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# Dietary nitrate and nitric oxide metabolism: mouth, circulation, skeletal muscle and exercise performance

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## Abstract

Nitric oxide (NO) is a gaseous signaling molecule that plays an important role in myriad physiological processes including the regulation of vascular tone, neurotransmission, mitochondrial respiration and skeletal muscle contractile function. NO may be produced via the canonical NOS-catalyzed oxidation of l-arginine and also by the sequential reduction of nitrate to nitrite and then NO. The body's nitrate stores can be augmented by the ingestion of nitrate-rich foods (primarily green leafy vegetables). NO bioavailability is greatly enhanced by the activity of bacteria residing in the mouth which reduce nitrate to nitrite, thereby increasing the concentration of circulating nitrite which can be reduced further to NO in regions of low oxygen availability. Recent investigations have focused on promoting this nitrate-nitrite-NO pathway to positively impact indices of cardiovascular health and exercise tolerance. It has been reported that dietary nitrate supplementation with beetroot juice lowers blood pressure in hypertensive patients whereas sodium nitrite supplementation improves vascular endothelial function and reduces stiffening of large elastic arteries in older humans. Nitrate supplementation has also been shown to enhance skeletal muscle function and to improve exercise performance in some circumstances. Recently, it has been established that nitrate concentration in skeletal muscle is much higher than that in blood and that muscle nitrate stores are exquisitely sensitive to dietary nitrate supplementation and deprivation. In this review, we consider the possibility that nitrate represents an essential storage form of NO and discuss the integrated function of the oral microbiome, the circulation and skeletal muscle in nitrate-nitrite-NO metabolism as well as the practical relevance for health and performance.

## Introduction

Nitric oxide (NO) is a small, diatomic gaseous signaling molecule that regulates an array of physiological functions essential for maintaining metabolic, neurological and cardiovascular integrity. The first known effects of NO were observed in the vasculature and several decades of research have confirmed that NO plays an essential role in vasodilation (and therefore the control of blood pressure and tissue blood flow) and also in blood clotting via its effects on platelet activation (1). However, NO has physiological effects well beyond the vasculature including, for example, in processes as diverse as neurotransmission (2), immune defense (3), mitochondrial respiration (4) and skeletal muscle contractile function (5). Given that NO has an extremely short half-life, of perhaps only a few milliseconds in biological tissues, it is essential that it is produced continuously at its sites of action.

Most tissues contain one or more isoforms of the nitric oxide synthase (NOS) enzyme, which catalyzes NO production through the conversion of the semi-essential amino acid l-arginine to l-citrulline in a reaction which requires the presence of oxygen (6). NO, as a short-lived free radical, is either used locally or is oxidized to nitrate ($NO_3^-$) in a reaction catalyzed by various ferrous oxyheme-containing proteins (such as oxyhemoglobin or oxymyoglobin; 7). It is important to recognize that the availability of oxygen varies for different tissues and there may even be regional differences in oxygen tension within an organ (8, 9). When oxygen availability is limited, NOS-derived NO generation may be inhibited or impaired. Relatively recently, it has been discovered that, rather than being an inert product of NO oxidation, nitrate can be reduced under appropriate physiological circumstances to nitrite ($NO_2^-$) and then NO (7, 10). Importantly, this ‘complementary’ nitrate-nitrite-NO generation pathway does not require the presence of oxygen and is in fact facilitated by a more acidic pH and lower oxygen tension. In this way, NO may be produced, and vasodilation and other NO effects may be sustained, across a wide range of tissue oxygenation states (7, 10).

Continuous NO generation is essential for the maintenance of cellular function and the overall health of the organism. Indeed, older age and several disease conditions are characterized by NOS dysfunction, lower NO bioavailability and reduced

perfusion of many organs (11, 12, 13). This has led to efforts to enhance NO availability through the diet. While oral l-arginine supplementation has not convincingly improved NO bioavailability or bioactivity, at least in healthy humans, dietary inorganic nitrate supplementation appears to be much more promising (14, 15). Indeed the 'substrate' for the nitrate-nitrite-NO pathway includes not only the nitrate generated from the endogenous oxidation of NO produced via NOS, as described above, but also exogenous inorganic nitrate from the diet, particularly that derived through the ingestion of green leafy vegetables such as rocket, kale, lettuce and spinach, as well as some root vegetables such as beetroot (16). These vegetables typically contain over 250 mg (or ~4 mmol) of nitrate per 100 g fresh weight produce (16), although it should be noted that the nitrate content of vegetables can vary considerably according to growth conditions (geography and season), time elapsed since harvest and food preparation (i.e. raw vs. cooked), (17).

Following ingestion, dietary nitrate is absorbed by the upper gastrointestinal tract into the bloodstream. Approximately 25% of this circulating nitrate is then absorbed by the salivary gland via active transporters, such as sialin, and is concentrated in the saliva (18). In the oral cavity, resident facultative and obligate anaerobic bacteria reduce some of the salivary nitrate to nitrite (19, 20). When subsequently swallowed, a portion of this nitrite is reduced to NO in the acidic environment of the stomach (21) but some of the remaining nitrite enters the systemic circulation and is distributed in blood and stored in various tissues, where it can undergo a one-electron reduction to yield NO. Following the acute ingestion of a nitrate bolus, the peak plasma nitrate and nitrite concentrations are reached after about 1 h and 2-3 h, respectively (22) with a clear 'dose-response' relationship between the quantity of nitrate ingested and the magnitude of the subsequent peak plasma nitrate and nitrite concentrations (22). The delayed peak in plasma nitrite, compared to nitrate, highlights the importance of the oral microbiome in the 'processing' of dietary nitrate. However, while it was previously thought that only bacteria possessed the ability to reduce nitrate into nitrite (20), native nitrate reductase activity has also been discovered in mammalian cells (23).

Despite questionable evidence in humans, dietary nitrate and nitrite have long been associated with an increased risk of gastric cancer, but this viewpoint is rapidly

changing (24). Indeed, there is increasing evidence that nitrate may be an essential molecule which supports human cardiovascular and metabolic health and contributes to optimal physical and cognitive function; a cherished nutrient that is extracted from the diet or otherwise preserved and which is stored in the blood and tissues as a 'NO reservoir' which can be drawn on to contribute to ongoing vascular and metabolic processes. This involves an intricate and elegant inter-organ system for absorbing, transporting, storing and metabolizing endogenous and exogenous sources of nitrate. Figure 1 provides a schematic by which this concept can be understood and which should be referred to in appreciating the connections between the 'compartments' and functions which are described in this review.

This article summarizes the proceedings of a scientific symposium entitled 'Dietary nitrate and nitric oxide metabolism: from mouth to muscle' which was presented at the 2019 American College of Sports Medicine annual meeting in Orlando, Florida. The session was convened and introduced by Dr Jones and featured presentations from Drs Vanhatalo, Seals, Piknova and Jonvik. The objective of this article is to highlight and summarize novel discoveries in this rapidly-evolving field. To this end, the review shall cover: the role of dietary nitrate and the oral microbiota as a source of NO; subsequent transport of nitrate and nitrite in the circulation and attendant implications for vascular function and health; skeletal muscle as a nitrate reservoir and its role in regulating systemic NO bioavailability and local metabolism; and practical applications of dietary nitrate supplementation including for the potential enhancement of exercise performance.

## The role of oral microbiota in nitric oxide homeostasis

The metabolic activity of the microbial community that inhabits the human alimentary canal can have far-reaching effects on host physiology. The recent emergence of high-throughput, cost-effective, next generation sequencing of the bacterial 16S ribosomal RNA hypervariable regions has transformed human microbiome research. Epidemiological studies have shown that a perturbed oral microbiota and poor oral health are associated with systemic conditions, such as cardiovascular, metabolic and kidney diseases, rheumatoid arthritis and Alzheimer's disease, and the aetiology of these diseases is inextricably linked with NO signalling and bioavailability (25, 26).

Inorganic nitrate is a natural micronutrient and is abundant in a vegetable-rich diet, but human cells have limited ability to 'activate' biologically inert nitrate. Instead, humans depend to a large extent, albeit not exclusively (27), upon the symbiotic bacteria residing in the mouth and in the alimentary canal to reduce ingested nitrate to bio-active nitrite (19), which is further reduced to NO in the circulation and other tissues, thus increasing systemic NO bioavailability. It is therefore conceivable that the capacity of the oral microbiota for NO production within this 'nitrate-nitrite-NO reduction pathway' represents the causative link that underpins the correlation between oral and systemic health.

Dietary nitrate supplementation has been shown to reduce blood pressure in both younger and older healthy adults (*e.g.* 28, 29, 30), and to improve muscle contractile function (31, 32), exercise economy and endurance exercise tolerance (33, 34, 35), and brain perfusion and cognitive function (36, 37). An individual's ability to benefit from ingested nitrate, however, may be affected by a dysfunctional oral microbiota, as illustrated by studies in which the use of bactericidal mouthwash blunted the increase in plasma nitrite concentration and the decrease in blood pressure following the ingestion of a standardized nitrate dose (38, 39). Epidemiological evidence suggests that regular mouthwash use is associated with elevated risk of developing pre-diabetes/diabetes during a 3-year follow-up, possibly due to chronic attenuation of microbial nitrate-reductase activity in the oral cavity (40). While short-term, twice-daily chlorhexidine mouthwash administration to healthy adults resulted in no effect on blood pressure in the face of reduced oral nitrate reduction capacity in one study (41), another study using the same chlorhexidine intervention revealed that blood pressure increased during mouthwash use in those subjects who also cleaned their tongues twice-daily and decreased in subjects who did not clean their tongues (42). Therefore, the regulation of the oral microbiome via oral hygiene practices may have far-reaching effects on systemic health.

There are marked differences between individuals in physiological responsiveness to nitrate supplementation and some of this variability likely stems from differences in oral nitrate-reduction capacity. The ability to reduce nitrate, *per se*, is not an uncommon property of facultative and obligate anaerobic oral bacteria, but the net nitrite production by an oral ecosystem is determined by complex interactions

between co-occurring bacterial species (43, 44). Doel et al. (19) used a double agar overlay method to identify nitrate-reducing colonies in oral samples collected from ten healthy volunteers which were incubated for 72 h. The most prevalent nitrate-reducing species identified in these *in vitro* colonies using 16S rRNA sequencing included *Actinomyces naeslundii, Veillonella atypica*, *Actinomyces odontolyticus, Veillonella dispar*, *Rothia dentocariosa* and *Rothia mucilaginosa* (19). In another study, tongue scraping samples collected from six healthy subjects were grown in a biofilm for four days and ranked according to nitrate-reduction activity to best, intermediate and worst nitrate-reducing biofilms (43). The best nitrate-reducing biofilms were found to contain bacteria belonging in genera *Neisseria, Veillonella, Haemophilus, Porphyromonas, Fusobacterium, Prevotella, Leptotrichia, Brevibacillus* and *Granulicatella* (43). While these studies provided significant novel information in identifying species that possess high nitrate-reduction potential *in vitro*, it is pertinent to consider that bacterial communities grown from oral samples *in vitro* tend to lose diversity very rapidly, such that after 72 h more than 80% of species present in the original sample may be lost (43).

Functional interpretation of host-microbiome interactions requires assessment of an *in vivo* oral bacterial community alongside physiological host characteristics. To this end, cross-sectional studies have identified various host characteristics and lifestyle factors that are associated with distinct oral microbiomes. Men had greater abundances of *Veillonella*, *Prevotella* and *Megasphaera* bacteria than women (45), current smokers had lower relative abundance of the phylum Proteobacteria, which includes genus *Neisseria*, compared with never smokers (46), and systemic disease in older age has been associated with increased prevalence of several oral pathogens (47). Cross-sectional studies comparing habitual diets have shown that the *Neisseria*-to-*Prevotella* ratio was elevated in vegans (n=78), with habitually high vegetable intake, compared to omnivores (n=82) (48); however, other studies have found no differences in the oral microbiomes of vegetarians or vegans compared to omnivores (22 vegetarians, 19 omnivores (41); 51 vegans, 55 omnivores, 55 vegetarians (49)). It should be noted that these studies used self-reported dietary records and nutritional analysis software programs to estimate macro-and micronutrient intake, and one study that quantified dietary nitrate intake (41) showed no difference between vegetarians and omnivores.

To test the hypotheses that dietary nitrate as a prebiotic dietary intervention might promote the proliferation of nitrate-reducing bacteria in humans, recent studies have utilized 16S rRNA sequencing of the oral microbiome from saliva and tongue swab samples. Six weeks of nitrate supplementation significantly altered the relative abundances of 78 taxonomic units in hypercholesterolemic patients (n=60), with appreciable increases in *Neisseria flavescens* and *Rothia mucilaginosa* (50). Ten days of nitrate supplementation in healthy young and older adults (n=18) resulted in changes in 52 taxonomic units, including increases in *Neisseria* and *Rothia* and decreases in *Prevotella, Veillonella,* and *Megasphaera* (44). In another study, seven days of nitrate supplementation in young men (n=11) increased the relative abundance of *Neisseria* and decreased the relative abundances of *Prevotella, Actinomyces* and *Streptococcus* (51). Despite differences between studies in sample medium (saliva, tongue swab), 16S rRNA hypervariable region (V1-3, V3-4), subject characteristics, supplementation regime and dietary control in free-living volunteers, the emerging evidence collectively indicates that dietary nitrate is a powerful modulator of the oral microbiome that particularly favors *Neisseria* and *Rothia* and disadvantages *Prevotella* and *Veillonella.*

The decrease in the relative abundances of some nitrate-reducing oral taxonomic units consequent to high dietary nitrate intake might reflect mutual co-occurrence and co-exclusion relationships among oral bacteria (45). Such relationships might also contribute to the diverse results of correlational analyses aiming to link individual or aggregate abundances of nitrate-reducing oral taxonomic units to physiological NO biomarkers. Two studies have shown that an aggregate sum of selected nitrate-reducing oral taxonomic units (from genera *Rothia*, *Neisseria*, *Haemophilus*, *Veillonella* and *Prevotella*) was related to saliva, but not systemic, NO bioavailability (51, 52). Another study found that the baseline abundance of *Prevotella* in tongue swab samples was inversely correlated with the magnitude of the subsequent plasma nitrite response to nitrate supplementation (44). The same study reported positive (*Neisseria, Rothia*) and inverse (*Prevotella, Veillonella*) single-taxon correlations between bacterial abundances in saliva and plasma NO biomarkers across pooled data from the placebo and nitrate conditions (44). Such relationships do not necessarily infer causality and it should be noted that sample sizes in these initial

studies have been relatively small (n<20; 44, 51, 52). In a larger cohort of men and women (n=281), standardized scores for 20 subgingival plaque bacteria previously identified as nitrate-reducing (19, 43) were inversely correlated with insulin resistance and plasma glucose and, among normotensive subjects (n=187), also with systolic blood pressure (53). Lower resting systolic blood pressure has also been reported to be correlated with the abundance of nitrate-reducing bacteria (42, 53). Collectively, these studies highlight the physiological significance of the oral bacterial ecosystem for human health and the considerable potential that exists for exploration of novel dietary and lifestyle interventions that might promote the nitrate-reduction capacity of the oral microbiota. Greater nitrate-to-nitrite conversion by the oral microbiota following nitrate ingestion has the potential to enhance circulating nitrite, and therefore to amplify NO bioavailability and its attendant physiological effects including those that are specific to the vasculature.

**Effects of dietary nitrite and nitrate on vascular health**

Cardiovascular diseases (CVD) remain the leading causes of morbidity and mortality in developed and most developing societies (54). By far, the strongest risk factor for CVD is (increasing) age, with adults >40 years of age bearing the great majority of the burden of CVD (55). Because the number of late middle-aged and older adults will continue to increase in coming decades and make up an increasingly larger percentage of the total population, both the prevalence and incidence of CVD are projected to increase progressively in the future (56). As such, it is imperative to establish effective, evidence-based strategies to lower the risk of age-associated CVD. A key initial question is how to best identify the most compelling targets for CVD prevention?

Much of the increase in CVD risk associated with advancing age is attributable to vascular dysfunction, including stiffening of the large elastic arteries (aorta and carotid arteries) and the development of endothelial dysfunction (57). Large elastic artery stiffening increases systolic blood pressure, pulse pressure, and blood flow and pressure pulsatility that damages the delicate microvasculature of tissues with consequent end-organ injury, leads to pathophysiological remodeling of the heart, including left ventricular hypertrophy and increased risk of heart failure, and is a

major risk factor for cognitive dysfunction, dementia and chronic kidney diseases (58, 59, 60, 61). In humans, large elastic artery stiffness is assessed by carotid-to-femoral pulse wave velocity (CFPWV; “aortic” PWV in rodents) and via measurement of carotid artery compliance using high-resolution ultrasound imaging to capture changes in arterial diameter of one carotid artery with simultaneous assessment of arterial pressure excursions in the contralateral carotid artery (62). Increases in CFPWV (decreases in carotid artery compliance) indicate greater arterial stiffness and vice-versa.

Endothelial dysfunction is the major clinical antecedent to atherosclerotic diseases, including coronary artery disease, occlusive stroke and peripheral artery disease (63, 64, 65). Endothelial function is assessed most commonly using the magnitude of the endothelium-dependent dilation (EDD) to a chemical or mechanical stimulus that evokes NO release from vascular endothelial cells, which, in turn, mediates vascular smooth muscle relaxation (64, 66). EDD is most commonly assessed by administering an endothelium-dependent dilating chemical (e.g., acetylcholine) or, in humans, by measuring brachial artery dilation to an increase in blood flow induced by a period of blood flow occlusion (64); both stimuli evoke an EDD that is predominantly mediated by NO (67). The greater the EDD, the greater the individual’s or group’s vascular endothelial function.

The predominant mechanisms mediating vascular dysfunction with advancing age are oxidative stress and chronic low-grade inflammation (57, 64). These states cause arterial stiffening by increasing vascular smooth muscle tone and by stimulating changes to the extracellular matrix of the arterial wall, including degradation of elastin fibers, compensatory deposition of collagen (fibrosis), and synthesis of advanced glycation end-products, which “cross-link” structural proteins and confer additional stiffening (57, 68). Oxidative stress induces endothelial dysfunction by reducing NO bioavailability via: a) excessive production of superoxide from the electron transport chain of dysfunctional mitochondria, increased activity and/or expression of the enzyme nicotinamide adenine dinucleotide phosphate oxidase [NADPH oxidase], and “uncoupled” endothelial nitric oxide synthase [eNOS], which readily reacts with NO producing peroxynitrite; and b) reducing NO production via uncoupled eNOS (66, 69, 70).

Comprehensive reviews on possible strategies for improving vascular dysfunction with aging can be found elsewhere (71, 72, 73). Briefly, lifestyle practices, including regular aerobic exercise and consuming a healthy diet (i.e., healthy energy intake and nutrient composition), are the most evidence-based strategies for preserving vascular function with aging (71, 72, 73). However, numerous barriers exist for consistent practice of healthy lifestyle strategies and, as a result, adherence to physical activity and dietary guidelines is poor at the population level. As a result, there is strong interest in what we refer to as healthy lifestyle-*inspired* strategies, i.e., approaches that are based on the molecular and cellular mechanisms responsible for the benefits of conventional healthy lifestyle practices (74).

One such strategy is based on the well-established observation that improvements in NO bioavailability are an important mechanism mediating the vascular benefits of healthy lifestyle practices (74). Theoretically, NO bioavailability might be increased by greater expression/activation of eNOS, conversion via the eNOS-independent "nitrate-nitrite-NO pathway", or both (75, 76). Because eNOS dysfunction often occurs with aging, the nitrate-nitrite-NO pathway has been viewed as the more promising approach (75, 76). Given that, biochemically, nitrite represents a *one*-step reduction to NO with a higher conversion efficiency than the two-step reduction of nitrate to NO, we have employed *sodium nitrite* supplementation as a strategy to improve vascular function with aging (13, 76, 77, 78).

In our preclinical investigations, we supplemented the drinking water of C57BL/6 mice with sodium nitrite for 3 weeks as the active treatment condition and used non-supplemented water as the control (13, 77). We found that nitrite bioavailability (the conventional measure of NO bioavailability given the short half-life of the gaseous NO molecule) was reduced in both the plasma and large arteries of old compared with young mice, and that sodium nitrite supplementation restored nitrite to concentrations at or above young controls (13). Aortic PWV was greater in the old compared with the young untreated mice, and sodium nitrite treatment reduced aortic PWV to levels not different than young mice (13, 77), (Figure 2A). The amelioration of arterial stiffening by sodium nitrite in the old mice was accompanied by a complete reversal of age-associated increases in aortic superoxide production, nitrotyrosine

abundance (a molecular biomarker of oxidative stress), expression of NADPH oxidase, and abundance of advanced glycation end-products (Figure 2B-D). *Ex vivo* experiments showed that in aortic segments from young mice, exposure to pyrogallol, a superoxide-generating chemical, induced an “aging-like” increase in advanced glycation end products (AGEs), and that direct treatment with AGEs caused stiffening - effects that were prevented by pre-incubation with sodium nitrite (77). Finally, proinflammatory cytokines were increased and total superoxide dismutase (antioxidant) enzyme activity was reduced in the aorta of the old compared with untreated mice and these differences were abolished by sodium nitrite treatment (13). Collectively, the results of these studies demonstrated that, at least in mice, nitrite supplementation can reverse large elastic artery stiffening with aging by reducing oxidative stress and associated formation of advanced glycations end-products, and these effects coincide with reversal of age-related arterial inflammation.

In studies investigating vascular endothelial function (13), we found that EDD of isolated carotid arteries in response to acetylcholine was impaired in the old compared with the young untreated mice as a result of reduced NO bioavailability (Figure 2E). Administration of a superoxide-scavenging compound restored EDD in the old animals to levels of young mice, suggesting that the reduced NO-mediated EDD was caused by excessive superoxide-induced oxidative stress (Figure 2F). Additional experiments demonstrated that increased activity/expression of NADPH oxidase and uncoupled eNOS were sources of the excessive superoxide, reduced NO bioavailability and consequent impairment in EDD (13). Most importantly, sodium nitrite supplementation completely reversed these age-related differences, restoring endothelial function of the old mice to young adult levels (Figure 2E-F).

We then sought to translate these observations to humans. We performed a small (n=34) randomized, double-blind, placebo-controlled, parallel group design pilot clinical trial of 10 weeks of sodium nitrite at 80 and 160 mg/d doses in a cohort of healthy adults 50-79 years of age (79). Compared with placebo, sodium nitrite supplementation increased plasma nitrite levels, acutely and chronically, in a dose-dependent manner and was well-tolerated. Resting blood pressure was not affected in these healthy men and women with normal baseline levels, but sodium nitrite

treatment significantly improved brachial artery flow mediated dilation (FMD; mean changes: +45% [160 mg/d]; +60% [80 mg/d]) (Figure 3A). Although sodium nitrite supplementation did not change CFPWV vs. placebo, carotid artery beta-stiffness index (a blood pressure-adjusted [reciprocal] expression of carotid artery compliance) was significantly reduced after the 80 mg/d treatment (Figure 3B). Together, these results in humans confirmed our findings in mice that nitrite supplementation may enhance vascular function with aging. Preliminary results from a recent larger clinical trial in healthy middle-aged and older men and women (ClinicalTrials.gov NCT02393742) are consistent with this conclusion (78).

There is substantial interest in nitrate supplementation via the diet as a more "natural" (healthy lifestyle-based) approach for improving vascular health, particularly in clinical populations at risk of CVD (80). A key clinical trial in patients with essential hypertension (18-85 years of age; n=68) (81) found that chronic dietary nitrate supplementation with beetroot juice (250 ml/d) increased plasma nitrite >5-fold, was well-tolerated, and significantly lowered blood pressure assessed in the clinic, under 24-hour ambulatory (free-living) conditions, and at home (self-measured) compared with placebo. Vascular endothelial function (brachial artery FMD) and CFPWV also were improved in the group treated with beetroot juice. Improvements in endothelial function and arterial stiffness have also been observed in patients with hypercholesterolemia (50), and in older adults free of clinical diseases (82) following chronic (i.e., >4 weeks) supplementation with beetroot juice. In heart failure patients with preserved ejection fraction, acute beetroot juice supplementation was reported to reduce systemic vascular resistance and increase cardiac output and exercise capacity (83). Although beneficial effects have not been observed in all scenarios (84), the majority of data suggest dietary approaches to enhancing circulating nitrite and NO bioavailability also hold great promise for improving CV health in humans (85). Indeed, a recently initiated clinical trial by our group seeks to extend these findings to patients with mild-to-severe chronic kidney disease (ClinicalTrials.gov NCT03826147), a group at markedly increased risk of CVD (86).

In conclusion, both pharmacological nitrite and dietary nitrate supplementation represent intriguing healthy lifestyle-inspired strategies for improving vascular function and potentially lowering the risk of incident CVD.

As outlined earlier in this article, dietary nitrate ingestion results in elevated circulating blood nitrate and nitrite levels which can exert effects on the vasculature. However, it is also important to understand if, how and where these ions are transported, stored and metabolized within the body (Figure 1).

## Skeletal muscle as a nitrate reservoir and potential regulator of NO homeostasis

The two NO-generating pathways, NOS-dependent and nitrate-dependent, are shown as a cycle in Figure 4. This cycle is an open system, with exogenous inputs of nitrate and nitrite from the diet and excretion of products by several organs but chiefly the kidneys and lungs. In addition, it is important to keep in mind that all these reactions take place in well-defined compartments – organs and tissue, with either diffusion or transporters being responsible for fluxes of molecules between these compartments.

In the past, blood and the vasculature, including the heart, were the main organs studied in the field of NO (and nitrite and nitrate) biology. The alternate pathway to NO via nitrite reduction was discovered when it was realized that there is a gradient of nitrate between the arterial and venous sides and that deoxyhemoglobin can act as a nitrite reductase (7). Nitrite reduction by other proteins, such as xanthine oxidoreductase (XOR) and aldehyde oxidase (AO), was also described (27, 87). Recently, mammalian nitrate reduction by XOR in the liver was reported (23, 88) and this represented an important step from the previous paradigm that humans rely solely on their commensal bacteria to reduce nitrate to nitrite (see ‘The role of oral microbiota in nitric oxide homeostasis’). Briefly, the accepted paradigm was that nitrate consumed through the diet, together with nitrate derived from oxidation of NO produced by NOS, are the main sources of nitrate in the body and that this nitrate, absorbed by the salivary glands and excreted into the oral cavity, is reduced to nitrite by the oral microbes. Subsequently, nitrite is distributed through the bloodstream into organs such as the heart or liver where nitrite reductases such as XOR, AO or deoxyhemoglobin (in the blood) reduce it to NO. The component parts of this cycle

(i.e., nitrate and nitrite) were considered to be “transient” – that is, easily absorbed from the diet and excreted with a half-life in the order of hours, with no longer-term storage of any of the biochemical entities involved.

Such a situation, which does not include a nitrate/nitrite reservoir, might be considered to be unwise if nitrate/nitrite-derived NO is of high importance for maintaining normal blood pressure and vascular function, as is suggested by previous research (30, 89). The realization that nNOS, one of the three isoforms of NOS, is highly expressed in skeletal muscle tissue, along with myoglobin, a heme protein that is known to be involved in NO metabolism (90, 91), led us to hypothesize that skeletal muscle tissue might play a role in nitrate-nitrite-NO metabolism. We therefore measured nitrate and nitrite levels in rat skeletal muscle and found significantly higher levels of nitrate in skeletal muscle tissue compared with other organs and blood, but with a smaller variation of nitrite distribution between tissues (92).

The discovery of significantly elevated nitrate levels in muscle and the presence of a muscle-to-blood-to-liver nitrate gradient led us to formulate a hypothesis that skeletal muscle serves as an endogenous nitrate reservoir. The natural question which follows is what are the sources of this nitrate and how is nitrate sequestered into muscle cells? The combination of known nitrate-generating pathways (see Figure 4) and the fact that muscle cells contain large amount of nNOS (perhaps especially in type II fibers (93)), myoglobin and XOR, led us to propose that NO produced by nNOS within the skeletal muscle cell is oxidized *in situ* into nitrate by oxymyoglobin. It is also known that nitrate can be directly produced by the so-called ‘futile cycle’ of nNOS (94, 95). To determine the role of nNOS, we measured nitrate in the muscle of wild-type and nNOS knock-out mice and found that the nNOS knock-out mouse had very little nitrate in its skeletal muscle (92). This was also true, but to a lesser extent, for the eNOS knock-out mouse (unpublished results). We also inhibited NOS in Wistar rats using L-NAME, which led to a significant decrease of nitrate levels (unpublished results). Moreover, myoglobin knock-out mice also showed significantly lower amounts of nitrate in their skeletal muscle when compared to their littermates (96). Together, these data suggested that NOS and NO-myoglobin systems are, indeed, endogenous sources of nitrate in skeletal muscle.

Next, we tested the possibility that nitrate can be transported into skeletal muscle from exogeneous, dietary sources. We fed Wistar rats low or high nitrate diets. Consistent with our hypothesis, the low nitrate diet decreased, and the high nitrate diet increased, the amount of nitrate present in rodent skeletal muscle tissue (97). Diet-derived nitrate is sequestered from the bloodstream and transported into muscle cells by anion transporters and, albeit to a lesser extent, by diffusion. There are several active mechanisms of nitrate transport into skeletal muscle cells, such as the nitrate transporter, sialin (98) and chloride channel protein 1 (CLC-1) mediated nitrate transport (99). Sialin is present in human myotubes (100) and the CLC-1 transporter is a chloride channel expressed exclusively in muscle tissue (99, 101). It should be noted, however, that while skeletal muscle has a relatively high nitrate concentration compared, for example, to blood, the nitrate concentration of fresh meat (~10-30 mg/kg) is only ~10% of the nitrate concentration of green leafy vegetables (102).

We also showed that skeletal muscle is not only a passive reservoir supplying necessary nitrate to other more active organs, such as the liver via the bloodstream, but muscle tissue itself is able to use this reservoir *in situ* (97, 103). We investigated whether skeletal muscle tissue contains known nitrate or nitrite reductases and also sought to confirm some earlier reports (104, 105) that XOR is present in skeletal muscle tissue in substantial amounts. We found that the skeletal muscle homogenate, even at neutral pH of 7.4 and oxygen level of 2% (~15 mmHg, which is within the usual level of oxygen for this tissue; 8), is able to reduce nitrate into nitrite and nitrite into NO (103). The process can be inhibited by oxypurinol (XOR inhibitor), but not by L-NAME (NOS inhibitor), and it is more efficient at pH of 6.5 than at pH of 7.4 (103). While this reaction occurs in skeletal muscle homogenate to a much lower extent than in liver homogenate for the same conditions, it is by no means negligible and, due to the substantial amount of skeletal muscle, might be very important for whole-body physiology.

It was of interest to consider whether skeletal muscle uses its endogenous nitrate when metabolic rate is elevated and blood flow requirements are greater, such as during exercise, when muscle oxygen utilization is accelerated and tissue pH

decreases. It had been known for over a century that blood flow into exercising muscle greatly increases and various mechanisms for this phenomenon have been proposed, including a contribution from NO (106). We hypothesized that exercise-induced hyperemia is, at least partially, due to the reduction of resident nitrate into nitrite and NO by skeletal muscle XOR. We exercised rats and measured nitrate and nitrite in skeletal muscle at baseline, immediately post-exercise and at 3 hours after exercise. We found that following exercise, nitrate levels in skeletal muscle decreased while nitrite levels increased (103). These data strongly support the idea that nitrate stored in skeletal muscle is an important source of NO generated during exercise.

Having observed that both exercise and consuming a low nitrate/nitrite diet decreases nitrate levels in skeletal muscle, we next investigated the influence of consuming a high nitrate diet on the muscle nitrate store. We used 7 days of a low nitrate diet to deplete the nitrate reservoir in muscle (nitrate starvation) followed up by 7 days of a high nitrate diet (97). Strikingly, we found that reintroducing nitrate to the diet for less than 3 days quickly and effectively restored muscle nitrate levels. In addition, and to our surprise, after 7 days of access to the high nitrate diet, muscle nitrate values greatly exceeded not only those observed at baseline but also values in muscle of rats that were consuming a high nitrate diet without being first subject to nitrate starvation (see Figure 3 in ref 97). This might be interpreted to indicate that access to nitrate is important to muscle and that nitrate homeostasis is a tightly regulated process, with nitrate deprivation triggering still-to-be-discovered mechanisms at the cellular and molecular levels leading to muscle nitrate "supercompensation" when nitrate is reintroduced to the diet.

Of great interest for the fields of human and exercise physiology is that the first reports on the nitrate content of human skeletal muscle seems to agree with data obtained so far in rodents (107, 108). Wylie et al. (107) reported that baseline nitrate and nitrite concentrations were appreciably higher in muscle than in plasma and that human muscle contains sialin. Ingestion of 13 mmol dietary nitrate was reported to significantly elevate muscle nitrate concentration and, following supplementation, muscle nitrate concentration was decreased by exercise (107). These results confirm the dynamic nature of the nitrate content of skeletal muscle and indicate that

skeletal muscle is sensitive to both nitrate supply and demand. It should be recognized, however, that these investigations are at an early stage, especially in humans, and significant further research is required to elucidate these effects and the mechanisms that underpin them (109). The extent to which muscle function and exercise performance are related to muscle nitrate or nitrite content and may be influenced by dietary nitrate supplementation are also important considerations for future research.

## Applications of dietary nitrate supplementation

The prevalence of dietary supplement use by athletes internationally has been estimated to range between 40-90%, with greater prevalence reported among elite athletes (110). Beetroot juice is one of the supplements that has been increasingly used over the past decade and this has been mirrored by significant research attention which has been aimed at establishing robust evidence for the enhancement of sports performance by dietary nitrate supplementation.

In 2007, Larsen et al. (33) made the remarkable discovery that sodium nitrate supplementation reduced the oxygen cost of submaximal cycling. Similar findings were confirmed using beetroot juice (34), where a ~5% reduction in oxygen uptake at a fixed submaximal power output was reported. These results imply that dietary nitrate permits more muscular work to be performed per unit time for the same energy cost, i.e., that the efficiency of skeletal muscle contraction is enhanced (see ref 111 for meta-analysis). Improved energy efficiency is an important factor for endurance sport performance. However, studies on nitrate supplementation, contraction efficiency and endurance sport performance have shown variable results and it is clear that nitrate supplementation is not beneficial in all instances (14, 112).

As research on nitrate supplementation and endurance sports expanded, it appeared rather difficult to induce ergogenic effects in more highly-trained individuals. For example, in a study comparing groups of different endurance training status, it was concluded that nitrate supplementation was only beneficial for recreational and not for highly-trained athletes with high values of maximal oxygen uptake (113). A recent meta-analysis has confirmed that individuals with high aerobic fitness (i.e. $VO_2$peak >

65 ml/kg/min) do not benefit from nitrate supplementation during endurance exercise (112). In recent years, however, new insights into the mechanisms underpinning the effects of nitrate supplementation on exercise performance has led to a shift in attention towards more high-intensity exercise protocols, where nitrate supplementation might have the potential to improve performance even in elite athletes (14).

The nitrate-nitrite-NO pathway is particularly stimulated under conditions of low pH and low oxygen availability (114), and therefore nitrate supplementation has been suggested to be most beneficial in hypoxia. This could include environmental hypoxia, such as exercise at altitude or underwater, or 'local hypoxia' within the muscle, such as during (partly anaerobic) high-intensity and intermittent exercise. The relatively low oxygen tension surrounding type II (fast-twitch) muscle fibers, may create optimal circumstances for the reduction of nitrite to NO (115), and animal work has shown that nitrate may largely benefit exercise performance through effects on contractile function (116) and blood flow (117) in type II muscle. Well-trained athletes competing in high-intensity sports, such as sprinting, track cycling, speed skating and field sports, likely have a high proportion of type II muscle fibers (118), theoretically increasing the ergogenic potential of nitrate. Furthermore, given the potential for nitrate to elicit a reduction in the oxygen cost of exercise (33, 34), one could argue that nitrate supplementation would be most beneficial in situations where oxygen demand exceeds oxygen supply, such as during very high-intensity exercise. In line with this, recent studies indicate that nitrate may enhance skeletal muscle contractility, power generation, and sprint and repeated sprint performance (for review, see ref. 14).

Repeated-sprint performance in moderately to well-trained subjects has been shown to improve after several days of nitrate supplementation (119, 120, 121, 122, 123). Wylie et al. (122) found no improvement in power during repeated 30-s sprints in recreational athletes, whereas the performance of shorter sprints was improved following nitrate supplementation. These observations may be explained by a predominant effect of nitrate on the initial force production of type II muscle fibers. This would be consistent with an enhanced early-phase force production following nitrate supplementation that has been reported in mouse fast-twitch muscle (116). In

humans, nitrate supplementation has also been reported to enhance force production during the initial phase of muscle contractions (124; see also 125). Furthermore, in animal models, nitrate supplementation can improve muscle calcium handling (116, 126) which may elicit a greater impact during the initial phase of contraction where the calcium saturation is normally incomplete. However, this has yet to be confirmed in humans. Whitfield et al. (32) found that beetroot juice supplementation increased force production at low-stimulation frequencies but without altering the expression of protein targets associated with calcium handling in human skeletal muscle. Obviously, for very high-intensity sport disciplines, a high proportion of, and the ability to recruit, type II muscle fibers may be important to success (127, 128). When comparing repeated 30-s sprint performance following nitrate supplementation between recreational, competitive, and elite sprint athletes, Jonvik et al. (129) found that the improvement in the time to reach peak power was not dependent on the athletes' competition level. As such, it can be suggested that even for elite athletes, in high-intensity sports where rapid acceleration is crucial, nitrate supplementation could result in reaching the top speed faster and improving actual sports performance.

For various reasons, most sports nutrition studies include recreational to well-trained athletes, while very few studies are undertaken on world-class elite athletes (118). To date, only seven studies have investigated the impact of nitrate supplementation (dose: 500-1200 mg/day) in elite or professional endurance athletes ($VO_2$max: >69 ml/kg/min), four of which found no effect on performance (130, 131, 132, 133) and three of which reported small performance-enhancing effects (129, 134, 135). It is obvious, however, that even minor benefits could be very relevant to elite athletes. A performance enhancement of less than 1% generally represents the difference between receiving the gold medal and not even reaching the podium, but such small differences in performance may be difficult to detect using existing scientific approaches (136).

There are several possible reasons why elite endurance athletes may benefit less from nitrate supplementation (137, 138). One possibility is that elite athletes already have high baseline blood nitrate and nitrite concentrations, due to training-induced NOS upregulation (139, 140), and therefore increases in these variables are attenuated

following nitrate supplementation. Consistent with this, Porcelli et al. (113) reported a blunted plasma response to nitrate supplementation in highly trained compared with lesser trained endurance athletes. However, Jonvik et al. (129) reported no difference in either baseline concentrations or the changes in plasma nitrate or nitrite following nitrate supplementation between recreational, competitive and elite sprint athletes. It has been speculated that elite athletes do not respond to nitrate supplementation due to high energy intake and, therefore, high habitual nitrate intake. However, in a large study of >550 well-trained to Olympic athletes (141), the habitual daily dietary nitrate intake of most athletes was substantially lower than that provided by a standard supplemental dose of nitrate. Therefore, a blunted effect of nitrate supplementation in elite athletes in unlikely to be caused by high habitual dietary nitrate intakes.

An open question is whether the type of sports discipline determines whether elite athletes could benefit from nitrate supplementation. Since nitrate supplementation may be most effective in type II muscle fibers and under conditions of low oxygen availability and low pH (115), elite endurance athletes may not benefit much, if at all, from nitrate supplementation. On the other hand, nitrate supplementation might benefit sports events performed at very high intensity which have a high dependency on type II muscle fibers. Consequently, nitrate supplementation may still have ergogenic potential in elite athletes when assessed under such conditions. It should also be considered whether limited effects of nitrate in elite athletes are related to methodological limitations in research conducted in this population. Small sample size and very subtle expected differences are inherent in studies of elite athletes. Furthermore, the potential for there to be minor improvements may be overshadowed by response variability, due to difficulties in standardization of training periods, busy schedules and conflicting priorities. Moreover, potential improvements in performance measures may be difficult to translate to actual sports performance due to a lack of sport-specific tests.

In summary, the ergogenicity of nitrate supplementation is complex, multifactorial and may be highly individual. A possible future approach would be to complete serial testing of the impact of nitrate supplementation on competition performance within the same athlete. This may provide information on the existence of potential responders and non-responders to nitrate supplementation, thereby enabling more tailored and

individualized nutritional approaches, e.g. whether an athlete should consider nitrate supplementation or not, and, if so, how.

*Practical Recommendations*

Based on the available evidence and best practice, the following recommendations for nitrate supplementation in athletes can be made, bearing in mind that we are still far from reaching consensus. The daily dose of nitrate supplementation should be >300 mg (~ 5 mmol), and perhaps considerably higher in more trained athletes. It appears that both acute and multiple-day supplementation protocols could be effective (112). To optimize benefits, the final dose of beetroot juice should be ingested at least 90 min prior to the event and nitrate-rich vegetables a minimum of 3 h prior to the event. It seems that a vegetable source is more effective than nitrate salts (142), taken as a supplement (e.g. 1-2 shots of concentrated beetroot juice) or through ~200-400 g nitrate-rich vegetables per day. A few studies have shown that the effects of nitrate can be achieved via meal ingestion (143, 144, 145). However, whether such large vegetable intakes are feasible still needs to be investigated, and for most athletes consuming a beetroot juice concentrate would be the more practical strategy. Figure 5 presents which athletes could benefit from nitrate supplementation. Nitrate is likely more beneficial under oxygen-limited conditions (114), involving 'local hypoxia' in the muscle. Oxygen will more often be limiting for less trained athletes, who could benefit from nitrate in several types of events, including endurance-type sports. Based on the current evidence, nitrate supplementation may be of limited benefit to elite endurance athletes, while elite athletes competing in very high-intensity exercise tasks may still benefit from nitrate. However, since doses, duration, timing and type of athletes vary substantially between studies, additional research is required to determine the optimal supplementation strategy for nitrate to enhance performance in various sports. Moreover, potential sex-based differences in responses to nitrate supplementation require elucidation (112, 146).

## Conclusions

This review has highlighted our emerging understanding of nitrate and nitrite as storage forms of, and important precursors to, NO production and therefore to human

cardiovascular, neuromuscular and metabolic health, as well as to physiological function. Nitrate and nitrite are continuously produced endogenously as products of NOS-mediated NO production but the body's nitrate and nitrite stores may be augmented exogenously via the diet and are used in the nitrate-nitrite-NO production pathway in situations where NOS function is impaired and when tissue oxygen availability is limited.

This integrated 'NO preservation system' is exquisitely coordinated to enable nitrate and nitrite, however they are produced, to be sequestered, transported, stored and recycled. Dietary nitrate (as well as nitrate produced endogenously which subsequently enters the entero-salivary system) is reduced to nitrite and therefore functionally 'activated' by symbiotic bacteria residing in the oral cavity. This has led to great efforts to identify the key nitrate-reducing bacterial species in the oral microbiota and how they respond to factors such as diet, ageing, and health, fitness and training. This might ultimately lead to the development of probiotic treatments designed to 'optimize' the oral microbiota for the purpose of nitrate-to-nitrite conversion. A high nitrate diet (and nitrate supplementation) increases levels of nitrate and nitrite in the circulation and has been shown to result in beneficial effects on the vasculature including reduced resting blood pressure, reduced large elastic artery stiffness and improved endothelial function. This may explain, in part, the success of diets which emphasize the consumption of fruits and vegetables (especially cruciferous varieties), such as the Mediterranean diet, on indices of cardiovascular health.

While the concentration of nitrate (and nitrite) in plasma and blood cells is greatly increased by nitrate ingestion, peak blood nitrate and nitrite concentrations are transient, with nitrate/nitrite either being excreted from the body (by the kidneys) or distributed into tissues, including skeletal muscle - which may be the main storage site for these NO precursors, due to its considerable total mass. This points to active transport of nitrate from blood to muscle and opens up the intriguing possibility that muscle can release nitrate into the bloodstream when required and perhaps contribute to functional hyperemia. Several recent observations indicate that nitrate may be essential to skeletal muscle, and perhaps wider biological, function: 1) during exercise, muscle nitrate stores are decreased; 2) a high nitrate diet increases the

765 muscle nitrate store whereas a low nitrate diet reduces it; and 3) the muscle nitrate
766 store becomes ‘super-compensated’ when nitrate is reintroduced to the diet following
767 a period of deprivation (akin to what has been established previously with
768 carbohydrate intake and muscle glycogen stores). This sensitivity of skeletal muscle
769 to nitrate availability and the dynamic changes in nitrate and nitrite during exercise
770 and subsequent recovery certainly hints at an under-appreciated physiological role
771 for nitrate with skeletal muscle being central to the maintenance of whole-body nitrate
772 ‘homeostasis’. While this picture of nitrate/nitrite dynamics in skeletal muscle relies
773 mostly on rodent studies to date, there are emerging signs that a similar system is
774 operative in humans.
775
776 If nitrate and/or nitrite are indeed essential for muscle function then the extent to
777 which muscle (and exercise) performance might be enhanced by increasing nitrate
778 stores via dietary nitrate supplementation becomes an intriguing question. It is
779 feasible that supplementation may be beneficial when muscle stores are low relative
780 to demand; on the other hand, there may be a storage ‘ceiling’ in terms of functional
781 outcomes, in which case supplementation would be futile. Over the last decade,
782 many studies have contributed to our understanding of the circumstances under
783 which nitrate supplementation may enhance exercise performance. Key
784 considerations in this regard appear to include: the age, health, sex, aerobic fitness,
785 training status and muscle fiber type of the individual; and the intensity, duration, and
786 nature of the sport or activity.
787
788 While the importance of NO to life is well established, it is clear that there is
789 complexity, redundancy and indeed splendor in the mechanisms by which it is
790 produced, processed, stored and utilized. Much remains to be discovered.
791

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## References


1. Jin RC, Loscalzo J. Vascular nitric oxide: formation and function. *J Blood Med*. 2010,1:147-162.

2. Philippu A. Nitric oxide: A universal modulator of brain function. *Curr Med Chem*. 2016;23:2643-2652.

3. Bogdan C. Nitric oxide and the immune response. *Nat Immun*. 2001;2:907-916.

4. Poderoso JJ, Helfenberger K, Poderoso C. The effect of nitric oxide on mitochondrial respiration. *Nitric Oxide* 2019;88:61-72.

5. Stamler JS, Meissner G. Physiology of nitric oxide in skeletal muscle. *Physiol Rev*. 2001;81(1):209-237.

6. Forstermann U, Sessa W. Nitric oxide synthases: regulation and function. *Eur. Heart J*. 2012;33:829-837.

7. Cosby K, Partovi KS, Crawford JH, Ptel RP, Reiter CD, Martyr S, et al. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med.* 2003;9(12):1498–1505.

8. Carreau A, El Hafny-Rahbi B, Matejuk A, Grillon C, Kieda C. Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *J Cell Mol Med*. 2011;15:1239-1253.

9. Hirai DM, Craig JC, Colburn TD, Eshima H, Kano Y, Sexton WL, et al. Skeletal muscle microvascular and interstitial $PO_2$ from rest to contractions *J Physiol*. 2018;596:869–883.

10. Modin A, Bjorne H, Herulf M, Alving K, Weitzberg E, et al. Nitrite-derived nitric oxide: a possible mediator of acidic–metabolic vasodilation. *Acta Physiol Scand.* 2001;171:9–16.

11. Lauer T,Heiss C, Balzer J, Kehmeier E,Mangold S, et al. Age-dependent endothelial dysfunction is associated with failure to increase plasma nitrite in response to exercise. *Basic Re. Cardiol.* 2008;103:291–97.

12. Kina-Tanada M, Sakanashi M, Tanimoto A, Kaname T, Matsuzaki T, Noguchi K, et al. Long-term dietary nitrite and nitrate deficiency causes the metabolic syndrome, endothelial dysfunction and cardiovascular death in mice. *Diabetologia* 2017;60:1138-1151.

13. Sindler AL, Fleenor BS, Calvert JW, Marshall KD, Zigler ML, Lefer DJ, et al. Nitrite supplementation reverses vascular endothelial dysfunction and large elastic artery stiffness with aging. *Aging Cell*. 2011;10(3):429-437.

14. Jones AM, Thompson C, Wylie LJ, Vanhatalo A. Dietary nitrate and physical performance. Annu Rev Nutr. 2018;38:303-328.

15. Lundberg JO, Carlstrom M, Larsen FJ,Weitzberg E. Roles of dietary inorganic nitrate in cardiovascular health and disease. *Cardiovasc Res.* 2010;89:525–32.

16. Hord NG, Tang Y, Bryan NS. Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *Am J Clin Nutr.* 2009;90:1–10.

17. Kyriacou MC, Soteriou GA, Colla G, Rouphael Y. The occurrence of nitrate and nitrite in Mediterranean fresh salad vegetables and its modulation by preharvest practices and postharvest conditions. *Food Chem*. 2019;285:468-477.

18. Govoni M, Jansson EA,Weitzberg E, Lundberg JO. The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide* 2008;19:333–37.

19. Doel JJ, Benjamin N, Hector MP, Rogers M, Allaker RP. Evaluation of bacterial nitrate reduction in the human oral cavity. *Eur J Oral Sci.* 2005;113:14-19

20. Duncan C, Dougall H, Johnston P, Green S, Brogan R, Leifert C, et al. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. Nat Med. 1995;1:546-551.

21. Benjamin N, O'Driscoll F, Dougall H, Duncan F, Smith L, et al. 1994. Stomach NO synthesis. *Nature* 1994;368:502.

22. Wylie LJ, Kelly J, Bailey SJ, Blackwell JR, Skiba PF, Winyard PG, Jeukendrup AE, Vanhatalo A, Jones AM. Beetroot juice and exercise: pharmacodynamic and dose-response relationships. *J Appl Physiol*. 2013;115(3):325-336.

23. Jansson EA, Huang L, Malkev R, Govoni M, Nihlen C, Olsson A, et al. A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis. *Nat Chem Biol*. 2008;4:411- 417.

24. Gilchrist M, Winyard PG, Benjamin N. Dietary nitrate--good or bad? *Nitric Oxide*. 2010;22:104-109.

25. Briskey D, Tucker PS, Johnson DW, Coombes JS. Microbiota and the nitrogen cycle: Implications in the development and progression of CVD and CKD. *Nitric Oxide* 2016;57:64-70.

26. Kumar PS. From focal sepsis to periodontal medicine: a century of exploring the role of the oral microbiome in systemic disease. *J Physiol*. 2017;595(2):465-476.

27. Maia LB, Moura JJG**.** Putting xanthine oxidoreductase and aldehyde oxidase on the NO metabolism map: Nitrite reduction by molybdoenzymes. *Redox Biol*. 2018;19:274-289.

28. Ashworth A, Mitchell K, Blackwell JR, Vanhatalo A, Jones AM. High-nitrate vegetable diet increases plasma nitrate and nitrite concentrations and reduces blood pressure in healthy women. *Public Health Nutr*. 2015;18:2669-78.

29. Kelly J, Fulford J, Vanhatalo A, Blackwell JR, French O, Bailey SJ, et al. Effects of short-term dietary nitrate supplementation on blood pressure, O2 uptake kinetics, and muscle and cognitive function in older adults. *Am J Physiol Regul Integr Comp Physiol*. 2013;304(2):R73-83.

30. Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, et al. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension*. 2008;51:784-790.

31. Coggan AR, Leibowitz JL, Spearie CA, Kadkhodayan A, Thomas DP, Ramamurthy S, et al. Acute dietary nitrate intake improves muscle contractile function in patients with heart failure: a double-blind, placebo-controlled,

897 randomized trial. *Circ Heart Fail*. 2015;8:914-920.

898 32. Whitfield J, Gamu D, Heigenhauser GJF, Van Loon LJC, Spriet LL, Tupling
899 AR, Holloway GP. Beetroot juice increases human muscle force without changing
900 Ca2+-handling proteins. *Med Sci Sports Exerc*. 2017;49:2016-2024.

33. Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B. Effects of dietary nitrate on oxygen cost during exercise. Acta Physiol. 2007;191(1):59-66.

901 34. Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, Dimenna FJ, Wilkerson DP,
902 et al. Dietary nitrate supplementation reduces the $O_2$ cost of low-intensity exercise
903 and enhances tolerance to high-intensity exercise in humans. *J Appl Physiol.*
904 2009;107:1144-1155.

905 35. Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Pavey TG, Wilkerson DP,
906 et al. Acute and chronic effects of dietary nitrate supplementation on blood
907 pressure and the physiological responses to moderate-intensity and incremental
908 exercise. *Am J Physiol Regul Integr Comp Physiol*. 2010;299(4):R1121-1131.

909 36. Justice JN, Johnson LC, DeVan AE, Cruickshank-Quinn C, Reisdorph N,
910 Bassett CJ, et al. Improved motor and cognitive performance with sodium nitrite
911 supplementation is related to small metabolite signatures: a pilot trial in middle-
912 aged and older adults. *Aging*. 2015;7(11):1004-1021.

913 37. Presley TD, Morgan AR, Bechtold E, Clodfelter W, Dove RW, Jennings JM, et
914 al. Acute effect of a high nitrate diet on brain perfusion in older adults. *Nitric Oxide*.
915 2011;4:34-42.

916 38. Kapil V, Haydar SM, Pearl V, Lundberg JO, Weitzberg E, Ahluwalia A.
917 Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free*
918 *Radic Biol Med*. 2013;55:93-100.

919 39. McDonagh ST, Wylie LJ, Winyard PG, Vanhatalo A, Jones AM. The effects of
920 chronic nitrate supplementation and the use of strong and weak antibacterial
921 agents on plasma nitrite concentration and exercise blood pressure. *Int J Sports*
922 *Med*. 2015;36:1177-1185.

923 40. Joshipura KJ, Muñoz-Torres FJ, Morou-Bermudez E, Patel RP. Over-the-
924 counter mouthwash use and risk of pre-diabetes/diabetes. *Nitric Oxide*.
925 2017;71:14-20.

926 41. Ashworth A, Cutler C, Farnham G, Liddle L, Burleigh M, Rodiles A, et al.
927 Dietary intake of inorganic nitrate in vegetarians and omnivores and its impact on
928 blood pressure, resting metabolic rate and the oral microbiome. *Free Radic Biol*
929 *Med*. 2019;138:63-72.

930 42. Tribble GD, Angelov N, Weltman R, Wang BY, Eswaran SV, Gay IC, et al.
931 Frequency of tongue cleaning impacts the human tongue microbiome composition
932 and enterosalivary circulation of nitrate. *Front Cell Infect Microbiol*. 2019;9:39.

933 43. Hyde ER, Andrade F, Vaksman Z, Parthasarathy K, Jiang H, Parthasarathy
934 DK, et al. Metagenomic analysis of nitrate-reducing bacteria in the oral cavity:
935 implications for nitric oxide homeostasis. *PLoS One*. 2014;9(3):e88645.

936 44. Vanhatalo A, Blackwell JR, L'Heureux JE, Williams DW, Smith A, van der
937 Giezen M, et al. Nitrate-responsive oral microbiome modulates nitric oxide
938 homeostasis and blood pressure in humans. *Free Radic Biol Med*. 2018;124:21-
939 30.

940 45. Zaura E, Brandt BW, Prodan A, Teixeira de Mattos MJ, Imangaliyev S, Kool J,
941 et al. On the ecosystemic network of saliva in healthy young adults. *ISME J*
942 2017;;11(5):1218-1231.

943 46. Wu J, Peters BA, Dominianni C, Zhang Y, Pei Z, Yang L, et al.
944 Cigarette smoking and the oral microbiome in a large study of American adults.
945 *ISME J*. 2016;10:2435-2446.

946 47. Feres M, Teles F, Teles R, Figueiredo LC, Faveri M. The subgingival
947 periodontal microbiota of the aging mouth. *Periodontol 2000*. 2016;72:30-53.

948 48. Hansen TH, Kern T, Bak EG, Kashani A, Allin KH, Nielsen T, et al. Impact of a
949 vegan diet on the human salivary microbiota. *Sci Rep*. 2018;8(1):5847.

950 49. De Filippis F, Vannini L, La Storia A, Laghi L, Piombino P, Stellato G, et al.
951 The same microbiota and a potentially discriminant metabolome in the saliva of
952 omnivore, ovo-lacto-vegetarian and Vegan individuals. *PLoS One*.
953 2014;9(11):e112373.

954 50. Velmurugan S, Gan JM, Rathod KS, Khambata RS, Ghosh SM, Hartley A, et
955 al. Dietary nitrate improves vascular function in patients with
956 hypercholesterolemia: a randomized, double-blind, placebo-controlled study. *Am J*

*Clin Nutr*. 2016;103:25-38.

51. Burleigh M, Liddle L, Muggeridge DJ, Monaghan C, Sculthorpe N, Butcher J, et al. Dietary nitrate supplementation alters the oral microbiome but does not improve the vascular responses to an acute nitrate dose. *Nitric Oxide*. 2019;89:54-63.

52. Liddle L, Burleigh MC, Monaghan C, Muggeridge DJ, Sculthorpe N, Pedlar CR et al. Variability in nitrate-reducing oral bacteria and nitric oxide metabolites in biological fluids following dietary nitrate administration: An assessment of the critical difference. *Nitric Oxide*. 2019;83:1-10.

53. Goh CE, Trinh P, Colombo PC, Genkinger JM, Mathema B, Uhlemann AC, et al. Association between nitrate-reducing oral bacteria and cardiometabolic Outcomes: results from ORIGINS. *J Am Heart Assoc*. 2019;8(23):e013324.

54. Benjamin EJ, Muntner P, Alonso A, Bittencourt MS, Callaway CW, Carson AP, et al. Heart Disease and Stroke Statistics-2019 Update: A Report From the American Heart Association. *Circulation*. 2019;139(10):e56-e528.

55. Joseph P, Leong D, McKee M, Anand SS, Schwalm JD, Teo K, et al. Reducing the Global Burden of Cardiovascular Disease, Part 1: The Epidemiology and Risk Factors. *Circ Res*. 2017;121(6):677-694.

56. Heidenreich PA, Trogdon JG, Khavjou OA, Butler J, Dracup K, Ezekowitz MD, et al. Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association. *Circulation*. 2011;123(8):933-944.

57. Lakatta EG. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part III: cellular and molecular clues to heart and arterial aging. *Circulation*. 2003;107(3):490-497.

58. Hughes TM, Craft S, Lopez OL. Review of 'the potential role of arterial stiffness in the pathogenesis of Alzheimer's disease'. *Neurodegener Dis Manag*. 2015;5(2):121-135.

59. Mitchell GF. Effects of central arterial aging on the structure and function of the peripheral vasculature: implications for end-organ damage. *J Appl Physiol*. 2008;105(5):1652-1660.

60. Scuteri A, Tesauro M, Appolloni S, Preziosi F, Brancati AM, Volpe M. Arterial stiffness as an independent predictor of longitudinal changes in cognitive function in the older individual. *J Hypertens*. 2007;25(5):1035-1040.

61. van Sloten TT, Sedaghat S, Laurent S, London GM, Pannier B, Ikram MA, et al. Carotid stiffness is associated with incident stroke: a systematic review and individual participant data meta-analysis. *J Am Coll Cardiol*. 2015;66(19):2116-2125.

62. Townsend RR, Wilkinson IB, Schiffrin EL, Avolio AP, Chirinos JA, Cockcroft JR, et al. Recommendations for improving and standardizing vascular research on arterial stiffness: A scientific statement from the Aamerican Heart Association. *Hypertension*. 2015;66(3):698-722.

63. Lind L, Berglund L, Larsson A, Sundstrom J. Endothelial function in resistance and conduit arteries and 5-year risk of cardiovascular disease. *Circulation*. 2011;123(14):1545-1551.

64. Seals DR, Jablonski KL, Donato AJ. Aging and vascular endothelial function in humans. *Clin Sci (Lond)*. 2011;120(9):357-375.

65. Yeboah J, Crouse JR, Hsu FC, Burke GL, Herrington DM. Brachial flow-mediated dilation predicts incident cardiovascular events in older adults: the Cardiovascular Health Study. *Circulation.* 2007;115(18):2390-2397.

66. Donato AJ, Morgan RG, Walker AE, Lesniewski LA. Cellular and molecular biology of aging endothelial cells. J Mol Cell Cardiol. 2015;89(Pt B):122-135.

67. Green DJ, Dawson EA, Groenewoud HM, Jones H, Thijssen DH. Is flow-mediated dilation nitric oxide mediated?: A meta-analysis. *Hypertension*. 2014;63(2):376-382.

68. Fleenor BS. Large elastic artery stiffness with aging: novel translational mechanisms and interventions. *Aging Dis*. 2013;4(2):76-83.

69. Donato AJ, Eskurza I, Silver AE, Levy AS, Pierce GL, Gates PE, et al. Direct evidence of endothelial oxidative stress with aging in humans: relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-kappaB. *Circ Res*. 2007;100(11):1659-1666.

70. Eskurza I, Myerburgh LA, Kahn ZD, Seals DR. Tetrahydrobiopterin augments endothelium-dependent dilatation in sedentary but not in habitually exercising older adults. *J Physiol*. 2005;568(Pt 3):1057-1065.

71. Nowak KL, Rossman MJ, Chonchol M, Seals DR. Strategies for achieving healthy vascular aging. *Hypertension*. 2018;71(3):389-402.

72. LaRocca TJ, Martens CR, Seals DR. Nutrition and other lifestyle influences on arterial aging. *Ageing Res Rev*. 2017;39:106-119.

73. Craighead DH, Freeberg KA, Seals DR. The protective role of regular aerobic exercise on vascular function with aging. *Curr Opin Physiol*. 2019;10:55-63.

74. Rossman MJ, LaRocca TJ, Martens CR, Seals DR. Healthy lifestyle-based approaches for successful vascular aging. *J Appl Physiol*. 2018;125(6):1888-1900.

75. Bryan NS. Nitrite in nitric oxide biology: cause or consequence? A systems-based review. *Free Radic Biol Med*. 2006;41(5):691-701.

76. Sindler AL, Devan AE, Fleenor BS, Seals DR. Inorganic nitrite supplementation for healthy arterial aging. *J Appl Physiol*. 2014;116(5):463-477.

77. Fleenor BS, Sindler AL, Eng JS, Nair DP, Dodson RB, Seals DR. Sodium nitrite de-stiffening of large elastic arteries with aging: role of normalization of advanced glycation end-products. *Exp Gerontol*. 2012;47(8):588-594.

78. Woodward KA, Santos-Parker JR, Lubieniecki KL, Nagy EE, Bryan NS, Chonchol M, et al. Sodium nitrite supplementation improves vascular endothelial function but not motor or cognitive function in middle-gged and older adults. *FASEB J*. 2019;33(1):833.13.

79. DeVan AE, Johnson LC, Brooks FA, Evans TD, Justice JN, Cruickshank-Quinn C, et al. Effects of sodium nitrite supplementation on vascular function and related small metabolite signatures in middle-aged and older adults. *J Appl Physiol*. 2016;120(4):416-425.

80. Ahluwalia A, Gladwin M, Coleman GD, Hord N, Howard G, Kim-Shapiro DB, et al. Dietary nitrate and the epidemiology of cardiovascular disease: report from a national heart, lung, and blood institute workshop. *J Am Heart Assoc*. 2016;5(7):e003402.

81. Kapil V, Khambata RS, Robertson A, Caulfield MJ, Ahluwalia A. Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2, double-blind, placebo-controlled study. *Hypertension*. 2015;65(2):320-327.

82. Rammos C, Hendgen-Cotta UB, Sobierajski J, Bernard A, Kelm M, Rassaf T. Dietary nitrate reverses vascular dysfunction in older adults with moderately increased cardiovascular risk. *J Am Coll Cardiol*. 2014;63(15):1584-1585.

83. Zamani P, Rawat D, Shiva-Kumar P, Geraci S, Bhuva R, Konda P, et al .Effect of inorganic nitrate on exercise capacity in heart failure with preserved ejection fraction. *Circulation*. 2015;131(4):371-380.

84. Broxterman RM, La Salle DT, Zhao J, Reese VR, Richardson RS, Trinity JD. Influence of dietary inorganic nitrate on blood pressure and vascular function in hypertension: prospective implications for adjunctive treatment. *J Appl Physiol*. 2019;127(4):1085-1094.

85. Jackson JK, Patterson AJ, MacDonald-Wicks LK, Oldmeadow C, McEvoy MA. The role of inorganic nitrate and nitrite in cardiovascular disease risk factors: a systematic review and meta-analysis of human evidence. *Nutr Rev*. 2018;76(5):348-371.

86. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med*. 2004;351(13):1296-1305.

87. Li H, Samouilov A, Liu X, Zweier JL. Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrite reduction. Evaluation of its role in nitric oxide generation in anoxic tissues. *J Biol Chem*. 2001;276:24482-24489.

88. Li H, Samouilov A, Liu X, Zweier JL. Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrite reduction. Evaluation of its role in nitric oxide generation in anoxic tissues. *Biochemistry*. 2003;42:1150-1159.

89. Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, et

al. Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertension*. 2010;56(2):274-281.

90. Ormerod JOM, Ashrafian H, Maher AR, Arif S, Steeples V, Born GVR, et al. The role of vascular myoglobin in nitrite-mediated blood vessel relaxation. *Cardiovasc Res*. 2011;89:560-565.

91. Totzeck M, Hendgen-Cotta UB, Kelm M, Rassaf T. Crosstalk between nitrite, myoglobin and reactive oxygen species to regulate vasodilation under hypoxia. *PLOS One.* 2014;9(8):e105951.

92. Piknova B, Park JW, Swanson KM, Dey S, Noguchi CT, Schechter AN. Skeletal muscle as an endogenous nitrate reservoir. *Nitric Oxide*. 2015;47:10-16.

93. Kobzik L, Reid MB, Bredt DS, Stamler JS. Nitric oxide in skeletal muscle. *Nature*. 1994;372(6506):546-548.

94. Santolini J, Meade AL, Stuehr DJ. Differences in three kinetic parameters underpin the unique catalytic profiles of nitric-oxide synthases I, II, and III. *J Biol Chem*. 2001;276:48887-48898.

95. Tejero J, Hunt AP, Santolini J, Lehnert N, Stuehr DJ. Mechanism and regulation of ferrous heme-nitric oxide (NO) oxidation in NO synthases. *J Biol Chem*. 2019;294:7904-7916.

96. Park JW, Piknova B, Dey S, Noguchi CT, Schechter AN. Compensatory mechanisms in myoglobin deficient mice preserve NO homeostasis. *Nitric Oxide*. 2019; 90:10-14.

97. Gilliard CN, Lam JK, Cassel KS, Park JW, Schechter AN, Piknova B. Effect of dietary nitrate levels on nitrate fluxes in rat skeletal muscle and liver. *Nitric Oxide*. 2018;75:1-7.

98. Qin L, Liu X, Sun Q, Fan Z, Xia D, Ding G, et al. Sialin (SLC17A5) functions as a nitrate transporter in the plasma membrane. *PNAS* 2012;109:13434-13439.

99. Rychkov GY, Pusch M, Roberts ML, Jentsch TJ, Bretag AH. Permeation and Block of the Skeletal Muscle Chloride Channel, ClC-1, by Foreign Anions. *J Gen Physiol*. 1998;111:653–665.

100. Srihirun S, Park JW, Teng R, Sawaengdee W, Piknova B, Schechter AN. Nitrate uptake and metabolism in human skeletal muscle cell cultures. *Nitric Oxide*. 2020;94:1-8.

101. Poroca DR, Pelis RM, Chappe VM. ClC Channels and Transporters: Structure, Physiological Functions, and Implications in Human Chloride Channelopathies. *Front Pharmacol*. 2017;8:151.

102. Kalaycıoğlu Z, Erim FB. Nitrate and Nitrites in Foods: Worldwide Regional Distribution in View of Their Risks and Benefits. *J Agric Food Chem*. 2019;67(26):7205-7222.

103. Piknova B, Park JW, Lam KKJ, Schechter AN. Nitrate as a source of nitrite and nitric oxide during exercise hyperemia in rat skeletal muscle. *Nitric Oxide*. 2016;55-56:54-61.

104. Apple FS; Hyde JE; Ingersoll-Stroubos AM, Theologides A. Geographic distribution of xanthine oxidase, free radical scavengers, creatine kinase, and lactate dehydrogenase enzyme systems in rat heart and skeletal muscle. *American Journal of Anatomy*. 1991;192(3): 319-323.

105. Dorion D, Zhong A, Chiu C, Forrest CR, Boyd B, Pang CY. Role of xanthine oxidase in reperfusion injury of ischemic skeletal muscles in the pig and human. *J Appl Physiol*. 1993;75(1):246-255.

106. Tschakovsky ME, Joyner MJ. Nitric oxide and muscle blood flow in exercise. *Appl Physiol Nutr Metab*. 2008;33(1):151-61.

107. Wylie LJ, Park JW, Vanhatalo A, Kadach S, Black MI, Stoyanov Z, et al. Human skeletal muscle nitrate store: influence of dietary nitrate supplementation and exercise. *J Physiol*. 2019;597(23):5565-5576.

108. Nyakayiru J, Kouw IWK, Cermak NM, Senden JM, van Loon LJC,

1142 Verdijk LB. Sodium nitrate ingestion increases skeletal muscle nitrate content in
1143 humans. *J Appl Physiol*. 2017;123:637–644.

1144

1145 109. Nyakayiru J, van Loon LJC, Verdijk LB. Could intramuscular storage of
1146 dietary nitrate contribute to its ergogenic effect? A mini-review. *Free Radic Biol*
1147 *Med*. 2020;152:295-300.

1148

1149 110. Knapik JJ, Steelman RA, Hoedebecke SS, Austin KG, Farina EK,
1150 Lieberman HR. Prevalence of Dietary Supplement Use by Athletes: Systematic
1151 Review and Meta-Analysis. *Sports Med*. 2016;46(1):103-123.

1152

1153 111. Pawlak-Chaouch M, Boissière J, Gamelin FX, Cuvelier G, Berthoin S,
1154 Aucouturier J. Effect of dietary nitrate supplementation on metabolic rate
1155 during rest and exercise in human: A systematic review and a meta-analysis.
1156 *Nitric Oxide*. 2016;53:65-76.

1157

1158 112. Senefeld JW, Wiggins CC, Regimbal RJ, Dominelli PB, Baker SE, Joyner
1159 MJ. Ergogenic effect of nitrate supplementation. A systematic review and meta-
1160 analysis. *Med Sci Sports Exerc*, in press.

113. Porcelli S, Ramaglia M, Bellistri G, Pavei G, Pugliese L, Montorsi M, et al. Aerobic Fitness Affects the Exercise Performance Responses to Nitrate Supplementation. *Med Sci Sports Exerc*. 2015;47(8):1643-1651.

114. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov*. 2008;7:156-167.

115. Jones AM, Ferguson SK, Bailey SJ, Vanhatalo A, Poole DC. Fiber Type-Specific Effects of Dietary Nitrate. *Exerc Sport Sci Rev*. 2016;44(2):53-60.

116. Hernandez A, Schiffer TA, Ivarsson N, Cheng AJ, Bruton JD, Lundberg JO, et al. Dietary nitrate increases tetanic [Ca2+]i and contractile force in mouse fast-twitch muscle. *J Physiol*. 2012;590(15):3575-3583.

117. Ferguson SK, Holdsworth CT, Wright JL, Fees AJ, Allen JD, Jones AM, et al. Microvascular oxygen pressures in muscles comprised of different fiber types: Impact of dietary nitrate supplementation. *Nitric Oxide*. 2015;48:38-43.

118. Burke LM. Practical Issues in Evidence-Based Use of Performance Supplements: Supplement Interactions, Repeated Use and Individual Responses. *Sports Med*. 2017;47(Suppl 1):79-100.

119. Wylie LJ, Mohr M, Krustrup P, Jackman SR, Ermiotadis G, Kelly J, et al. Dietary nitrate supplementation improves team sport-specific intense intermittent exercise performance. *Eur J Appl Physiol*. 2013;113:1673-1684.

120. Thompson C, Wylie LJ, Fulford J, Kelly J, Black MI, McDonagh STJ, et al. Dietary nitrate improves sprint performance and cognitive function during prolonged intermittent exercise. *Eur J Appl Physiol*. 2015;115(9):1825-1834.

121. Thompson C, Vanhatalo A, Jell H, Fulford J, Carter J, Nyman L, et al. Dietary nitrate supplementation improves sprint and high-intensity intermittent running performance. *Nitric Oxide*. 2016;61:55-61.

122. Wylie LJ, Bailey SJ, Kelly J, Blackwell JR, Vanhatalo A, Jones AM. Influence of beetroot juice supplementation on intermittent exercise performance. *Eur J Appl Physiol.* 2016;116(2):415-425.

123. Nyakayiru J, Jonvik KL, Trommelen J, Pinckaers PJ, Senden JM, van Loon LJ, et al. Beetroot Juice Supplementation Improves High-Intensity Intermittent Type Exercise Performance in Trained Soccer Players. *Nutrients.* 2017;9(3).

124. Haider G, Folland JP. Nitrate supplementation enhances the contractile properties of human skeletal muscle. *Med Sci Sports Exerc*. 2014;46(12):2234-2243.

125. Coggan AR, Leibowitz JL, Kadkhodayan A, Thomas DP, Ramamurthy S, Spearie CA, et al. Effect of acute dietary nitrate intake on maximal knee extensor speed and power in healthy men and women. *Nitric Oxide*. 2015;48:16-21.

126. Bailey SJ, Gandra PG, Jones AM, Hogan MC, Nogueira L. Incubation with sodium nitrite attenuates fatigue development in intact single mouse fibres at physiological PO2. *J Physiol*. 2019;597(22):5429-5443.

127. Mero A, Komi PV, Gregor RJ. Biomechanics of sprint running. A review. *Sports Med*. 1992;13(6):376-392.

128. Lockie RG, Murphy AJ, Knight TJ, Janse de Jonge XA. Factors that differentiate acceleration ability in field sport athletes. *J Strength Cond Res*. 2011;25(10):2704-2714.

129. Jonvik KL, Nyakayiru J, van Dijk JW, Maase K, Ballak SB, Senden JM, et al. Repeated-sprint performance and plasma responses following beetroot juice supplementation do not differ between recreational, competitive and elite sprint athletes. *Eur J Sport Sci*. 2018;18(4):524-533.

130. Peacock O, Tjonna AE, James P, Wisloff U, Welde B, Bohlke N, et al. Dietary nitrate does not enhance running performance in elite cross-country skiers. *Med Sci Sports Exerc*. 2012;44:2213-2219.

131. Christensen PM, Nyberg M, Bangsbo J. Influence of nitrate supplementation on VO(2) kinetics and endurance of elite cyclists. *Scand J Med Sci Sports*. 2013;23:e21-31.

132. Boorsma RK, Whitfield J, Spriet LL. Beetroot juice supplementation does not improve performance of elite 1500-m runners. *Med Sci Sports Exerc*. 2014;46(12):2326-2334.

133. Sandbakk SB, Sandbakk O, Peacock O, James P, Welde B, Stokes K, et al. Effects of acute supplementation of L-arginine and nitrate on endurance and sprint performance in elite athletes. *Nitric Oxide*. 2015;48:10-15.

134. Peeling P, Cox GR, Bullock N, Burke LM. Beetroot Juice Improves On-Water 500 M Time-Trial Performance, and Laboratory-Based Paddling Economy in National and International-Level Kayak Athletes. *Int J Sport Nutr Exerc Metabol*. 2015;25(3):278-284.

135. Jonvik KL, Van Dijk JW, Senden JMG, Van Loon LJC, Verdijk LB. The Effect of Beetroot Juice Supplementation on Dynamic Apnea and Intermittent Sprint Performance in Elite Female Water Polo Players. *Int J Sport Nutr Exerc Metab*. 2018;28(5):468-473.

136. Hopkins WG, Schabort EJ, Hawley JA. Reliability of power in physical performance tests. *Sports Med*. 2001;31(3):211-234.

137. Jones AM. Influence of dietary nitrate on the physiological determinants of exercise performance: a critical review. *Appl Physiol Nutr Metab*. 2014;39(9):1019-1028.

138. Jonvik KL, Nyakayiru J, van Loon LJ, Verdijk LB. Can elite athletes benefit from dietary nitrate supplementation? *J Appl Physiol*. 2015;119(6):759-761.

139. McAllister RM, Laughlin MH. Vascular nitric oxide: effects of physical activity, importance for health. *Essays Biochem* 2006;42:119–131.

140. McConell GK, Bradley SJ, Stephens TJ, Canny BJ, Kingwell BA, Lee-Young RS. Skeletal muscle nNOS mu protein content is increased by exercise training in humans. *Am J Physiol Regul Integr Comp Physiol* 2007;293(2):R821–R828.

141. Jonvik KL, Nyakayiru J, Van Dijk JW, Wardenaar FC, Van Loon LJ, Verdijk LB. Habitual Dietary Nitrate Intake in Highly Trained Athletes. *Int J Sport Nutr Exerc Metab*. 2017;27(2):148-157.

142. Jonvik KL, Nyakayiru J, Pinckaers PJ, Senden JM, van Loon LJ, Verdijk LB. Nitrate-Rich Vegetables Increase Plasma Nitrate and Nitrite Concentrations and Lower Blood Pressure in Healthy Adults. *J Nutr.* 2016;146(5):986-993.

143. Hobbs DA, Kaffa N, George TW, Methven L, Lovegrove JA. Blood pressure-lowering effects of beetroot juice and novel beetroot-enriched bread products in normotensive male subjects. *Br J Nutr*. 2012;108:2066-2074.

144. Liu AH, Bondonno CP, Croft KD, Puddey IB, Woodman RJ, Rich L, et al. Effects of a nitrate-rich meal on arterial stiffness and blood pressure in healthy volunteers. *Nitric Oxide*. 2013;35:123-130.

145. Porcelli S, Pugliese L, Rejc E, Pavei G, Bonato M, Montorsi M, La Torre A, Rasica L, Marzorati M. Effects of a Short-Term High-Nitrate Diet on Exercise Performance. Nutrients. 2016; 8(9):534.

146. Wickham KA, Spriet LL. No longer beeting around the bush: a review of potential sex differences with dietary nitrate supplementation (1). *Appl Physiol Nutr Metab*. 2019;44(9):915-924.

## Figure Legends

**Figure 1.** The ‘nitrate cycle’. Schematic illustration, based on the ‘Wasserman gears’ concept, of an integrated system involving multiple organs for the processing and preservation of the nitric oxide precorsors, nitrate and nitrite. Some of the nitrate produced endogenously via oxidation of the products of the NOS-catalyzed synthesis of NO or which is introduced to the body via the diet can be reduced to nitrite by facultative bacteria in the oral cavity. These ions enter the bloodsteam via the upper intestine and maintain a circulating reservoir of NO intermediaries which can be delivered to other organs or used in physiological processes such as vasodilation. Nitrate and nitrite can be taken up by (and are produced by) various tissues (of which skeletal muscle is the largest by mass) and stored, where they may contribute to metabolism and organ function or be released into the circulation for transportation. This theoretical model decribes a dynamic system which is sensitive to NOS activity and dietary nitrate ingestion and functions to maintain sufficient ‘substrate’ for NO production.

**Figure 2.** Aortic pulse wave velocity (A), superoxide production (electron paramagnetic resonance [EPR] spectroscopy signal) (B), and abundance of ntirotyrosine (C) and advanced glycation end-products (AGEs) (D), and isolated carotid artery endothelium-dependent dilation to acetylcholine (ACh) (E) and nitric oxide (NO)-dependent dilation (maximal dilation with ACh - maximal dilation with ACh in the presence of an NO synthase inhibitor) (F) in young control, old control and old nitrite-supplemented (Old Nitrite) mice. Values are means ± SE. *$P < 0.05$ Old Control vs. Old Nitrite. Data were adapted from (13, 77).

**Figure 3.** Brachial artery flow-mediated dilation (A) and carotid artery beta-stiffness index (B) at baseline (pre) and after (post) placebo or sodium nitrite (80 mg/day or 160 mg/day) supplementation. Values are means ± SE. *$P < 0.05$ vs. pre-supplementation within group. Data were adapted from (79).

**Figure 4**. Summary of nitrite and nitrate reduction and oxidation pathways and NO generation cycle with most of known proteins and reactions involved. This cycle is as an open system, with exogenous and endogenous inputs from NOS, diet and

bacteria and excretion by kidneys and lungs. Concentrations marked for NO, nitrite and nitrate are those detected (nitrite, nitrate) or estimated (NO) in bloodstream. Mo-Co proteins is a family of molybdenum-containing proteins which is mammals consists of xanthine oxidoreductase (XOR), aldehyde oxidase, sulfite oxidase, MARC-1 and MARC-2 proteins. $Fe^{II}$-heme proteins is a large family of proteins including, but not limited to hemoglobin, myoglobin and cytochromes.

**Figure 5**. Athletes likely to benefit from nitrate supplementation. The top section presents sports where oxygen is the limiting factor and where nitrate supplementation is more likely beneficial for performance. The bottom section presents increasing rate of effect in different athlete groups; no effect in elite endurance, small effect in elite sprint/power, medium effect in recreational endurance and large effect in recreational sprint/power athletes.