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The role of inorganic nitrate and nitrite in CVD

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Abstract

CVD is the leading cause of death worldwide, a consequence of mostly poor lifestyle and dietary behaviours. Although whole fruit and vegetable consumption has been consistently shown to reduce CVD risk, the exact protective constituents of these foods are yet to be clearly identified. A recent and biologically plausible hypothesis supporting the cardioprotective effects of vegetables has been linked to their inorganic nitrate content. Approximately 60–80 % inorganic nitrate exposure in the human diet is contributed by vegetable consumption. Although inorganic nitrate is a relatively stable molecule, under specific conditions it can be metabolised in the body to produce NO via the newly discovered nitrate–nitrite–NO pathway. NO is a major signalling molecule in the human body, and has a key role in maintaining vascular tone, smooth muscle cell proliferation, platelet activity and inflammation. Currently, there is accumulating evidence demonstrating that inorganic nitrate can lead to lower blood pressure and improved vascular compliance in humans. The aim of this review is to present an informative, balanced and critical review of the current evidence investigating the role of inorganic nitrate and nitrite in the development, prevention and/or treatment of CVD. Although there is evidence supporting short-term inorganic nitrate intakes for reduced blood pressure, there is a severe lack of research examining the role of long-term nitrate intakes in the treatment and/or prevention of hard CVD outcomes, such as myocardial infarction and cardiovascular mortality. Epidemiological evidence is needed in this field to justify continued research efforts.

Key words: **Nitrate: Nitrite: CVD**

Introduction

Despite major medical research advancements over the past 50 years, CVD remains the leading cause of death worldwide and is responsible for 39 % of non-communicable disease deaths in populations aged under 70 years[1]. The leading non-communicable disease risk factor is hypertension, which is responsible for 13 % of global deaths each year and is a major risk factor for coronary artery disease (CAD), IHD and stroke[1].

The pathogenesis of CVD is influenced by a variety of risk factors that can be broadly categorised as either modifiable or non-modifiable[2]. Non-modifiable risk factors cannot be controlled through intervention and include advancing age, sex (men at greater risk than premenopausal women; post-menopausal women at greater risk than men), ethnicity and family history of CVD[2]. Modifiable risk factors, on the other hand, have the ability to be manipulated through intervention in order to control, treat or modify the risk factor[2]. Established modifiable risk factors for CVD include hypertension, tobacco use, raised blood glucose, physical inactivity, unhealthy diet, raised blood cholesterol/lipids and overweight and obesity[2].

Implementation of various lifestyle strategies which target specific modifiable risk factors can reduce the risk of CVD by up to 80 %[1,2], thus indicating that CVD is a chronic and mostly lifestyle-induced disease, to which the majority of current mortality is the consequence of previous exposures to behavioural risk factors such as inappropriate nutrition, insufficient physical activity and tobacco exposure[2–5]. In addition, excess weight and central obesity, increased blood pressure, dyslipidaemia, diabetes and low cardiorespiratory fitness are among the factors contributing principally to CVD risk[2,6].

Given the scope and prevalence of CVD within our current food and lifestyle environment, it is clear that preventative measures are the most appropriate to deal with this global health issue in order to reduce the costs to both the community (through improved quality of life) and governments through a reduction in hospitalisations, medication use and rehabilitation[2]. Although behavioural factors such as smoking cessation and increased physical activity appear relatively straightforward targets for public health preventative interventions, the definition of a perceived 'healthy' diet has changed over time, leading to a general sense of public confusion and uncertainty surrounding the topic[7,8].

Abbreviations: ADMA, asymmetric dimethylarginine; CAC, circulating angiogenic cells; CAD, coronary artery disease; DASH, Dietary Approaches to Stop Hypertension; MI, myocardial infarction; NOS, nitric oxide synthase; NO_x, nitrate/nitrite.

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Currently, the most compelling dietary evidence for CVD prevention is linked to whole-diet approaches such as the Mediterranean and Dietary Approaches to Stop Hypertension (DASH) diets[7,9]. Although the cardioprotective effects of these diets may be credited to a whole-diet/whole-food effect, some individual nutritive components of these foods have also been extensively investigated.

The investigation of single nutritive components demonstrates that the evidence is less clear; this is especially noticeable for fruit and vegetable constituents. While whole fruit and vegetable consumption has been consistently shown to reduce CVD risk, as evidenced by various prospective studies showing a direct inverse association between fruit and vegetable intakes and the development of CVD events such as myocardial infarction (MI) and stroke[10–13], the various constituents of fruits and vegetables such as vitamin C, polyphenols, fibre and antioxidants are yet to clearly demonstrate a beneficial link or a physiological pathway for their individual effect[14–18].

A recent and biologically plausible hypothesis for the cardioprotective and blood pressure-lowering effect of vegetables has been linked to their inorganic nitrate (NO_3^-)/nitrite (NO_2^-) content[19]. Support for this hypothesis has been implied in studies indicating that nitrate-rich green leafy vegetables and vitamin C-rich fruits and vegetables contribute most to the apparent cardiovascular protective effect of total fruit and vegetable intake[20,21]. Additionally, cardioprotective diets including the DASH, Mediterranean and traditional Japanese diets have been shown to naturally contain high quantities of inorganic nitrate (147–1222 mg/d) relative to a typical Western-style diet (about 75 mg/d)[22–24].

Within the human body, inorganic nitrate/nitrite (NO_x) can be metabolised to produce NO (Fig. 1)[25,26]. NO is a highly valuable signalling molecule and has been demonstrated to mediate favourable effects on blood pressure control, platelet function, vascular health and exercise performance[27–30]. In addition, the utility of inorganic NO_x as an NO donor may be of particular relevance given that one serving of nitrate-rich vegetables (such as beetroot) has been estimated to produce more NO under specific conditions than can be endogenously formed by the classical L-arginine–nitric oxide synthase (NOS) pathway each day[19,31,32] (Fig. 1[33]).

Currently, the true effect that dietary/inorganic NO_x may have on CVD risk factors and outcomes is poorly understood, but it is a highly worthwhile line of investigation given that an increased daily consumption of nitrate intake represents a potential low-cost and simple treatment option for reducing CVD burden.

Production of nitric oxide in the body

Endogenous production via the L-arginine–nitric oxide synthase pathway

The notion that NO_x could be produced endogenously in the body was first considered in the early 1980s, upon finding that NO_x excretion was exceeding quantities of ingestion in animal and human models[34,35]. Later it was demonstrated that L-arginine was the substrate for synthesising nitrogen oxides endogenously via the action of NOS enzymes[36].

In healthy individuals the L-arginine–NOS pathway can produce sufficient quantities of NO to maintain health (approximately 1·7 mmol/d)[31,32]. However, conditions such as diabetes mellitus, ageing, hypercholesterolaemia and tobacco exposure have been found to make an impact on the bioactivity of endogenously produced NO via one or more of the following functions[37–42]:

(1) Increased degradation of NO[38,42,43];
(2) Altered phosphorylation and activation of NOS[38,43];

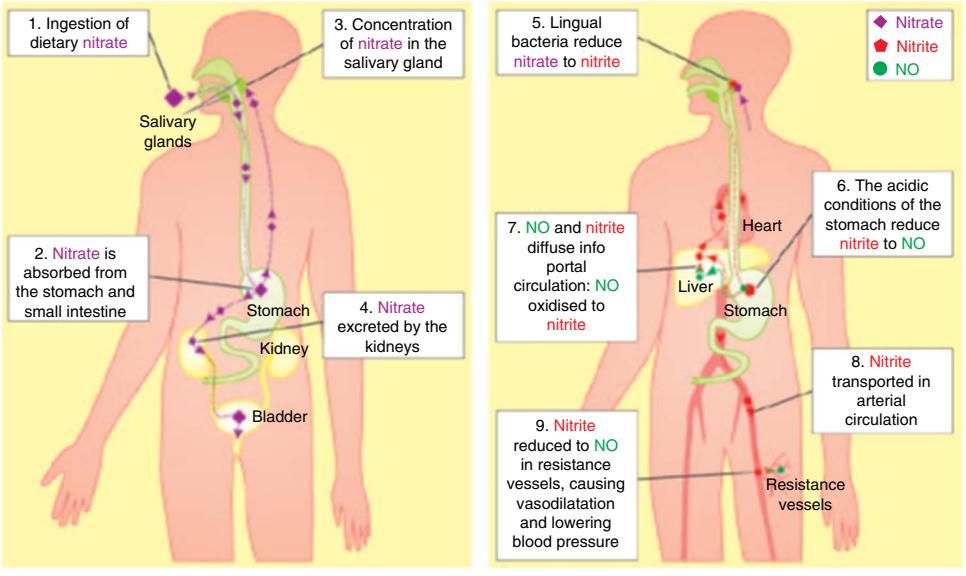

Fig. 1. The fate of dietary nitrate. Nitrate is systematically absorbed becoming concentrated in the salivary glands and part of the salivary circulation. Salivary nitrate is reduced to nitrite by oral bacteria. In the stomach nitrite may produce NO. Nitrite transported in arterial circulation can be reduced to NO in low oxygen concentrations which can lead to vasodilation and reductions in blood pressure (Webb A, Patel N, Loukogeorhakis S, *et al.* Acute blood pressure lowering,vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension*, vol. 51, pp. 784–790, from http://hyper.ahajournals.org/content/51/3/784.short[33]).

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(3) Increased production of NOS inhibitors (for example, asymmetric dimethylarginine; ADMA), leading to disruption of NOS activation[38,39,41–43];
(4) Deficiency of the NOS substrate, L-arginine[34,38,41];
(5) Reduced availability of one or more cofactors essential for NOS function[34,38].

While appropriate medical management, consumption of a healthy diet and moderate exercise can somewhat reverse these effects, it has been postulated that supplementing parts of the NOS pathway may enhance NOS activity and NO production[38,41,43]. This has been of particular importance given that increased ADMA levels inhibit NOS function and have been cited as the strongest risk predictor of cardiovascular events, and all-cause and cardiovascular mortality in individuals with CAD[44]. Although it remains unclear whether a change in ADMA can alter CVD risk, interventions such as L-arginine supplementation have been shown to improve endothelial-mediated vasodilation in individuals with elevated ADMA levels[41,44].

As a result, the effect of L-arginine supplementation has been investigated and short-term supplementation has been shown to improve endothelial function and relieve symptoms in patients with CHD[45]. Long-term (6 months) supplementation, however, demonstrated no beneficial effect[46]. In fact the long-term L-arginine supplementation led to increased rates of death and less cardiovascular improvement compared with the placebo due to the development of arginine toxicity and hyperkalaemia (abnormally high serum K)[47,48]. In addition, the utility of supplementing arginine is questionable given that arginine is classified as a 'semi-essential' or 'conditionally essential' amino acid, depending on the developmental stage or health status of the individual[49]. However, it is generally accepted that healthy adults should not need to supplement with arginine as their bodies produce physiologically sufficient amounts[48]. Arginine is also highly abundant in the diet, as rich dietary sources include meat, dairy products, vegetables, legumes and whole grains[48,49].

The 'arginine paradox' appears to address this notion, as it refers to the phenomenon that exogenous arginine causes NO-mediated biological effects, despite the fact that NOS are theoretically saturated in the substrate L-arginine[49]. A recently published cross-sectional study including 2771 men and women investigated whether regular dietary intakes of L-arginine were associated with serum NO_x, as an indicator of systemic NO production[50]. This study found that increased dietary L-arginine intakes were strongly associated with serum NO_x, which was independent of the overall dietary patterns of the study participants and other dietary factors, including intakes of high-nitrate-containing foods (probably due to collection of fasting blood samples)[50]. Therefore, although there may be some utility in consuming adequate amounts of arginine, which is readily achieved by consumption of a healthy balanced diet, there appears to be no great benefit for the general population to be using arginine supplements. However, dietary intervention to also consume nitrate-rich foods holds much promise for supplementing the NOS pathway via the alternative nitrate–nitrite–NO pathway.

The nitrate–nitrite–nitric oxide pathway

Up until the early 1990s, plasma NO_x were considered to be biologically inactive endproducts of NO production in the human body. However, it is now clear that under specific conditions nitrate and nitrite anions can be recycled *in vivo* back to NO[26,27,51,52].

With a bioavailability of 100 %, ingested inorganic nitrate is swiftly absorbed in the proximal small intestine leading to significantly raised plasma nitrate concentrations for a period of up to 5–6 h post-nitrate ingestion[27,33,53–55]. About 75 % of this nitrate is excreted at the kidneys; however, the other 25 % of plasma nitrate is actively extracted by the salivary glands, leading to salivary nitrate concentrations which are ten to twenty times higher than plasma nitrate concentrations[27,43,55–57]. Salivary nitrate accumulation must occur in order for nitrate to be reduced to nitrite, as anaerobic bacteria in the oral cavity use nitrate as an alternative electron acceptor to oxygen during respiration[27,55,56,58]. When this nitrite-rich saliva is swallowed it is reduced in the acidic stomach to produce nitrogen oxides including NO[26,27,52,59]. Today, this process is widely known as the nitrate–nitrite–NO pathway, and is thought to be one of the body's major sources of NO generation, especially in situations when NO bioavailability via the conventional L-arginine–NOS pathway is compromised. In addition it has been suggested that the nitrate–nitrite–NO pathway may play a significant role in maintaining levels of bioactive NO and may be critical for maintaining cardiovascular homeostasis in the body[27,53,60].

Noteworthy factors other than inorganic nitrate and nitrite consumption which have been shown to facilitate the nitrate–nitrite–NO pathway include:

(1) Entero-salivary nitrate cycling. Approximately 25 % of plasma nitrate is actively taken up by the salivary glands leading to significant nitrate accumulation in the saliva. Within the oral cavity, anaerobic bacteria reduce nitrate to nitrite via the action of nitrate-reductive enzymes. Nitrite-rich saliva must be swallowed to produce NO in the acidic stomach. The importance of this salivary nitrate cycling has been demonstrated in studies where subjects spat after a dietary load of inorganic nitrate, preventing the opportunity for nitrate to accumulate in the saliva and be reduced to nitrite, therefore preventing NO production and any beneficial effects[25,33,61].
(2) Presence of anaerobic bacteria. Mammalian bacteria can utilise nitrate as an alternative electron acceptor to oxygen during respiration, and is a vital component of the nitrate–nitrite–NO pathway as human cells lack the required nitrate reductase enzymes[61]. The importance of these bacteria has been further established in studies of germ-free rats, in which gastric NO formation was negligible post-dietary nitrate load[62]. Additionally, human studies have demonstrated that the use of commercial antibacterial mouthwash in human subjects abolished any blood pressure-lowering effects of a dietary nitrate load, indicating that the mouthwash killed off the commensal facultative bacteria in the mouth, thus preventing the production of nitrite and NO leading to a loss of beneficial health effects[63–65].

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(3) Hypoxic conditions. The rate in which nitrate is reduced to nitrite is thirty times greater during conditions of low oxygen tension, as the oral bacteria use salivary nitrate as an alternative electron acceptor to oxygen during respiration[65]. Xanthine oxidoreductase has also been shown to catalyse the reduction of nitrite to NO in hypoxic conditions[66–68]. This could also account for the increased production and utility of NO seen in exercising skeletal muscle or during myocardial ischaemia[52,61,69]. It is also important to note that plasma nitrite can be reduced to NO along the physiological oxygen gradient of the circulatory system[70]. Specifically, deoxygenated Hb in the peripheral circulation can act as a nitrite reductase for NO production, as it has been revealed that as Hb deoxygenation increases, more NO is produced[71–73]. This provides an explanation for how various human studies have observed vasodilation after a NO_x load, in healthy subjects at rest[33,74].

(4) Acidic conditions. Nitrite in the acidic stomach has been shown to spontaneously decompose to NO, a reaction that appears to increase in conditions of reduced pH (increased acidity)[26]. The importance of an acidic stomach for this reaction has been demonstrated in a study showing that NO production via nitrite protonation was inhibited in individuals using proton pump inhibitors (medications which reduce the acidity of gastric juices)[75].

(5) Presence of reducing agents including vitamin C and polyphenols. Both vitamin C and polyphenols are abundant in a vegetable-rich diet, and their presence in the diet has been shown to favour the formation of NO via the nitrate–nitrite–NO pathway and prolong the half-life of NO in the stomach[76,77].

Sources of dietary inorganic nitrate and nitrite

N is vital to life on Earth and can undergo many chemical and biological changes in order to be amalgamated into living and non-living material. An essential form of environmental N includes inorganic nitrate, as an adequate nitrate supply in the soil is essential for plant growth[43,78].

The two major determining factors of the nitrate content of vegetables and fruit include their species and the amount of available nitrate in the soil[43]. Some species of vegetables such as green leafy vegetables (mean nitrate about 975–3624 mg/kg) and beetroot (mean nitrate about 1992 mg/kg) are naturally high in nitrate; however, environmental factors can lead to great variation among samples[22]. These factors include seasonal differences and disruption to normal plant growth, leading to nitrate accumulation in the plant leaves, stems and stalks, due to changes in the photosynthetic conversion of plant nitrate to amino acids[78–80]. Therefore, established factors shown to effect the normal growth of plants include drought conditions, high temperatures, shady and cloudy conditions, deficiency of soil nutrients, and excessive soil N[43]. Additionally, farming practices leading to damaged produce, early harvest, storage and transport conditions, processing and cooking practices will also result in significant variation in vegetable and fruit nitrate content[43].

European-based studies have demonstrated that organically grown vegetables have a lower nitrate content than conventionally grown crops, despite the fact that organic fertilisers may cause high nitrate levels in vegetables, depending on the types and amount of organic fertilisers applied[81]. A California-based study by Muramoto[81] reiterated this notion, as it found that spinach grown and harvested during the same season and under the same farming practices had a wide range of nitrate contents. This range appeared greatest in organic spinach, in which the maximum nitrate content measured was 3000 mg/kg, which was five times higher than the minimum (600 mg/kg)[81]. However, this study also demonstrated that conventionally grown spinach contained on average 30 % more nitrate than spinach grown organically, a result most probably explained due to the wide use of N-containing fertilisers in conventional farming[81].

Muramoto[81] also found a statistically significant seasonal difference in the nitrate content of iceberg lettuce, as winter samples were found to have on average 52 % more nitrate than summer samples[81]. This finding is consistent with Ekart *et al.*[82], which found lettuce harvested during summer had a statistically significant lower nitrate content than lettuce harvested during winter (summer harvest: 1209 mg/kg; winter harvest: 2164 mg/kg). In addition, Ekart *et al.*[82] found that washing leafy greens reduced the nitrate content of foods on average by 19 %. Other processing, such as boiling, blanching and sautéing, were found to significantly reduce the nitrate content of spinach by 53, 36 and 30 %, respectively[82], a finding which could be partly explained due to the water-soluble nature of inorganic nitrate[83].

Due to the high variability of nitrate within plant species, accurate and reliable nitrate intake measured from fruit and vegetable consumption is difficult to predict. Despite this, combined vegetable and fruit intake is the major source of exogenous inorganic nitrate exposure and is predicted to constitute 30–90 % of total nitrate intake[84]. Other sources of nitrate intake include drinking water and meat products; however, their nitrate content is highly regulated to comply with strict government limits[85–89].

Nitrate occurs naturally in the water supply; however, in most developed countries water nitrate is generally present in concentrations much lower than allowed in the water guidelines (≤50 mg/l)[85,86,88]. Therefore, nitrate from the water supply is unlikely to contribute significantly to total nitrate intake in comparison with food sources.

Nitrate and nitrite salts (for example, potassium nitrite/sodium nitrate) have been used as food additives in cured meats for many years due to their effectiveness in ensuring microbial safety and their ability to enhance the flavour and appearance of the product[43]. The maximum levels of nitrate and nitrite allowed as food additives have been defined (Table 1)[85,90–92].

It has been estimated that approximately 60–80 % of dietary nitrates are derived from vegetables (mainly green leafy and root vegetables), indicating that vegetable intake tends to contribute the greatest quantities of dietary nitrate (Table 2)[22,93]. This has been further implied by dietary patterns such as the DASH diet, Mediterranean, vegetarian and traditional Japanese diets which tend to include high quantities of vegetables (five or more serves per d) and provide approximately 147–1222 mg nitrate per d[22–24]. This is a relatively high nitrate intake

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Table 1. Permissions for nitrate and nitrite in food products*

Product	Additive	Maximum permitted level (mg/kg)
Cheese and cheese products	Nitrite salt	150[90]
	Nitrate salt	50[85,90]
Commercially sterile canned dried meat	Nitrite salt	50–150[85,91]
Dried meat	Nitrite salt	125[90]
	Nitrate salt	150[85]
Slow-dried cured meat	Nitrite salt	125–200[85,90]
	Nitrate salt	175–500[85,92]
Processed comminuted meat, poultry and game products	Nitrite salt	125–175[85,90]
	Nitrate salt	150–300[90,91]
Fermented, uncooked processed comminuted meat product	Nitrite salt	150[90]
	Nitrate salt	500[85,90]

* Nitrate salt: potassium nitrate and sodium nitrate. Nitrite salt: potassium nitrite and sodium nitrite.

compared with the typical Western-style diet which tends to be low in vegetables (one to three serves per d) and provides about 60–75 mg nitrate per d[24]. In addition, processed and cured meats are frequently cited as the major dietary source of nitrite (Table 3)[22,25,84,94], followed by various fruits and vegetables (Tables 2, 4 and 5) that have been physically damaged or poorly stored, as enzymes present in the plant tissues and/or contaminating bacteria facilitate the reduction of nitrate to nitrite[43,85].

Nitric oxide in the cardiovascular system

Within the cardiovascular system, basal endothelial NO has a critical role in maintaining cardiovascular health as it controls vascular tone, smooth muscle cell proliferation and growth, platelet activity and aggregation, leucocyte trafficking, expression of adhesion molecules and inflammation[34,94–99]. However, when

Table 2. Vegetable sources of nitrate and nitrite with estimated nitrate and/or nitrite contents* (Mean values and ranges)

	Nitrate content (mg/kg)			Nitrite content (mg/kg)		
Vegetable type	Mean	Range	Reference	Mean	Range	Reference
Rocket	3624	1550–7316	[111,191]	NA		
Turnip greens	3467		[192–194]	NA		
Spinach	2485	2–6700	[22,79,85,111,191,193–205]	15	ND–162	[22,85,200,202,203,205]
Swiss chard	2363		[199]	NA		
Turnip	2174	10–4800	[111,194,195,197,201]	NA		
Rhubarb	1999	55–6500	[191,193,194,196,197,201,204]	NA		
Celery	1964	19–5300	[85,191,193–199,201,203]	2·5	ND–6	[85,191]
Beetroot	1992	100–8100	[85,111,193–201,203,204,206–208]	1·7	ND–110	[85,199,203,209]
Chinese cabbage	1855	111–8050	[201,202,206,208,210]	0·9	ND–14·3	[206,208]
Radish	1773	60–9000	[111,191,193–196,201]	NA		
Lettuce	1689	10–13000	[79,85,111,191,193–199,201–206,208,209]	0·8	ND–5	[85,203,205,206,208]
Watercress	1640	890–2790	[203]	2·5	ND–5	[203]
Buk choy	1620	1023–3098	[202]	20	0·09–30	[202]
Kale/mustard greens	1318	19–5500	[22,191–194,197,205]		0·03–0·64	[22,205]
Silver beet	1255	190–1770	[203,209]	2·5	ND–5	[203,209]
Endive	975	10–3800	[194,199]	NA		
Broccoli	793	ND–2300	[22,85,193,194,196–199,203,204]	3	ND–110	[22,85,203]
Cabbage	756	1–3100	[85,193–199,201,203,204,207–210]	0·8	ND–26	[85,203,208]
Cauliflower	547	ND–4500	[191,193–199,201]	NA		
Mixed salad	540	80–821	[22,111,191,201]	1·3		[22]
Eggplant	479	31–1500	[191,194,195,198,199]	NA		
Leek	399	56–841	[111,195]	NA		
Pumpkin/squash	389	ND–2200	[85,191,194–199,201,203]	6	ND–194	[85,203]
Green onion	366	4–1676	[111,201]	NA		
Fennel	363		[199]	NA		
Green beans	315	6–1100	[85,111,193,195,197,199,208]	7	0·16–57	[85,208]
Cucumber	184	1–1236	[85,111,191,194,195,198,199,208–210]	3	ND–1164	[85,208]
White potato	184	ND–5521	[22,85,111,191,193–198,201,203,207–210]	1	ND–10·3	[22,85,203,208]
Carrot	182	ND–2800	[22,85,111,191,193–199,201,203–205,207,208]	0·7	ND–7·5	[22,85,203,205,208]
Garlic	163	1–462	[111,191,199]	NA		
Lima beans	160	54–310	[193,195,198]	NA		
Brussels sprouts	118	ND–170	[194]	NA		
Onion	100	ND–2300	[85,191,194–196,199,201]	0·5	ND–2·2	[85]
Mushroom	92	ND–400	[85,191,194]	NA		
Asparagus	84	13–700	[194,196,198]	NA		
Tomato	71	ND–392	[22,85,111,191,193–196,198,199,201,204,207–210]	0·6	ND–13	[22,85,208]
Sweet potato	55	ND–66	[191,193–195,198]	NA		
Peas	32	ND–124	[85,191,193–195,198,199]		ND–22	[85]
Dry beans	30	9–68	[195,198]	NA		
Maize	30	ND–45	[85,195,198]		ND–7·5	[85]
Artichoke	30		[199]	NA		

NA, data not available; ND, not detected.
* Data are combined nitrate and nitrite estimates from various published papers, government documents and reviews.

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Table 3. Meat-based sources of nitrate and nitrite with estimated nitrate and/or nitrite contents* (Mean values and ranges)

	Nitrate content (mg/kg)			Nitrite content (mg/kg)		
Meat type	Mean	Range	Reference	Mean	Range	Reference
Salami	94	ND–450	(85,202,203,211–213)	31	ND–108	(85,202,203,211–213)
Bolonga	65	4–98	(211,214,215)	14	ND–55	(211,214,215,216)
Frankfurter/hot dog	64	8–81	(22,85,202,203)	39	0·5–95	(22,85,202,203)
Shelf-stable, canned cured meat	63	ND–840	(211,212,214)	31	ND–19	(211,212,214)
Sausages	58	15–240	(85,202,211,214,217,218)	33	ND–940	(91,202,211,214,216–220)
Ham	55	ND–1400	(22,85,202,203,211,215,217,221)	47	ND–640	(22,85,202,203,211,217,219,221,222)
Bacon	42	ND–310	(22,85,202,203,211,214,215)	29	ND–430	(22,85,202,203,211–215,218,219,222–224)
'Luncheon meat'	32	<10–70	(85,203,215)	31	ND–130	(85,203,215)
Pork	21	ND–19	(22,215)		ND–8	(22,215)
Corned beef	14	4–36	(203,215)	3	ND–8	(203,215)
Minced beef	12	ND–24	(202,203)	NA		

ND, not detected; NA, data not available.
* Data are combined nitrate and nitrite estimates from various published papers, government documents and reviews.

Table 4. Fruit sources of nitrate and nitrite with estimated nitrate and/or nitrite contents* (Mean values and ranges)

	Nitrate content (mg/kg)			Nitrite content (mg/kg)		
Fruit type	Mean	Range	Reference	Mean	Range	Reference
Melon	325	38–600	(194,195,196,199,201)	NA		
Strawberries	172	96–233	(85)	18	8–80	(85)
Banana	76	45–200	(22,85)	2	ND–11	(22,85)
Apple	20	ND–56	(85)		ND–7·5	(85)
Grapes	19	ND–52	(85)	10	ND–19·4	(85)
Sultanas	16	9–22	(85)	0·8	ND–5·5	(85)
Peach	10	7–18	(85)	17	ND–22	(85)
Orange	9	ND–21	(22,85)	0·2	ND–7·5	(85)
Mango	9	ND–12	(85)	6	ND–15	(85)
Watermelon	8	7–18	(85)		ND–16·4	(85)
Pineapple	7	ND–12	(85)	17	10–22	(85)

NA, data not available; ND, not detected.
* Data are combined nitrate and nitrite estimates from various published papers, government documents and reviews.

Table 5. Nitrate- and nitrite-containing herbs with estimated nitrate and/or nitrite contents* (Mean values and ranges)

	Nitrate content (mg/kg)			Nitrite content (mg/kg)		
Herb type	Mean	Range	Reference	Mean	Range	Reference
Dill	2590	2236–3267	(200,201)	102		(200)
Parsley	1304	ND–4467	(85,194–196,200,201)		ND–94	(85,200)
Tea	3	2–3	(85)		ND–0·3	(85)

ND, not detected.
* Data are combined nitrate and nitrite estimates from various published papers, government documents and reviews.

the bioavailability of NO is compromised, the beneficial effects of NO are lost and endothelial dysfunction predominates due to the imbalance created between the release of vasoconstrictors and vasodilators (such as NO)[53,100,101]. This idea has been supported in a study conducted by Kleinbongard *et al.*[102] which found that plasma nitrite levels are a reliable indicator of endothelial dysfunction and correlate with cardiovascular risk factors in humans. Additionally, endothelial dysfunction has been strongly linked with atherosclerosis development and a number of cardiovascular disorders such as hypertension, CAD, congestive heart failure and peripheral artery disease in multiple longitudinal studies[53,101,103–107].

While in the past most of the evidence suggesting a relationship between endothelial dysfunction and clinical events from atherosclerosis development was considered 'circumstantial', more recently conducted cross-sectional studies have indicated that severe endothelial dysfunction of the arteries can trigger events of unstable angina and MI[108,109]. Al Suwaidi *et al.*[104] studied 157 patients with mild CAD for 2·3 years, and found an increased incidence of cardiovascular

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events in patients with impaired endothelium-dependent vasodilation (NO production of endothelium) of the coronary arteries. In another study by Katz *et al.*[110], 259 subjects with chronic heart failure were assessed prospectively, to which endothelial dysfunction in chronic heart failure was found to significantly increase risk of mortality, thus supporting the notion that coronary endothelial dysfunction plays a role in the pathogenesis of coronary atherosclerosis, risk of cardiac events and death[104,110].

Many factors are known to predispose to endothelial dysfunction, due to reductions in NO concentrations and bioavailability in humans[34,111,112]. These factors are consistent with the modifiable and non-modifiable risk factors for CVD, including hypertension, hypercholesterolaemia, diabetes, tobacco use, physical inactivity, consumption of unhealthy diets and increased age and sex (NO bioavailability is reduced in post-menopausal women, a period in which CVD risk is drastically increased in women)[34,112–120]. Interestingly, improved endothelial function is a common feature of experimental intervention studies, which have shown reductions in cardiovascular risk and improvements in endothelial-dependent vasodilation in the coronary and peripheral circulation[108]. Such interventions commonly include the use of lipid- and blood pressure-lowering medications, smoking cessation and increased physical activity[108,117,121–124]. However, the notion that inorganic nitrate and nitrite either consumed from dietary sources such as green leafy vegetables or supplements is relatively new, and their therapeutic potential as an NO donor via the nitrate-nitrite–NO pathway remains unclear[112,125].

Cardiovascular protective actions of nitric oxide

NO is non-polar and can diffuse freely across cell plasma membranes and is a key signalling molecule capable of many important functions, acting primarily by stimulating intra-cellular receptors within the target cell[126].

Within the vasculature of the cardiovascular system, the primary role for NO's action is for the regulation of vascular function and blood pressure, a notion which has been clearly demonstrated in animal models in which synthesis of NO was blocked leading to persistently elevated blood pressure[112,127]. In addition, this interaction has been demonstrated in some recently conducted short-term dietary nitrate trials in human subjects, which showed that peak blood pressure-lowering effects were achieved in synchronisation with peak plasma concentrations of NO (NO_x) after a dietary nitrate load[28,33,128].

The cellular pathway in which NO exerts this vasodilatory action is well established. NO rapidly diffuses across vascular smooth muscle cell membranes. Within the smooth muscle cells, NO binds to and activates guanylyl cyclase to produce cyclic GMP[126]. Once produced, cyclic GMP can have a number of effects in the cells, but many of these effects are mediated through the activation of protein kinase G. Activation of protein kinase G via cyclic GMP leads to the activation of myosin phosphatase which in turn leads to smooth muscle cell relaxation and vasodilation[126,127].

In addition to regulating vascular tone, NO can facilitate many other important functions preventing the development of atherosclerosis, which include antiplatelet effects, anti-proliferative effects, anti-inflammatory, and antioxidant effects[127,129,130]. Although the cellular pathways for these actions are yet to be clearly defined, it is clear that NO is capable of binding to or reacting with a variety of chemical modalities within the cellular environment, including metal-containing proteins, membrane receptors, ion channels, enzymes, transcription factors and oxygen species[127,131].

Other nitric oxides and possible mechanisms in the cardiovascular system

While NO is the most widely cited bioactive metabolite underpinning the cardiovascular therapeutic benefits of dietary inorganic nitrates and nitrites, it has been suggested that other nitric oxides also play a role[25,93]. This may be expected, given that dietary constituents in the stomach may react with each other in order to form a variety of bioactive compounds[25]. Examples of such compounds include nitrated fatty acids, nitrosothiols and ethyl nitrite[25].

While the biological significance of these compounds is yet to be made clear, the following actions have been suggested:

(1) Ethyl nitrite. Rat models have shown that ethanol from alcoholic drinks can interact with salivary-derived nitrite in the acidic stomach, leading to the production of ethyl nitrite[25,132]. Ethyl nitrate is a potent smooth muscle relaxant and may have a vasodilatory role in the cardiovascular system[132].
(2) Nitrosothiols. In the stomach, nitrite has been shown to induce *S*-nitrosation within the gastric compartment. *S*-nitrosothiols are thought to represent a circulating endogenous reservoir of NO acting as an NO donor[25].
(3) Nitrated fatty acids (nitroalkenes). Nitric oxides can react with unsaturated fatty acids to produce nitroalkenes. Analysis of synthetic nitroalkenes derivatives of oleic, linoleic and arachidonic acids reveals that these species possess unique chemical reactions which may support multiple cell signalling events such as vasodilation and reduced inflammation[25]. Such events may be mediated through their NO donor capabilities.

Currently the systemic capabilities of these bioactive N compounds remain uncertain; however, it highlights a possible whole-diet effect for exerting a beneficial effect on NO and other relevant cardiovascular signalling molecules. This notion is highlighted by Lundberg and Weitzberg[25,93], indicating that various dietary constituents of the Mediterranean diet may interact in the stomach to produce these potentially therapeutic compounds, and may provide an additional explanation for the cardiovascular health benefits/protection seen with this dietary pattern.

Inorganic *v.* organic nitrate and nitrite

Organic nitrates such as glyceryl trinitrate and isosorbide mononitrate represent the first class of NO donors to reach the clinical setting and have been used extensively in the treatment of various cardiovascular conditions including angina, CAD and heart failure[83].

Unlike inorganic nitrates which are relatively simple molecules and naturally occurring in fruits and vegetables, organic nitrates are synthetic compounds produced by a reaction

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between nitric acid and an alcohol group[83]. Organic nitrates are complex, non-polar hydrocarbon chains attached to a nitro-oxy-radical (–ONO_2), which is responsible for its biological effects (Fig. 2)[83].

Once organic nitrates are introduced to the blood system, levels rise quickly leading to the rapid onset of their action[83]. At low doses (≤1·25 mg/kg body weight) organic nitrate has been demonstrated to dilate large conductance veins and large arteries, while at high doses (2·5–5 mg/kg body weight) organic nitrates can also induce dilation of the arterioles of the micro-circulation[83]. These vasodilatory effects of organic nitrates have been shown to reduce cardiac work and lower myocardial oxygen requirements, which may alleviate or even prevent cases of MI[133]. In addition, it has been suggested that organic nitrates have anti-aggregatory properties in patients with stable and unstable angina[133].

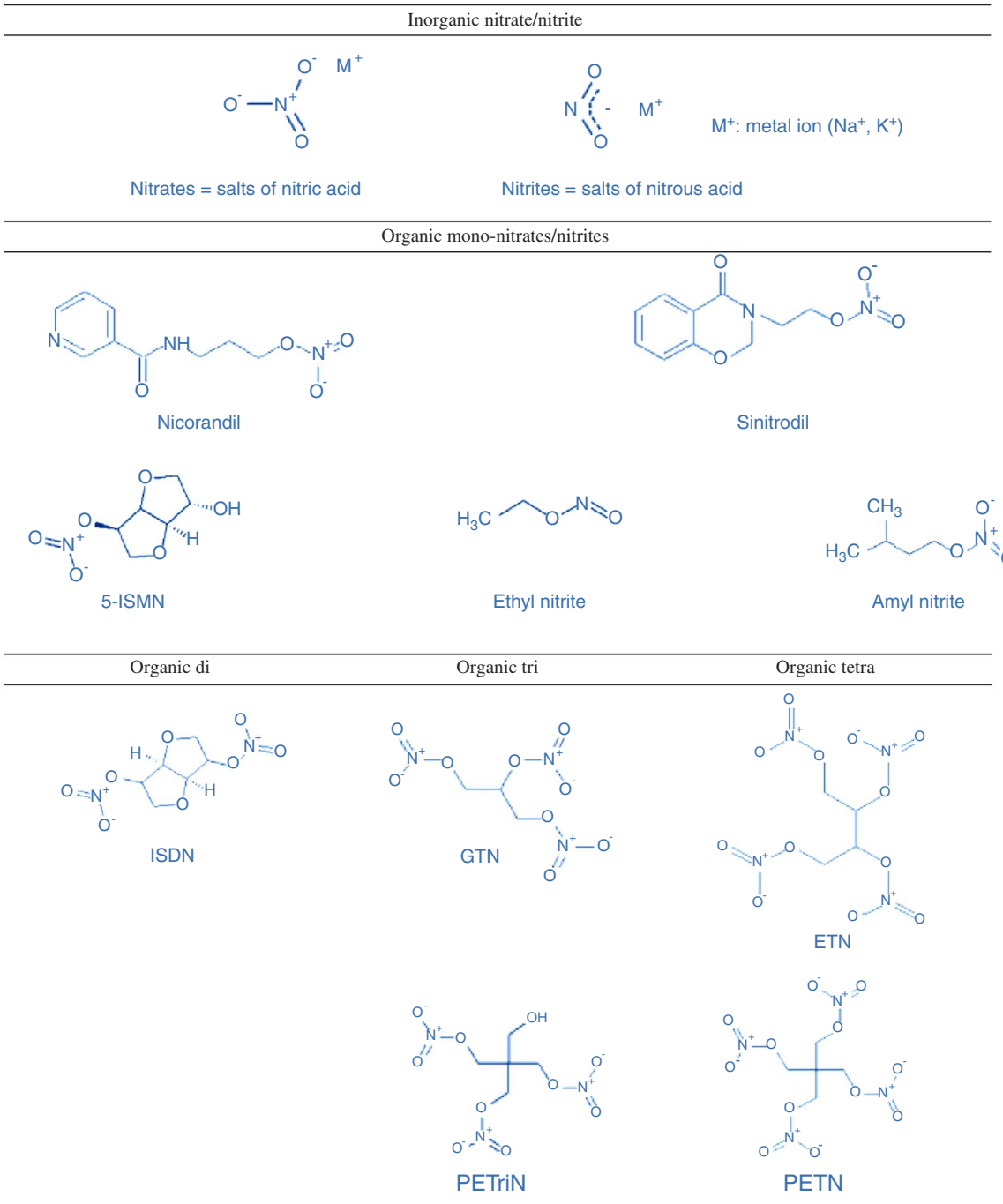

Fig. 2. Chemical structure of inorganic nitrate/nitrite compared with organic mono-, di-, tri- and tetra-nitrates/nitrites. 5-ISMN, isosorbide-5-mononitrate; ISDN, isosorbide dinitrate; GTN, glyceryl trinitrate; ETN, erythritol tetranitrate; PETriN, pentaerythrityl trinitrate; PETN, pentaerythritol tetranitrate. Reprinted from Omar *et al.*[83], with permission from Elsevier.

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Today in clinical practice short-acting organic nitrates, most notably in the form of glyceryl trinitrate, are administered during the symptomatic treatment of MI and angina[83,133]. Glyceryl tri-nitrates are generally administered in the form of either a mouth spray or intravenous infusion, to which onset of action is rapid (2–3 min)[133]. Although short-term treatment with organic nitrates has some positive impact on endothelial function, acute side effects of their use include hypotension, dizziness, nausea and headache[83]. Also, despite the high potency of organic nitrates and their long history as being used to treat various CVD, nitrate tolerance is a huge limitation and an undesirable side effect of their use[83,133].

Nitrate tolerance is a complex phenomenon and is poorly understood; however, it is clearly a result of chronic organic nitrate use to which nitrovasodilator responsiveness is lost[83]. Nitrate tolerance has been reported to occur within 1–3 d of continuous glyceryl trinitrate treatment in patients with MI, stable angina and chronic congestive heart failure[133]. Further, chronic organic nitrate use has also been linked to endothelial dysfunction, increased production of free radicals and the development of vascular tolerance to other endothelium-dependent vasodilators[83]. Although this phenomenon is poorly understood, recent animal and human studies indicate that increased vascular production of the superoxide anion (O_2^-) underlies the mechanism for tolerance[133]. This oxidative stress hypothesis of nitrate tolerance is supported by numerous reports demonstrating that the tolerance is prevented by co-administration of antioxidants (for example, vitamin C, vitamin E and folic acid) and interventions which inhibit reactive oxygen species formation (lipid- and blood pressure-lowering medications)[133–136].

It is interesting to note that the phenomenon of tolerance is not exhibited with the consumption of inorganic nitrates/nitrites; however, despite showing promise in preventing or treating certain cardiovascular conditions, such as hypertension, they have received little attention by the medical community[27].

Inorganic nitrate and nitrite: from dietary contaminant to potential therapeutic nutrient

Throughout history, cases of accidental toxic exposure to nitrate and nitrite have been documented; however, the health risk of excessive inorganic nitrate and nitrite consumption appears specific to population subgroups[22]. One of these subgroups includes infants aged less than 6 months, to which excessive nitrite exposure has been linked to cases of methaemoglobinaemia (blue baby syndrome)[137]. As a result, strict regulatory limits have been established to govern the NO_x content of the drinking water supply and their use as an additive to processed and cured meats in order to limit exposure to the population[85,86].

Methaemoglobinaemia can occur when nitrite oxidises ferrous Fe (Fe^{2+}) in Hb to the ferric state (Fe^{3+}), resulting in methaemoglobin. Methaemoglobin is incapable of binding molecular oxygen, and impairs oxygen delivery to the tissues, causing hypoxia and cyanosis[137]. While most cases of methomeoglobinaemia have been attributed to the consumption of well water (prone to high nitrate accumulation) used for the preparation of infant formula, there have been reported cases of nitrate poisoning in infants from the ingestion of plant nitrates[86,137]. While Martinez *et al.*[138] found that the use of certain high-nitrate vegetables (herbs and green leafy vegetables) in infant homemade vegetable purée increased methaemoglobinaemia in infants (herbs: OR 5·2, 95 % CI 1·1, 24·6; and green leafy vegetables: OR 2·0, 95 % CI 0·4, 8·7), the most important factor increasing methaemoglobinaemia was the time lapse between vegetable purée preparation and consumption (OR 17·4, 95 % CI 3·5, 86·3 if purée was prepared 24–48 h before; and OR 24·9, 95 % CI 3·3, 187·6 if prepared >48 h before)[138].

To date, human nitrate and nitrite exposure studies have failed to prove a direct link with methaemoglobinaemia, suggesting that NO_x exposure alone may not be responsible for methaemoglobinaemia development[139,140].

Another population subgroup that is thought to be at health risk due to excessive NO_x exposure is high consumers of cured and processed meats[22,141]. It has been theorised that nitrates and nitrites from processed meats generate *N*-nitroso compounds which can be carcinogenic[142].

In October 2015 the International Agency for Research on Cancer (IARC) summarised more than 800 studies conducted globally, and determined that 50 g of processed meat per d increased the risk of colorectal cancer by 18 %, and therefore concluded that processed meats are carcinogenic[141]. In animal studies *N*-nitrosamines and related *N*-nitrosamides have been shown to be carcinogenic in a variety of molecular structures[143,144]. However, such direct evidence demonstrating nitrate and nitrite as human carcinogens is severely lacking. This has been reflected in the conclusions of the FAO expert committee who found no consistent increased risk of cancer with increasing consumption of nitrate, as available epidemiological studies did not provide evidence that nitrate is carcinogenic to humans[145].

Currently, researchers are interested in understanding whether the health risks associated with inorganic nitrates/nitrites outweigh the recently discovered health benefits; however, there is a growing consensus that any weak and inconclusive data on inorganic NO_x and cancer associations are far outweighed by the potential health benefits of restoring NO homeostasis[22,84,139,143]. In particular this has been demonstrated in various animal and human experimental studies, in which inorganic NO_x has been shown to improve outcomes such as blood pressure, endothelial function, platelet function, ischaemia–reperfusion injury, exercise performance and host defence[143,146–151].

Evidence of cardiovascular benefit from animal studies

Intakes of dietary inorganic nitrate have been shown to be strongly cardioprotective in animal studies. Carlström *et al.*[152] indicated this in a four-arm dietary intervention trial in rats. The rats were placed on either a normal-salt diet (control), a high-salt diet, a high-salt diet supplemented with a nutritional (low) dose of nitrate, and a high-salt diet supplemented with a pharmacological (high) dose of nitrate for 8–11 weeks[152]. As expected, results demonstrated that chronic consumption of

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a high-salt diet develops hypertension; however, when combined with a low nitrate dose, blood pressure was non-statistically significantly lower[152]. On the other hand, the higher nitrate dose lowered blood pressure by a significant 24 mmHg compared with the plain high-salt diet, a magnitude of blood pressure reduction considerably magnified compared with blood pressure reductions observed in another study of healthy normotensive rats using the same nitrate dose[152,153]. Similar results were reported by Kanematsu *et al.*[154], finding that in hypertensive rats, antihypertensive effects were only apparent with the highest dose of nitrate, yet there was a strong tissue-protective effect seen with lower doses equivalent to modest dietary intakes. Ferguson *et al.*[155] demonstrated clinically significant reductions in mean arterial pressure with beetroot juice supplementation in exercising rats (control: 137 (SEM 3); beetroot juice: 127 (SEM 4) mmHg; $P < 0{\cdot}05$), indicating that clinically significant blood pressure reductions may be achievable in doses attained from dietary sources[155].

In addition to significant blood pressure control, Carlström *et al.*[152] found that dietary nitrate supplementation can partly prevent the development of cardiac hypertrophy and high nitrate doses significantly reduced the fibrotic changes which were observed in the high-salt group, two factors which are major predictors of heart failure[152]. Two other studies found that mice ingesting inorganic nitrate led to a significantly reduced infarct size during myocardial ischaemia, an important finding given that reduced infarct size is associated with lower heart failure risk post-MI and mortality[156–158].

When Baker *et al.*[149] treated rats with an intravenous bolus of sodium nitrite across various doses (0·04, 0·4, 1·0, 4·0, 7·0 and 10·0 mg/kg), before initialising a blockage of the coronary artery, there was a clear dose-dependent effect of nitrite on infarct size. However, it was intriguing to note that protection was only found in doses up to 4·0 mg/kg, an effect which was absent at higher doses[149]. Rats administered with 4·0 mg/kg nitrite exhibited a significant 32 % reduction in infarct size compared with controls[149]. Nitrite was also found most effective when administered before and/or during the ischaemic event, but not at the onset of reperfusion[149]. Further, equivalent doses of sodium nitrate had no effect on infarct size, indicating that administration timing and doses are key considerations for nitrite protection from MI[149].

Thrombosis is largely a result of platelet adhesion, activation and aggregation, and is a common pathology underlying IHD and ischaemic stroke[159,160]. NO plays a key role in preventing thrombosis development[161]. Park *et al.*[161] demonstrates this notion upon discovering an inverse correlation between NO_x levels and platelet activity/aggregation in mice. In addition, Apostoli *et al.*[162] examined the effect of inorganic nitrite on platelet aggregation in endothelial NOS-deficient mice. This study found that inorganic nitrite exerts an antiplatelet effect during endothelial NOS deficiency and suggested that dietary nitrate may reduce platelet hyperactivity during endothelial dysfunction[162].

Pulmonary hypertension can lead to the remodelling of the artery wall, causing abnormalities of elastic fibres, intimal fibrosis and medial hypertrophy[163]. This can result in vascular stiffness and is a condition linked to the development of chronic heart failure[163]. Sodium nitrite interventions in lamb and mouse models have shown reductions in pulmonary hypertension specifically during hypoxic conditions[164,165]. However, Casey *et al.*[166] found that intravenous injections of sodium nitrite during normoxic conditions could lead to reductions in pulmonary and systemic arterial pressure and increased cardiac outputs in adult male rats. This suggests that sodium nitrite may have a role in reducing the workload of the heart during pulmonary hypertension, thus protecting the heart and vascular system from associated damage and dysfunction[166].

Hendgen-Cotta *et al.*[167] pre-treated mice with nitrate before inducing chronic limb ischaemia, and nitrate supplementation was found to enhance revascularisation and increased mobilisation of circulating angiogenic cells (CAC), which are important for the recovery and maintenance of healthy endothelial function[167]. Heiss *et al.*[168], on the other hand, injected inorganic nitrite into healthy mice, and found that nitrite significantly increased CAC at 1 h compared with controls. It is interesting to note, however, that when this test was repeated in endothelial NOS-deficient mice, no CAC mobilisation was observed, indicating that NOS may be required to take part in nitrate-mediated CAC mobilisation[168].

In a study conducted by Sindler *et al.*[169] the effect of nitrite in aged, but healthy, mice was investigated and high dietary nitrite doses were found to reverse age-related vascular dysfunction, arterial stiffness and reduce levels of oxidative stress. This is in line with Carlström *et al.*[152] who found that key plasma and urinary oxidative stress markers (malondialdehyde, type VI isoprostane ($iPF_{2\alpha}$-VI) and 8-oxo-2'-deoxyguanosine (8-OHdG)) were significantly reduced (despite co-consumption of a high-salt diet) with both low- (0·1 mmol nitrate/d) and high- (1·0 mmol nitrate/d) dose dietary nitrate supplementation, which may be useful in preventing NO degradation and endothelial dysfunction[152,170]. This is an interesting finding, given that oxidative stress is directly linked with an inflammatory response which is thought to have a central role in the development of atherosclerosis[93].

Stokes *et al.*[171] found that mice fed cholesterol-enriched diets for 3 weeks tend to develop clear signs of vascular disease pathology, including elevated leucocyte adhesion and endothelial dysfunction, an effect which was prevented with nitrite supplementation in the drinking water. In another study by Carlström *et al.*[172] it was demonstrated that several features of the metabolic syndrome (including visceral fat and circulating TAG, which are strong risk factors for CVD) can be reversed by dietary nitrate supplementation, in amounts which correspond to those derived from endothelial NOS under normal healthy conditions or a vegetable-rich diet[172].

Evidence of cardiovascular benefit from human studies

In 2003, Cosby *et al.*[71] conducted one of the first studies demonstrating a relationship between inorganic nitrite supplementation and blood pressure reductions in healthy human subjects. This study chose to use sodium nitrite ($NaNO_2^-$) infusions providing approximately 75 mg $NaNO_2^-$ over two 15-min periods, a dose which was found to significantly reduce mean blood pressure by 7 mmHg ($P < 0{\cdot}01$)[71]. Similar findings

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were later established using sodium nitrate ($NaNO_3^-$) in a study conducted by Larsen *et al.*[173]. In this study healthy subjects consumed $NaNO_3^-$ (8·5 mg/kg per d for 3 d) as a dietary supplement, and although systolic blood pressure was not changed during this time compared with placebo (sodium chloride), diastolic blood pressure was significantly reduced on average by 3·7 mmHg ($P<0·02$) and mean arterial pressure was lowered by 3·2 mmHg ($P<0·03$)[173]. Soon after, Webb *et al.*[33] investigated this topic further using beetroot juice (containing approximately 1400 mg inorganic nitrate). Results from Webb *et al.*[33] showed a peak reduction in systolic blood pressure of 10·4 (SEM 3) mmHg ($P<0·01$), a reduction in diastolic blood pressure of 8·1 (SEM 2·1) mmHg ($P<0·01$) and mean arterial pressure reduction of 8·0 (SEM 2·1) mmHg ($P<0·01$), thus indicating that significant blood pressure reductions are possible with the acute consumption of dietary inorganic nitrate in healthy subjects. This is a notion which has been further supported by a recently conducted systematic review and meta-analysis which found that inorganic nitrate and beetroot juice consumption was associated with greater changes in systolic blood pressure (–4·4 (95 % CI –5·9, –2·8) mmHg; $P<0·001$) than diastolic blood pressure (–1·1 (95 % CI –2·2, 0·1) mmHg; $P=0·06$)[174]. However, it is important to note that these findings have not been consistent across the literature, as a few recently conducted randomised controlled trials have found that inorganic nitrate consumption from either beetroot juice or from a high-nitrate diet (rich in green leafy vegetables) for 1–2 weeks had little/no effect on the blood pressure of study subjects[57,175,176]. The exact cause of this variation across studies remains unclear, yet could be due to methodological differences including the study population (for example, healthy subjects *v.* hypertensive subjects) or the conditions in which NO_x was consumed (for example, food *v.* supplement, dosing or altered environmental conditions such as exercise stress). Nevertheless, this question remains unclear and will require further investigation, in order to better understand the usefulness of dietary/inorganic NO_x within the general population.

While the acute effects of dietary inorganic nitrate on blood pressure have been extensively investigated, very few studies have investigated long-term effects. Sobko *et al.*[23] investigated the effects of a traditional Japanese diet on blood pressure which provided approximately 1140 mg of nitrate per d for a 10 d period. The traditional Japanese diet led to a lower diastolic blood pressure than seen in the non-Japanese diet group (71·3 (SD 7·9) *v.* 75·8 (SD 7·8) mmHg; $P=0·0066$), indicating that dietary inorganic nitrate consumption for longer-periods of time may have some blood pressure-lowering effects in healthy individuals; however, a 10 d intervention can hardly be classified as a long-term intervention[23]. In another 4-week intervention, Kapil *et al.*[29] assigned hypertensive patients to receive a daily dose of either 250 ml of beetroot juice or placebo (nitrate-depleted beetroot juice). Notably, Kapil *et al.*[29] found that daily dietary nitrate supplementation significantly reduced mean clinic blood pressure (7·7/2·4 mmHg (range 3·6–11·8/0·0–4·9 mmHg); $P<0·001$, $P=0·05$), mean 24 h ambulatory blood pressure (7·7/5·2 mmHg (range 4·1–11·2/2·7–7·7 mmHg); $P<0·001$ for both) and mean home blood pressure (8·1/3·8 mmHg (range 3·8–12·4/0·7–6·9 mmHg); $P<0·001$, $P<0·01$)[29].

Currently, the longest intervention study conducted in this area is a 10-week intervention trial from DeVan *et al.*[125]. In this study, healthy 50- to 79-year-old subjects were recruited to consume either 0, 80 or 160 mg of sodium nitrite per d for a 10-week period[125]. Results indicated no significant changes in blood pressure at week 10 compared with baseline blood pressure values; however, a significant time × treatment effect for carotid diameter in the nitrite groups was detected, as well as improved endothelial function of the brachial artery, suggesting improved vascular function with chronic inorganic nitrite supplementation despite a lack of an effect seen with blood pressure[125]. However, it is worth noting that the only prospective cohort study on this topic conducted by Golzarand *et al.*[177] found that higher dietary intakes of nitrate-containing vegetables (about 427·6 g/d) in normotensive individuals may have a protective effect against the development of hypertension (highest tertile of nitrate-containing vegetables, OR 0·63 (95 % CI 0·41–0·98); $P=0·05$).

Endothelial dysfunction is one of the key early events involved in the development of atherosclerosis[178]. Flow-mediated dilatation is commonly used as a measure of endothelial function as reduced flow-mediated dilatation is an indicator of endothelial dysfunction (caused by reduced NO bioavailability) and has been associated with increased severity and duration of blood pressure elevations[179]. More recently, dietary inorganic nitrate interventions have been shown to significantly improve flow-mediated dilatation in healthy and hypertensive human subjects consuming spinach, beetroot juice or sodium nitrate capsules[29,168,180,181]. Joris & Mensink[182] tested the effects of beetroot juice (containing approximately 500 mg nitrate) with a dietary load of fat (56·6 g fat) in overweight and obese subjects (BMI 30·1 (SD 1·9) kg/m^2). While the control drink group saw impaired flow-mediated dilatation with dietary fat intake, the consumption of beetroot juice appeared to attenuate this impairment (beetroot juice: –0·37 (SD 2·92) % *v.* control: –1·56 (SD 2·9) %; $P=0·03$)[182]. Additionally, flow-mediated dilatation has been shown to be reduced by approximately 40 % after vascular ischaemia; however, Ingram *et al.*[183] demonstrated that sodium nitrite pre-conditioning (providing a nitrite dose before ischaemic event) will prevent ischaemic reperfusion injury by preventing reductions in flow-mediated dilatation and endothelial dysfunction. Similar findings have been reported by Kapil *et al.*[29] and Webb *et al.*[33] with beetroot juice pre-conditioning, indicating that higher plasma NO_x concentrations achieved by inorganic NO_x consumption may have a role for improving cardiovascular outcomes after vascular ischaemic events[29,33].

In addition to flow-mediated dilatation, CAC have been identified as an important indicator of vascular endothelial function, as they have a critical role in vascular repair[184]. The number of CAC have also been shown to predict the occurrence of CVD and death[168]. Therefore it is of interest to note that Heiss *et al.*[168] have indicated an important role for dietary nitrate for increasing CAC, showing that a single dose of sodium nitrate (12·7 mg/kg body weight) can double the number of CAC 1–2 h post-nitrate ingestion.

Pulse wave velocity and augmentation index are accepted measurements of arterial stiffness and atherosclerosis, to which higher readings are associated with increased CVD risk[185,186].

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The role of dietary inorganic nitrate in preventing arterial stiffness has been established, as Kapil *et al.*[29] found that a 4-week beetroot juice intervention reduced pulse wave velocity and augmentation index in hypertensive subjects. Zamani *et al.*[187] also saw a significantly reduced augmentation index with beetroot juice consumption in patients with symptomatic heart failure (beetroot juice: 132·2 (SD 16·7) %; placebo: 141·2 (SD 21·9) %; mean change –9·1 (SD 15·4) %; $P=0·03$). Rammos *et al.*[188] investigated the effect of a 4-week sodium nitrate supplementation trial in elderly volunteers with mild hypertension, and found that vascular stiffness was significantly improved in the nitrate-supplemented volunteers. This is a very significant finding given that vascular stiffness tends to naturally increase with age[189].

In an randomized controlled trial conducted by Jones *et al.*[190], participants prone to MI and undergoing primary percutaneous coronary intervention (non-surgical intervention to treat stenosis) were administered with either a high-dose bolus injection of $NaNO_2^-$ (1·8 μmol) or NaCl placebo. The nitrite group experienced a significantly ($P=0·05$) improved myocardial salvage index (established indicator of cardioprotective benefit) relative to placebo[190]. In addition, a subset of participants who exhibited a blocked blood vessel experienced a 19% reduction in infarct size with nitrite treatment compared with placebo[190]. A 1-year follow-up of study participants also found that the nitrite group experienced a significant reduction in major adverse cardiac events ($NaNO_2^-$: 2·6% *v.* NaCl: 15·8%; $P=0·04$)[190].

Conclusion

CVD remains the major killer from any disease across the developed world. Currently the available evidence indicates a role for dietary nitrate for improving CVD risk factors, a highly valuable finding given that dietary nitrate from beetroot and green leafy vegetables could represent a relatively simple and cost-effective treatment/preventative strategy for reducing CVD and its sequelae. However, at present it remains unclear whether incidence of CVD morbidity or mortality can be reduced with long-term dietary intakes of inorganic nitrate, as such evidence investigating this question directly has not yet been published. At present, there is an overwhelming need for epidemiological research to be conducted to identify the potential long-term effects of sustained inorganic nitrate and nitrite consumption on the development of CVD and its consequences.

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