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Nitrate, the oral microbiome and cardiovascular health: a systematic literature review of human and animal studies

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Short running head: Nitrate, oral microbiome and cardiovascular health

Abbreviations: ABP, ambulatory blood pressure; AC, adenylate cyclase; Ach, acetylcholine; ADI, acceptable daily intake; AIx, augmentation index; BH_4, tetrahydrobiopterin; cGMP, cyclic guanosine monophosphate; CKD, chronic kidney disease; DASH, dietary approaches to stop hypertension; DBP, diastolic blood pressure; eNOS, endothelial nitric oxide synthase; FMD, flow-mediated dilatation; GTP, guanosine triphosphate; nitrous acid, HNO_2; MAP, mean arterial pressure; NO, nitric oxide; NOS, nitric oxide synthase; PI3K, phosphoinoside 3-kinase; PWV, pulse wave velocity; sGC, soluble guanylate cyclase; SBP, systolic blood pressure.

ABSTRACT

Background: Dietary nitrate is an important source of nitric oxide (NO), a molecule critical for cardiovascular health. Nitrate is sequentially reduced to NO through an enterosalivary nitrate-nitrite-NO pathway that involves the oral microbiome. This pathway is considered an important adjunct pathway to the classical L-arginine-NO synthase pathway. The objective of this study was to systematically assess the evidence for dietary nitrate intake and improved cardiovascular health from both human and animal studies.

Methods: A systematic literature search was performed according to PRISMA guidelines using key search terms in Medline and EMBASE databases and defined inclusion and exclusion criteria.

Results: Thirty-seven articles were included on humans and fourteen articles on animals from 12,541 screened references. Data on the effects of dietary nitrate on blood pressure, endothelial function, ischaemic reperfusion injury, arterial stiffness, platelet function, and cerebral blood flow in both human and animal models were identified. Beneficial effects of nitrate on vascular health have predominantly been observed in healthy human populations while effects in populations at risk of cardiovascular disease are less clear. Few studies have investigated the long-term effects of dietary nitrate on cardiovascular disease clinical endpoints. In animal studies, there is evidence that nitrate improves blood pressure and endothelial function particularly in animal models with reduced NO bioavailability. Nitrate dose seems to be a critical factor as there is evidence of cross-talk between the two pathways of NO production.

Conclusion: Evidence for a beneficial effect in humans at risk of cardiovascular disease is limited. Furthermore, there is a need to investigate the long-term effects of dietary nitrate on cardiovascular disease clinical endpoints. Further animal studies are required to elucidate the mechanisms behind the observed effects.

Keywords: vegetables, nitrate, nitric oxide, oral microbiome, cardiovascular diseases

Introduction

Cardiovascular disease is the number one cause of death globally and contributes a major burden to public health systems worldwide (1). Several observational cohort studies have found plant-based diets rich in vegetables to be associated with a lower incidence of cardiovascular disease clinical endpoints (2-4). Specific vegetable groups, such as green leafy vegetables, have been shown to be the most beneficial (5-9). There are many bioactive components in green leafy vegetables that may benefit cardiovascular health. One component that has gained research interest in the last decade is nitrate (10).

Nitrate is present in all vegetables at various concentrations; however, the richest sources of nitrate are beetroot and green leafy vegetables (11). Increasing nitrate intake through the diet is one potential strategy to increase nitric oxide (NO) bioavailability (12). NO plays an important role in vascular tone and integrity, and is a vital molecule for cardiovascular health (12). Reduced NO bioavailability has been observed in individuals with cardiovascular disease (13). Strategies to increase NO in healthy individuals and those at risk of cardiovascular disease may reduce cardiovascular-related events in the wider population.

Due to the increased research interest in the vascular benefits of dietary nitrate, the aim of this review is to provide an overview of dietary nitrate as a source of NO, the importance of the oral microbiome in the nitrate-nitrite-NO pathway, and dietary sources of nitrate. We have also systematically compiled evidence to date on the effects of nitrate ingestion on blood pressure, arterial stiffness, endothelial function, platelet function, and cerebral blood flow in human and animal studies. This systematic literature search was conducted using criteria outlined in the PRISMA checklist. Key search terms used in Medline and EMBASE databases are outlined in **Supplemental Table 1** and inclusion and exclusion criteria in **Supplemental Table 2**. The PRISMA flow charts for human studies can be found in

Supplemental Figure 1 and animal studies in **Supplemental Figure 2**. Articles were excluded if full texts could not be accessed or the articles were not in English.

Two pathways to nitric oxide

Nitric oxide is an important cell signalling molecule critical for vascular homoeostasis (13). A powerful vasodilator, NO relaxes smooth muscle tissue and increases regional blood flow (14). Nitric oxide also inhibits platelet and leukocyte adhesion to the vessel wall, delaying the onset of atherogenesis (15). Nitric oxide is generated through the L-arginine-NOS pathway and the recently described enterosalivary nitrate-nitrite-NO pathway.

L-arginine-NOS pathway

Nitric oxide is synthesised predominantly through the classical L-arginine NO synthase (NOS) pathway (16) which involves three types of NOS isoforms. These include neuronal NOS (nNOS or NOS-1), cytokine-inducible NOS (iNOS or NOS-2), and endothelial NOS (eNOS or NOS-3) (17). Due to the large mass of the endothelium within the body, eNOS is a major contributor to NO production. The regulation of eNOS activity is via intracellular calcium (Ca^{2+}) (18) and several signal transduction pathways, including phosphoinoside 3-kinase (PI3K) and adenylate cyclase (AC) pathways (19). An increase in shear stress, cyclic strain or receptor activation of vascular endothelium by biochemical stimuli (bradykinin, acetylcholine, thrombin, adenosine diphosphate, and serotonin) causes a release of Ca^{2+} from intracellular stores, stimulating eNOS activity (17, 20). Phosphorylation of several residues on the eNOS dimer is also an important requirement for activation (19). Equimolar amounts of NO and L-citrulline are produced using L-arginine and molecular oxygen together with tetrahydrobiopterin (BH_4) in a complex oxygen-dependent five electron-transfer reaction (18, 21).

Nitric oxide synthesised from L-arginine in the endothelium diffuses across the cell membrane to nearby smooth muscle cells stimulating soluble guanylate cyclase (sGC) (18).

This results in the synthesis of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP), triggering the relaxation of smooth muscle cells (18). Uncoupling of eNOS, by reduced bioavailability of BH_4 or the substrate L-arginine, can lead to the production of superoxide or H_2O_2 (22). Furthermore, studies have demonstrated that reduced tissue levels of BH_4 and increased superoxide generation are associated with risk factors for atherosclerosis (23-25).

Nitrate-nitrite-NO pathway

Historically, nitrate and nitrite have been considered to be environmental pollutants and potential carcinogenic residues in the food chain (26). Now, however, nitrate and nitrite are considered important molecules for cardiovascular health (27).

Vegetables are a major source of nitrate consumed in the human population (28). When nitrate is ingested, it is absorbed in the proximal area of the small intestine (12). Nitrate then enters the bloodstream and mixes with endogenous sources of nitrate (mainly derived from oxidation of NO through the L-arginine-NOS pathway). Approximately 75% of circulating nitrate is excreted by the kidneys. The rest (~25%) is actively taken up by the salivary glands where nitrate is concentrated in saliva and secreted in the oral cavity (29, 30). Nitrate is then reduced to nitrite by facultative anaerobic bacteria found in the deep clefts on the dorsal surface of the tongue (31). The commensal bacteria in the oral cavity use nitrate as an alternative electron acceptor to oxygen during respiration, reducing nitrate to nitrite by nitrate reductases (32). Once swallowed, a proportion of nitrite is rapidly protonated forming nitrous acid (HNO_2) in the acidic environment of the stomach (33). Nitrous acid decomposes further to form NO, having localised benefits (33). This non-enzymatic reduction of nitrite to NO is enhanced by vitamin C and polyphenols (34, 35). The remaining nitrate and nitrite in the stomach enter the small intestine and are absorbed into the bloodstream where they mix with

endogenous forms of nitrate and nitrite (mainly derived from oxidation of NO through the L-arginine-NOS pathway).

The one-electron reduction of nitrite to NO in the blood and tissues is catalysed by both enzymatic and non-enzymatic pathways (10). Enzymatic pathways include a number of proteins and enzymes including globins (such as haemoglobin, myoglobin, cytoglobin, and neuroglobin), xanthine oxidoreductase, cytochrome P450, mitochondrial proteins, carbonic anhydrase, aldehyde oxidase and eNOS (10). Non-enzymatic pathways include protons, polyphenols, and vitamin C (10). Both enzymatic and non-enzymatic reductions of nitrite to NO are enhanced during hypoxia and at a low pH (10, 36). Recent evidence suggests that the acidic environment of the stomach plays an important role in the reduction of nitrite to NO (37).

The nitrate-nitrite-NO pathway and the L-arginine-NOS pathway are interconnected through the anions, nitrate and nitrite. Nitrate and nitrite are the oxidation end products of NO metabolism through the L-arginine-NOS pathway but can also be derived from the diet (32). Nitrate and nitrite, derived from the diet and derived as oxidation end products of NO metabolism, are both recycled through the nitrate-nitrite-NO pathway. Both pathways become a storage pool for NO production. Because the L-arginine-NOS pathway requires molecular oxygen to produce NO, nitrite reduction to NO via the nitrate-nitrite-NO pathway may form as a backup system for NO production during hypoxia. A crucial step in the nitrate-nitrite-NO pathway is nitrate to nitrite reduction by the oral microbiome.

The oral microbiome

The oral microbiome is the second most diverse microbial community in the human body comprising 50 – 100 billion bacteria, from over 700 prokaryotic taxa, as well as a fungal and viral flora (38). Disturbances to the composition, and therefore function, of the oral microbiome are thought to play a role in a number of diseases, including cardiovascular

disease (38). Whether this link is related in part to the nitrate-nitrite-NO pathway is garnering research interest. An important step in the nitrate-nitrite-NO pathway is the reduction of nitrate to nitrite by facultative anaerobic bacteria found in the oral cavity. Reduced oral bacterial nitrate to nitrite reduction, both in the presence and absence of dietary nitrate intake, could have detrimental effects on the circulating NO pool with subsequent vascular effects. In the presence of nitrate intake, interrupting the nitrate-nitrite-NO pathway with an antibacterial mouthwash or spitting out of saliva, prevented the resultant increase in salivary and plasma nitrite and the associated decrease in blood pressure (39, 40). In the absence of dietary nitrate intake, increases in blood pressure with concomitant decreases in salivary and plasma nitrite were observed with daily chlorhexidine based antibacterial mouthwash use in both healthy volunteers (41) and treated hypertensives (42). This could be explained by the fact that nitrate and nitrite, produced as end-products of NO metabolism, are recycled through the nitrate-nitrite-NO pathway back into the circulating NO pool. Thus nitrate to nitrite reduction by the oral microbiome could play a key role in blood pressure control. The influence on other measures of vascular health has yet to be determined.

The fundamental role of the oral microbiome in the nitrate-nitrite-NO pathway and possibly blood pressure control makes understanding all the factors that influence oral nitrate to nitrite reduction an important research area. Indeed, there is evidence of a considerable variation between individuals in the nitrate-reducing capacity of the oral microbiome (43). The first set of factors to consider is the use of anti-bacterial mouthwashes, anti-bacterial toothpastes, and antibiotics. Given the results of the studies described above, the widespread use of daily mouthwash in the general population is of potential concern. The mouthwash used in these studies, however, contained chlorhexidine, a strong antibacterial agent. Different effects have been observed with other types and strengths of antibacterial mouthwashes (44). To date only one study has examined the effect of antibacterial toothpaste, containing triclosan, on oral

nitrate to nitrite reduction (45), with no effect observed. These results need to be confirmed in additional studies examining the effect of mouthwash and toothpaste on oral nitrate reduction. Interestingly, epidemiological studies show that regular tooth brushing and mouthwash use, indicative of good oral hygiene, is associated with a decreased risk of hypertension and cardiovascular disease (46, 47). The effect of antibiotic use on oral nitrate to nitrite reduction has yet to be ascertained.

Other important factors are those inherent to the complex oral microbial community such as bacterial genetics, the presence and influence of other microorganisms and environmental pressures. There are a number of potential nitrate-reducing taxa present in the oral microbiome. Doel et al (48) identified *Veillonella* spp as the most abundant nitrate-reducing genus followed by *Actinomyces, Rothia* and *Staphylococcus* spp (48). Hyde et al (49) confirmed *Veillonella* spp as the most abundant nitrate-reducing genus present but also detected *Prevotella, Neisseria* and *Haemophilus* at a higher abundance than *Actinomyces* spp. Nitrate to nitrite reduction by these bacteria is highly variable both within and between bacterial species and needs to be examined in the context of the huge interdependent microbial network in which they exist. This network comprises a heterogenous microbial community within a biofilm which communicates using a process called quorum sensing. These communities are highly complex, with all members influencing its health and vitality. Interestingly, the presence of nitrite reducers may prevent the accumulation of nitrite in the saliva and as such have a negative influence on the nitrate-nitrite-NO pathway (49).

Microbial nitrate metabolism can also be altered by environmental influences such as pH and oxygen tension. A low pH in an oral microenvironment together with increased nitrate and nitrite concentration, can select for nitrate-reducing bacteria (50). Nitrate-reducing bacteria are facultative anaerobes. A low or no oxygen environment will therefore result in the nitrate reductive pathway being utilised for respiration. Other potential factors influencing nitrate to

nitrite reduction that requires future investigation include host factors such as age, diet, and oral health.

The evidence of the link between oral health and cardiovascular disease being related to the nitrate-nitrite-NO pathway is strongly suggestive. Future studies will need to examine this relationship in the context of the large number of factors that could influence oral nitrate to nitrite reduction.

Dietary sources of nitrate and nitrite

Vegetables contribute approximately 80% of dietary nitrate intake in the human population (28, 51-54). Nitrate ingested in the diet can also be derived from other food sources such as fruits, grains, and animal products with the remainder coming from drinking water. Many countries have strict regulations to maintain low levels of nitrate in drinking water due to underlying health concerns, such as methaemoglobinaemia (55). High levels, however, have been detected in private wells in rural areas due to nitrogen-based fertiliser use in agricultural areas (56). Another controversial health concern is the addition of nitrate and nitrite to meat and their potential to form N-nitrosoamines, which are potential carcinogens (29). Compounds such as polyphenols, vitamins C and E and other antioxidants inhibit the formation of N-nitrosoamines (56). These compounds are abundant in vegetables. A large number of countries have also set maximum levels for nitrate in vegetables, particularly for lettuce and spinach, which are known to accumulate high amounts of nitrate (57). These maximum levels vary across harvest period, being higher in winter and if grown under cover, and lower in summer and if grown in open air (57).

Dietary nitrite, on the other hand, contributes only a small amount to human exposure and is mainly consumed from animal-based foods such as cured meats and bacon (52). Nitrite is added to these products as a preservative and to enhance taste and appearance (52). Although a small amount of nitrite is consumed from these food sources, the majority of nitrite

exposure (70-90%) is derived from the *in vivo* conversion of nitrate to nitrite through endogenous pathways (58).

The nitrate content of vegetables depends on many different factors including the biological properties of plants, fertiliser use, soil conditions, sun exposure, and cooking and storage methods. The biological properties of plants can influence the amount of nitrate that accumulates in that plant. For example, nitrate accumulates in different parts of the plants with the leaf and stem having the highest concentrations, and the bulb and fruit having the lowest (28). In our recently developed reference database for assessing dietary nitrate in vegetables (11), leafy vegetables were found to have the highest nitrate content, with Chinese flat cabbage and arugula containing the highest concentrations of nitrate (3000 mg/kg fresh weight). Corn, mushroom, and peas had the lowest nitrate content (<50 mg/kg fresh weight). Nitrate concentration in vegetables also differs between varieties. For example, Chinese lettuce has a 3-fold higher nitrate value than iceberg lettuce (11).

Nitrogen-based fertilisers enhance the growth of plants, and thus, have an impact on how much nitrate accumulates in vegetables. Nitrate located in the soil of a growing vegetable is transported via the plant xylem system to the leaves of the vegetables (52). As organic vegetables tend to be grown in fertilisers containing less nitrogen, by comparison conventionally grown vegetables tend to accumulate higher nitrate levels (11, 59).

Other factors such as handling, storage, and processing, as well as temperature and light intensity can also influence the amount of nitrate in vegetables (52). Higher nitrate levels are observed in vegetables grown in winter compared to summer, and vegetables grown under cover contain higher nitrate levels than those grown outdoors in the same season and the same region (11, 52).

Storage in ambient temperature can also reduce the nitrate content of fresh vegetables. Under refrigerated and frozen storage conditions nitrate levels appear to be unaffected (52).

Endogenous nitrate reductase activity and the amount of bacterial contamination due to post-harvest storage and wilting processes reduce nitrate and subsequently increase nitrite in fresh vegetables (52). Being water soluble, nitrate is also reduced with washing and cooking methods by approximately 10-15% and 50%, respectively (52). As nitrate is also found in the skin of vegetables, peeling of the skin can also reduce nitrate levels by roughly 20-34% (52).

Nitrate ingestion and its effects on vascular function

Dietary nitrate is now considered an important alternative source of NO. Human and animal studies to date have focused on the effects of nitrate ingestion on blood pressure, arterial stiffness, endothelial function, platelet function, and cerebral blood flow, as discussed below. A summary of the beneficial effects of nitrate ingestion on these cardiovascular-related outcomes in human and animal studies is shown in **Figure 1**. Benefits of nitrate ingestion on exercise performance will not be covered in this review.

Blood pressure

Evidence that decreased NO production was associated with hypertension raised the possibility that nitrate, through the nitrate-nitrite-NO pathway, could partially account for the blood pressure lowering effects of green leafy vegetables. Randomised controlled trials such as the Dietary Approaches to Stop Hypertension (DASH) trial have been shown to reduce blood pressure (60). It has been suggested that the high nitrate content of the DASH diet contributes to the blood pressure lowering effects observed (28). The DASH diet has been estimated to include as much as 1,222 mg (19.7 mmol) of nitrate per day (28). This amount can, however, differ by as much as 700% due to the wide variation of nitrate in vegetables (28). An Acceptable Daily Intake (ADI) of 3.7 mg nitrate per kg body weight was set by the Joint Food and Agricultural Organisation and World Health Organisation (52). For an average person weighing 70 kg, this is calculated to be 259 mg of nitrate. The DASH diet can provide up to 500% more nitrate than this ADI.

The DASH diet is associated with reductions of 4.5 mmHg in systolic blood pressure (SBP) (61). This blood pressure reduction is similar to that seen in a meta-analysis demonstrating that consumption of inorganic nitrate and nitrate-rich beetroot juice is associated with a SBP reduction of 4.4 mmHg (62). There is now substantial evidence from human intervention trials to demonstrate blood pressure reductions with short-term intake of dietary nitrate in healthy populations (62). However, the effects of chronic nitrate intake on blood pressure in older populations and populations at risk of cardiovascular disease remain uncertain (50, 63-68).

Human studies

Our systematic literature search revealed 27 acute studies (≤24 hours) (**Table 1**) (40, 50, 63, 69-85) and 15 chronic studies (>1 day) (**Table 2**) (50, 65-68, 86-93) in 32 publications investigating the effects of nitrate ingestion on blood pressure. Beetroot juice was the most common nitrate source used in both acute and chronic studies. Twenty-four hour ambulatory blood pressure (24-hour ABP), the preferred diagnostic method for assessing hypertension (94, 95), was used in 10 studies (65-68, 77, 80-82, 87, 90). Clinic blood pressure was used in 34 studies (40, 50, 63, 65, 67, 69-76, 78, 79, 83-93) and four studies used home blood pressure monitoring (66, 67, 87, 90).

Acute studies

The acute effects of nitrate ingestion on blood pressure were investigated between 2-24 hours with nitrate doses ranging from 68-1488 mg (1.1-24 mmol) (Table 1). Five studies showed a significant reduction in SBP only (78, 82-85) and four studies showed a significant reduction in only diastolic blood pressure (DBP) (71, 77, 79, 80). Eleven studies showed significant reductions in both SBP and DBP (40, 50, 71, 72, 81, 85). Acute reductions in SBP ranged from 2.7 to 22.2 mmHg and 2.6 to 23.6 mmHg for DBP. Reductions in blood pressure were seen across the entire range of nitrate doses investigated and in subjects that were healthy (40,

71, 72, 77, 78, 80-85), overweight (79), and hypercholesterolaemic (50). Sample sizes of these populations ranged from 6 to 67 participants. Blood pressure reductions were not seen in seven studies (63, 69, 70, 73-76). These populations consisted of subjects that were healthy (69, 70, 73-76) and subjects with heart failure (63). Sample sizes of these populations ranged from 5 to 40 participants.

Chronic studies

The chronic effects of nitrate ingestion on blood pressure were investigated in 15 studies from 3 to 42 days (6 weeks) with nitrate doses ranging from 155-1104 mg/d (2.5-17.8 mmol/d) (Table 2). Three studies showed a significant reduction in SBP (88-90) and three other studies showed a significant reduction in DBP (86, 92, 93). Only one study showed a significant reduction in both SBP and DBP (87). In total, seven studies demonstrated a significant reduction in blood pressure. It is worth noting, the study conducted by Ashworth et al (88) was not clear whether the significant reductions in blood pressure were acute or chronic as the subjects were advised to eat high nitrate vegetables 2-3 hours before blood pressure was taken on the final day. Reductions in SBP ranged from 4.0 to 8.1 mmHg and reduction in DBP ranged from 2.4 to 12 mmHg with nitrate doses ranging from 165-1104 mg/d (2.7-17.8 mmol/d). Reductions in blood pressure were seen in one study using 24-hour ABP monitoring (87), two studies using home blood pressure (87, 90) and six studies using clinic blood pressure (86-89, 92, 93). Blood pressure reductions were seen in subjects that were healthy (86, 88, 92, 93), at moderate cardiovascular risk (89), older and overweight (90), and grade 1 hypertensive (treated and untreated) (87). These studies were a mix of young (mean age <37 y) (86, 88, 92, 93) and older cohorts (mean age >56 y) (87, 89, 90). Most studies demonstrating reductions in blood pressure were of low sample size (n range 6-25), except Kapil et al (87), which had a sample size of n=64.

Blood pressure reductions were not seen in eight studies (50, 65-68, 91). These populations consisted of subjects that were older (91), pre-hypertensive (67), treated hypertensive (66), overweight and obese (65), type 2 diabetic (68), and hypercholesterolemic (50). These populations were all older adult populations (mean age >60 y) with larger sample sizes (n range 27-67) (50, 65-68), apart from one study which had a sample size of n=8 (91).

There is now clear and convincing evidence that nitrate reduces blood pressure within hours of ingestion. The evidence of chronic ingestion of nitrate on blood pressure is less clear. Studies suggest that chronic intake of nitrate lowers blood pressure in young healthy individuals; however, these blood pressure lowering effects are not seen in older individuals and individuals at risk of cardiovascular disease. Recent evidence suggests possible interactions between sulphate and nitrate which may explain some of these inconsistencies (96). However, research is in need to further investigate this theory.

Animal studies

We identified 17 studies, in 12 publications, that assessed the effect of nitrate supplementation on blood pressure in an animal model (**Table 8**). Nitrate sources included $NaNO_3$ (n=10), KNO_3 (n=1), and $Mg(NO_3)_2$ (n=1) supplemented drinking water. Nitrate doses ranged from 0.1-4.27 mmol/kg/d and treatment time ranged from 1 week to 12 months. The number of animals in each treatment group ranged from 5 to 23. Nine studies reported a decrease in blood pressure after nitrate supplementation and five studies reported no change in blood pressure. Only one study reported an increase in blood pressure; Carlstrom et al reported a significant increase in mean arterial pressure (MAP) in healthy rats after 8 weeks of nitrate supplementation (1 mM/kg/d) (97). In the same study, a decrease in blood pressure was seen with a 0.1 mM dose of nitrate. In two studies where high blood pressure was induced, either by the use of spontaneously hypertensive rats (98, 99) or by administration of a high-fructose diet (100), nitrate supplementation prevented the increase in blood pressure

observed in the control group. In a study by Henzel et al, a decrease in MAP and SBP was only seen in old (22 months) Sprague-Dawley rats and not in young (3 months) rats (101). It is important to note that although both groups were receiving the same concentration of nitrate in their drinking water, the younger rats were receiving a much higher dose of nitrate (776 µmol/kg/d vs 290 µmol/kg/d), due to their higher water intake and lower body weight. In a study by Khalifi et al, a decrease in SBP was only seen in diabetic Wistar rats and not their healthy counterparts (102). This may be due to positive effects of nitrate supplementation on NO status and oxidative stress, which would have been compromised in the diabetic rats but not the healthy rats. Other studies have shown that higher doses of nitrate can reduce blood pressure in animal models that have been shown to have reduced NO bioavailability (100, 101, 103).

Endothelial function

The endothelium lines the entire vascular system and plays an essential role in the maintenance of vascular homoeostasis (104). Dysfunction of the endothelium has been identified in the development of atherosclerotic-related diseases (105). Flow-mediated dilatation (FMD) via non-invasive ultrasound measures the endothelial function of the brachial artery (106, 107). It is the gold standard method for assessing conduit artery endothelial function (106) and is significantly associated with cardiovascular disease events (108, 109). It has previously been shown from a meta-analysis of 14 prospective cohort studies that the risk of experiencing a cardiovascular event is reduced by 13% for every 1% higher in FMD (110). The degree of endothelial function is determined by the change in brachial artery diameter before and after a shear stress stimulus, induced by reactive hyperaemia (108). In the forearm vasculature, FMD provides a measure of endothelium-derived NO bioavailability (111).

Human studies

Our systematic literature search revealed seven acute studies (≤24 hours) (**Table 3**) (50, 75, 76, 79, 83, 84, 112) and four chronic studies (>1 day) (**Table 4**) (50, 68, 87, 89) in 10 publications investigating the effects of nitrate ingestion on FMD. Beetroot juice was the most common nitrate source used in both acute and chronic studies.

Acute studies

The acute effects of nitrate ingestion on FMD were investigated between 1.5-4 hours with nitrate doses ranging from 6-772 mg (0.1-12.4 mmol) (Table 3). The lower nitrate dose in this range was estimated using the global average body weight of 62 kg as no average body weight was reported in this study (75). Six studies demonstrated a significant improvement in FMD (50, 75, 76, 79, 83, 112) and one study demonstrated no effect (84). Improvements in FMD ranged from 0.5 to 4.0% were seen across the entire range of nitrate doses investigated. Beetroot juice was also found to attenuate the postprandial impairment of FMD following a high-fat meal (79). Improvements in FMD were seen in mainly healthy populations (75, 76, 83, 112). Other populations where improvements in FMD were seen included hypercholesterolaemic (50) and overweight (79) subjects. These healthy and at risk populations consisted of three studies in younger cohorts (mean age ≤27 y) (75, 76, 112) and three studies in older cohorts (mean age >45 y) (50, 79, 83) with an overall sample size ranging from 5 to 67. No effects on FMD were observed in one healthy population of 14 participants aged 28 y (84).

Chronic studies

The chronic effects of nitrate ingestion on FMD were investigated ranging from 14 to 42 days (2 to 6 weeks) with nitrate doses ranging from 375 to 577 mg/d (6.0 to 9.3 mmol/d) (Table 4). The higher nitrate dose in this range was estimated using the global average body weight of 62 kg as no average body weight was reported in this study (89). Three studies showed a significant improvement in FMD (50, 87, 89) and one study had no effects (68). In particular,

Rammos et al (89) demonstrated dietary nitrate reversed vascular dysfunction in older adults with moderately increased cardiovascular risk. Improvements in FMD ranged from 0.5 to 1.1% and were seen across the entire range of nitrate doses investigated. Increases in FMD (~1%) were seen in two studies (50, 87) using similar nitrate doses from beetroot juice (375 mg/d and 398 mg/d). Ingestion of a slightly higher nitrate dose of 577 mg/d (9.3 mmol/d) using sodium nitrate showed a 0.5% improvement (89). Improvements in FMD were seen in subjects with hypercholesterolemia (50), treated and untreated hypertension (87), and moderate cardiovascular risk (89). All populations were older adult populations (mean age >50 y) with large sample sizes (>60), except one study that had a sample size of 11 (89). No effects on FMD were observed after 14 days of nitrate ingestion (beetroot juice) in 27 subjects with type 2 diabetes mellitus (68).

Animal studies

Numerous studies have reported that blood vessels with a damaged endothelium have impaired vasorelaxation in response to acetylcholine (ACh) (Table 8) (113, 114). We identified three animal studies, from two publications, investigating the effects of dietary nitrate supplementation on endothelial function (97, 115). Bakker et al (115) demonstrated that although supplementation with very high dose nitrate (10 mmol/kg/d) had no effect on Ach-mediated vessel relaxation in a mouse model of atherosclerosis, low (0.1 mmol/kg/d) and moderate (1 mmol/kg/d) dose nitrate supplementation significantly improved the endothelial dysfunction associated with this mouse model. In addition, Carlstrom et al (97) reported that dietary supplementation with a high dose of nitrate (1 mmol/kg/d) was associated with attenuated acetylcholine-mediated vasorelaxation. These observations are in support of the theory proposed by Carlstrom et al that there is cross-talk between the two pathways of NO production. They suggest that high doses of dietary nitrate may inhibit production of NO through the L-arginine-NOS pathway, leading to a net decrease in the

amount of NO reaching the smooth muscle cells of the blood vessel (97). Although Bakker et al showed improvements with a 1 mmol/kg/d dose of nitrate and Carlstrom et al reported no improvements with the same dose, the animal model used is likely an important factor as the Apolipoprotein-E knock-out mice used in the study by Bakker et al (115) have reduced NO bioavailability.

Ischaemic reperfusion injury

Ischaemic reperfusion injury is tissue damage caused by a period of ischemia or lack of oxygen. Lack of oxygen during an ischaemic period results in inflammation and oxidative damage leading to microvascular dysfunction (116). Local and systemic tissue ischemia remains the major cause of death from cardiovascular disease (1). As the nitrate-nitrite-NO pathway is enhanced in times of hypoxia, this pathway may provide a back up to the classical L-arginine-NO synthase pathway.

Human studies

Our systematic literature search revealed three acute studies (two publications) investigating the effects of nitrate ingestion on ischaemic reperfusion injury (**Table 5**) (40, 85). Beetroot juice was the most common nitrate source used. The acute effects of nitrate ingestion on ischaemic reperfusion injury were investigated between 2-3 hours with nitrate doses ranging from 341-1488 mg (5.5-24 mmol) (Table 5). Benefits were also seen in all studies where beetroot juice (40, 85) and potassium nitrate (85) attenuated ischaemia reperfusion-induced endothelial dysfunction measured using FMD. Improvements were seen in young (mean age <28 y), healthy populations (40, 85) with an overall sample size ranging from 10 to 12.

Animal studies

We found only one study describing the effects of dietary nitrate supplementation on ischaemia-induced revascularisation in an animal model (Table 8). In a study by Hendgen-Cotta et al, mice were treated with either nitrate (1 g/L $NaNO_3$ in drinking water) or NaCl

(control) for 14 days (117). Perfusion recovery in the ischaemic hind limb was significantly improved in mice treated with nitrate compared with controls via a significant increase in capillary density. These results suggest that dietary nitrate supplementation may represent a novel strategy to enhance ischaemia-induced revascularization.

Arterial stiffness

Pulse wave velocity (PWV) is a measure of aortic stiffness and is a strong predictor of cardiovascular events (118-120). Pulse wave velocity is recognised as the most simple, non-invasive, robust and reproducible technique to determine arterial stiffness and is considered the gold-standard measurement of arterial stiffness (121). Pulse wave velocity measures arterial stiffness by dividing the estimated distance between the carotid and femoral arteries by the pulse transit time, the time delay between the carotid and femoral waveforms. A tonometer is used to capture the carotid waveform and a cuff is placed around the femoral artery to capture the femoral waveform. Augmentation index (AIx) is another measure of arterial stiffness which provides a composite measure of elastic plus muscular artery stiffness and wave reflection. Augmentation index has also been shown to be an independent predictor of future cardiovascular disease events (122).

Human studies

Our systematic literature search revealed seven acute studies (≤24 hours) (50, 70, 72, 78-80, 84) and 5 chronic studies (>1 day) (50, 65, 67, 87, 89) in 10 publications investigating the effects of nitrate consumption on arterial stiffness (**Table 6**). Beetroot juice was the most common nitrate source used in both acute and chronic studies.

Acute studies

The acute effects of nitrate ingestion on arterial stiffness were investigated between 2-6 hours with nitrate doses ranging from 68-583 mg (1.1-9.4 mmol) (Table 6). Three studies demonstrated a significant decrease in arterial stiffness (50, 72, 84) and four studies

demonstrated no effect (70, 78-80). A significant decrease of 0.3 m/s in PWV was observed in two studies (50, 84) with a nitrate dose of 375 mg (6 mmol) from beetroot juice (50) and 496 mg (8 mmol) from potassium nitrate (84). The study by Velmurugan et al (50) consisted of a large sample size of 67 hypercholesterolaemic men and women with a mean age of 53 y, whereas the study by Bahra et al (84) consisted of a smaller sample of 14 healthy individuals with a mean age 28 y. Hughes et al (72) demonstrated a reduced AIx in young, but not old, adults following a nitrate dose of 583 mg (9.4 mmol). No effect was seen in four studies with nitrate doses ranging from 68-500 mg (1.1-8.1 mmol) using beetroot juice (70, 79), beetroot-enriched bread (80), and spinach (78). These studies consisted of healthy (70, 78, 80) and overweight (79) subjects.

Chronic studies

The chronic effects of nitrate ingestion on arterial stiffness were investigated from 7 to 42 days (1 to 6 weeks) with nitrate doses ranging from 300-600 mg/d (4.8-9.7 mmol/d) (Table 6). Three studies demonstrated a significant decreased in arterial stiffness after nitrate ingestion (50, 87, 89) and two studies demonstrated no effect (65, 67). Studies found a significant decrease of 0.2-1.2 m/s in PWV with nitrate doses ranging from 375-577 mg/d (6-9.3 mmol/d) using beetroot juice and sodium nitrate (577 mg/d was estimated using the global average body weight of 62 kg as no average body weight was reported in this study (89)). The populations where an effect was observed had moderate cardiovascular risk (89), untreated and treated hypertension (87), and hypercholesterolemia (50). No effect was seen in two studies with nitrate doses of 300 mg/d (4.8 mmol/d) from green leafy vegetables (67) and 600 mg/d (9.7 mmol/d) from beetroot juice (65); populations that were overweight and obese (65) and pre-hypertensive (67). It has been demonstrated that for every 3.4 m/s in increase in PWV, the risk of experiencing a cardiovascular event is increased by 17% (118). Therefore, a

514 decrease of 0.2-1.2 m/s in PWV is likely to provide a small but significant reduction in the
515 risk of experiencing a cardiovascular disease event.

516 ***Animal studies***

517 Upon search of the literature, we found no animal studies investigating the effects of dietary
518 nitrate supplementation on arterial stiffness.

519 **Platelet function**

520 Platelets play a major role in the acute complications of atherosclerosis in the late stages of
521 the disease, which can subsequently lead to atherosclerotic-related events (123). Nitric oxide
522 has been shown to inhibit platelet aggregation and adhesion to the endothelial wall (124) and
523 there is now evidence to suggest dietary nitrate may repress platelet reactivity.

524 ***Human studies***

525 Our systematic literature search identified five acute studies (≤24 hours) (40, 125, 126) and
526 one chronic study (>1 day) (50), in four publications, investigating the effects of nitrate
527 intake on platelet function (**Table 7**). Potassium nitrate was the most common nitrate source
528 used in acute studies whilst beetroot juice was used in the chronic study.

529 *Acute studies*

530 The acute effects of nitrate ingestion on platelet function were investigated between 2.5-3
531 hours with nitrate doses between 31-1054 mg (0.5-17 mmol) (Table 7). All five studies
532 demonstrated reductions in platelet aggregation and reactivity (40, 125, 126). Velmurugan et
533 al (125) demonstrated that nitrate ingestion decreased platelet reactivity in healthy males, but
534 not in healthy females. This was observed with both beetroot juice (192 mg or 3.1 mmol) and
535 potassium nitrate (496 mg or 8 mmol). Further studies using beetroot juice (1054 mg or 17
536 mmol) (40) and potassium nitrate (31 mg and 124 mg or 0.5 and 2 mmol) (126) demonstrated
537 reductions in platelet aggregation. All cohorts consisted of young healthy populations and

were of small sample sizes (n<25). Further acute studies are needed to replicate these findings in older adult populations at risk of developing cardiovascular disease.

Chronic studies

The chronic effects of nitrate ingestion on platelet function were investigated in only one study (Table 7) (50). Velmurugan et al (50) demonstrated a reduction in platelet-monocyte aggregates after 42 days of daily beetroot juice ingestion with a nitrate dose of 375 mg/d (6 mmol/d). This study had a large sample size (n=67) of older male and female adults aged 53 y with hypercholesterolemia. There is a strong need for further chronic studies to investigate the effects of nitrate ingestion on platelet function in healthy populations and to replicate findings in older adult populations at risk of cardiovascular disease.

Animal studies

Only one animal study has been published investigating the effects of dietary nitrate supplementation on platelet function (Table 8). In this study, wild-type C57BL/6 mice were supplemented with 1 g/L $NaNO_3$ in their drinking water for 1 week, placed on a low nitrate diet or continued on standard mice chow (control) (127). Platelet aggregation was significantly decreased in the group supplemented with nitrate and was significantly increased in the group on the low nitrate diet, in comparison to the control group. These findings demonstrate that manipulation of nitrate levels in blood, via supplementation or dietary restriction, could affect platelet function in mice, although further studies are required to corroborate this finding.

Cerebral blood flow

The effect of dietary nitrate on cerebral blood flow has been investigated in several studies due to the observed effects of dietary nitrate on vasodilation and increases in blood flow. Diminished blood flow to the brain is likely to contribute to the pathophysiological processes underlying vascular cognitive impairment (128).

Human studies

Our systematic literature search identified one acute study (≤24 hours) (129) and one chronic study (>1 day) (130) in two publications investigating the effect of nitrate ingestion on cerebral blood flow (**Table 9**). Sodium nitrate and a high nitrate diet were used as nitrate sources.

Acute studies

Presley et al (129) demonstrated consuming a high nitrate diet (769 mg or 12.4 mmol of nitrate) over a 24 hour period increased regional cerebral perfusion in frontal lobe white matter, in older adults with a mean age of 75 y (Table 9). This was particularly evident in the dorsolateral prefrontal cortex and anterior cingulate cortex. In the same study, however, the acute effects of a high nitrate diet did not modify global cerebral perfusion.

Chronic studies

Aamand et al (130) demonstrated no effects after 3 days of sodium nitrate ingestion (477 mg/d or 7.7 mmol/d of nitrate, based on study mean weight of 77kg) on cerebral blood flow in 20 healthy men (Table 9).

Animal studies

No animal studies investigating the effects of dietary nitrate supplementation on blood flow were found.

Summary: nitrate ingestion and its effects on vascular function

Human intervention studies have now demonstrated ingestion of nitrate lowers blood pressure and improves endothelial function. These studies are predominantly in healthy populations and are of short duration. It is yet to be established whether nitrate ingestion has the same effects in populations at higher risk of cardiovascular disease as few studies have been conducted and findings are inconsistent. Further research is also needed to understand the long-term effects of nitrate intake on cardiovascular clinical endpoints.

588 **Epidemiological evidence**

589 Epidemiological studies have found plant-based diets rich in vegetables are associated with
590 lower rates of cardiovascular disease (2, 4, 131-136). In particular, cohort studies have shown
591 specific vegetable groups high in nitrate, such as green leafy vegetables, to be most beneficial
592 (6-9). The exact mechanisms for the protective effects shown in these studies are still
593 unknown. The Mediterranean diet (3, 137), the DASH diet (60, 138) and a vegetarian diet
594 (139, 140), all rich in vegetables, have been shown to be particularly beneficial towards
595 cardiovascular health. These diets are likely to contain substantially higher amounts of nitrate
596 than the average Western diet. Thus, nitrate is one possible candidate for explaining
597 cardiovascular health benefits seen with higher vegetable intakes (141).
598 There are very few observational epidemiological studies investigating nitrate intake and
599 cardiovascular-related health outcomes (**Table 10**). Although databases have been established
600 to calculate the nitrate intake in observational epidemiological studies (142-144), there was a
601 strong need for a more comprehensive database with compiled up-to-date data. Our recently
602 developed database on the nitrate content of vegetables (11) now gives researchers the
603 opportunity to conduct more observational epidemiological studies with an adequate
604 assessment of nitrate intake.
605 To date, there have been two articles published utilising the nitrate content of vegetables
606 database (11). We have demonstrated nitrate intake to be inversely associated with
607 atherosclerotic vascular disease mortality in a cohort of older adult women (mean age 75 ± 3
608 y) (53). In comparison to lower intakes of nitrate from vegetables <53 mg/d (median 39
609 mg/d), the inverse relationship with atherosclerotic vascular disease mortality plateaued at
610 intakes of 53-76 mg/d (median 63 mg/d) (53). In the same cohort of older adult women, we
611 also observed an inverse relationship between nitrate intake from vegetables and common
612 carotid artery intima-media thickness, as well as ischaemic cerebrovascular disease events

(hospitalisation or death) (54). The inverse relationship with ischaemic cerebrovascular disease events also plateaued at intakes of 53-76 mg/d (median 63 mg/d) (54).

Prior to these studies being published, the Tehran Lipid and Glucose Study reported on the relationship between consumption of nitrate-containing vegetables and risk of hypertension (145) and chronic kidney disease (CKD) (146), both risk factors for cardiovascular disease. These studies investigated nitrate intake by assessing whole vegetables containing nitrate. The authors further categorised nitrate-containing vegetables into low-nitrate, medium-nitrate and high-nitrate vegetables. It is worth noting that these studies essentially investigated whole vegetables and then different types of vegetables according to their nitrate levels and not nitrate as a separate entity. It is, however, difficult to separate nitrate intake from vegetable intake as the two can be highly correlated; as we have previously demonstrated (r=0.75, P<0.001) (53). Golzarand et al (145) found a significant inverse association between the intake of nitrate-containing vegetables and 3-year incidence of hypertension in the highest tertile compared with the lowest tertile of nitrate-containing vegetables. There were no significant associations observed between low-nitrate, medium-nitrate and high-nitrate containing vegetables and 3-year risk of hypertension. As no associations were found between categories of nitrate-containing vegetables, it is difficult to determine whether the inverse association demonstrated with total nitrate-containing vegetables is due to vegetable intake alone. This cohort consisted of 1,546 Iranian men and women (57% women), aged 38±12 years, without hypertension at baseline. In the same cohort, Mirmiran et al (146) found that the highest compared to the lowest tertile of nitrate-containing vegetables was associated with a lower estimated glomerular filtration rate and a higher prevalence of CKD at baseline. This could be a demonstration of reverse causality bias where the diagnosis of chronic disease has altered dietary intake. There was no association with the occurrence of CKD after 3 years of follow-up after excluding patients with CKD at baseline. Lastly, Bahadoran et al

638 (147) recently reported findings on the potential effects of dietary nitrate and nitrite on the
639 occurrence of type 2 diabetes in the same cohort of Iranian men and women (Tehran Lipid
640 and Glucose Study). Bahadoran et al (147) reported on 2,139 adults free of type 2 diabetes at
641 baseline with a median follow-up of 5.8 y. Nitrate and nitrite values were determined from a
642 recent survey conducted on frequently consumed food items among Iranians (148). Nitrate
643 and nitrite concentrations of 87 foods were determined using spectrophotometric methods.
644 The authors found no associations between nitrate intake and the risk of developing type 2
645 diabetes. However, the authors demonstrated an increased risk of type 2 diabetes among
646 participants with higher intakes of total and animal-based nitrite in the presence of low
647 vitamin C intake. The same was not observed in participants with high intakes of vitamin C
648 (>108 mg/d) (147), suggesting that diets high in vitamin C may counteract the suggested
649 adverse effects of nitrite on type 2 diabetes. However, higher intakes of total and animal-
650 based nitrite in the presence of low vitamin C intake may be a marker of an unhealthy diet
651 and lifestyle that may also be associated with a higher prevalence of type 2 diabetes.
652 There is a lingering concern that nitrate and nitrite may form cancerous compounds such as
653 nitrosamines (10). The majority of epidemiological studies to date have investigated
654 relationships between nitrate intake and cancer outcomes. A report compiled by the
655 International Agency for Research on Carcinogenicity concluded “Ingested nitrate or nitrite
656 under conditions that result in endogenous nitrosation is probably carcinogenic to humans
657 (Group 2A)” (149). Conditions that increase endogenous nitrosation are complex but could
658 involve interactions between the amount of nitrate and nitrite consumed, stomach acidity,
659 smoking status, medical conditions and the low intakes of nutrients that are likely to decrease
660 the potential for nitrosation such as polyphenols, vitamin C and vitamin E (56).
661 Now that there is a comprehensive database on the nitrate content of vegetables available,
662 researchers have the opportunity to further investigate the associations between chronic

663 intake of nitrate and health outcomes. Further research is needed to elucidate the relationships
664 amongst different populations including young vs. older age groups, low vs. higher
665 background nitrate intakes, and healthy vs. at risk populations.

666 **Conclusion**

667 There is now strong evidence to suggest that dietary nitrate derived from vegetables can
668 reduce blood pressure and other markers of vascular function in healthy populations. There is
669 a need for further research to investigate whether similar effects are observed in populations
670 at risk of developing cardiovascular disease. Few studies have investigated the long-term
671 effects of dietary nitrate on cardiovascular disease clinical endpoints; large observational
672 follow-up studies are required to address this. Further animal studies are required to elucidate
673 the mechanisms behind the observed beneficial effects. Increasing nitrate in the diet through
674 the consumption of nitrate-rich vegetables may prove to be an achievable and cost effective
675 way to reduce the risk of cardiovascular disease.

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Table 1. Intervention studies investigating the acute effects of inorganic nitrate on blood pressure in humans.

	Blood pressure effect	**Nitrate source**	**Nitrate dose**	**Duration**	**Subjects**	**Screening/baseline blood pressure**	**Reference**
Effect	↓ Clinic SBP ↓ Clinic DBP	Beetroot juice	583 mg (9.4 mmol)	3 h	Young: 25±4 y (10 M; 3 F) Old: 64±5 y (9 M; 3 F) Healthy	Optimal/normal	Hughes 2016 (72)
	↓ Clinic DBP	Sodium nitrate	800 mg (12.9 mmol)	5 h	28±1 y (11 M; 7 F) Healthy	Optimal/Normal	Jonvik 2016 (71)
	↓ Clinic SBP ↓ Clinic DBP	Beetroot juice					
	↓ Clinic SBP ↓ Clinic DBP	Rocket salad beverage					
	↓ Clinic SBP ↓ Clinic DBP	Spinach beverage					
	↓ Clinic SBP ↓ Clinic DBP	Beetroot juice	375 mg (6 mmol)	3 h	Nitrate: 53±10 y (12 M; 21 F) Placebo: 53±12 y (12 M; 22 F) Hypercholesterolaemic	Normal	Velmurugan 2016 (50)
	↓ Clinic SBP	Spinach	220 mg (3.5 mmol)	3.5 h	58.8±7.6 y (6 M; 20 F) Healthy	Optimal	Liu 2013 (78)
	↓ Clinic DBP	Beetroot juice	500 mg (8.1 mmol)	2 h	61±7 y(20 M) Overweight	High-normal	Joris 2013 (79)
	↓ Clinic SBP	Spinach	182 mg (2.9 mmol)	3.3 h	47±14 y (6 M; 24 F) Healthy	Optimal	Bondonno 2012 (83)
	↓ Clinic SBP	Potassium nitrate	496 mg (8 mmol)	3 h	28±2 y (14) Healthy	Optimal	Bahra 2012 (84)
	↓ Clinic SBP ↓ Clinic DBP	Potassium nitrate	1488 mg (24 mmol)	24 h	23±1 y (8 M; 12 F) Healthy	Optimal	Kapil 2010 (85)

↓ Clinic SBP ↓ Clinic DBP	Potassium nitrate	248 mg, 744 mg (4 mmol, 12 mmol)	3 h	29±2 y (6) Healthy	Optimal	
↓ Clinic SBP	Beetroot juice	341 mg (5.5 mmol)	3 h	25±1 y (9) Healthy	Normal	
↓ Clinic SBP ↓ Clinic DBP	Beetroot juice	1395 mg (22.5 mmol)	24 h	26 ± 5 y (9 M; 5 F) Healthy	Optimal	Webb 2008 (40)
↓ Ambulatory DBP in T carriers only	Beetroot bread	68 mg (1.1 mmol)	6 h	34±9 y (14 M) Healthy	Normal	Hobbs 2014 (77)
↓ Ambulatory DBP	Beetroot bread	68 mg (1.1 mmol)	6 h	31±2 y (23 M) Healthy	Normal	Hobbs 2013 (80)
↓ Ambulatory SBP ↓ Ambulatory DBP	Beetroot juice	0-707 mg (0-11.4 mmol)	24 h	31±3 y (18 M) Healthy	High-normal	Hobbs 2012 (81)
↓ Ambulatory SBP ↓ Ambulatory DBP	White beetroot-enriched bread	99 mg (1.6 mmol)	24 h	25±1 y (14 M) Healthy	High-normal	
↓ Ambulatory SBP ↓ Ambulatory DBP	Red beetroot-enriched bread	112 mg (1.8 mmol)				
↓ Ambulatory SBP (M only)	Beetroot juice	465 mg (7.5 mmol)	24 h	43±3 y (15 M; 15 F) Healthy	High-normal	Coles and Clifton 2012 (82)

No effect	No effect on clinic BP	Beetroot gel	391 mg (6.3 mmol)	3 h	27±2 y (4 M; 1 F) Healthy	Optimal	da Silva 2016 (73)
	No effect on clinic BP	Beetroot juice	341 mg (5.5 mmol)	2.5 h	Nitrate: 21±1 y (5 M; 15 F) Placebo: 21±1 y (7 M; 13 F) Healthy	Optimal	Wightman 2015 (74)
	No effect on clinic BP	Sodium nitrate	0.1-10 mg/kg body weight	4 h	25±1 y (15 M) Healthy	Optimal	Rodriguez-Mateos 2015 (75)
	No effect on clinic BP	Beetroot juice	694 mg (11.2 mmol)	2 h	57±10 y (5 M; 4 F) Heart failure	Optimal	Coggan 2015 (63)
	No effect on clinic BP	Beetroot juice	310 mg (5 mmol)	3 h	25±5 y (7 M; 4 F) Healthy	Optimal	Bakker 2015 (76)
	No effect on clinic BP	Beetroot juice	738 mg (11.9 mmol)	3 h	Young: 27±6 y (11 M; 5 F) Old: 59±6 y (8 M; 7 F) Healthy	Normal/high-normal	Shepherd 2016 (69)
	No effect on clinic BP	Beetroot juice	403-434 mg (6.5-7.0 mmol)	2 h	23±3 y (20 M) Healthy	Optimal	Lefferts 2016 (70)

Screening/baseline blood pressure was based on criteria in the Australian guidelines for the diagnosis and management of hypertension in adults (150). BP, blood pressure; DBP, diastolic blood pressure; SBP, systolic blood pressure.

Table 2. Intervention studies investigating the chronic effects of inorganic nitrate on blood pressure in humans.

	Blood pressure effect	**Nitrate source**	**Nitrate dose**	**Duration**	**Subjects**	**Screening/baseline blood pressure**	**Reference**
Effect	↓ Clinic DBP	Beetroot juice	450 mg/d (7.3 mmol/d)	3 d	24±1 y (6 M) Healthy	Normal	Keen 2015 (86)
	↓ Clinic, home and ambulatory SBP ↓ Clinic, home and ambulatory DBP	Beetroot juice	398 mg/d (6.4 mmol/d)	28 d	n=64 (26 M; 38 F) Nitrate: 58±14 y Placebo: 56±16 y Drug-naïve and treated hypertensive	Grade 1 hypertension	Kapil 2015 (87)
	↓ Clinic SBP	High nitrate vegetables	339±133 mg/d (5.5±2.1 mmol/d)	7 d	20±2 y (19 F) Healthy	Optimal	Ashworth 2015 (88)
	↓ Home SBP No effect on clinic and ambulatory BP	Beetroot juice	300-400 mg/d (4.8-6.4 mmol/d)	21 d	n=21 (12 M; 9 F) Beetroot:63±2 y Placebo: 61±1 y Older overweight	Normal/high-normal	Jajja 2014 (90)
	↓ Clinic SBP	Sodium nitrate	9.3 mg/kg body weight/d	28 d	63±6 y (4 M; 7 F) Moderate cardiovascular risk	High-normal	Rammos 2014 (89)

	↓ Clinic DBP	Japanese traditional diet	18.8 mg/kg/body weight/d	10 d	36±10 y (10 M; 15 F) Healthy	Optimal	Sobko 2010 (92)
	↓ Clinic DBP	Sodium nitrate	6.2 mg/kg body weight/d	3 d	24 y (15 M; 2 F) Healthy	Optimal	Larsen 2006 (93)
No effect	No effect on clinic BP	Beetroot juice	375 mg/d (6 mmol/d)	42 d	Nitrate: 53±10 y (12 M; 21 F) Placebo: 53±12 y (12 M; 22 F) Hypercholesterolaemic	Normal	Velmurugan 2016 (50)
	No effect on clinic and ambulatory BP	Beetroot juice	600 mg/d (9.7 mmol/d)	7 d	62±5 y (14 M; 16 F) Overweight and obese	Normal/high-normal	Lara 2015 (65)
	No effect on home and ambulatory BP	Beetroot juice	434 mg/d (7 mmol/d)	7 d	63±4 y (10 M; 17 F) Treated hypertensive	High-normal	Bondonno 2015 (66)
	No effect on clinic, home and ambulatory BP	Green leafy vegetables	300 mg/d (4.8 mmol/d)	7 d	61±7 y (12 M; 26 F) Pre-hypertensive	High-normal	Bondonno 2014 (67)
	No effect on ambulatory BP	Beetroot juice	465 mg/d (7.5 mmol/d)	14 d	67±5 y (18 M; 9 F) T2DM	Grade 1 hypertension	Gilchrist 2013 (68)
	No effect on clinic BP	High nitrate diet	155 mg/d (2.5 mmol/d)	3 d	73±5 y (3 M; 5 F) Older	High-normal	Miller 2012 (91)
		Beetroot juice	527 mg/d (8.5 mmol/d)	3 d		Normal	
		Combination	682 mg/d (11 mmol/d)	3 d		High-normal	

Screening/baseline blood pressure was based on criteria in the Australian guidelines for the diagnosis and management of hypertension in adults (150). BP, blood pressure; DBP, diastolic blood pressure; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus.

Table 3. Intervention studies investigating the acute effects of inorganic nitrate on endothelial function in humans.

	FMD effect	Nitrate source	Nitrate dose	Duration	Subjects	Reference
Effect	↑ FMD	Beetroot juice	375 mg (6 mmol)	3 h	Nitrate: 53±10 y (12 M; 21 F) Placebo: 53±12 y (12 M; 22 F) Hypercholesterolaemic	Velmurugan 2016 (50)
	↑ FMD	Beetroot juice	310 mg (5 mmol)	3 h	25±5 y (7 M; 4 F) Healthy	Bakker 2015 (76)
	↑ FMD	Sodium nitrate	0.1-10 mg/kg body weight	4 h	24±1 y (15 M) Healthy	Rodriguez-Mateos 2015 (75)
	↑ FMD	Beetroot juice	500 mg (8.1 mmol)	2 h	61±7 y (20 M) Overweight	Joris 2013 (79)
	↑ FMD	Sodium nitrate	9.3 mg/kg body weight	1.5 h	26±1 y (5 M; 5 F) Healthy	Heiss 2012 (112)
	↑ FMD	Spinach	182 mg (2.9 mmol)	4 h	47±14 y (6 M; 24 F) Healthy	Bondonno 2012 (83)
No effect	No effect	Potassium nitrate	496 mg (8 mmol)	3 h	28±2 y (14) Healthy	Bahra 2012 (84)

FMD, flow-mediated dilatation.

Table 4. Intervention studies investigating the chronic effects of inorganic nitrate on endothelial function in humans.

	FMD effect	Nitrate source	Nitrate dose	Duration	Subjects	Reference
Effect	↑ FMD	Beetroot juice	375 mg/d (6 mmol/d)	42 d	Nitrate: 53±10 y (12 M; 21 F) Placebo: 53±12 y (12 M; 22 F) Hypercholesterolaemic	Velmurugan 2016 (50)
	↑ FMD	Beetroot juice	398 mg/d (6.4 mmol)	28 d	n=64 (26 M; 38 F) Nitrate: 58±14 y Placebo: 56±16 y Drug-naïve and treated hypertensive	Kapil 2015 (87)
	↑ FMD	Sodium nitrate	9.3 mg/kg body weight/d	28 d	63±6 y (4 M; 7 F) Moderate cardiovascular risk	Rammos 2014 (89)
No effect	No effect	Beetroot juice	465 mg/d (7.5 mmol/d)	14 d	67±5 y (18 M; 9 F) T2DM	Gilchrist 2013 (68)

FMD, flow-mediated dilatation; T2DM, type 2 diabetes mellitus.

Table 5. Intervention studies investigating the acute effects of inorganic nitrate on ischemic reperfusion in humans.

	Ischemic reperfusion effect	**Nitrate source**	**Nitrate dose**	**Duration**	**Subjects**	**Reference**
Effect	Attenuated IR-induced endothelial dysfunction	Potassium nitrate	1488 mg (24 mmol)	3 h	25±1 y (12) Healthy	Kapil 2010 (85)
		Beetroot juice	341 mg (5.5 mmol)	3 h		
	Attenuated IR-induced endothelial dysfunction	Beetroot juice	1395 mg (22.5 mmol)	2 h	27±7 y (4 M; 6 F) Healthy	Webb 2008 (40)

IR, ischemic reperfusion.

Table 6. Intervention studies investigating the effects of inorganic nitrate on arterial stiffness in humans.

	Arterial stiffness effect	Nitrate source	Nitrate dose	Duration	Subjects	Reference
Effect	↓ AIx (young only)	Beetroot juice	583 mg (9.4 mmol)	Acute (3 h)	Young: 25±4 y (10 M; 3 F) Old: 64±5 y (9 M; 3 F) Healthy	Hughes 2016 (72)
	↓ PWV	Potassium nitrate	496 mg (8 mmol)	Acute (3 h)	28±2 y (14) Healthy	Bahra 2012 (84)
	↓ PWV ↓ AIx	Beetroot juice	375 mg/d (6 mmol)	Acute (3 h)	Nitrate: 53±10 y (12 M; 21 F) Placebo: 53±12 y (12 M; 22 F) Hypercholesterolaemic	Velmurugan 2016 (50)
	↓ PWV			Chronic (42 d)		
	↓ PWV ↓ AIx	Beetroot juice	398 mg/d (6.4 mmol/d)	Chronic (28 d)	n=64 (26 M; 38 F) Nitrate: 58±14 y Placebo: 56±16 y Drug-naïve and treated hypertensive	Kapil 2015 (87)
	↓ PWV ↓ AIx	Sodium nitrate	9.3 mg/kg body weight/d	Chronic (28 d)	63±6 y (4 M; 7 F) Moderate cardiovascular risk	Rammos 2014 (89)
No effect	No effect on PWV and AIx	Beetroot juice	403-434 mg (6.5-7.0 mmol)	Acute (2 h)	23±3 y (20 M) Healthy	Lefferts 2016 (70)
	No effect on PWV and AIx	Beetroot bread	68 mg (1.1 mmol)	Acute (6 h)	31±2 y (23 M) Healthy	Hobbs 2013 (80)
	No effect on PWV and AIx	Beetroot juice	500 mg (8.1 mmol)	Acute (2 h)	61±7 y (20 M) Overweight	Joris 2013 (79)
	No effect on PWV and AIx	Spinach	220 mg (3.5 mmol)	Acute (3.5 h)	59±8 y (6 M; 20 F) Healthy	Liu 2013 (78)
	No effect on PWV	Beetroot juice	600 mg/d (9.7 mmol/d)	Chronic (7 d)	62±5 y (14 M; 16 F) Overweight and obese	Lara 2015 (65)

No effect on PWV and AIx	Green leafy vegetables	300 mg/d (4.8 mmol/d)	Chronic (7 d)	61±7 y (12 M; 26 F) Pre-hypertensive	Bondonno 2014 (67)

AIx, augmentation index; PWV, pulse wave velocity.

Table 7. Intervention studies investigating the effects of inorganic nitrate on platelet function in humans.

	Platelet effect	Nitrate source	Nitrate dose	Duration	Subjects	Reference
Effect	↓ in platelet reactivity in males but not females	Beetroot juice	192 mg (3.1 mmol)	Acute (3 h)	M: 26±1 y (12) F: 24±2 y (12) Healthy	Velmurugan 2013 (125)
	↓ in platelet reactivity in males but not females	Potassium nitrate	496 mg (8 mmol)	Acute (3 h)	M: 27±1 y (12) F: 29±2 y (12) Healthy	
	↓ in platelet aggregation	Beetroot juice	1054 mg (17 mmol)	Acute (2.5 h)	31±2 y (5 M; 1 F) Healthy	Webb 2008 (40)
	↓ in platelet aggregation	Potassium nitrate	124 mg (2 mmol)	Acute (2.5 h)	18-44 y (4 M; 3 F)	Richardson 2002 (126)
	↓ in platelet aggregation	Potassium nitrate	31 mg, 124 mg (0.5 mmol, 2 mmol)	Acute (2.5 h)	18-44 y (3 M; 3 F)	
	↓ in platelet-monocyte aggregates	Beetroot juice	375 mg/d (6 mmol/d)	Chronic (42 d)	Nitrate: 53±10 y (12 M; 21 F) Placebo: 53±12 y (12 M; 22 F) Hypercholesterolaemic	Velmurugan 2016 (50)

Table 8. Intervention studies investigating the effects of inorganic nitrate in animals.

	Effect	Nitrate source	Background diet	Nitrate dose	Duration	Animals	Reference
Blood pressure	↓ MAP (6.7 mmol dose only) No change in MAP	KNO_3 in drinking water	Not described	2.5 or 6.7 mmol/kg/d	3 w	Hypoxia WT male mice (n≥8) Hypoxia eNOS KO male mice (n≥8)	Baliga 2012 (151)
	↓ MAP	$NaNO_3$ in drinking water	Not described	0.1 mmol/kg/d	8 w	Rats (5≤n≥15)	Carlstrom 2010 (152)
	↓ MAP (1mM dose only)	supplemented with $NaNO_3$	High-salt diet	0.1 or 1 mmol/kg/d	8-11 w	UNX Male Sprague–Dawley rats	Carlstrom 2011 (103)
	Prevented ↑ in MAP No change in MAP	$NaNO_3$ in drinking water	Not described	1 mmol/kg/d	8 w	Male SH rats (n=6) Normotensive Wistar Kyoto rats (n=6)	Chien 2014 (98)
	↓ MAP	Supplemented with $NaNO_3$	High-fructose diet	1.8 mmol/kg/d	6 w	Male Sprague–Dawley rats (n=8)	Essawy 2014 (100)
	Prevented ↑ in MAP				10 w from start	Male Sprague–Dawley rats (n=8)	
	↓ MAP	$NaNO_3$ in drinking water	Standard chow	0.2 mmol/kg/d	1 w	Male Sprague-Dawley rats (n=7)	Petersson 2009 (153)
	↓ MAP and ↓ DBP				5 d	Male Sprague-Dawley rats	
	No change in MAP or SBP	$NaNO_3$ in drinking water	Standard chow	0.8 mmol/kg/d	2 w	Young male Sprague–Dawley rats (n=8)	Hezel 2016 (101)
	↓ MAP and ↓ SBP			0.3 mmol/kg/d		Old male Sprague–Dawley rats (n=5)	

	No change in SBP ↓ SBP	$NaNO_3$ in drinking water	Standard chow	0.1 g/L	8 w	Male Wistar rats (n=8) Diabetic Male Wistar rats (n=8)	Khalifi 2015 (102)
	No change in BP	$NaNO_3$ in drinking water	Western-type diet	0.2 mmol/d	14 w	LDL receptor KO mice (n=15)	Marsch 2016 (154)
	Smaller rise in SBP	$Mg(NO_3)_2$ in drinking water	Not described	0.3 mmol/kg/d	4 w	Male SH rats (n=7)	Vilskersts 2014 (99)
	↓ MAP (0.1mM dose only) ↑ MAP (1mM dose only)	$NaNO_3$ in drinking water	Standard chow	0.1 or 1 mmol/kg/d	8-10 w	Male Sprague–Dawley rats (n=5-12)	Carlstrom 2015 (97)
Vascular function	↓ Ach-mediated vasorelaxation (1mM dose only) No vasorelaxation				2-4 w	WT C57BL/6 mice (n=5-12) eNOS KO mice (n=5-12)	
	↑ Ach-mediated vessel relaxation (0.1 and 1 mmol dose only)	$NaNO_3$ in drinking water	High-fat diet	0.1, 1 or 10 mmol/kg/d	10 w	Male ApoE KO mice (n= 8-12)	Bakker 2016 (115)
Ischaemic reperfusion	↑ Perfusion recovery	$NaNO_3$ in drinking water	Not described	5.0 mmol/kg/d	2 w	Male NMRI mice or C57BL/6 mice (n=21-23)	Hendgen-Cotta 2012 (117)
Platelet function	↓ collagen induced platelet aggregation	$NaNO_3$ in drinking water	Standard chow	1 g/L	1 w	WT C57BL/6 mice (n≥5) eNOS KO mice (n≥5)	Park 2013 (127)

Ach, acetylcholine; ApoE, apolipoprotein e; eNOS, endothelial nitric oxide synthase; KO, knock-out; LDL, low density lipoprotein; MAP, mean arterial pressure; NMRI, Naval Medical Research Institute; NO, nitric oxide; NOS, nitric oxide synthase; SBP, systolic blood pressure; SH, spontaneously hypertensive; UNX, uninephrectomized; WT, wild-type.

Table 9. Intervention studies investigating the effects of inorganic nitrate on cerebral blood flow in humans.

	Cerebral blood flow effect	Nitrate source	Nitrate dose	Duration	Subjects	Reference
Effect	↑ regional cerebral perfusion in frontal lobe white matter but no effect on global cerebral perfusion	High nitrate diet	769 mg (12.4 mmol)	Acute (24 h)	75±7 y (14) Older	Presley 2011 (129)
No effect	No effect on cerebral blood flow	Sodium nitrate	6.2 mg/kg body weight/d	Chronic (3 d)	25±1 y (20 M) Healthy	Aamand 2014 (130)

NOS, nitric oxide synthase.

Table 10. Observational epidemiological studies of dietary nitrate and cardiovascular-related health outcomes

Study design and population	Nitrate intake assessment	Primary outcome	Adjusted variables	Results	Reference
15 y follow-up study n=1226 Australian female older adults Diabetes and ASVD-free 75.1±2.7 y	FFQ	ASVD mortality	Model 1: Unadjusted. Model 2: Age and energy. Model 3: Age, BMI, physical activity, alcohol intake, history of smoking, socioeconomic status, calcium supplementation group, organic nitrate medication, antihypertensive medication, statin medication, low-dose aspirin, renal function, and energy intake.	↓ ASVD mortality	Blekkenhorst 2017 (53)
15 y follow-up study n=1226 Australian female older adults Diabetes and ASVD-free 75±3 y	FFQ	Ischaemic cerebrovascular disease hospitalisation and death	Model 1: Unadjusted. Model 2: Age and energy. Model 3: Age, BMI, energy intake, alcohol intake, energy expended in physical activity, antihypertensive medication, statin medication, low-dose aspirin medication, organic nitrate medication, history of smoking, and treatment.	↓ ischaemic cerebrovascular disease hospitalisation and death	Bondonno 2017 (54)
Cross-sectional and 3 y follow-up study n=1538 cross-sectional n=1229 follow-up Iranian male and female adults (57% female)	FFQ	eGFR and CKD	Model 1: age, sex, and BMI. Model 2: Additional adjustment for smoking, education, physical activity, diabetes, and hypertension.	↓ eGFR, ↑ CKD (cross-sectional) No association for 3 year	Mirmiran 2016 (146)

38.0±12.0 y			Model 3: Additional adjustment for dietary intake of energy, fibre, and potassium.	follow-up of CKD	
5.8 y follow-up study n=2139 Iranian male and female adults (54.6% male) T2DM-free 38.9±12.6 y	FFQ	T2DM	Model 1: Diabetes risk score. Model 2: Additional adjustment for dietary total fat, fibre, and vitamin C.	No association	Bahadoran 2017 (147)
3 y follow-up study Iranian male and female adults (57% female) 38±12 y	FFQ	Hypertension	Model 1: Adjusted for age and sex. Model 2: Additional adjustment for weight, 3-year weight change, smoking, education, physical activity, baseline SBP and DBP. Model 3: Additional adjustment for dietary intake of energy, fibre, sodium, potassium and processed meat.	No association	Golzarand 2016 (145)

ASVD, atherosclerotic vascular disease; BMI, body mass index; CKD, chronic kidney disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FFQ, food frequency questionnaire; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus.

Figure legends:

Figure 1. Observed beneficial effects of nitrate ingestion on cardiovascular-related health outcomes in human and animal studies. ASVD, atherosclerotic vascular disease; CVD, cardiovascular disease.