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Does dietary nitrate say NO to cardiovascular ageing? Current evidence and implications for research

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CVD are characterised by a multi-factorial pathogenesis. Key pathogenetic steps in the development of CVD are the occurrence of endothelial dysfunction and formation of atherosclerotic lesions. Reduced nitric oxide (NO) bioavailability is a primary event in the initiation of the atherosclerotic cascade. NO is a free radical with multiple physiological functions including the regulation of vascular resistance, coagulation, immunity and oxidative metabolism. The synthesis of NO proceeds via two distinct pathways identified as enzymatic and non-enzymatic. The former involves the conversion of arginine into NO by the NO synthases, whilst the latter comprises a two-step reducing process converting inorganic nitrate (NO_3^-) into nitrite and subsequently NO.

Inorganic NO_3^- is present in water and food, particularly beetroot and green leafy vegetables. Several investigations have therefore used the non-enzymatic NO pathway as a target for nutritional supplementation (NO_3^- salts) or dietary interventions (high-NO_3^- foods) to increase NO bioavailability and impact on cardiovascular outcomes. Some studies have reported positive effects of dietary NO_3^- on systolic blood pressure and endothelial function in patients with hypertension and chronic heart failure. Nevertheless, results have been inconsistent and the size of the effect appears to be declining in older individuals. Additionally, there is a paucity of studies for disorders such as diabetes, CHD and chronic kidney failure. Thus, whilst dietary NO_3^- supplementation could represent an effective and viable strategy for the primary and secondary prevention of age-related cardiovascular and metabolic diseases, more large-scale, robust studies are awaited to confirm or refute this notion.

Nitric oxide: Nutrition: Ageing: Endothelial function: Dietary nitrate: CVD

CVD are the leading cause of death worldwide and a major cause of morbidity and disability[1]. In the UK, cardiovascular mortality accounts for 19 and 28 % of premature deaths among women and men, respectively[2]. CVD are characterised by a multifactorial pathogenesis including genetic, diet and lifestyle factors[3]. The clinical outcomes of CVD, such as heart failure, atrial fibrillation and cerebrovascular disease, are largely attributed to a

Abbreviations: BP, blood pressure; ED, endothelial dysfunction; EF, endothelial function; NO, nitric oxide; eNOS, endothelial NOS; NOS, nitric oxide synthase enzyme; ROS, reactive oxygen species.

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reduction in the blood supply to associated organs and tissues. This occurs secondary to thickening of the walls of the blood vessels and formation of obstructive atherosclerotic plaques[4]. Endothelial dysfunction (ED) appears to be a critical step in the initiation of the atherosclerotic process[4].

The endothelium

The endothelium is a monolayer of cells separating the vascular lumen from the rest of the blood vessel. It is now recognised that the endothelium has vital paracrine, endocrine and autocrine functions[5]. Therefore, in addition to helping maintain blood flow, the main function of the endothelium is to serve as an endocrine organ[6]. The endothelium generates several extracellular messengers that mediate multiple functions including preserving haemostatic balance[7]. In addition to insulating the thrombogenic sub-endothelial layers, the endothelium secretes molecules that inhibit the inappropriate formation of thrombus including nitric oxide (NO), prostacyclin I_2, tissue plasminogen activator and protein C/protein S[7]. However, in cases of vessel damage and exposure to certain pro-inflammatory substances, the balance is shifted towards a procoagulant/prothrombotic state[5]. This stimulates the endothelium to secrete agents that help with platelet aggregation and clot formation including platelet activating factor, von Willebrand factor and thromboxane A_2[5,8].

Normal vascular endothelium has anti-proliferative and anti-apoptotic properties that are mediated through the activity of NO, prostacyclin I_2 and C-type natriuretic peptide. Moreover, endothelial cells secrete factors that promote proliferation of smooth muscle cells and the formation of new blood vessels, e.g. vascular endothelial growth factor, angiopoietins and adropins[9]. Further, NO secreted by the normal endothelium prevents inflammatory response in the vascular wall secondary to local injury. Dysfunction in the endothelium is characterised by disturbed vasodilator and anticoagulant function, increased adhesiveness of the vessel wall for platelets and leucocytes (inflammation), reduced fibrinolytic activity and breakdown of barrier function causing leakage and oedema formation[10].

Nitric oxide and endothelial function

NO is a free radical gas molecule that is involved in the regulation of multiple physiological processes such as blood pressure (BP), glucose metabolism, inflammation and coagulation[11]. Reduced availability of NO contributes to pathological conditions including hypertension, diabetes, chronic heart failure or kidney failure[12]. NO is regarded as one of the most important molecules secreted by the endothelium. It is a highly diffusible molecule with a very short half-life (<1 s)[13]. The production of NO is catalysed by the nitric oxide synthase enzyme (NOS). There are three isoforms of this enzyme including: endothelial (eNOS), neuronal and inducible[14]. The eNOS is a homodimeric enzyme expressed constitutively in the endothelial cells that facilitate the conversion of the amino acid L-arginine into L-citrulline and NO[6]. This process requires molecular oxygen and reduced NADPH as co-substrates, and the following cofactors: FAD, FMN, tetrahydrobiopterin, haeme and $Ca^{2\pm}$–calmodulin[15] (see Fig. 1).

The triggers for NO synthesis and release are either mechanical stretching of the vessel wall or release of receptor-mediated agonists such as bradykinin, acetylcholine or histamine[16]. These signals lead to an increase in intracellular calcium concentration. Intracellular Ca^{2+} binds to calmodulin to form Ca^{2+}–calmodulin complex that mobilises eNOS from its binding to caveolin, thereby allowing the activated eNOS to catalyse the synthesis of NO from L-arginine[16]. Because of the gaseous nature of NO, it diffuses from where it is synthesised in the endothelium to the vascular smooth muscle where it activates soluble guanylate cyclase leading to increasing intracellular cyclic guanosine monophosphate. The cyclic guanosine monophosphate causes smooth muscle relaxation and, eventually, arterial dilation[17].

In addition to arterial dilation, NO has many other vital protective functions in blood vessels including decreasing: (1) smooth muscle proliferation; (2) platelet aggregation; (3) endothelin production; (4) monocytes and platelets adhesion; (5) expression of adhesion molecules; and (6) oxidation of LDL[10]. Because of the vital role of NO, researchers have suggested that reduced NO availability is the major cause of ED. This deficiency activates atherogenic processes in the vessel wall, which include vasoconstriction, monocyte activation and adherence to vascular endothelium, proliferation of smooth muscle cells, thrombosis and impaired coagulation and, eventually, atherosclerosis[18].

Many factors modulate NO synthesis and degradation, and therefore, affect endothelial function (EF). Asymmetric dimethyl L-arginine is a product of protein metabolism formed secondarily to methylation of L-arginine[19]. Asymmetric dimethyl L-arginine decreases the synthesis of NO by reducing the expression and/or activity of eNOS. Asymmetric dimethyl L-arginine is increased in many pathological conditions such as hypercholesterolaemia, atherosclerosis, hypertension, chronic heart failure, diabetes mellitus and chronic renal failure[20]. Further, uncoupling of eNOS as a result of the oxidation of tetrahydrobiopterin or depletion of L-arginine and the accumulation of endogenous methylarginines may lead to reduced formation of NO, i.e. the eNOS enzyme is converted from NO-producing enzyme to O_2^--producing enzyme[21]. Overproduction of reactive oxygen species (ROS) is the major cause of reduced NO availability in CVD. NO reacts with superoxide anion with high affinity forming the harmful free radical peroxynitrite ($ONOO^-$)[5]. Lipid peroxyl radicals and oxidised LDL react with endothelial NO before it reaches the vascular smooth muscle cells and, therefore, inhibit NO from dilating blood vessels[5].

The ability of the endothelium to maintain the integrity of the vessel wall can be affected by both the biochemical and pathophysiological states of the rest of

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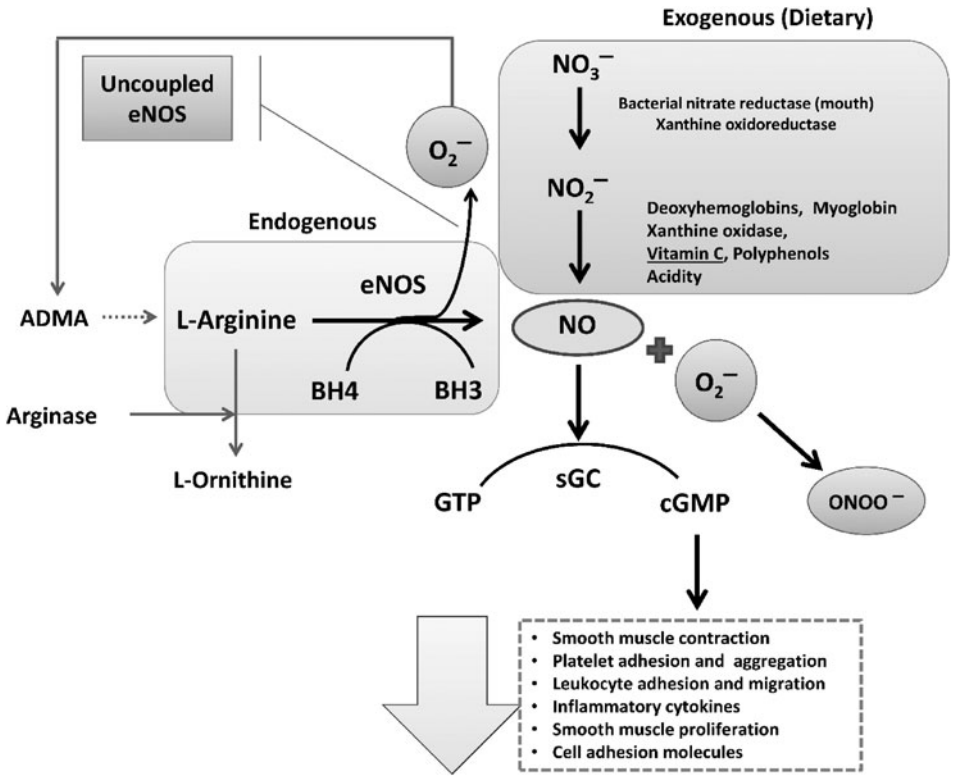

Fig. 1. Exogenous and endogenous sources of nitric oxide (NO). NO is produced by a family of enzymes known as NO synthases (NOS) which utilise the substrate L-arginine. The $NO_3^- - NO_2^-$ – NO pathway has been proposed as an alternative pathway for NO generation. ADMA, asymmetric dimethylarginine; BH4, tetrahydrobiopterin; cGMP, cyclic GMP; $ONOO^-$, peroxynitrite; sGC, soluble guanylate cyclase.

the body. For example, chronic smoking deteriorates EF by decreasing NO production and enhancing its degradation via the generation of oxygen-free radicals[22]. Further, hypercholesterolaemia and high homocysteinaemia may reduce the availability of NO secondary to oxidative stress[23,24].

Endothelial dysfunction and hypertension

ED has been demonstrated both in the resistance and conduit arteries of several animal models of hypertension[25]. In human subjects, reduced forearm blood flow responses to endothelium-dependent vasodilator agonists, such as acetylcholine and bradykinin[26,27], and increased vasoconstrictor responses to locally administered NOS inhibitors[28] have been observed in hypertensive patients. The cause of ED associated with hypertension is speculated to be a reduction in NO bioavailability (increased degradation by oxidative stress, reduced production by eNOS inactivation) and abundance of vasoconstrictor agents in the circulation such as angiotensin II and prostaglandins[25].

Vascular ageing

The ageing process is characterised by a progressive decline of cellular integrity and function resulting from the structural modification of macromolecules including formation of oxidised lipid species, advanced glycated products, nitrosylated proteins and DNA mutations[29,30]. The accumulation of modified molecules and their incorporation into cellular components are responsible for the structural and functional deterioration of tissues and organs with time[31].

Whilst the complexity of the biological mechanisms contributing to the ageing process is still poorly understood, a comprehensive summary of some of these mechanisms has been proposed recently by Lopez-Otin *et al.*[32]. The authors proposed the following set of hallmarks of ageing: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication[32]. Many factors contribute to the age-related molecular damage, but it seems likely that much damage is due to three common stressors including oxidative stress/redox changes, inflammation and metabolic stress[33] (see Fig. 2).

Ageing and CVD risk

Age-specific mortality rates from heart disease and stroke increase exponentially with age and account for more than 40 % of all deaths worldwide among individuals

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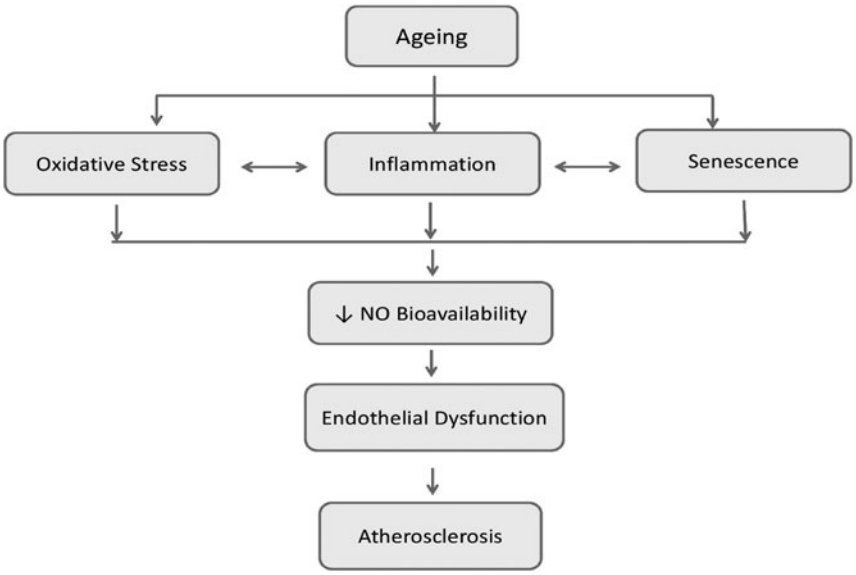

Fig. 2. Factors associated with vascular ageing and atherosclerosis. The triad of oxidative stress, inflammation and endothelial cell senescence contribute to reduced nitric oxide (NO) availability, endothelial dysfunction and the subsequent atherosclerosis.

aged 65–74 years and almost 60 % at age 85 years and older[34]. In the UK, although death rates from CVD have been declining over the past four decades, IHD is ranked as the number one for years of life lost due to premature mortality[2]. Importantly, key cardiovascular risk factors including lifestyle factors such as smoking, poor diet and lack of physical activity are the major causes of morbidity measured by disability-adjusted life years[35].

Ageing is associated with complex structural and functional changes in all tissues including the vascular system, and these changes increase CVD risk independent of other risk factors such as hypertension, diabetes or hypercholesterolaemia[36]. These functional changes include widespread ED, dilation of the central arteries and increased arterial stiffness[37,38]. Development of strategies to attenuate ageing of the vascular system could make a substantial contribution to lowering CVD risk and improving the quality of life of older people[39].

Factors that impair endothelial function with ageing

Oxidative stress

ROS encompass a large family of oxidant molecules such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH.) and $ONOO^-$. The accumulation of ROS and the resulting oxidative modification of cellular macromolecules (lipids, proteins and nucleic acids/DNA) have been suggested to contribute to ageing in all organisms[40]. Indeed, increased production of free radicals, secondary to mitochondrial dysfunction, causes oxidative damage to cells including vascular cells[41]. ROS formation can also lead to a propagation of the activity where the effect of a single reactive molecule can be amplified due to a series of chain reactions causing further damage and the loss of cell homeostasis[42]. Beside their damaging effect, ROS are also important secondary messengers in physiological process regulating enzymatic activity, gene expression and have a key role in response to pathogens infections[43]. For this reason, ROS production is tightly regulated by key antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase that when not jeopardised are able to keep the balance between the production and elimination of these oxidant species[44].

The major sources of ROS in CVD are represented by NADPH[45,46], mitochondrial respiration[47], xanthine oxidase[48], lipoxygenase and uncoupled NOS[49]. The putative mechanism by which the dysregulated enzymatic functions are linked to CVD are thought to be connected to the excessive O_2^- generation that may act as a NO scavenger causing both a reduction of NO bioavailability in the vascular tissue and the production of the highly reactive $ONOO^-$, which in turn can negatively modulate protein functions through nitrosylation of tyrosine residues. Mitochondria also represent an important source of ROS (mtROS) that have been associated with CVD pathogenesis[50], and the role of nitrate (NO_3^-) and nitrite (NO_2^-) in the regulation of mitochondrial function and ROS generation is becoming an area of interest in the context of CVD prevention[51].

Inflammation

Chronic inflammation is a driver of ageing and contributes to the pathology of many age-related diseases including atherosclerosis[52]. Observational and experimental studies have demonstrated the importance of inflammation as a determinant of an unhealthy ageing phenotype. For example, the Whitehall II study reported that a high level of IL-6 almost halved the odds of successful ageing after 10 years (OR 0·53) and increased the

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risk of cardiovascular events and non-cardiovascular mortality[53]. Growing evidence suggests important cross-talk between oxidative stress, inflammatory processes and the onset of ED prior to atherosclerosis[34]. ROS induce pro-inflammatory changes in the vascular endothelium, described as endothelial activation, which involves secretion of autocrine/paracrine factors, leucocyte-endothelial interaction and the up-regulation of expression of cellular adhesion molecules[54]. Oxidative stress activates redox-sensitive transcription factors including the activator protein and NF-κB, increasing the expression of cytokines (TNF-α, IL-1 and IL-6), adhesion molecules (intercellular adhesion molecule and vascular cell adhesion molecule) and pro-inflammatory enzymes (inducible NOS and cyclooxygenase-2)[39].

Ageing is associated with higher circulating concentrations of cytokines, especially TNF-α, IL-1β and IL-6, which mediate the acute phase protein C-reactive protein[39]. These factors contribute significantly to the pro-inflammatory microenvironment and facilitate the development of vascular dysfunction[39]. Among middle-aged and older adults, the Framingham heart study showed that brachial flow-mediated dilation is inversely related to C-reactive protein, IL-6 and intercellular adhesion molecule inflammatory markers[55]. Further, inhibition of NF-κB signalling improved EF significantly in middle-aged and older adults[56].

Senescence

Cellular senescence is characterised by telomere shortening and permanent loss of mitotic capability, which are associated with morphological and functional changes and impaired cellular homeostasis[57]. Risk factors for atherosclerosis including oxidative stress, inflammation, smoking, diabetes and hypertension have all been associated with accelerated telomere shortening[58]. Telomere length in endothelial cells is inversely proportional to patient age[59], and this shortening is exacerbated in older aged patients with coronary artery disease[60]. Cross-sectional studies demonstrate that those with increased arterial stiffness, an indicator of vascular ageing, have shorter telomeres[61]. Hypertensive patients have shorter telomeres than their normotensive peers and hypertensives with shorter telomeres are more likely to develop atherosclerosis over 5 years follow-up[39]. The afore-mentioned observations suggest that telomere length might be a potential candidate marker for cardiovascular ageing. Vascular endothelial cell senescence *in vivo* has also been observed[62]. The development of more senescent endothelial cells has been linked to a shift from an anti-atherosclerotic phenotype (characterised by decreased levels of NO, eNOS activity and shear stress-induced NO production) to a pro-atherosclerotic phenotype (indicated by increased ROS, thromboxane A_2 and endothelin-1). These observations implicate endothelial cell senescence in the initiation and progression of atherosclerosis[63]. NO increases telomerase activity and promotes mobilisation of endothelial progenitors cells, which have the potential to delay endothelial cell ageing by replacing damaged endothelial cells to maintain physical and functional integrity of the endothelium[64].

Ageing-associated nitric oxide insufficiency

Vascular NO insufficiency in older people is mediated in part by decreased NO production by eNOS[65]. There is evidence that eNOS activity is reduced with age because of post-translational modification such as acylation, nitrosylation, glycation or phosphorylation[65]. Additionally, this reduction in eNOS activity might be secondary to the deficiency of cofactors required in the process of NO production (e.g. tetrahydrobiopterin)[66]. The age-associated increase in arginase activity may compete with eNOS for the critical substrate required in NO production, L-arginine[66]. Further, excessive O_2^- production with ageing may contribute to NO insufficiency. The interaction of O_2^- with NO produces the highly reactive $ONOO^-$[67]. Due to its ability to restore reduced NO bioavailability, inorganic NO_3^- represents a potential therapeutic strategy to treat age-associated vascular dysfunction[67].

The nitrate–nitrite−nitric oxide pathway

Epidemiological studies have consistently shown a protective effect of higher intake of fruit and vegetables and reduced risk of CVD[68]. Whilst the exact mechanism/s through which a fruit- and vegetable-rich diet reduces CVD risk remains to be fully elucidated, an increase in NO bioavailability is likely to be important. Eighty-five per cent of the dietary NO_3^- is derived from vegetables and the remaining is mostly from drinking-water. Dietary intake of NO_2^- principally comes from cured meat, to which NO_2^- salts are added to prevent the development of botulinum toxin and to maintain product taste and colour[69]. Vegetables can be categorised according to their NO_3^- contents into three categories: (1) high NO_3^- contents: e.g. rocket, spinach, lettuce and beetroot (>1000 mg/kg); (2) medium NO_3^- contents: e.g. turnip, cabbage, green beans, cucumber and carrot (100–1000 mg/kg); and (3) low NO_3^- contents: e.g. onion and tomato (<100 mg/kg)[70]. The concentration of NO_3^- in drinking-water varies according to the geographical location and regional rules regarding safe levels of NO_3^- in tap or bottled water[71].

It is thought that the beneficial effect of NO disappears in a few seconds as this gasotransmitter is oxidised to NO_2^- and then to NO_3^-. NO_3^- is then excreted in urine as a cumulative by-product of NO metabolism and dietary NO_3^- intake[72]. Interestingly, in the past two decades, scientists discovered an alternative pathway for NO source other than the classical L-arginine–eNOS–NO pathway[73]. The other source of NO was found to be NO_2^-, which can be converted back to NO by the action of several enzymes and molecules including deoxyhaemoglobin, deoxymyoglobin, xanthine oxidoreductase, protons, polyphenols and ascorbic acid[74]. Of note, this pathway is more active and efficient in cases of hypoxia in which the level of both oxygen and NO are low[75].

Dietary NO_3^- is well absorbed in the upper gastrointestinal tract with approximately 100 % bioavailability and

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plasma concentration of NO_3^- peaking after 1 h[70]. About 25 % of the circulating pool of NO_3^- is actively taken up from the blood via an anion exchange channel called sialin and secreted by the salivary glands into the saliva[76]. The salivary NO_3^- is reduced to NO_2^- by facultative anaerobic bacteria in the oral cavity, particularly those residing on the dorsal surface of the tongue[74]. This NO_2^- and other inorganic NO_3^- travel to the stomach where they are converted to NO with the help of ascorbic acid. In this strong acidic environment of the stomach, NO_2^- is protonated to form nitrous acid (HNO_2)[76]. Nitrous acid can spontaneously give rise to the generation of NO through the following sequence of reactions: $2HNO_2 \rightarrow H_2O + N_2O_3$ and $N_2O_3 \leftrightarrow NO + NO_2^-$[77]. The liberated NO has been found to be protective for the gastric mucosa, i.e. enhances blood supply[74]. Moreover, the remaining NO, NO_2^- and NO_3^- diffuse to the general circulation and contribute to NO pool[78].

In the circulation, NO_2^- may function as a source of NO that is activated in hypoxia and acidic conditions to increase blood flow and regulate BP[79,80]. There are many mechanisms involved in the bioconversion of NO_2^- to NO in the blood[81]. The most common is the reaction of deoxyhaemoglobin ($HbFe^{2+}$) with NO_2^- in acidic environment, which will liberate NO ($NO_2^- + HbFe^{2+} + H^+ \rightarrow NO + HbFe^{3+} + OH^-$)[81]. In addition to $HbFe^{2+}$, there are many enzymes and proteins that enhance the conversion of NO_2^- to NO such as myoglobin, cytochrome C oxidase, eNOS and xanthine oxidoreductases[81].

Therapeutic effects of inorganic nitrate in patients with CVD

NO_3^- has been used in the treatment of CVD including angina and digital ischaemia since medieval times[77,82]. In the past 30 years, since the discovery of the $NO_3^- - NO_2^- - NO$ pathway and its contribution to the overall NO pool[83], there has been a renewal in using NO_3^- and NO_2^- in experiments and in clinical trials focused on the prevention of CVD. Larsen *et al.*[84] in a pioneer study demonstrated the beneficial effect of inorganic NO_3^- in BP reduction; the investigators administered 0·1 mmol sodium NO_3^-/kg body weight daily to healthy participants (which corresponds to an intake of 100–300 g of NO_3^--rich vegetables daily) and found after 3 d of NO_3^- supplementation, a 4 mmHg reduction in diastolic BP[84]. Administration of the same dose of NO_3^- to a larger group of individuals produced significant reductions in both systolic and diastolic BP[85]. After the publication of these seminal studies, there has been a growing interest in the protective effects of dietary NO_3^- on cardio-metabolic outcomes. However, the majority of the studies have been conducted in healthy populations and the evidence on the effects of dietary NO_3^- supplementation in patients with CVD is still limited. A summary of the dietary NO_3^- and NO_2^- interventions conducted in patients with CVD is provided in Table 1.

Four weeks of NO_3^- supplementation (9 mg/kg) to older individuals at higher CVD risk significantly lowered systolic BP by 8 mmHg in comparison with placebo[86]. Supplementing beetroot juice (providing a NO_3^- dose of 300–400 mg) to older overweight, but otherwise healthy, participants for 3 weeks lowered daily home-measured systolic BP by 7 mmHg[87]. However, BP values were found to have returned to pre-intervention values, 1 week after stopping the beetroot supplementation. Kapil *et al.*[88] conducted the largest and longest trial in stage 1 hypertensive patients and found that dietary NO_3^- improved both systolic and diastolic BP (measured by 24 h monitoring, home monitoring and clinic resting) and EF (measured by flow-mediated dilation and arterial stiffness). In contrast, studies in treated hypertensive patients did not show significant improvement of BP with beetroot administration[89]. Moreover, an individual participant meta-analysis (eighty-five participants) showed that beetroot supplementation lowered 24 h ambulatory BP significantly in younger participants only (<65 years)[90]. Two meta-analyses have demonstrated a significant reduction of systolic BP (−4·4 mm Hg)[91] and a significant improvement of EF[92] with inorganic NO_3^- or beetroot consumption.

The discovery of the contribution of dietary NO_3^- to NO bioavailability has provided a rationale for the use of NO_3^- to reverse ED secondary to NO insufficiency in cardiovascular and metabolic diseases[82]. Inorganic NO_2^- supplementation reversed ED significantly in a murine model of hypercholesterolaemia[93]. In human subjects, dietary NO_3^- supplementation has been found to improve flow-mediated dilation and arterial stiffness in hypercholesterolaemic patients[94] and reduce TAG concentrations in patients at higher CVD risk[95].

Data from animal studies have also shown promising results regarding the effect of dietary NO_3^- on biomarkers of metabolic diseases. Supplementation of eNOS-deficient mice suffering from metabolic syndrome with inorganic NO_3^- for 10 weeks reduced visceral fat and circulating TAG concentration and reversed the pre-diabetic phenotype[96]. Further, supplementing diabetic rats with sodium NO_3^- for 2 months produced significant improvements in glucose homeostasis, lipid profile and oxidative stress markers[97]. However, NO_3^- supplementation in human subjects showed no evidence of improvement in glucose and insulin homeostasis in diabetics[98–100] or non-diabetic participants[101]. Potassium NO_3^- supplementation did not improve glucose tolerance in young and older obese individuals but reduced oxidative stress during hyperglycaemia in older individuals[102].

Inorganic NO_2^- reversed ageing-related arterial stiffness in older mice. In one study, the plasma NO_2^- concentration in older mice was found to be restored to youthful concentrations with inorganic NO_2^- supplementation[103]. In healthy human subjects, Bahra *et al.*[104] observed a significant reduction in arterial stiffness 3 h after NO_3^- ingestion. Further, one study found that daily consumption of NO_3^- (900 mg) for 4 weeks reduced pulse wave velocity in older people at increased CVD risk[86]. However, in another study, arterial compliance increased with no change in pulse wave velocity after 220 mg NO_3^- supplementation in twenty-eight healthy participants[105].

Inorganic NO_3^- administration inhibits platelet aggregation, and therefore, may reduce thrombotic events

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Table 1. Summary of nutritional interventions testing the effects of dietary nitrate supplementations in patients with CVD

	First Author, reference	Population	Design	Intervention (duration)	Control	Outcomes	Main results
Hypertension	Gosh[118]	Fifteen drug-naïve hypertensive subjects	R, CO, P	BJ (24 h)	Water	Clinic resting BP	At 24 h, clinic SBP was significantly lower than the control
	Bondonno[119]	Thirty-eight pre-hypertensive subjects	R, CO	High-nitrate green leafy vegetables (7 d)	Low-nitrate vegetables	24 h ABPM, home resting BP and clinic resting BP	Ambulatory, home and clinic were not different between the high-nitrate diet and the low-nitrate diet
	Rammos[86]	Eleven older subjects with moderate cardiovascular risk (HeartScore 4·7)	R, PAR, DB, P	Sodium nitrate (4 weeks)	Sodium chloride	FMD, arterial stiffness and clinic resting BP	Dietary nitrate improved FMD, vascular stiffness and reduced SBP
	Kapil[88]	Sixty-eight drug-naive (*n* 34) and treated (*n* 34) patients with stage 1 hypertension	R, PAR, DB, P	BJ (8 weeks)	Nitrate-free BJ	24 h ABPM, home resting BP and clinic resting BP; FMD and arterial stiffness	Dietary nitrate improved both SBP and DBP (measured by the three methods) and endothelial function (FMD and arterial stiffness)
	Kerley[120]	Eleven subjects had controlled BP whilst 8 had uncontrolled BP	Pre-post intervention	BJ (14 d)	None	24 h ABPM, arterial stiffness	Significant improvement in night-time DBP and arterial stiffness were only observed in patients with uncontrolled hypertension
	Shaltout[121]	Twenty-six patients with controlled stage 1 hypertension	R, PAR, DB, P	Exercise + BJ (6 weeks)	Exercise + Nitrate-free BJ	Muscular performance, clinic resting BP, 24 h ABPM, cardiac haemodynamics	Significant decrease in systolic BP after exercise and nitrate supplementation
Diabetes	Gilchrist[99]	Twenty-seven older patients with diabetes	R, CO, P, DB	BJ (2 weeks)	Nitrate-free BJ	Insulin sensitivity, 24 h ABPM, FMD and microvascular blood flow	Dietary nitrate supplementation was not associated with significant changes in BP, macrovascular or microvascular endothelial function or insulin sensitivity
	Cermak[98]	Seventeen patients with diabetes	R, CO, P, DB	Sodium nitrate (2 h)	Sodium Chloride	Insulin and glucose concentrations	A single dose of dietary nitrate does not improve oral glucose tolerance in patients with type 2 diabetes
	Shepherd[100]	Forty-eight patients with diabetes	R, CO, P, DB	BJ (4 d)	Nitrate-free BJ	Physical performance, clinic resting BP	Dietary nitrate supplementation does not modulate the response to exercise and BP in patients with diabetes
Dyslipidaemia	Zand[95]	Thirty patients older than 40 years with ≥3 cardiovascular risk factors	R, PAR, P, DB	Nitrate + nitrite supplement (30 d)	Placebo*	Cholesterol, TAG and C-reactive protein	Significant reduction in TAG in patients with elevated baseline levels (>150 mg/dl)
	Velmugaran[94]	Sixty-nine patients with untreated hypercholesterolaemia	R, CO, P, DB	BJ (6 weeks)	Nitrate-free BJ	Clinic resting BP, FMD, PWV, platelet aggregation	Dietary nitrate was associate with small but significant improvements in FMD, PWV and platelet aggregation
CHD	Schwarz[111]	Seventy patients with stable angina	R, CO, P, DB	Sodium nitrate (7 d)	Sodium Chloride	1 mm ST depression on electrocardiogram treadmill test	Sodium nitrate treatment may confer a modest exercise capacity benefit in patients with chronic angina who are taking other background medication
	Burnley-Hall[108]	Twenty coronary artery disease patients on clopidogrel therapy	R, CO, P, DB	Nitrate supplement (2 h)	Placebo*	Extracellular vesicles and platelet aggregation	Nitrate supplementation reduced platelet-derived extracellular vesicles in coronary artery disease patients on clopidogrel therapy

CHF	Coggan[122]	Nine patients with heart failure	R, CO, P, DB	BJ (2 h)	Nitrate-free BJ	Physical performance, clinic resting and exercise BP	Inorganic nitrate increased peak knee extensor power but did not modify BP
	Eggebeen[123]	Twenty patients with heart failure with preserved ejection fraction	R, CO, P, DB	BJ (2 h for acute assessment and then continued for 1 week)	Nitrate-free BJ (only used for acute 2-h experiment)	Physical performance, clinic resting and exercise BP	One week of daily dosing with BJ significantly improves submaximal aerobic endurance and blood pressure in elderly patients with heart failure
	Chirinos[124]	Seventeen patients with heart failure with preserved ejection fraction	R, CO, P, DB	BJ (2 h)	Nitrate-free BJ	Clinic resting BP, arterial stiffness, blood flow of carotid and left ventricle	Inorganic nitrate reduced wave reflections but did not reduce blood pressure, carotid bed vascular resistance or carotid characteristic impedance
	Coggan[113]	Eight patients with heart failure with reduced ejection fraction	R, CO, P, DB	BJ (2 h)	Nitrate-free BJ	Physical performance, clinic resting and exercise BP	Dietary nitrate improved muscle performance but did not have an effect on resting and exercise BP
	Shaltout[121]	Nineteen patients with heart failure with preserved ejection fraction	R, PAR, DB, P	Exercise + BJ (4 weeks)	Exercise + Nitrate-free BJ	Muscular performance, clinic resting and exercise BP, cardiac haemodynamics	There were no additional benefits of dietary nitrate over exercise on the study outcomes
	Zamani[114]	Twelve subjects with heart failure with preserved ejection fraction	R, PAR, DB, P	Potassium nitrate (2 weeks)	Potassium chloride (2 weeks)	Muscular performance, clinic resting BP	Inorganic nitrate improved exercise tolerance and also had a significant effect on SBP
PAD	Kenjale[112]	Eight patients with peripheral arterial disease	R, CO, P	BJ (3 h)	Orange Juice	Muscular performance, clinic resting and exercise BP	Dietary nitrate increased peripheral tissue oxygenation in areas of hypoxia and exercise tolerance and lowered DBP
CKD	Kemmer[125]	Seventeen patients with chronic kidney disease	R, CO, P	BJ (4 h)	Water	Renal vascular resistance, clinic resting BP	Peripheral systolic and diastolic blood pressure as well as renal vascular resistance were significantly reduced after the dietary nitrate load

R, randomised; CO, cross-over; P, placebo; BJ, beetroot juice; BP, blood pressure; SBP, systolic blood pressure; ABPM, ambulatory blood pressure monitoring; PAR, parallel; DB, double blind; FMD, flow-mediated dilation; DBP, diastolic blood pressure; PWV, pulse wave velocity; CHF, chronic heart failure; PAD, peripheral arterial disease; CKD, chronic kidney disease.
Electronic search conducted on PubMed on 24 January 2018 using the following algorithm: 'dietary nitrate' OR beetroot OR beet root OR 'inorganic nitrate'. Number of articles retrieved by primary search: 1649.
One author screened all articles to include studies that investigated effects of dietary nitrate supplementation in patients with CVD.
* Placebo was not defined.

in both human subjects and experimental animals[106,107]. Two studies have found a positive effect of dietary NO_3^- supplementation on platelet aggregation in hypercholesterolaemic patients[94] and platelet-derived extracellular vesicles in coronary artery disease patients on clopidogrel therapy[108]. The restoration of blood to a tissue after a period of ischaemia is sometimes associated with severe tissue injury due to a high release of free radicals. Animal studies have demonstrated that the prior administration of inorganic NO_3^- reduces the infarct size in a model of ischaemic-reperfusion injury[109]. Moreover, low-dose sodium NO_2^- attenuated myocardial ischaemia and vascular reperfusion injury in a human experimental study[110]. However, Schwarz *et al.*[111] showed that supplementation with sodium NO_3^- marginally improved exercise performance in patients with chronic angina on prescribed medications. Conversely, positive effects of dietary NO_3^- supplementation were found on exercise tolerance and onset of *claudication intermittens* in eight patients with peripheral arterial disease[112]. Dietary NO_3^- supplementation appears to have positive effects on exercise performance and oxygen consumption in patients with chronic heart failure[113,114], whereas the effects on BP, at rest and during exercise, and cardiac haemodynamics are less replicable[88–91,99,115–117].

Directions for future research

There is currently limited evidence to support the protective effects of inorganic NO_3^- supplementation on cardiovascular and metabolic outcomes in patients at higher CVD risk. Several studies have been conducted in patients with hypertension and chronic heart failure, but the results have been contrasting, whereas for other cardiovascular disorders such as diabetes, CHD or chronic kidney failure, there is simply a paucity of studies. In addition, the evidence is further weakened by the pilot nature of these studies both in terms of short duration (longest trial is 8 weeks)[83] of the interventions and small sample size (largest population is seventy patients)[111]. Future research efforts should be therefore directed at the conduction of more robust, confirmatory trials to provide strong and unbiased evidence on the effects of dietary NO_3^- on cardiovascular outcomes. In consideration of the larger number of studies and overall supportive effects of dietary NO_3^- on BP, priority might be given to the design of trials testing the effects of dietary NO_3^- in larger populations of hypertensive patients with and without anti-hypertensive medications to evaluate whether dietary NO_3^- provides additive effects to background pharmacological treatments of BP. These studies may also take into consideration the recruitment of patients with more severe hypertension (stage 2 or 3) and evaluate whether ethnicity could be a modifying factor of the BP response to dietary NO_3^- supplementation.

Conclusions

NO influences several physiological functions involved in the pathogenesis of CVD such as ROS generation, inflammation and platelet aggregation. Increasing inorganic NO_3^- intake, via supplementation of NO_3^- salts or increased high-NO_3^- food consumption (i.e. beetroot, green leafy vegetables) could represent a viable and effective strategy for the prevention of age-related chronic cardiovascular and metabolic diseases. The evidence from randomised clinical trials has so far suggested positive effects of systolic BP and EF but the size of the effect appears to be declining in older patients at higher cardiovascular risk. Therefore, until larger and more robust trials are conducted in patients at higher CVD risk, dietary NO_3^- supplementation cannot be recommended as a nutritional or clinical strategy for the primary and secondary prevention of CVD.

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Conflicts of Interest

None.

Authorship

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