to the muscles with an improved balance between blood flow and oxygen uptake. While beetroot juice is high in nitrate, the physiological and cardiovascular effects observed after ingestion could be due to the presence of other bioactive compounds such as betaine and polyphenols. Lansley et al developed a placebo nitrate-depleted beetroot juice. Blood pressure reductions and improved O₂ cost of low, moderate and high intensity exercise were observed after acute and chronic nitrate-rich beetroot juice intake compared to the low nitrate placebo beetroot juice (Lansley et al., 2011a; Lansley et al., 2011b). These findings could be beneficial not only for athletes but also for people with diseases that have reduced oxygen availability such as heart disease and chronic obstructive pulmonary disease.

POTENTIAL FOR DETRIMENTAL EFFECTS OF HIGH NITRATE INTAKE

There are conflicting opinions in the literature about the toxicity of nitrate and nitrite in food and water. In the last decade numerous health benefits have been ascribed to nitrate and nitrite as detailed above, but there is lingering concern among some researchers about their potential toxicity. One recent report stated “the presence of nitrate in vegetables, as in water and generally in other foods, is a serious threat to man’s health” (Santamaria, 2006). This concern arises from evidence of methaemoglobinaemia, cancer and cardiovascular disease in relation to nitrate and nitrite consumption.

Methaemoglobinaemia (or blue baby syndrome) can result from the reaction between nitrite and haemoglobin rendering the haemoglobin incapable of carrying oxygen (Knobeloch et al., 2000). While levels of methaemoglobin less than 1% of normal haemoglobin levels are normal, levels greater than 10% can cause cyanosis through to asphyxia, which is potentially fatal (Santamaria, 2006). Concern about nitrate in relation to methaemoglobinaemia was first raised in the 1940's when methaemoglobinaemia and cyanosis was observed in infants fed formula made with well water that had a high nitrate content (Comly, 1945). The high nitrate content of the water was due to faecal contamination. In contrast, infants fed formula made from well water with a low nitrate level rarely developed methaemoglobinaemia (Walton, 1951). It has since been argued that nitrate per se was not the cause but rather faecal bacteria present in the well water (Avery, 1999). Intestinal infection with faecal bacteria causes an increase in NO production in the gastrointestinal system and NO has the ability to convert haemoglobin to methaemoglobin (Addiscott, 2005). Indeed, in a study in 1948 infants and older adults given a dose of 50 or 100 mg nitrate/kg/d nitrate did not develop methaemoglobinaemia (Cornblath and Hartmann, 1948). Nitrite exposure studies in adults have since confirmed this finding (Dejam et al., 2007). A high nitrate level in drinking water may therefore be a marker of contamination rather than being toxic itself. Nevertheless, in Western countries millions of dollars are spent lowering the nitrate content of drinking water to levels established by the World Health Organisation in 1970 and reviewed in 2004. Whether the recommended level of nitrate in drinking water should be increased is a controversial issue (Powlson et al., 2008).

Cancer in relation to nitrate and nitrite consumption has been a concern among researchers since the 1970's when it was demonstrated that dietary nitrate had the potential to form carcinogenic

N-nitrosoamines (Spiegelhalder et al., 1976; Tannenbaum et al., 1976). *N*-nitrosoamines, which react with nucleic acids, are highly carcinogenic in laboratory animals (Gangolli et al., 1994) and could also be associated with human cancer (Bartsch and Montesano, 1984; Craddock, 1983). A report published in 1979 that dietary nitrite caused lymphomas in rats (Newberne, 1979) triggered extensive animal and epidemiological studies to determine if there was a link between dietary nitrate consumption and cancer. While some epidemiological studies reported a weak link, a review of all studies in 2003 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) found no evidence of an increased risk of cancer with dietary nitrate consumption (Speijers and Brandt, 2003). Indeed, a diet rich fruit and vegetables, which far exceeds the WHO acceptable daily intake of nitrate, is not associated with an increase in deaths from cancer (Hung et al., 2004). Since both nitrate and nitrite are formed endogenously, it seems unlikely that exogenous sources would be toxic. Additionally, vegetables rich in nitrate also contain high levels of antioxidants, such as vitamins C and E, which could prevent *N*-nitrosoamine formation. One study, however, raises the possibility that certain sub groups of the population may be at risk. Winter et al (Winter et al., 2007) demonstrated increased formation of *N*-nitrosoamines within the oesophagus of patients with Barrets oesophagus, a condition with severe gastro-oesophageal reflux and a higher incidence of gastric cancer.

There is good evidence of increased risk of cardiovascular disease with consumption of processed meats. A recent meta-analysis of 20 studies found that processed meat intake but not red meat was associated with higher incidence of cardiovascular disease and diabetes mellitus (Micha et al., 2010). Nitrate is added to meat as an antioxidant, to maintain the red colour and enhance flavour. Sodium nitrite is added to processed meat as an antimicrobial agent, flavour

enhancer and colourant. Whether the increased risk in cardiovascular disease is related to the presence nitrate, nitrite and the potential formation of nitrosamines is controversial. There is little evidence for this link.

CONCLUSION

Clearly NO is a critical molecule in cardiovascular health. Cardiovascular disease is associated with decreased production and /or bioavailability of NO which is produced mainly via the classical L-arginine NO synthase pathway. The discovery of an alternate pathway of NO production, the enterosalivary nitrate-nitrite-NO pathway, has resulted in a change of perspective of the role of dietary nitrate in cardiovascular health. Dietary nitrate is now highlighted as a potential candidate for the cardioprotective effect of a diet rich in vegetables. Indeed, improvements in blood pressure, endothelial function, ischaemia-reperfusion injury, arterial stiffness, platelet function and exercise performance with concomitant augmentation of NO status, have been observed in clinical trials with nitrate. These important findings are not without controversy. There is still concern about nitrate in relation to methaemoglobinaemia and cancer. Good evidence from epidemiological studies, however, is lacking due to inherent difficulties in linking nitrate intake with health or disease outcomes. Nevertheless, results from clinical trials to date suggest that increasing dietary nitrate intake may be an effective strategy in the prevention of cardiovascular disease. Future studies are still required to determine if dietary nitrate is a potential new therapy for people at risk for cardiovascular disease such as those with hypertension or impaired vascular function.

ACKNOWLEDGEMENTS, CONFLICTS OF INTEREST AND DISCLOSURE STATEMENT:

The authors have nothing to disclose, and there are no conflicts of interest. C.P. Bondonno acknowledges the support of an Australian Postgraduate Award. J.M. Hodgson acknowledges the support of a National health and Medical Research Council Senior Research Fellowship.

REFERENCES

- Aamand, R., Dalsgaard, T., Jensen, F. B., Simonsen, U., Roepstorff, A., and Fago, A. (2009). Generation of nitric oxide from nitrite by carbonic anhydrase: a possible link between metabolic activity and vasodilation. *Am. J. Physiol. Heart Circ. Physiol.* **297**: H2068-H2074.
- Addiscott, T. M. (2005). Nitrate, agriculture and the environment. CABI Publishing, Oxford.
- Alderton, W. K., Cooper, C. E., and Knowles, R. G. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* **357**: 593.
- Alheid, U., Frolich, J. C., and Forstermann, U. (1987). Endothelium-derived relaxing factor from cultured human endothelial cells inhibits aggregation of human platelets. *Throm. Res.* **47**: 561-571.
- Anjana, U. S., and Abrol, Y. P. (2007). Are nitrate concentrations in leafy vegetables within safe limits? *Curr. Sci.* **92**: 355-360.
- Appel, L. J., Moore, T. J., Obarzanek, E., Vollmer, W. M., Svetkey, L. P., Sacks, F. M., Bray, G. A., Vogt, T. M., Cutler, J. A., Windhauser, M. M., Lin, P. H., and Karanja, N. (1997). A clinical trial of the effects of dietary patterns on blood pressure. *N. Engl. J. Med.* **336**: 1117-1124.
- Avery, A. A. (1999). Infantile methemoglobinemia: reexamining the role of drinking water nitrates. *Environ. Health Perspect.* **107**: 583.
- Awtry, E. H., and Loscalzo, J. (2000). Aspirin. *Circulation.* **101**: 1206-1218.

- Bahra, M., Kapil, V., Pearl, V., Ghosh, S., and Ahluwalia, A. (2012). Inorganic nitrate ingestion improves vascular compliance but does not alter flow-mediated dilatation in healthy volunteers. *Nitric Oxide*. **26**: 197-202.
- Bailey, S. J., Fulford, J., Vanhatalo, A., Winyard, P. G., Blackwell, J. R., DiMenna, F. J., Wilkerson, D. P., Benjamin, N., and Jones, A. M. (2010). Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *J. Appl. Physiol.* **109**: 135-148.
- Bailey, S. J., Winyard, P., Vanhatalo, A., Blackwell, J. R., Dimenna, F. J., Wilkerson, D. P., Tarr, J., Benjamin, N., Jones, A. M., Bailey, S. J., Winyard, P., Vanhatalo, A., Blackwell, J. R., Dimenna, F. J., Wilkerson, D. P., Tarr, J., Benjamin, N., and Jones, A. M. (2009). Dietary nitrate supplementation reduces the O₂ cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J. Appl. Physiol.* **107**: 1144-1155.
- Baker, J. E., Su, J., Fu, X., Hsu, A., Gross, G. J., Tweddell, J. S., and Hogg, N. (2007). Nitrite confers protection against myocardial infarction: Role of xanthine oxidoreductase, NADPH oxidase and KATP channels. *J. Mol. Cell Cardiol.* **43**: 437-444.
- Barbotin-Larrieu, F., Mazmanian, M., Baudet, B., Detruit, H., Chapelier, A., Libert, J. M., Dartevelle, P., and Herve, P. (1996). Prevention of ischemia-reperfusion lung injury by inhaled nitric oxide in neonatal piglets. *J. Appl. Physiol.* **80**: 782-788.
- Bartsch, H., and Montesano, R. (1984). Relevance of nitrosamines to human cancer. *Carcinogenesis*. **5**: 1381.

- Basireddy, M., Isbell, T. S., Teng, X., Patel, R. P., and Agarwal, A. (2006). Effects of sodium nitrite on ischemia-reperfusion injury in the rat kidney. *Am. J. Physiol. Renal Physiol.* **290**: F779-F786.
- Bazzano, L. A., Serdula, M. K., and Liu, S. (2003). Dietary intake of fruits and vegetables and risk of cardiovascular disease. *Curr. Atheroscler. Rep.* **5**: 492-499.
- Beckman, J. S. (2009). Understanding peroxynitrite biochemistry and its potential for treating human diseases. *Arch. Biochem. Biophys.* **484**: 114.
- Benetos, A., Laurent, S., Hoeks, A. P., Boutouyrie, P. H., and Safar, M. E. (1993). Arterial alterations with aging and high blood pressure. A noninvasive study of carotid and femoral arteries. *Arterioscler. Thromb. Vasc. Biol.* **13**: 90-97.
- Benjamin, N., O'Driscoll, F., Dougall, H., Duncan, C., Smith, L., Golden, M., and McKenzie, H. (1994). Stomach NO synthesis. *Nature.* **368**: 502.
- Bjorne, H. H., Petersson, J., Phillipson, M., Weitzberg, E., Holm, L., and Lundberg, J. O. (2004). Nitrite in saliva increases gastric mucosal blood flow and mucus thickness. *J. Clin. Invest.* **113**: 106-114.
- Bondonno, C. P., Croft, K. D., Puddey, I. B., Considine, M. J., Yang, X., Ward, N. C., and Hodgson, J. M. (2012a). Nitrate causes a dose-dependent augmentation of nitric oxide status in healthy women. *Food Funct.* **3**: 522-527.
- Bondonno, C. P., Yang, X., Croft, K. D., Considine, M. J., Ward, N. C., Rich, L., Puddey, I. B., Swinny, E., Mubarak, A., and Hodgson, J. M. (2012b). Flavonoid-rich apples and nitrate-rich spinach augment nitric oxide status and improve endothelial function in healthy men and women: a randomized controlled trial. *Free Radic. Biol. Med.* **52**: 95-102.

- Bredt, D. S. (1999). Endogenous nitric oxide synthesis: biological functions and pathophysiology. *Free Radical Res.* **31**: 577-596.
- Bredt, D. S. (2003). Nitric oxide signaling specificity-the heart of the problem. *J. Cell Sci.* **116**: 9-15.
- Bryan, N. S., Calvert, J. W., Elrod, J. W., Gundewar, S., Ji, S. Y., and Lefer, D. J. (2007). Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. *Proc. Natl. Acad. Sci. U.S.A.* **104**: 19144-19149.
- Bryan, N. S., Fernandez, B. O., Bauer, S. M., Garcia-Saura, M. F., Milsom, A. B., Rassaf, T., Maloney, R. E., Bharti, A., Rodriguez, J., and Feelisch, M. (2005). Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. *Nat. Chem. Biol.* **1**: 290-297.
- Bryan, N. S., and Grisham, M. B. (2007). Methods to detect nitric oxide and its metabolites in biological samples. *Free Radic. Biol. Med.* **43**: 645-657.
- Bryan, N. S., and Hord, N. G. (2010). Dietary nitrates and nitrites: The physiological context for potential health benefits. *Food, Nutrition and the Nitric Oxide Pathway: Biochemistry and Bioactivity*. NS Bryan, ed. DEStech Publications Inc., Lancaster PA.
- Bryan, N. S., Rassaf, T., Maloney, R. E., Rodriguez, C. M., Saijo, F., Rodriguez, J. R., and Feelisch, M. (2004). Cellular targets and mechanisms of nitros (yl) ation: an insight into their nature and kinetics in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **101**: 4308.
- Cannon, R. O., Schechter, A. N., Panza, J. A., Ognibene, F. P., Pease-Fye, M. E., Waclawiw, M. A., Shelhamer, J. H., and Gladwin, M. T. (2001). Effects of inhaled nitric oxide on

- regional blood flow are consistent with intravascular nitric oxide delivery. *J. Clin. Invest.* **108**: 279-288.
- Celermajer, D. S., Sorensen, K. E., Gooch, V. M., Sullivan, I. D., Lloyd, J. K., Deanfield, J. E., and Spiegelhalter, D. J. (1992). Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *The Lancet.* **340**: 1111-1115.
- Clerc, P., Rigoulet, M., Leverve, X., and Fontaine, E. (2007). Nitric oxide increases oxidative phosphorylation efficiency. *J. Bioenerg. Biomembr.* **39**: 158-166.
- Comly, H. H. (1945). Cyanosis in infants caused by nitrates in well water. *JAMA.* **129**: 112-116.
- Cornblath, M., and Hartmann, A. F. (1948). Methemoglobinemia in young infants. *J. Pediatr.* **33**: 421.
- Cornelissen, V. A., and Fagard, R. H. (2005). Effects of endurance training on blood pressure, blood pressure-regulating mechanisms, and cardiovascular risk factors. *Hypertension.* **46**: 667-675.
- Cosby, K., Partovi, K. S., Crawford, J. H., Patel, R. P., Reiter, C. D., Martyr, S., Yang, B. K., Waclawiw, M. A., Zalos, G., Xu, X., Huang, K. T., Shields, H., Kim-Shapiro, D. B., Schechter, A. N., Cannon, R. O., and Gladwin, M. T. (2003). Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat. Med.* **9**: 1498-1505.
- Craddock, V. M. (1983). Toxicology: Nitrosamines and human cancer: proof of an association? *Nature.* **306**: 638-638.
- Davi, G., and Patrono, C. (2007). Platelet Activation and Atherothrombosis. *N. Engl. J. Med.* **357**: 2482-2494.

- Dejam, A., Hunter, C. J., Tremonti, C., Pluta, R. M., Hon, Y. Y., Grimes, G., Partovi, K., Pelletier, M. M., Oldfield, E. H., Cannon, R. O., Schechter, A. N., and Gladwin, M. T. (2007). Nitrite infusion in humans and nonhuman primates. *Circulation*. **116**: 1821-1831.
- Duranski, M. R., Greer, J. J., Dejam, A., Jaganmohan, S., Hogg, N., Langston, W., Patel, R. P., Yet, S. F., Wang, X., Kevil, C. G., Gladwin, M. T., and Lefer, D. J. (2005). Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *J. Clin. Invest.* **115**: 1232-1240.
- Dyckhuizen, R. S., Frazer, R., Duncan, C., Smith, C. C., Golden, M., Benjamin, N., and Leifert, C. (1996). Antimicrobial effect of acidified nitrite on gut pathogens: importance of dietary nitrate in host defense. *Antimicrob. Agents Chemother.* **40**: 1422-1425.
- Endemann, D. H., and Schiffrin, E. L. (2004). Endothelial dysfunction. *J. Am. Soc. Nephrol.* **15**: 1983-1992.
- Feelisch, M., Fernandez, B. O., Bryan, N. S., Garcia-Saura, M. F., Bauer, S., Whitlock, D. R., Ford, P. C., Janero, D. R., Rodriguez, J., and Ashrafian, H. (2008). Tissue processing of nitrite in hypoxia: an intricate interplay of nitric oxide-generating and -scavenging systems. *J. Biol. Chem.* **283**: 33927-33934.
- Ford, P. C., Wink, D. A., and Stanbury, D. M. (1993). Autoxidation kinetics of aqueous nitric oxide. *Febs Lett.* **326**: 1-3.
- Fox-Robichaud, A., Payne, D., Hasan, S. U., Ostrovsky, L., Fairhead, T., Reinhardt, P., and Kubes, P. (1998). Inhaled NO as a viable antiadhesive therapy for ischemia/reperfusion injury of distal microvascular beds. *J. Clin. Invest.* **101**: 2497.

- Fuster, V., Kelly, B. B., and Vedanthan, R. (2011). Promoting global cardiovascular health. *Circulation*. **123**: 1671-1678.
- Gangolli, S. D., van den Brandt, P. A., Feron, V. J., Janzowsky, C., Koeman, J. H., Speijers, G. J. A., Spiegelhalder, B., Walker, R., and Wishnok, J. S. (1994). Nitrate, nitrite and N-nitroso compounds. *Eur. J. Pharmacol. (Environ. Toxicol. Pharmacol. Sect.)*. **292**: 1-38.
- Giansante, C., and Fiotti, N. (2006). Insights into human hypertension: the role of endothelial dysfunction. *J. Hum. Hypertens*. **20**: 725-726.
- Gilchrist, M., Shore, A. C., and Benjamin, N. (2011). Inorganic nitrate and nitrite and control of blood pressure. *Cardiovasc. Res*. **89**: 492-498.
- Gilchrist, M., Winyard, P. G., Aizawa, K., Anning, C., Shore, A., and Benjamin, N. (2013). Effect of dietary nitrate on blood pressure, endothelial function, and insulin sensitivity in type 2 diabetes. *Free Rad Biol Med*.
<http://dx.doi.org/10.1016/j.freeradbiomed.2013.01.024>.
- Giuffrè, A., Stubauer, G., Brunori, M., Sarti, P., Torres, J., and Wilson, M. T. (1998). Chloride bound to oxidized cytochrome c oxidase controls the reaction with nitric oxide. *J. Biol. Chem*. **273**: 32475-32478.
- Giustarini, D., Milzani, A., Dalle-Donne, I., and Rossi, R. (2007). Detection of S-nitrosothiols in biological fluids: A comparison among the most widely applied methodologies. *J. Chromatogr. B*. **851**: 124-139.
- Gladwin, M. T., Schechter, A. N., Kim-Shapiro, D. B., Patel, R. P., Hogg, N., Shiva, S., Cannon, R. O., 3rd, Kelm, M., Wink, D. A., Espey, M. G., Oldfield, E. H., Pluta, R. M., Freeman,

- B. A., Lancaster, J. R., Jr., Feelisch, M., and Lundberg, J. O. (2005). The emerging biology of the nitrite anion. *Nat. Chem. Biol.* **1**: 308-314.
- Gladwin, M. T., Shelhamer, J. H., Schechter, A. N., Pease-Fye, M. E., Waclawiw, M. A., Panza, J. A., Ognibene, F. P., and Cannon, R. O. (2000). Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans. *Proc. Natl. Acad. Sci. U.S.A.* **97**: 11482.
- Govoni, M., Jansson, E. A., Weitzberg, E., and Lundberg, J. O. (2008). The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide.* **19**: 333-337.
- Gow, A., Doctor, A., Mannick, J., and Gaston, B. (2007). S-Nitrosothiol measurements in biological systems. *J. Chromatogr. B.* **851**: 140-151.
- Green, D. (2005). Point: Flow-mediated dilation does reflect nitric oxide-mediated endothelial function. *J. Appl. Physiol.* **99**: 1233-1234.
- Grisham, M. B., Johnson, G. G., and Lancaster, J. R. (1996). Quantitation of nitrate and nitrite in extracellular fluids. *Methods Enzymol.* **268**: 237-246.
- Hambrecht, R., Adams, V., Erbs, S., Linke, A., Kränkel, N., Shu, Y., Baither, Y., Gielen, S., Thiele, H., and Gummert, J. F. (2003). Regular physical activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase. *Circulation.* **107**: 3152-3158.
- Hausladen, A., Rafikov, R., Angelo, M., Singel, D. J., Nudler, E., and Stamler, J. S. (2007). Assessment of nitric oxide signals by triiodide chemiluminescence. *Proc. Natl. Acad. Sci. U.S.A.* **104**: 2157.

- Heiss, C., Meyer, C., Totzeck, M., Hendgen-Cotta, U. B., Heinen, Y., Luedike, P., Keymel, S., Ayoub, N., Lundberg, J. O., and Weitzberg, E. (2012). Dietary inorganic nitrate mobilizes circulating angiogenic cells. *Free Rad Biol Med.* **52**: 1767-1772.
- Hendgen-Cotta, U. B., Luedike, P., Totzeck, M., Kropp, M., Schicho, A., Stock, P., Rammos, C., Niessen, M., Heiss, C., and Lundberg, J. O. (2012). Dietary nitrate supplementation improves revascularization in chronic ischemia. *Circulation.* **126**: 1983-1992.
- Herrmann, J., and Lerman, A. (2008). The endothelium-the cardiovascular health barometer. *Herz.* **33**: 343-353.
- Hill, B. G., Dranka, B. P., Bailey, S. M., Lancaster, J. R., and Darley-Usmar, V. M. (2010). What part of NO don't you understand? Some answers to the cardinal questions in nitric oxide biology. *J. Biol. Chem.* **285**: 19699-19704.
- Hobbs, D. A., Kaffa, N., George, T. W., Methven, L., and Lovegrove, J. A. (2012). Blood pressure-lowering effects of beetroot juice and novel beetroot-enriched bread products in normotensive male subjects. *Br. J. Nutr.* **1**: 1-9.
- Hord, N. G., Tang, Y., and Bryan, N. S. (2009). Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *Am. J. Clin. Nutr.* **90**: 1-10.
- Hu, F. B., and Willett, W. C. (2002). Optimal diets for prevention of coronary heart disease. *JAMA.* **288**: 2569-2578.
- Hull Jr, S. S., Vanoli, E., Adamson, P. B., Verrier, R. L., Foreman, R. D., and Schwartz, P. J. (1994). Exercise training confers anticipatory protection from sudden death during acute myocardial ischemia. *Circulation.* **89**: 548-552.

- Hung, H. C., Joshipura, K. J., Jiang, R., Hu, F. B., Hunter, D., Smith-Warner, S. A., Colditz, G. A., Rosner, B., Spiegelman, D., and Willett, W. C. (2004). Fruit and vegetable intake and risk of major chronic disease. *J. Natl. Cancer Inst.* **96**: 1577-1584.
- Ignarro, L. J. (1989). Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circul. Res.* **65**: 1-21.
- Jaffrey, S. R., Erdjument-Bromage, H., Ferris, C. D., Tempst, P., and Snyder, S. H. (2001). Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat. Cell Biol.* **3**: 193-197.
- Jansson, E. A., Huang, L., Malkey, R., Govoni, M., Nihlen, C., Olsson, A., Stensdotter, M., Petersson, J., Holm, L., Weitzberg, E., and Lundberg, J. O. (2008). A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis. *Nat. Chem. Biol.* **4**: 411-417.
- Jean A.T, P. (1998). Dietary exposure models for nitrates and nitrites. *Food Control.* **9**: 385-395.
- Jia, L., Bonaventura, J., and Stamler, J. S. (1996). S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature.* **380**: 21.
- Jin, R. C., and Loscalzo, J. (2010). Vascular nitric oxide: formation and function. *J. Blood Med.* **1**: 147-162.
- Joshipura, K. J., Ascherio, A., Manson, J. E., Stampfer, M. J., Rimm, E. B., Speizer, F. E., Hennekens, C. H., Spiegelman, D., and Willett, W. C. (1999). Fruit and vegetable intake in relation to risk of ischemic stroke. *JAMA.* **282**: 1233-1239.
- Joshipura, K. J., Hu, F. B., Manson, J. E., Stampfer, M. J., Rimm, E. B., Speizer, F. E., Colditz, G., Ascherio, A., Rosner, B., Spiegelman, D., and Willett, W. C. (2001). The effect of

- fruit and vegetable intake on risk for coronary heart disease. *Ann. Intern. Med.* **134**: 1106-1114.
- Jung, K. H., Chu, K., Ko, S. Y., Lee, S. T., Sinn, D. I., Park, D. K., Kim, J. M., Song, E. C., Kim, M., and Roh, J. K. (2006). Early intravenous infusion of sodium nitrite protects brain against in vivo ischemia-reperfusion injury. *Stroke*. **37**: 2744-2750.
- Kapil, V., Haydar, S., Pearl, V., Lundberg, J. O., Weitzberg, E., and Ahluwalia, A. (2013). Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free Rad Biol Med.* **55**: 93-100.
- Kapil, V., Milsom, A. B., Okorie, M., Maleki-Toyserkani, S., Akram, F., Rehman, F., Arghandawi, S., Pearl, V., Benjamin, N., Loukogeorgakis, S., Macallister, R., Hobbs, A. J., Webb, A. J., and Ahluwalia, A. (2010a). Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertension*. **56**: 274-281.
- Kapil, V., Webb, A. J., and Ahluwalia, A. (2010b). Inorganic nitrate and the cardiovascular system. *Heart*. **96**: 1703-1709.
- Kelly, J., Fulford, J., Vanhatalo, A., Blackwell, J. R., French, O., Bailey, S. J., Gilchrist, M., Winyard, P. G., and Jones, A. M. (2013). Effects of short-term dietary nitrate supplementation on blood pressure, O₂ uptake kinetics, and muscle and cognitive function in older adults. *Am J Physiol Regul Integr Comp Physiol*. **304**: R73-R83.
- Kelm, M. (1999). Nitric oxide metabolism and breakdown. *Biochim. Biophys. Acta*. **1411**: 273-289.
- Kenjale, A. A., Ham, K. L., Stabler, T., Robbins, J. L., Johnson, J. L., VanBruggen, M., Privette, G., Yim, E., Kraus, W. E., and Allen, J. D. (2011). Dietary nitrate supplementation

- enhances exercise performance in peripheral arterial disease. *J. Appl. Physiol.* **110**: 1582-1591.
- Keys, A. (1995). Mediterranean diet and public health: personal reflections. *Am. J. Clin. Nutr.* **61**: 1321S-1323S.
- Kleinbongard, P., Dejam, A., Lauer, T., Jax, T., Kerber, S., Gharini, P., Balzer, J., Zotz, R. B., Scharf, R. E., Willers, R., Schechter, A. N., Feelisch, M., and Kelm, M. (2006). Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans. *Free Radic. Biol. Med.* **40**: 295-302.
- Knobeloch, L., Salna, B., Hogan, A., Postle, J., and Anderson, H. (2000). Blue babies and nitrate-contaminated well water. *Environ. Health Perspect.* **108**: 675.
- Koshland, D. E. (1992). The molecule of the year. *Science.* **258**: 1861.
- Kozlov, A. V., Dietrich, B., and Nohl, H. (2003). Various intracellular compartments cooperate in the release of nitric oxide from glycerol trinitrate in liver. *Br. J. Pharmacol.* **139**: 989-997.
- Kozlov, A. V., Staniek, K., and Nohl, H. (1999). Nitrite reductase activity is a novel function of mammalian mitochondria. *Febs Lett.* **454**: 127-130.
- Kumar, D., Branch, B. G., Pattillo, C. B., Hood, J., Thoma, S., Simpson, S., Illum, S., Arora, N., Chidlow, J. H., Langston, W., Teng, X., Lefer, D. J., Patel, R. P., and Kevil, C. G. (2008). Chronic sodium nitrite therapy augments ischemia-induced angiogenesis and arteriogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **105**: 7540-7545.

- Lajous, M., and Willett, W. (2011). Nutritional Epidemiology of Nitrogen Oxides: What do the Numbers Mean? **In:** Nitrite and Nitrate in Human Health and Disease, pp. 85-96. Bryan, N. S., and Loscalzo, J. (Eds.), Springer Science+ Business Media.
- Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992). Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natl. Acad. Sci. U.S.A.* **89**: 6348.
- Lansley, K. E., Winyard, P. G., Bailey, S. J., Vanhatalo, A., Wilkerson, D. P., Blackwell, J. R., Gilchrist, M., Benjamin, N., and Jones, A. M. (2011a). Acute dietary nitrate supplementation improves cycling time trial performance. *Med Sci Sports Exerc.* **43**: 1125-1131.
- Lansley, K. E., Winyard, P. G., Fulford, J., Vanhatalo, A., Bailey, S. J., Blackwell, J. R., DiMenna, F. J., Gilchrist, M., Benjamin, N., and Jones, A. M. (2011b). Dietary nitrate supplementation reduces the O₂ cost of walking and running: a placebo-controlled study. *J. Appl. Physiol.* **110**: 591-600.
- Larsen, F. J., Ekblom, B., Sahlin, K., Lundberg, J. O., and Weitzberg, E. (2006). Effects of dietary nitrate on blood pressure in healthy volunteers. *N. Engl. J. Med.* **355**: 2792-2793.
- Larsen, F. J., Schiffer, T. A., Borniquel, S., Sahlin, K., Ekblom, B., Lundberg, J. O., and Weitzberg, E. (2011). Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell metabolism.* **13**: 149-159.
- Larsen, F. J., Weitzberg, E., Lundberg, J. O., and Ekblom, B. (2007). Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol.* **191**: 59-66.

- Larsen, F. J., Weitzberg, E., Lundberg, J. O., and Ekblom, B. (2010). Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise. *Free Radic. Biol. Med.* **48**: 342-347.
- Li, H., Cui, H., Kundu, T. K., Alzawahra, W., and Zweier, J. L. (2008). Nitric oxide production from nitrite occurs primarily in tissues not in the blood. *J. Biol. Chem.* **283**: 17855-17863.
- Liu, V. W. T., and Huang, P. L. (2008). Cardiovascular roles of nitric oxide: A review of insights from nitric oxide synthase gene disrupted mice *Cardiovasc. Res.* **77**: 19-29.
- Liu, X., Miller, M. J. S., Joshi, M. S., Sadowska-Krowicka, H., Clark, D. A., and Lancaster Jr, J. R. (1998). Diffusion-limited reaction of free nitric oxide with erythrocytes. *J. Biol. Chem.* **273**: 18709-18713.
- Lloyd-Jones, D. M., Hong, Y., Labarthe, D., Mozaffarian, D., Appel, L. J., Van Horn, L., Greenlund, K., Daniels, S., Nichol, G., and Tomaselli, G. F. (2010). Defining and setting national goals for cardiovascular health promotion and disease reduction the American Heart Association's strategic impact goal through 2020 and beyond. *Circulation.* **121**: 586-613.
- Loscalzo, J. (2001). Nitric oxide insufficiency, platelet activation, and arterial thrombosis. *Circul. Res.* **88**: 756-762.
- Lu, P., Liu, F., Yao, Z., Wang, C. Y., Chen, D. D., Tian, Y., Zhang, J. H., and Wu, Y. H. (2005). Nitrite-derived nitric oxide by xanthine oxidoreductase protects the liver against ischemia-reperfusion injury. *Hepatobiliary Pancreat. Dis. Int.* **4**: 350-355.
- Lundberg, J. O., Carlstrom, M., Larsen, F. J., and Weitzberg, E. (2011). Roles of dietary inorganic nitrate in cardiovascular health and disease. *Cardiovasc. Res.* **89**: 525-532.

- Lundberg, J. O., Feelisch, M., Bjorne, H., Jansson, E. A., and Weitzberg, E. (2006). Cardioprotective effects of vegetables: is nitrate the answer? *Nitric Oxide*. **15**: 359-362.
- Lundberg, J. O., Gladwin, M. T., Ahluwalia, A., Benjamin, N., Bryan, N. S., Butler, A., Cabrales, P., Fago, A., Feelisch, M., and Ford, P. C. (2009). Nitrate and nitrite in biology, nutrition and therapeutics. *Nat. Chem. Biol.* **5**: 865-869.
- Lundberg, J. O., and Govoni, M. (2004). Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic. Biol. Med.* **37**: 395-400.
- Lundberg, J. O., and Weitzberg, E. (2005). NO generation from nitrite and its role in vascular control. *Arterioscler. Thromb. Vasc. Biol.* **25**: 915-922.
- Lundberg, J. O., Weitzberg, E., and Gladwin, M. T. (2008). The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat. Rev. Drug Discov.* **7**: 156-167.
- Lundberg, J. O., Weitzberg, E., Lundberg, J. M., and Alving, K. (1994). Intra-gastric nitric oxide production in humans: measurements in expelled air. *Gut*. **35**: 1543-1546.
- MacArthur, P. H., Shiva, S., and Gladwin, M. T. (2007a). Measurement of circulating nitrite and S-nitrosothiols by reductive chemiluminescence. *J Chromatogr B Analyt Technol Biomed Life Sci.* **851**: 93-105.
- MacArthur, P. H., Shiva, S., and Gladwin, M. T. (2007b). Measurement of circulating nitrite and S-nitrosothiols by reductive chemiluminescence. *J. Chromatogr. B.* **851**: 93-105.
- Mahmud, A., and Feely, J. (2003). Effect of Smoking on Arterial Stiffness and Pulse Pressure Amplification. *Hypertension*. **41**: 183-187.
- Marletta, M. A. (1994). Nitric oxide synthase: aspects concerning structure and catalysis. *Cell*. **78**: 927-930.

- Micha, R., Wallace, S. K., and Mozaffarian, D. (2010). Red and processed meat consumption and risk of incident coronary heart disease, stroke, and diabetes mellitus. *Circulation*. **121**: 2271-2283.
- Modena, M. G., Bonetti, L., Coppi, F., Bursi, F., and Rossi, R. (2002). Prognostic role of reversible endothelial dysfunction in hypertensive postmenopausal women. *J. Am. Coll. Cardiol.* **40**: 505-510.
- Moncada, S., and Erusalimsky, J. D. (2002). Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat. Rev. Mol. Cell Biol.* **3**: 214-220.
- Munzel, T., Daiber, A., Ullrich, V., and Mulsch, A. (2005). Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase. *Arterioscler. Thromb. Vasc. Biol.* **25**: 1551-1557.
- Murray, C. J. L., and Lopez, A. D. (1997). Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *The Lancet*. **349**: 1498-1504.
- Ness, A. R., and Powles, J. W. (1997). Fruit and vegetables, and cardiovascular disease: a review. *Int. J. Epidemiol.* **26**: 1-13.
- Neunteufl, T., Katzenschlager, R., Hassan, A., Klaar, U., Schwarzacher, S., Glogar, D., Bauer, P., and Weidinger, F. (1997). Systemic endothelial dysfunction is related to the extent and severity of coronary artery disease. *Atherosclerosis*. **129**: 111-118.
- Newberne, P. M. (1979). Nitrite promotes lymphoma incidence in rats. *Science*. **204**: 1079-1081.
- O'Donnell, V. B., and Freeman, B. A. (2001). Interactions between nitric oxide and lipid oxidation pathways: implications for vascular disease. *Circul. Res.* **88**: 12-21.

- O'Rourke, M. F., and Hashimoto, J. (2007). Mechanical factors in arterial aging: a clinical perspective. *J. Am. Coll. Cardiol.* **50**: 1-13.
- Palmer, R. M. J., Ashton, D. S., and Moncada, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature.* **333**: 664-666.
- Pennington, J. A. T. (1998). Dietary exposure models for nitrates and nitrites. *Food Control.* **9**: 385-395.
- Petersen, A., and Stoltze, S. (1999). Nitrate and nitrite in vegetables on the Danish market: content and intake. *Food Addit. Contam.* **16**: 291 - 299.
- Petersen, M. G., Dewilde, S., and Fago, A. (2008). Reactions of ferrous neuroglobin and cytoglobin with nitrite under anaerobic conditions. *J. Inorg. Biochem.* **102**: 1777-1782.
- Petersson, J., Carlström, M., Schreiber, O., Phillipson, M., Christoffersson, G., Jägare, A., Roos, S., Jansson, E. Å., Persson, A. E. G., Lundberg, J. O., and Holm, L. (2009). Gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash. *Free Radic. Biol. Med.* **46**: 1068-1075.
- Petersson, J., Phillipson, M., Jansson, E. A., Patzak, A., Lundberg, J. O., and Holm, L. (2007). Dietary nitrate increases gastric mucosal blood flow and mucosal defense. *Am. J. Physiol. Gastrointest. Liver Physiol.* **292**: G718-724.
- Powlson, D. S., Addiscott, T. M., Benjamin, N., Cassman, K. G., De Kok, T. M., Van Grinsven, H., L'Hirondel, J. L., Avery, A. A., and Van Kessel, C. (2008). When does nitrate become a risk for humans? *J. Environ. Qual.* **37**: 291-295.

- Radomski, M. W., Palmer, R. M., and Moncada, S. (1990). An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. U.S.A.* **87**: 5193-5197.
- Radomski, M. W., Palmer, R. M. J., and Moncada, S. (1987). Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium *The Lancet*. **330**: 1057-1058.
- Rafikova, O., Sokolova, E., Rafikov, R., and Nudler, E. (2004). Control of plasma nitric oxide bioactivity by perfluorocarbons. *Circulation*. **110**: 3573-3580.
- Rassaf, T., Kleinbongard, P., Preik, M., Dejam, A., Gharini, P., Lauer, T., Erckenbrecht, J., Duschin, A., Schulz, R., Heusch, G., Feelisch, M., and Kelm, M. (2002). Plasma nitrosothiols contribute to the systemic vasodilator effects of intravenously applied NO: experimental and clinical study on the fate of NO in human blood. *Circ. Res.* **91**: 470-477.
- Rassaf, T., Lauer, T., Heiss, C., Balzer, J., Mangold, S., Leyendecker, T., Rottler, J., Drexhage, C., Meyer, C., and Kelm, M. (2007). Nitric oxide synthase-derived plasma nitrite predicts exercise capacity. *Br. J. Sports Med.* **41**: 669-673.
- Richardson, G., Hicks, S. L., O'Byrne, S., Frost, M. T., Moore, K., Benjamin, N., and McKnight, G. M. (2002). The ingestion of inorganic nitrate increases gastric S-nitrosothiol levels and inhibits platelet function in humans. *Nitric Oxide*. **7**: 24-29.
- Rocks, S. A., Davies, C. A., Hicks, S. L., Webb, A. J., Klocke, R., Timmins, G. S., Johnston, A., Jawad, A. S. M., Blake, D. R., and Benjamin, N. (2005). Measurement of S-nitrosothiols in extracellular fluids from healthy human volunteers and rheumatoid arthritis patients,

- using electron paramagnetic resonance spectrometry. *Free Radic. Biol. Med.* **39**: 937-948.
- Rogers, S. C., Khalatbari, A., Gapper, P. W., Frenneaux, M. P., and James, P. E. (2005). Detection of human red blood cell-bound nitric oxide. *J. Biol. Chem.* **280**: 26720-26728.
- Rouse, I., Armstrong, B., Beilin, L., and Vandongen, R. (1983). Blood pressure-lowering effect of a vegetarian diet: controlled trial in normotensive subjects. *The Lancet.* **321**: 5-10.
- Santamaria, P. (2006). Nitrate in vegetables: toxicity, content, intake and EC regulation. *J. Sci. Food Agr.* **86**: 10-17.
- Schmitt, M., Avolio, A., Qasem, A., McEniery, C. M., Butlin, M., Wilkinson, I. B., and Cockcroft, J. R. (2005). Basal NO locally modulates human iliac artery function in vivo. *Hypertension.* **46**: 227-231.
- Sessa, W. C., Pritchard, K., Seyedi, N., Wang, J., and Hintze, T. H. (1994). Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circulation Research.* **74**: 349-353.
- Shimbo, D., Grahame-Clarke, C., Miyake, Y., Rodriguez, C., Sciacca, R., Di Tullio, M., Boden-Albala, B., Sacco, R., and Homma, S. (2007). The association between endothelial dysfunction and cardiovascular outcomes in a population-based multi-ethnic cohort. *Atherosclerosis.* **192**: 197-203.
- Shiva, S., Huang, Z., Grubina, R., Sun, J., Ringwood, L. A., MacArthur, P. H., Xu, X., Murphy, E., Darley-Usmar, V. M., and Gladwin, M. T. (2007a). Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration. *Circul. Res.* **100**: 654-661.

- Shiva, S., Sack, M. N., Greer, J. J., Duranski, M., Ringwood, L. A., Burwell, L., Wang, X., MacArthur, P. H., Shoja, A., Raghavachari, N., Calvert, J. W., Brookes, P. S., Lefer, D. J., and Gladwin, M. T. (2007b). Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. *J. Exp. Med.* **204**: 2089-2102.
- Shiva, S., Wang, X., Ringwood, L. A., Xu, X., Yuditskaya, S., Annavajjhala, V., Miyajima, H., Hogg, N., Harris, Z. L., and Gladwin, M. T. (2006). Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. *Nat. Chem. Biol.* **2**: 486-493.
- Sobko, T., Marcus, C., Govoni, M., and Kamiya, S. (2010). Dietary nitrate in Japanese traditional foods lowers diastolic blood pressure in healthy volunteers. *Nitric Oxide.* **22**: 136-140.
- Speijers, G., and Brandt, P. A. V. D. (2003). Nitrate. **In:** Food Additives Series. Geneva: Joint FAO/WHO Expert Committee on Food Additives
- Spiegelhalder, B., Eisenbrand, G., and Preussmann, R. (1976). Influence of dietary nitrate on nitrite content of human saliva: Possible relevance to in vivo formation of N-nitroso compounds. *Food Cosmet. Toxicol.* **14**: 545-548.
- Stamler, J. S., Jaraki, O., Osborne, J., Simon, D. I., Keaney, J., Vita, J., Singel, D., Valeri, C. R., and Loscalzo, J. (1992). Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. *Proc. Natl. Acad. Sci. U.S.A.* **89**: 7674-7677.
- Stamler, J. S., and Meissner, G. (2001). Physiology of nitric oxide in skeletal muscle. *Physiol. Rev.* **81**: 209-237.

- Stewart, A. D., Millasseau, S. C., Kearney, M. T., Ritter, J. M., and Chowienczyk, P. J. (2003). Effects of inhibition of basal nitric oxide synthesis on carotid-femoral pulse wave velocity and augmentation index in humans. *Hypertension*. **42**: 915-918.
- Takahashi, Y., Kobayashi, H., Tanaka, N., Sato, T., Takizawa, N., and Tomita, T. (1998). Nitrosyl hemoglobin in blood of normoxic and hypoxic sheep during nitric oxide inhalation. *Am. J. Physiol. Heart Circ. Physiol.* **274**: H349-H357.
- Tanaka, H., DeSouza, C. A., and Seals, D. R. (1998). Absence of Age-Related Increase in Central Arterial Stiffness in Physically Active Women. *Arterioscl Thromb Vasc Biol.* **18**: 127-132.
- Tannenbaum, S. R., Weisman, M., and Fett, D. (1976). The effect of nitrate intake on nitrite formation in human saliva. *Food Cosmet. Toxicol.* **14**: 549-552.
- Thomas, D. D., Liu, X., Kantrow, S. P., and Lancaster, J. R. (2001). The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O₂. *Proc. Natl. Acad. Sci. U.S.A.* **98**: 355.
- Tripatara, P., Patel, N. S., Webb, A., Rathod, K., Lecomte, F. M., Mazzon, E., Cuzzocrea, S., Yaqoob, M. M., Ahluwalia, A., and Thiemermann, C. (2007). Nitrite-derived nitric oxide protects the rat kidney against ischemia/reperfusion injury in vivo: role for xanthine oxidoreductase. *J. Am. Soc. Nephrol.* **18**: 570-580.
- Troncy, E., Francoeur, M., Salazkin, I., Yang, F., Charbonneau, M., Leclerc, G., Vinay, P., and Blaise, G. (1997). Extra-pulmonary effects of inhaled nitric oxide in swine with and without phenylephrine. *Brit. J. Anaesth.* **79**: 631-640.

- Tsikas, D. (2000). Simultaneous derivatization and quantification of the nitric oxide metabolites nitrite and nitrate in biological fluids by gas chromatography/mass spectrometry. *Anal. Chem.* **72**: 4064-4072.
- Vallance, P., and Chan, N. (2001). Endothelial function and nitric oxide: clinical relevance. *Heart.* **85**: 342-350.
- Vallance, P., Collier, J., and Moncada, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *The Lancet.* **334**: 997-1000.
- van't Veer, P., Jansen, M. C., Klerk, M., and Kok, F. J. (2000). Fruits and vegetables in the prevention of cancer and cardiovascular disease. *Public Health Nutr.* **3**: 103-107.
- van Velzen, A. G., Sips, A. J. A. M., Schothorst, R. C., Lambers, A. C., and Meulenbelt, J. (2008). The oral bioavailability of nitrate from nitrate-rich vegetables in humans. *Toxicol. Lett.* **181**: 177-181.
- Vanhatalo, A., Bailey, S. J., Blackwell, J. R., DiMenna, F. J., Pavey, T. G., Wilkerson, D. P., Benjamin, N., Winyard, P. G., and Jones, A. M. (2010). Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *Am J Physiol Regul Integr Comp Physiol.* **299**: R1121-R1131.
- Vanhoutte, P. M. (2009). Endothelial dysfunction: the first step toward coronary arteriosclerosis. *Circ. J.* **73**: 595-601.
- Vanin, A. F., Bevers, L. M., Slama-Schwok, A., and Van Faassen, E. E. (2007). Nitric oxide synthase reduces nitrite to NO under anoxia. *Cell. Mol. Life Sci.* **64**: 96-103.

- Walton, G. (1951). Survey of literature relating to infant methemoglobinemia due to nitrate-contaminated water. *Am. J. Public Health Nations Health.* **41**: 986-996.
- Wang, X., Bryan, N. S., MacArthur, P. H., Rodriguez, J., Gladwin, M. T., and Feelisch, M. (2006). Measurement of nitric oxide levels in the red cell. *J. Biol. Chem.* **281**: 26994-27002.
- Webb, A., Bond, R., McLean, P., Uppal, R., Benjamin, N., Ahluwalia, A., Webb, A., Bond, R., McLean, P., Uppal, R., Benjamin, N., and Ahluwalia, A. (2004). Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proc. Natl. Acad. Sci. U.S.A.* **101**: 13683-13688.
- Webb, A. J., Patel, N., Loukogeorgakis, S., Okorie, M., Aboud, Z., Misra, S., Rashid, R., Miall, P., Deanfield, J., Benjamin, N., MacAllister, R., Hobbs, A. J., and Ahluwalia, A. (2008). Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension.* **51**: 784-790.
- Widlansky, M. E., Gokce, N., Keaney Jr, J. F., and Vita, J. A. (2003). The clinical implications of endothelial dysfunction. *J. Am. Coll. Cardiol.* **42**: 1149-1160.
- Wilkinson, I. B., Prasad, K., Hall, I. R., Thomas, A., MacCallum, H., Webb, D. J., Frenneaux, M. P., and Cockcroft, J. R. (2002a). Increased central pulse pressure and augmentation index in subjects with hypercholesterolemia. *J. Am. Coll. Cardiol.* **39**: 1005-1011.
- Wilkinson, I. B., Qasem, A., McEniery, C. M., Webb, D. J., Avolio, A. P., and Cockcroft, J. R. (2002b). Nitric oxide regulates local arterial distensibility in vivo. *Circulation.* **105**: 213-217.

- Willum Hansen, T., Staessen, J. A., Torp-Pedersen, C., Rasmussen, S., Thijs, L., Ibsen, H., and Jeppesen, J. (2006). Prognostic value of aortic pulse wave velocity as index of arterial stiffness in the general population. *Circulation*. **113**: 664-670.
- Wink, D. A., Grisham, M. B., Mitchell, J. B., and Ford, P. C. (1996). Direct and indirect effects of nitric oxide in chemical reactions relevant to biology. *Methods Enzymol*. **268**: 12-31.
- Winter, J. W., Paterson, S., Scobie, G., Wirz, A., Preston, T., and McColl, K. E. L. (2007). N-Nitrosamine generation from ingested nitrate via nitric oxide in subjects with and without gastroesophageal reflux. *Gastroenterology*. **133**: 164-174.
- Witte, D. R., Westerink, J., de Koning, E. J., van der Graaf, Y., Grobbee, D. E., and Bots, M. L. (2005). Is the association between flow-mediated dilation and cardiovascular risk limited to low-risk populations? *J. Am. Coll. Cardiol*. **45**: 1987-1993.
- Yang, X., Bondonno, C. P., Indrawan, A., Hodgson, J. M., and Croft, K. D. (2013). An Improved Mass Spectrometry Based Measurement Of No Metabolites In Biological Fluids. *Free Rad Biol Med*. **56**: 1-8.
- Ysart, G., Miller, P., Barrett, G., Farrington, D., Lawrance, P., and Harrison, N. (1999). Dietary exposures to nitrate in the UK. *Food Addit. Contam*. **16**: 521 - 532.
- Zhang, Z., Naughton, D. P., Blake, D. R., Benjamin, N., Stevens, C. R., Winyard, P. G., Symons, M. C., and Harrison, R. (1997). Human xanthine oxidase converts nitrite ions into nitric oxide (NO). *Biochem. Soc. Trans*. **25**.
- Zweier, J. L., Wang, P., Samouilov, A., and Kuppusamy, P. (1995). Enzyme-independent formation of nitric oxide in biological tissues. *Nat. Med*. **1**: 804-809.

Table 1: Isoforms of nitric oxide synthases (NOS), their site of synthesis, characteristics and action of NO derived. (Bredt, 1999; Hill et al., 2010; Jin and Loscalzo, 2010)

| Isoform | Site of synthesis | Characteristics | Action of NO |
|----------------|---|--|--|
| NOS I / nNOS | Neurons | Constitutively expressed Ca ²⁺ dependent Specifically and highly regulated at transcriptional and post-translational levels Moderate production range (nM to μM) | Messenger molecule |
| NOS II / iNOS | Macrophages, neutrophils, platelets, vascular smooth muscle cells and other nonvascular cells | Ca ²⁺ independent Regulated transcriptionally High production range (μM) | Cytotoxic effect in large amounts produced as part of a rapid response to pathogens by the immune system |
| NOS III/ eNOS | Endothelial cells | Constitutively expressed Ca ²⁺ dependent Specifically and highly regulated at transcriptional and post-translational levels Low production range (pM to nM) | Vasodilation Anti-thrombotic effect Anti-inflammatory effect Anti-proliferative effect |

Table 2. Sources, half-lives and basal plasma concentrations of nitrate, nitrite and NO

| | Sources | Half-life | Fasting plasma level |
|--------------|--|---|------------------------------|
| Nitrate | 1. Diet | 5-8 h ¹ | 20 – 40 µmol/L ² |
| | 2. L-arginine-NO synthase pathway | | |
| Nitrite | 1. Diet | 1-5 min (<i>ex vivo</i>) ¹ | 50 – 300 nmol/L ² |
| | 2. L-arginine-NO synthase pathway | 20-45 min (<i>in vivo</i>) ³ | |
| | 3. Enterosalivary pathway of nitrate | | |
| | 4. Mammalian nitrate reductases ⁴ | | |
| Nitric oxide | 1. L-arginine-NO synthase pathway | 1-2 ms | < 1 nmol/L ⁵ |
| | 2. Endogenous nitrite and S-nitrosothiols | | |

¹(Lundberg et al., 2008)²(Lundberg et al., 2008)³(Dejam et al., 2007)⁴(Jansson et al., 2008)⁵(Lundberg and Weitzberg, 2005)

Table 3. Fate of NO once produced, effect and subsequent action

| Fate of NO | Effect | Action |
|---|---------------|--|
| Binds with haeme moiety on sGC | Direct | Increases cGMP levels in underlying smooth muscle cells and platelets(Ignarro, 1989) |
| Binds with haeme moiety on haemoglobin | Indirect | Originally thought to be inactivated by haemoglobin but now, controversially, it is thought that a small proportion is converted to nitrite and possibly S-nitrosated derivative of oxyhaemoglobin (SNOHb) (Jia et al., 1996). |
| Binds with cytochrome <i>c</i> oxidase | Indirect | Modulation of mitochondrial respiration (Giuffre' et al., 1998) |
| Reacts with superoxide anion to form peroxy nitrite anion | Indirect | Major mediator of pathological effects associated with NO such as inflammation. Peroxynitrite decomposes to nitrate and nitrite or can react further to oxidise and nitrosate other substrates (Beckman, 2009) |
| Oxidised to nitrite in presence of oxygen | Indirect | Preserves NO from local inactivation and allows its effects to be transferred to distant targets. (Shiva et al., 2006) |
| Reacts with thiol groups on proteins to form nitrosothiol compounds | Indirect | Preserves NO from local inactivation and allows its effects to be transferred to distant targets.(Stamler et al., 1992) |
| Reacts with lipid peroxy radicals | Indirect | Vascular protection (O'Donnell and Freeman, 2001) |

Table 4 Summary of intervention studies investigating the effect of nitrate on blood pressure

| Nitrate Source | Nitrate Dose | Acute/Chronic | Subjects | BP Effect | References |
|--------------------------------|--------------------------|----------------|----------------------|-----------------|--------------------------|
| Beetroot juice | 1400 mg | Acute | Healthy M & F (n=14) | SBP, DBP, MAP ↓ | (Webb et al., 2008) |
| | 385 mg | Acute | Healthy M (n=9) | SBP ↓ | (Lansley et al., 2011a) |
| | 350 mg | Acute | Healthy M & F (n=14) | SBP ↓ | (Kapil et al., 2010a) |
| | 322 mg | Acute | Healthy M & F (n=8) | SBP, DBP, MAP ↓ | (Vanhatalo et al., 2010) |
| | 560 mg | Acute | PAD M & F (n=8) | DBP ↓ | (Kenjale et al., 2011) |
| | 340 mg | Chronic (6 d) | Healthy M (n=8) | SBP ↓ | (Bailey et al., 2009) |
| | 316 mg | Chronic (6 d) | Healthy M (n=7) | SBP, DBP, MAP ↓ | (Bailey et al., 2010) |
| | 385 mg | Chronic (6 d) | Healthy M (n=9) | SBP ↓ | (Lansley et al., 2011b) |
| | 322 mg | Chronic (15 d) | Healthy M & F (n=8) | SBP, DBP, MAP ↓ | (Vanhatalo et al., 2010) |
| | 465 mg | Chronic (14 d) | T2DM M & F (n=27) | No effect | (Gilchrist et al., 2013) |
| | 595 mg | Chronic (3 d) | Healthy M & F (n=12) | SBP, DBP ↓ | (Kelly et al., 2013) |
| Beetroot juice (dose response) | 143 mg | Acute | Healthy M (n=4) | SBP, DBP ↓ | (Hobbs et al., 2012) |
| | 353 mg | | Healthy M (n=4) | SBP, DBP ↓ | |
| | 707 mg | | Healthy M (n=4) | SBP, DBP ↓ | |
| Red beetroot enriched bread | 112 mg | Acute | Healthy M (n=5) | SBP ↓ | (Hobbs et al., 2012) |
| White beetroot enriched bread | 99 mg | | Healthy M (n=5) | No effect | |
| Spinach | 182 mg | Acute | Healthy M & F (n=30) | SBP ↓ | (Bondonno et al., 2012b) |
| Japanese traditional diet | 18.8 mg/kg (±1200 mg) | Chronic (10 d) | Healthy M & F (n=25) | DBP ↓ | (Sobko et al., 2010) |

ACCEPTED MANUSCRIPT

| | | | | | |
|-------------------|------------------------|---------------|----------------------|------------|-----------------------|
| Sodium nitrate | 6.2 mg/kg (±400 mg) | Chronic (3 d) | Healthy M & F (n=17) | DBP, MAP ↓ | (Larsen et al., 2006) |
| | 6.2 mg/kg (±400 mg) | Chronic (3 d) | Healthy M (n=9) | SBP, DBP ↓ | (Larsen et al., 2007) |
| Potassium nitrate | 1488 mg | Acute | Healthy M & F (n=21) | SBP, DBP ↓ | (Kapil et al., 2010a) |
| | 500 mg | Acute | Healthy M & F (n=21) | SBP ↓ | (Bahra et al., 2012) |

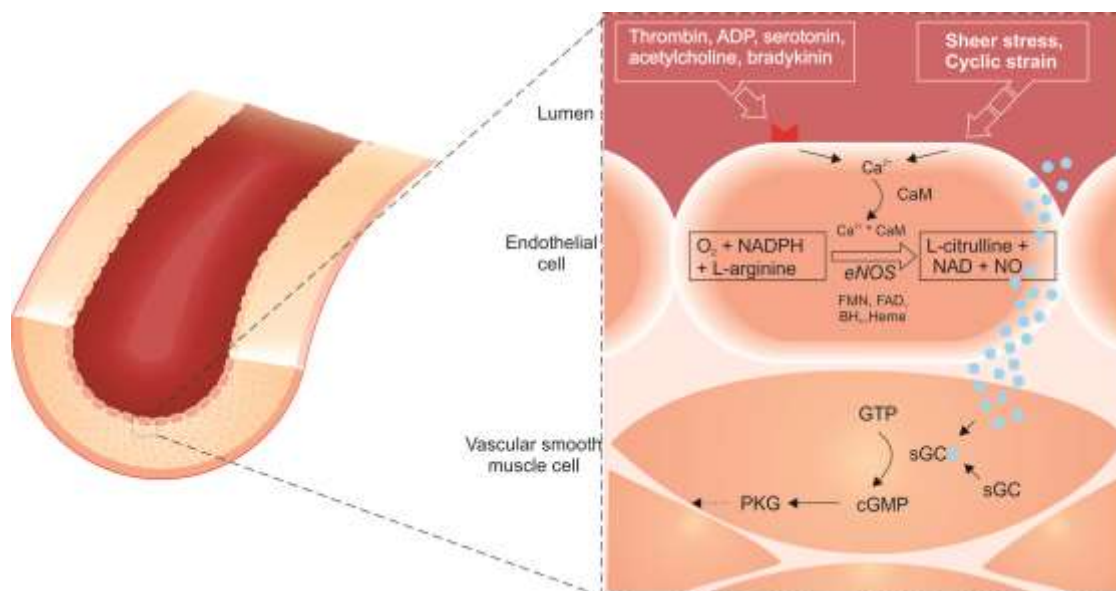


Figure 1: The L-arginine nitric oxide synthase (NOS) pathway. Endothelial cells lining the lumen of blood vessels produce endothelial NOS (eNOS) in response to biochemical and mechanical stimuli. Nitric oxide (NO) diffuses into the underlying smooth vascular cells where it activates soluble guanylate cyclase (sGC) resulting in a rise of cyclic guanosine monophosphate (cGMP) mediating smooth muscle relaxation. ADP: adenosine diphosphate, Ca: calcium, CaM: calmodulin, O₂: oxygen, NADPH: nicotinamide adenine dinucleotide phosphate, NAD: nicotinamide adenine dinucleotide, FMN: flavin mononucleotide, FAD: flavin adenine dinucleotide, BH₄: tetrahydrobiopterin, GTP: guanosine triphosphate, PKG: protein kinase G.

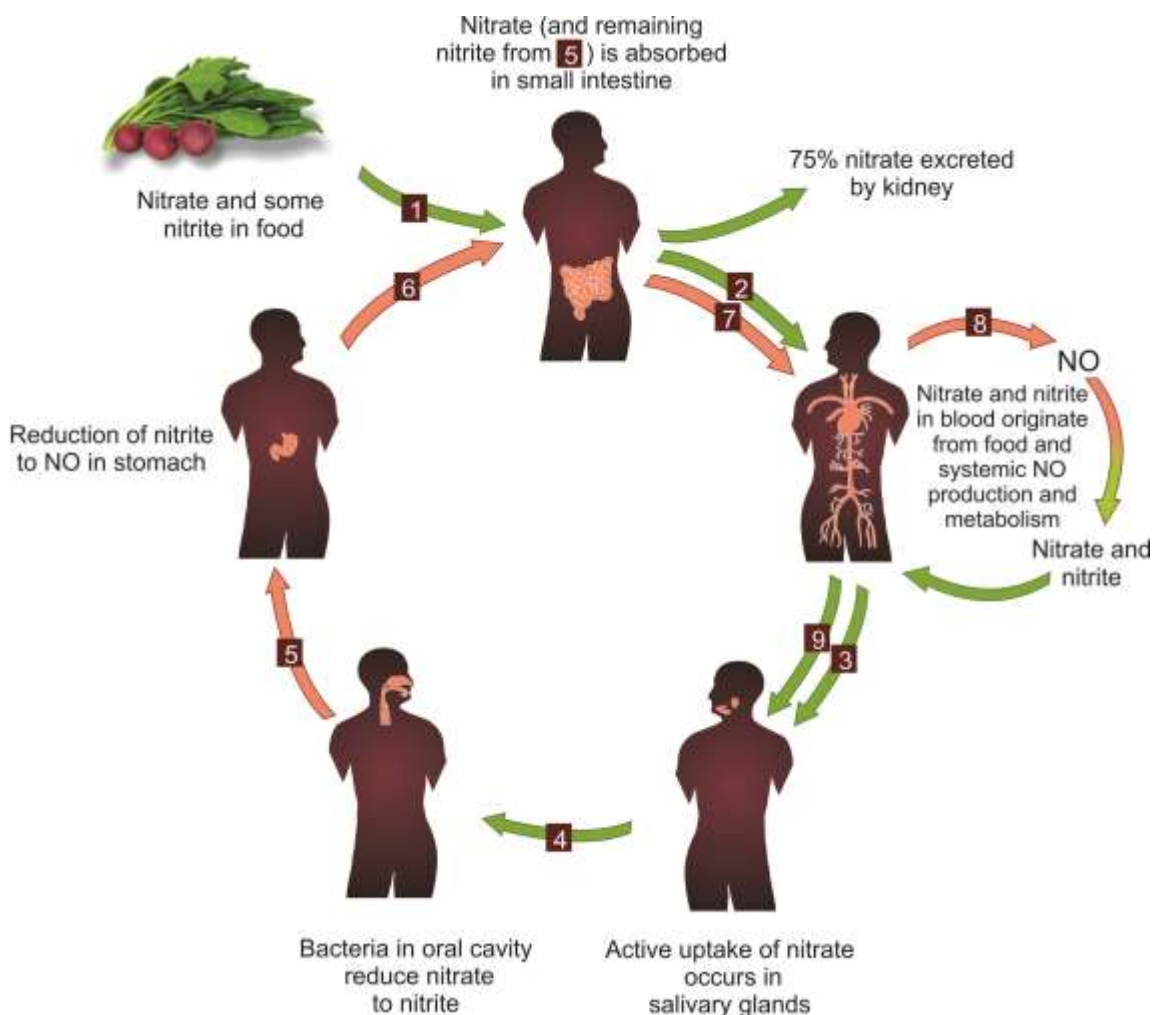


Figure 2: The nitrate-nitrite-nitric oxide (NO) pathway. After ingestion nitrate is absorbed in the small intestine (1) from where it enters the circulation (2). Approximately 75% of nitrate is excreted via the kidneys. There is an active uptake of the remaining nitrate by the salivary glands (3). In the oral cavity the nitrate is reduced to nitrite by nitrate reducing bacteria found predominantly on the dorsal surface of the tongue (4). Some of this nitrite is reduced to NO in

the acidic environment of the stomach (5). The remaining nitrite is absorbed in the small intestine (6) from where it enters the circulation (7). In the circulation, nitrite can be reduced to NO (8). Nitrate and nitrite can be formed by systemic NO metabolism. Nitrate thus formed could enter the cycle together with ingested nitrate (9).

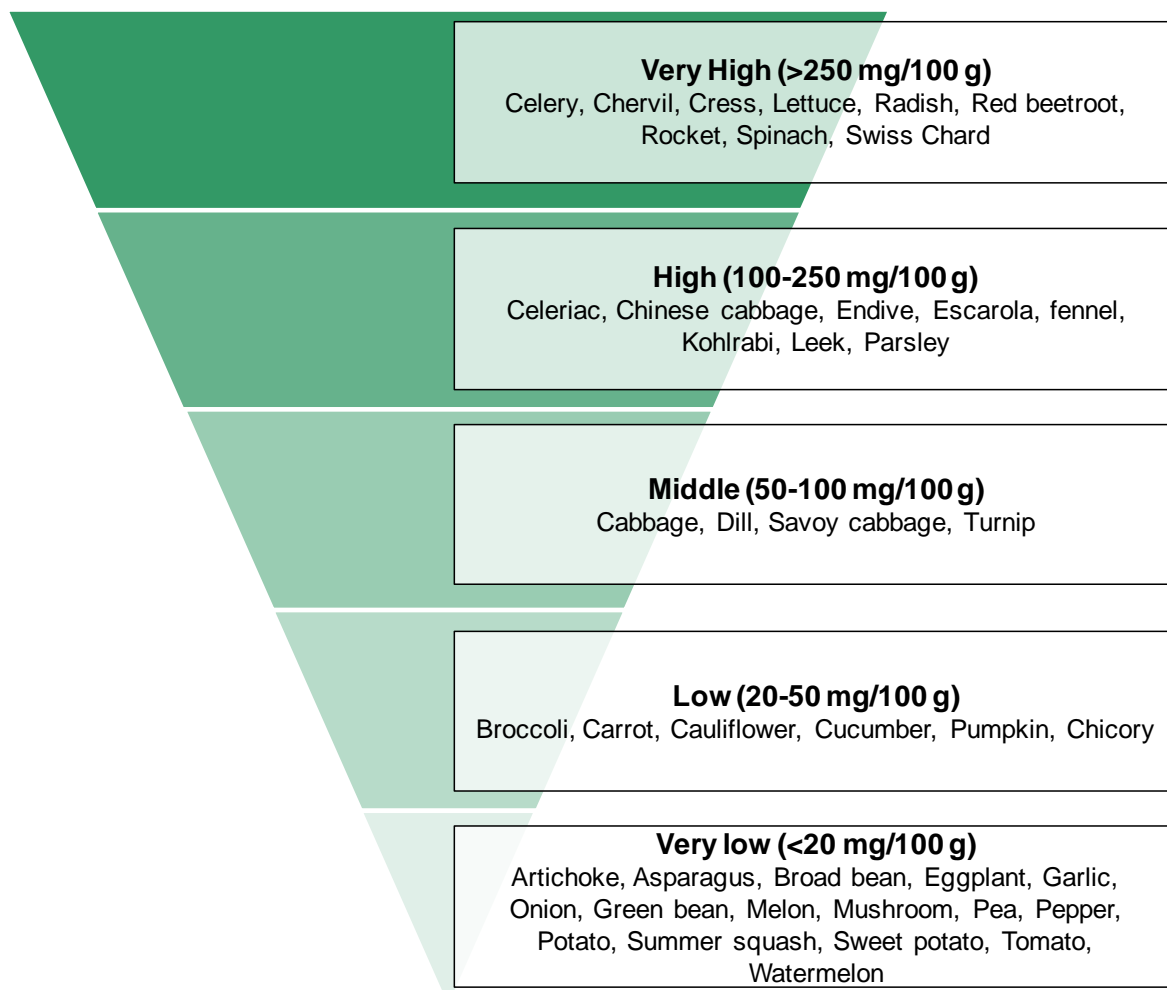


Figure 3: Dietary sources of nitrate arranged according to nitrate content.

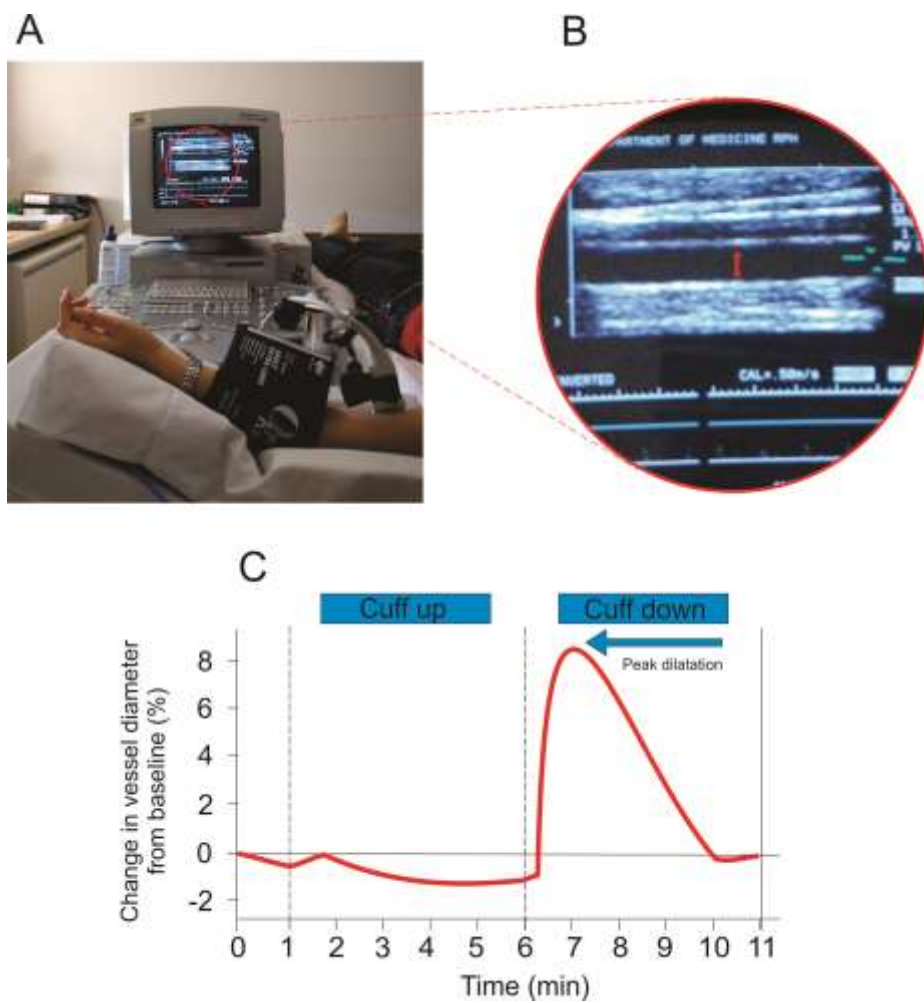


Figure 4: Flow mediated dilatation (FMD) of the brachial artery. A. Ultrasound probe set up. B. Ultrasound image of the brachial artery. C. A representation of time course percentage change in vessel diameter during cuff inflation and deflation.