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# The Noncanonical Pathway for In Vivo Nitric Oxide Generation: The Nitrate-Nitrite-Nitric Oxide Pathway

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**ABBREVIATIONS:** ABP, ambulatory blood pressure; ADI, acceptable daily intake; AMI, acute myocardial infarction; AMPK, AMP-activated protein kinase; AO, aldehyde oxidase; ApoE, apolipoprotein E; BNP, brain natriuretic peptide; BP, blood pressure; CABG, coronary artery bypass grafting; CCL2, chemokine (C-C motif) ligand 2; cGMP, cyclic GMP; COPD, chronic obstructive pulmonary disease; C-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; CV, cardiovascular; CVD, cardiovascular disease; CXCL2, chemokine (C-X-C motif) ligand 2; DASH, Dietary Approaches to Stop Hypertension; DBP, diastolic blood pressure; deoxyHb, deoxyhemoglobin; deoxyMb, deoxymyoglobin; dP/dt, rate of change of pressure; DOCA, deoxycorticosterone acetate; EDRF, endothelium-derived relaxing factor; eNOS, endothelial nitric oxide synthase; Fe(II), ferrous; Fe(III), ferric; FMD, flow-mediated dilation; GAG, glycosaminoglycan; GC-1, soluble guanylyl cyclase; GI, gastrointestinal; GTN, glyceryl trinitrate; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFREF, heart failure with reduced ejection fraction; ICAM-1, intercellular adhesion molecule-1; IDO1, indoleamine 2,3-dioxygenase 1; IL, interleukin; iNO, inhaled-NO; iNOS, inducible nitric oxide synthase; I/R, ischemia/reperfusion;  $K_{ATP}$ , ATP-sensitive  $K^+$ ; KO, knockout; L-NAME, *N*( $\gamma$ )-nitro-L-arginine methyl ester; LV, left ventricular; LVEF, left ventricular ejection fraction; MAP, mean arterial pressure; mARC, mitochondrial amidoxime reductase; Mb, myoglobin; MI, myocardial infarction; MRI, magnetic resonance imaging; ·NO, nitric oxide; NOS, nitric oxide synthase; NOx, nitrate and nitrite; NSAID, nonsteroidal anti-inflammatory drug; NT-proBNP, N-terminal portion of BNP; NYHA, New York Heart Association of Heart Failure; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; OSA, obstructive sleep apnea syndrome; oxyHb, oxyhemoglobin; oxyMb, oxymyoglobin;  $p_{50}$ , partial pressure to achieve 50% saturation; PAD, peripheral arterial disease; PAH, pulmonary arterial hypertension; PAP, pulmonary arterial pressure; PCI, percutaneous coronary intervention; PCr, phosphocreatine; PDE, phosphodiesterase; Pi, inorganic phosphate; PKG, protein kinase G; PVR, pulmonary vascular resistance; PWV, pulse wave velocity; rIPC, remote ischemic preconditioning; ROS, reactive oxygen species; R-state, relaxed state; SAH, subarachnoid hemorrhage; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat; SO, sulfite oxidase; SR, strain rate; TAC, thoracic aortic constriction; TIMI, thrombolysis in myocardial infarction; TNF $\alpha$ , tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine triphosphate nick-end labeling; T-state, tense state;  $VO_2$ , oxygen uptake; VASP, vasodilator-stimulated phosphoprotein;  $VO_{2max}$ , maximal oxygen consumption; WHO, World Health Organization; WT, wild type; XOR, xanthine oxidoreductase.

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**Abstract**—In contrast to nitric oxide, which has well established and important roles in the regulation of blood flow and thrombosis, neurotransmission, the normal functioning of the genitourinary system, and the inflammation response and host defense, its oxidized metabolites nitrite and nitrate have, until recently, been considered to be relatively inactive. However, this view has been radically revised over the past decade and more. Much evidence has now accumulated demonstrating that nitrite serves as a storage form of nitric oxide, releasing nitric oxide preferentially under acidic and/or hypoxic conditions but also occurring under physiologic conditions: a phenomenon that is catalyzed by a number of distinct mammalian nitrite reductases. Importantly, preclinical studies demonstrate that reduction of nitrite to nitric oxide results in a number of beneficial effects, including vasodilatation of blood vessels and lowering of blood pressure, as well as cytoprotective effects that limit the extent of damage caused by an ischemia/reperfusion insult, with this latter issue having been translated more recently to the clinical setting. In addition, research has demonstrated that the other main metabolite of the oxidation of nitric oxide (i.e., nitrate) can also be sequentially reduced through processing

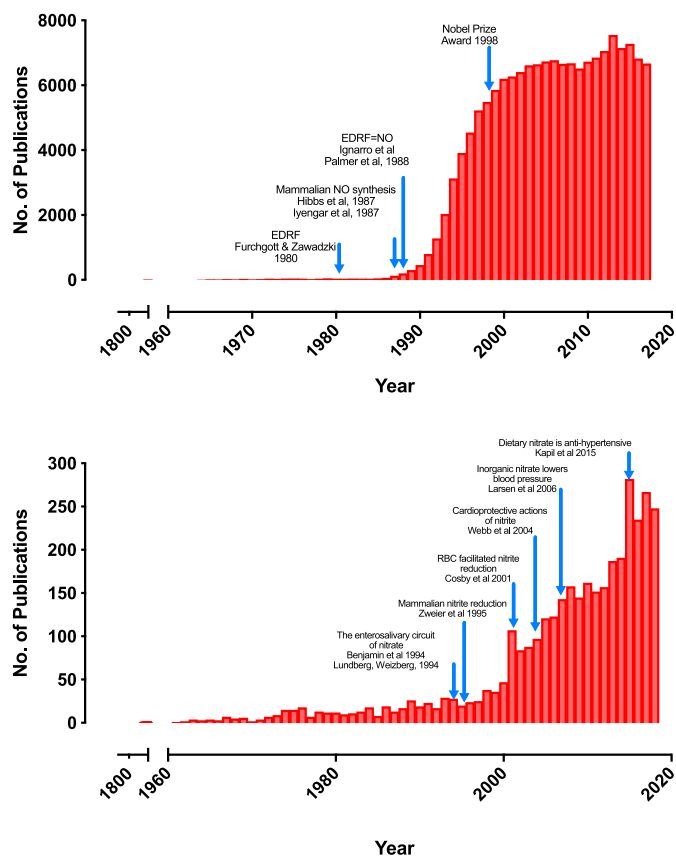
in vivo to nitrite and then nitrite to nitric oxide to exert a range of beneficial effects—most notably lowering of blood pressure, a phenomenon that has also been confirmed recently to be an effective method for blood pressure lowering in patients with hypertension. This review will provide a detailed description of the pathways involved in the bioactivation of both nitrate and nitrite in vivo, their functional effects in preclinical models, and their mechanisms of action, as well as a discussion of translational exploration of this pathway in diverse disease states characterized by deficiencies in bioavailable nitric oxide.

**Significance Statement**—The past 15 years has seen a major revision in our understanding of the pathways for nitric oxide synthesis in the body with the discovery of the noncanonical pathway for nitric oxide generation known as the nitrate-nitrite-nitric oxide pathway. This review describes the molecular components of this pathway, its role in physiology, potential therapeutics of targeting this pathway, and their impact in experimental models, as well as the clinical translation (past and future) and potential side effects.

## I. Introduction

Nitric oxide (·NO) was discovered by the British chemist Joseph Priestley simultaneous with his discovery of oxygen in 1776. For the next 200 years, ·NO was largely thought of as an unwanted, atmospheric pollutant (Spicer, 1977). However, the discovery of mammalian ·NO production and its protean effects on biologic systems profoundly changed this view. The magnitude of the importance of this shift in view is reflected by the award of the Nobel Prize in Physiology or Medicine in 1998 to Furchgott, Ignarro, and Murad, which is shared for their seminal roles in the discovery “concerning NO as a signaling molecule in the cardiovascular system” (<https://www.nobelprize.org/prizes/medicine/1998/>

summary). A PubMed search of the term ·NO identifies one publication in 1816, which describes the unfortunate event of poisoning with ·NO of quicksilver, rising to a colossal 6670 publications in 2017, with the highest number to date occurring in 2015, with 7277. These numbers clearly demonstrate that interest in this molecule and its biologic function really started to heat up after the seminal 1980 paper describing “The obligatory role of the endothelium...” and increasing exponentially after the Nobel Prize award. But perhaps more pertinently for this review, by the early 2000s, interest in ·NO, although still high, had plateaued until the more recent 5–10 years, in which it is clear that there has been a sudden boost in interest. We suggest that this reignited interest comes in part from discoveries demonstrating



**Fig. 1.** Number of publications per year for (upper)  $\cdot\text{NO}$  and (lower) nitrate and nitrite. Figures highlight only some of the key observations in the fields, and this is not an exhaustive list. Please see the text for greater detail. EDRF, endothelium derived relaxing factor.

the significant biologic activity of the nitrate-nitrite- $\cdot\text{NO}$  pathway, which we now describe as the noncanonical pathway for in vivo  $\cdot\text{NO}$  generation. Moreover, it seems to us that interest in  $\cdot\text{NO}$ , by virtue of these discoveries, is not going to abate any time soon (Fig. 1).

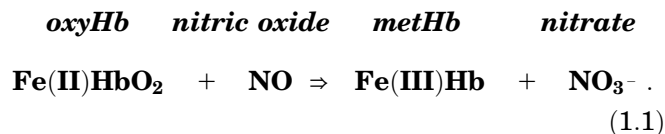
### A. Chemistry of $\cdot\text{NO}$ and Its Metabolism to Nitrite and Nitrate

$\cdot\text{NO}$  is a small, diatomic, amphipathic, free-radical molecule that is freely diffusible and membrane-permeable. Although one of the simplest biologic molecules,  $\cdot\text{NO}$  has relevant functions in almost every human physiologic system, including critical roles in neurotransmission, gastrointestinal (GI) physiology, genitourinary function, innate immunity, and the cardiovascular (CV) system (Moncada and Higgs, 1993).

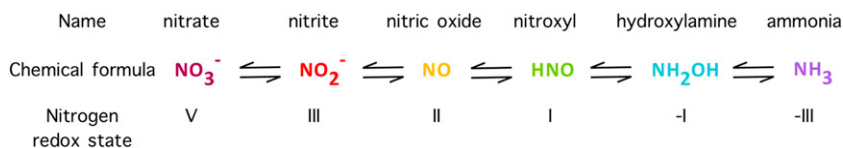
There are three core properties of  $\cdot\text{NO}$  that determine its physical and chemical interactions and enable

generation of a range of  $\cdot\text{NO}$  and other nitrogen species (Fig. 2): it is electrically neutral, it is small, and it has an unpaired electron in a nonbonding molecular orbital. The fundamental chemistry of  $\cdot\text{NO}$  is unique among all other small-molecule signaling agents. Since  $\cdot\text{NO}$  possesses an unpaired electron (i.e., it is paramagnetic), it readily reacts with many other species that are also paramagnetic. For example,  $\cdot\text{NO}$  reacts with dioxygen ( $\text{O}_2$ ), superoxide ( $\cdot\text{O}_2^-$ ), and nitrogen dioxide ( $\text{NO}_2$ ), as well as oxidizing carbon/nitrogen-centered radicals that would otherwise oxidize biologic molecules, potentially causing an “oxidative stress” (vide infra). Indeed, the ability of  $\cdot\text{NO}$  to react with and quench oxidizing radical species is the basis of its antioxidant properties.

$\cdot\text{NO}$  also reacts with transition metals and, therefore, in biologic systems, has numerous possible targets, i.e., molecules containing an iron heme moiety. The coordination chemistry of  $\cdot\text{NO}$  is unique compared with other small, diatomic molecules (e.g.,  $\text{O}_2$ ,  $\text{CO}$ ) in that it can react with both ferric [ $\text{Fe(III)}$ ] and ferrous [ $\text{Fe(II)}$ ] heme proteins (that contain an open coordination site). Reaction with  $\text{Fe(III)}$ -hemes often results in reduction to  $\text{Fe(II)}$ -hemes, with consequent generation of nitrite (Fukuto et al., 2000). The coordination of  $\cdot\text{NO}$  with  $\text{Fe(II)}$ -heme typically leads to the formation of a relatively stable  $\text{Fe(II)-NO}$  complex. The primary biologic target for  $\cdot\text{NO}$  is, indeed, the  $\text{Fe(II)}$ -heme protein soluble guanylyl cyclase (Hobbs and Stasch, 2010) (GC-1, or more commonly known as sGC [Alexander et al., 2019]). Coordination of  $\cdot\text{NO}$  to the  $\text{Fe(II)}$ -heme of GC-1 results in activation of the enzyme triggering generation of cyclic GMP (cGMP). Importantly, the coordination chemistry of  $\cdot\text{NO}$  with  $\text{Fe(II)}$ -hemes is unique among all small diatomics; thus, only  $\cdot\text{NO}$  is capable of significantly activating GC-1 via heme coordination.  $\cdot\text{NO}$  can also react with  $\text{O}_2$  bound to  $\text{Fe(II)}$ -heme [ $\text{Fe(II)-hemeO}_2$ ], such as that found in oxyhemoglobin [oxyHb,  $\text{Fe(II)HbO}_2$ ] or oxymyoglobin [oxyMb,  $\text{Fe(II)MbO}_2$ ], to give ferric Hb or ferric myoglobin (Mb) [ $\text{Fe(III)Hb}$ ,  $\text{Fe(III)Mb}$ , respectively] and nitrate, a reaction described first by Hermann (1865), cited in Gladwin et al. (2005) (eq. 1.1). Oxidative termination of  $\text{NO}$  activity with Hb is as follows:



It is now understood that the reaction of  $\cdot\text{NO}$  with  $\text{Fe(II)HbO}_2$  is relatively fast and provides a sink ( $6\text{--}8 \times 10^7$



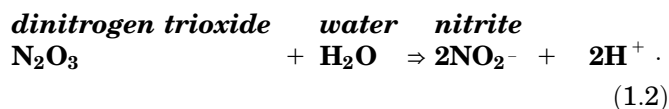
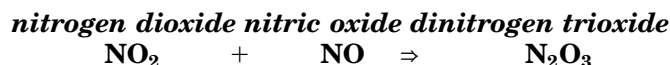
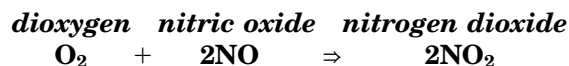
**Fig. 2.** Redox relationship of  $\text{NO}$  and other nitrogen species.

$M^{-1} s^{-1}$ ) for  $\cdot NO$  (Feelisch and Noack, 1987; Eich et al., 1996; Herold et al., 2001). Since this reaction is so fast, there were originally concerns that there was little chance of  $\cdot NO$  expressing functional activity in vivo (Lancaster, 1994); however, the discovery of a cell-free layer of blood flowing adjacent to the endothelium (Liao et al., 1999) and the recognition that the encapsulation of Hb within the erythrocyte limits the speed of the reaction because of the need for diffusion through the erythrocyte membrane (Liu et al., 1998; Vaughn et al., 1998, 2000; Han et al., 2002) have allayed these concerns, for the most part.

Like all biologic molecules,  $\cdot NO$  moves only inasmuch that the probability of random diffusion is greater for movement into a compartment with a lower concentration, resulting in net movement along a concentration gradient. Hence,  $\cdot NO$  molecules move away from a specific point of generation. Since  $\cdot NO$  is electrically neutral, it has a high diffusion coefficient [ $10^{-7} \text{ cm}^2 \text{ s}^{-1}$  (Vanderkooi et al., 1994)] (Malinski et al., 1993) and may travel far (100–200  $\mu\text{m}$ ) in a short time frame (Lancaster, 1994, 1997); however, because of its numerous interactions with a number of diverse molecules (i.e., something to which it can chemically react), this can greatly reduce its sphere of biologic action (Lancaster, 1997).

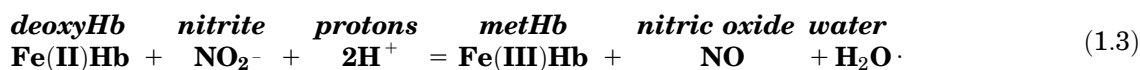
$\cdot NO$  does not dimerize in gas or in solution and is poorly soluble in water (1.57  $\text{mmol dm}^{-3}$  at 35°C) (Hughes, 2008) and does not react with water (i.e.,  $\cdot NO$  is not electrophilic).  $\cdot NO$  is lipophilic, which allows it to freely enter and accumulate in cell membranes. Although  $\cdot NO$  has been characterized as a “highly reactive” molecule, it is inherently stable in the pure form. However, it can react readily with specific species, such as those that also possess an unpaired electron. As discussed previously, the unpaired electron of  $\cdot NO$  makes it reactive with other paramagnetic species, including  $O_2$ ,  $NO_2$  (Bonner and Hughes, 1988; Ignarro et al., 1993), superoxide (Blough and Zafiriou, 1985),

of  $\cdot NO$  with  $O_2$  initially leads to the formation of  $NO_2$ , which is also a radical that is a relatively strong oxidant and, like  $\cdot NO$ , will also react with other radicals such as  $\cdot NO$ . The reaction of  $\cdot NO$  and  $NO_2$  results in the formation of  $N_2O_3$  (a nonradical species). In a purely aqueous system,  $N_2O_3$  can react with water to give nitrite. This series of chemical reactions is shown below (eq. 1.2). Oxidation of  $NO$  in aqueous solutions is as follows:



At biologically relevant levels of  $\cdot NO$ , this process is very slow, as the overall reaction kinetics are second order with respect to  $\cdot NO$ , making the  $O_2$ -dependent oxidation of  $\cdot NO$  to nitrite of limited importance (Li and Lancaster, 2012). In pure aqueous solutions,  $\cdot NO$  is slowly converted to nitrite, although there is evidence in humans of a nitrite synthase function of the multifunction copper-containing protein caeruloplasmin facilitating this reaction (Shiva et al., 2006). The importance of this reaction in vivo for termination of  $\cdot NO$  activity and regulating basal  $\cdot NO$  levels is currently unclear. The same reactivity of  $\cdot NO$  for transition metals has spurred interest in developing transition metal-based scavengers of  $\cdot NO$ , such as ruthenium complexes that are being developed for use in situations in which large amounts of inducible  $\cdot NO$  synthase (iNOS)-derived  $\cdot NO$  may be detrimental, such as in tumor growth (Flitney et al., 2011).

In addition, and of particular relevance to this review, is the potential for reduction of nitrite by deoxyHb

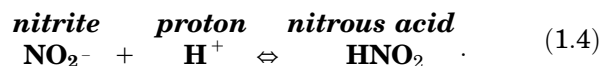


and other “radicals,” such as those involved in deleterious oxidative events (Rubbo et al., 1996). In spite of the fact that  $\cdot NO$  possesses an unpaired electron (and is considered to be a “radical” species), it is important to note that it is not an oxidizing radical (i.e., it does not readily take electrons or abstract H-atoms) and, therefore, is distinct from other known radical oxidants, such as  $NO_2$  or hydroxyl radical ( $HO\cdot$ ). Indeed, besides coordination to metals,  $\cdot NO$  tends to react only with other radicals that already exist or have been preformed in solution (e.g.,  $O_2$ , superoxide,  $NO_2$ , etc.). The reaction

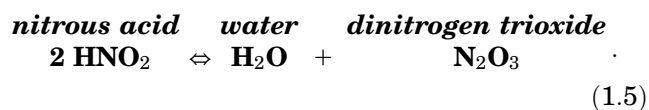
[Fe(II)Hb] to give  $\cdot NO$  (Cosby et al., 2003; Huang et al., 2005b; Grubina et al., 2007), described below (eq. 1.3). The reaction of  $NO_2^-$  with deoxyHb is as follows:

However, how  $\cdot NO$  derived from nitrite reduction within the erythrocyte in the presence of an excess of oxyHb (even in relative hypoxia), a rapid  $\cdot NO$  scavenger, escapes the erythrocyte is not clear. Some have suggested that  $\cdot NO$  bioactivity is exported from erythrocytes as dinitrogen trioxide after a nitrite anhydrase reaction involving Hb (eqs. 1.4–1.6) and that, once

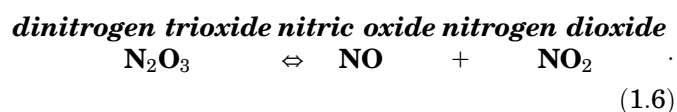
outside, the erythrocyte can dissociate to  $\cdot\text{NO}$  and nitrogen dioxide (Basu et al., 2007; Hopmann et al., 2011). It should be noted that  $\cdot\text{NO}$  made via these reactions represents a disproportionation (one species is oxidized, whereas the other is reduced) and not an overall reduction reaction.  $\text{NO}_2^-$  acidification to form  $\text{HNO}_2$  is as follows:



Dehydration of  $\text{HNO}_2$  is



$\text{N}_2\text{O}_3$  dissociation to  $\text{NO}$  is



Since the early proposals that Hb may be a site for nitrite reduction, a number of other nitrite reductases have been identified, and these and the chemistry involved are discussed in more detail in this review.

It is worthwhile to briefly discuss the potential for nitrite to be generated via the reactions of  $\cdot\text{NO}$  with  $\text{O}_2$ -derived species such as superoxide. Although it was previously thought that the reaction of  $\cdot\text{NO}$  with superoxide was of toxicological/pathophysiological concern, since the product, peroxynitrite ( $\text{ONOO}^-$ ), is a potential oxidant, this idea has not been well supported. The generation of peroxynitrite from this reaction may simply serve to limit the signaling associated with  $\cdot\text{NO}$  and/or superoxide. However, it is also worth considering that the  $\cdot\text{NO}$ /superoxide reaction can serve as a source of nitrite since peroxynitrite in the presence of excess  $\cdot\text{NO}$  or superoxide will lead to the formation of nitrite (Jourdh'euil et al., 2001).

Thus, unless  $\cdot\text{NO}$  and superoxide were generated at the same time, same flux, and in the same place in a cell (a highly unlikely scenario), nitrite generation will be the primary fate of this reaction. This also indicates that the spatial and temporal aspects of nitrite generation (via the  $\cdot\text{NO}$ /superoxide reaction) can be governed by the place and time of reactive oxygen species (ROS) [superoxide via xanthine oxidoreductase (XOR), for example] and  $\cdot\text{NO}$  generation. Moreover, it has been reported that  $\cdot\text{NO}$  can be metabolized by cells to give nitrite (Thomas et al., 2001) (although the chemical reactions responsible for the cellular conversion of  $\cdot\text{NO}$  to nitrite have not been established). Finally, just as the formation of nitrite from  $\cdot\text{NO}$  can be dependent on the timely and localized generation of ROS, the reverse process (formation of  $\cdot\text{NO}$  from nitrite) can also be dependent on  $\text{O}_2$  and  $\text{O}_2$ -derived species as well (Wink, 2003).

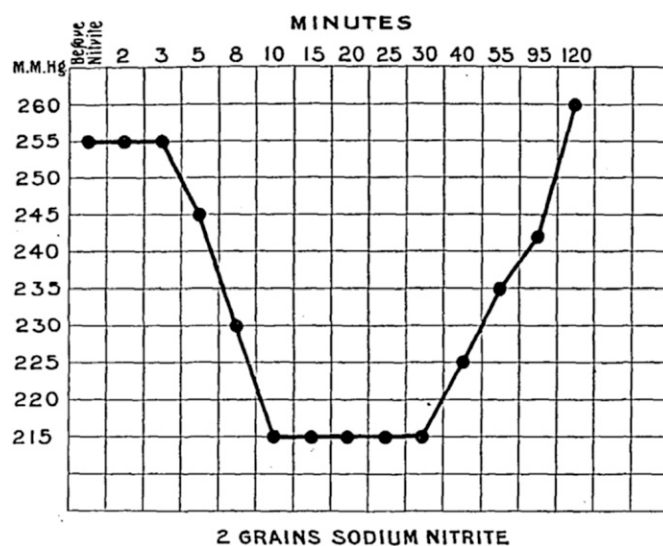


Fig. 3. Oral inorganic nitrite and acute BP response (Matthew, 1909).

## II. Inorganic Nitrite and Nitrate

### A. Historical Uses of Inorganic Nitrite

The use of nitrovasodilators by Lauder Brunton and Murrell led to the exploration of related chemicals that included salts of inorganic nitrite. Below are the words that Reichert used to start his detailed monograph on the actions of potassium nitrite to explain why he was using a related substance to amyl nitrite (Reichert and Mitchell, 1880):

“The very great value of amyl nitrite in warding off impending paroxysms of epileptic convulsions, angina pectoris, and asthma, has been so generally recognized by the profession, that the discovery of a new salt whose physiological action is identical with... that of amyl nitrite, but whose effects would be more permanent and therefore suitable for maintaining a continuous systemic influence, we would have an addition to our materia medica which would fill a very apparent therapeutic void.”

The experiments that Reichert was describing in this work demonstrated that, in dogs and cats, potassium nitrite caused profound hypotension, leading to death, and perhaps more importantly, that in humans potassium nitrite had a synonymous effect to the organic amyl nitrite (Reichert and Mitchell, 1880). These observations were followed by a detailed comparative analysis of the effects of sodium nitrite and organic nitrites, including amyl nitrite and glyceryl trinitrate (GTN). In normotensive subjects, the blood pressure (BP)-lowering effects of organic nitrites and nitrates were observed for up to 30 minutes postadministration, but a similar level of BP reduction was maintained for up to 60 minutes after inorganic nitrite dosing, indicating some differences in biologic activity between the two compound classes. The effects of sodium nitrite were much larger in patients with significant hypertension (see Fig. 3), with maximal systolic BP (SBP) reductions up to 50 mm Hg

(Matthew, 1909; Wallace and Ringer, 1909). It was after this, in the early part of the 20th century, that inorganic nitrite was used for the treatment of BP appearing in *materiae medicae* and being produced by several pharmaceutical suppliers for the treatment of patients with hypertension (Butler and Feelisch, 2008). This occurred despite an absence of a clear understanding of the mechanisms involved. However, the use of nitrite salts for BP management never really took off, and this relates to concerns regarding the reactions between nitrite and oxyHb generating metHB (eq. 1.3). If metHB levels rise >10%, the fraction of bound oxygen to Hb becomes insufficient for metabolic demand, thus provoking symptomatic hypoxemia despite adequate oxygenation (Skold et al., 2011). Indeed, concerns relating to this continue to be raised in relation to the use of both organic and inorganic nitrites in clinical practice (Pierce and Nielsen, 1989; Finan et al., 1998; Modarai et al., 2002).

### B. Historical Uses of Inorganic Nitrate

Inorganic nitrate has been used in traditional Chinese medicine to treat cardiovascular disease (CVD) for over a millennium. The following passage is a translation from an 8th century CE manuscript discovered in the Mogao caves in Gansu Province, China (Butler and Moffett, 2005):

“Putting under the tongue to cause heart qi to flow freely for treating symptoms such as struck by evil, acute heart pains and cold in the hands and feet which can kill a patient in an instant. Look at the patient’s fingers and those with greenish-black nails are such cases. Take saltpeter [xiaoishi, potassium nitrate] (five measures of a bi spoon) and realgar [xiongshi, arsenic sulphide] (one measure of a bi spoon) and combine the two into a fine powder. Lift the patient’s tongue and sprinkle one measure of a bi spoon under the tongue. If saliva is produced, have the patient swallow it. This is a certain cure.”

Interestingly, the comment regarding the production of saliva and the importance of swallowing appears to relate to knowledge of the bioactivation of inorganic nitrate by the oral microbiota (see below). Although it is difficult to tell whether there was widespread use of inorganic nitrate in traditional Chinese medicine over the proceeding centuries, it was much later, in the early 20th century, that Western physicians explored the utility of the same chemicals. Edward Stieglitz produced a body of work in Chicago with bismuth subnitrate [chemical formula:  $\text{Bi}_5\text{O}(\text{OH})_9(\text{NO}_3)_4$ ]. At that time, bismuth subnitrate was being used as an established and recommended treatment of peptic ulcer disease and diarrhea, with the caveat and caution against prolonged use because of the risk of hypotension (Frick, 1924). Stieglitz was aware of evidence from bacteriologists that enteric bacteria could convert nitrate to nitrite (Salen, 1925; Zobell, 1932). He thus proposed the following (Stieglitz, 1927):

“The idea to use bismuth subnitrate as an auxiliary to break the vicious circle of vascular fatigue arose from the observation of three cases of nitrate poisoning resulting from the liberal use of bismuth subnitrate in severe diarrhoeas... Therefore, theoretically, small frequent doses should lead to the liberation of small amounts of nitrite, uniformly and continuously absorbed. The effect of this is quite different from the violent, very transient vasodilatory effect of other forms of nitrite, such as nitroglycerol, amyl nitrite and sodium nitrite. The action to be expected is a gradually increasing vascular relaxation, with localized physiological rest to the arteriolar musculature.”

He reported, in almost 1000 patients, sustained hypotensive effects of bismuth subnitrate (Stieglitz, 1927, 1928, 1930, 1932). Furthermore, he determined that, although the basal level of nitrite in blood was 110–220 nmol/l, this was increased after an inorganic nitrate load and that, although nitrite was not a normal constituent of fresh urine, it could be detected after an oral inorganic nitrate load (Stieglitz and Palmer, 1934, 1936, 1937). Lastly, he expended much effort exploring the nitrate-reducing activity of bacteria *in vitro*, resulting with his hypothesis that bacterial nitrate reduction might be responsible for significant physiologic effects *in vivo* (Stieglitz and Palmer, 1936). There was not widespread uptake of this therapy, in part because of concerns regarding methemoglobinemia (Comly, 1945; Walton, 1951) and the prevailing view that nitrate was an inert end product of oxidative ·NO metabolism (Bonner and Hughes, 1988; Ignarro et al., 1993). However, of course, all of this changed in the late 2000s with the realization that nitrate via the noncanonical pathway offers an alternative approach to ·NO delivery and potential BP lowering.

## III. Sources and Pharmacokinetics of Nitrate

There are two major sources of nitrate in humans, i.e., through the oxidation of ·NO and directly from exogenously derived (environment and diet) sources.

### A. Endogenously Derived Nitrate

Assessments of the contribution of endogenously derived nitrate to daily bodily exposure has suggested that ~25% of bodily nitrate comes from endogenous sources (Green et al., 1981). Plasma nitrate levels in healthy, fasted persons have been shown to be in the order of 20–40  $\mu\text{mol/l}$  (Gladwin et al., 2000; Lundberg and Govoni, 2004; Webb et al., 2008b). In long-term nitrogen balance studies lasting 3 months, in healthy participants given <180  $\mu\text{mol}$  nitrate daily, endogenous nitrate biosynthesis estimated from 24-hour urine collection was between 0.5 and 1 mmol daily (Green et al., 1981), although with the contribution of iNOS in inflammatory states, this can be much higher (Stichtenoth et al., 1994).

## B. Exogenous Sources of Nitrate

Humans are exposed to exogenously derived nitrate on a daily basis. There are two primary sources: drinking water and diet.

1. *Dietary Intake.* The predominant dietary source of nitrate comes from vegetable intake. Using the World Health Organization (WHO) recommended intake of mixed vegetables of 400 g daily as a guide would lead to a total daily nitrate intake of ~2.5 mmol, although in reality, most people consume less than this, with estimates at 1.5–2 mmol (or 93–124 mg) nitrate daily (World Cancer Research Fund/American Institute for Cancer Research, 2007; European Food Safety Authority, 2008). However, in vegetable-rich diets, such as the proposed diet based upon the Dietary Approaches to Stop Hypertension (DASH) study, estimates suggest that intake levels can easily be increased to somewhere in the region of 6–20 mmol daily (Hord et al., 2009). Other dietary patterns, such as the traditional Japanese diet, may also have significantly higher nitrate intakes (Sobko et al., 2010) compared with European or United States diets. This variation in intake is driven by the differences in nitrate levels found in vegetables. In general, large green leafy vegetables contain the largest amounts of nitrate per gram (European Food Safety Authority, 2008), and this source is thought to be responsible for the elevated amounts consumed within the traditional Japanese diet. Importantly, because of concerns associated with toxicity (see later), the WHO has set an acceptable daily intake (ADI) recommendation for dietary nitrate at 3.7 mg/kg daily, which equates to ~4 mmol nitrate daily for an average 70-kg person (European Food Safety Authority, 2008).

2. *Water Supplies.* Because of historical concerns regarding toxicity from nitrate in drinking water supplies, mostly concerning the risk of infantile methemoglobinemia (see later), there exists regulatory control of the nitrate level in water at <50 mg/l (U.S. Public Health Service, 1962). The levels of nitrate in drinking water derived from surface water do not exceed 10 mg/l in most countries. In such conditions, the contribution of drinking water to nitrate intake is usually less than 14% (World Health Organization, 2011), and therefore, vegetables are the main source of nitrate intake (Chilvers et al., 1984; European Centre for Ecotoxicology and Toxicology of Chemicals, 1988).

In some particularly agricultural areas, however, concentrations of nitrate in drinking water are higher because of runoff and the discharge of sewage effluent and certain industrial wastes. In 15 European countries, evidence was shown indicating that the percentage of the population exposed to nitrate levels in drinking water above 50 mg/l ranged from 0.5% to 10% (European Centre for Ecotoxicology and Toxicology of Chemicals, 1988). Individual wells in agricultural areas throughout the world often contain

drinking water with nitrate levels exceeding 50 mg/l (World Health Organization, 2011), and in such circumstances, drinking water will be the major source of total nitrate intake, especially for bottle-fed infants (World Health Organization, 2011).

## C. Pharmacokinetics of Nitrate

After the oral ingestion of nitrate, it is rapidly absorbed across the upper GI tract (Hawksworth and Hill, 1971; Witter et al., 1979; Miyoshi et al., 2003) and does not undergo transformation with first-pass metabolism, thereby having almost 100% bioavailability (van Velzen et al., 2008). It is not clear how nitrate is able to cross the upper GI tract, and no putative mechanism or transporter has been identified to date.

After ingestion of a single inorganic nitrate load as a salt or in dietary form (i.e., vegetable), significant elevation in circulating plasma nitrate levels can be detected within 15 minutes, with peak levels achieved by 30–60 minutes postdose (McKnight et al., 1997; Lundberg and Govoni, 2004; van Velzen et al., 2008; Webb et al., 2008b). The effective half-life for nitrate in the plasma after consumption of different vegetable sources is ~6 hours (van Velzen et al., 2008), with a slow reduction over time and significant elevation in circulating plasma nitrate levels even 24 hours post-single-dose administration (Webb et al., 2008b; Kapil et al., 2010).

The fate of nitrate in the plasma is 2-fold. Studies with radiolabeled [<sup>15</sup>N]nitrate reveal that two-thirds is excreted in the urine. Peak excretion of nitrate occurs ~6 hours after supplementation with small amounts and has been accounted for in sweat (up to 10%) or feces (<1%) (Green et al., 1981; Wagner et al., 1983, 1984; Bartholomew and Hill, 1984; Packer et al., 1989; Pannala et al., 2003). Nitrate is freely filtered at the glomerulus, and clearance from the plasma has been estimated as ~20 ml/min in healthy subjects (Wennmalm et al., 1993). This relatively low rate of clearance (taking into consideration that a normal glomerular filtration rate is between 100 and 125 ml/min) suggests that much of the filtered nitrate is reabsorbed and explains its relatively long half-life (Kahn et al., 1975; Rahma et al., 2001).

As such, renal reabsorption of nitrate increases with increasing filtered nitrate level, with no clear transport limit (Godfrey and Majid, 1998). In clearance experiments, both mannitol and furosemide inhibited tubular reabsorption of nitrate, suggesting that nitrate reabsorption occurs across the whole tubule. Further stop-flow experiments also suggest that nitrate is avidly reabsorbed at the same location as sodium in the distal tubule, suggesting cotransport (Rahma et al., 2001), although the transporters for such action have not been identified, and this remains one of the major unknowns in the field.



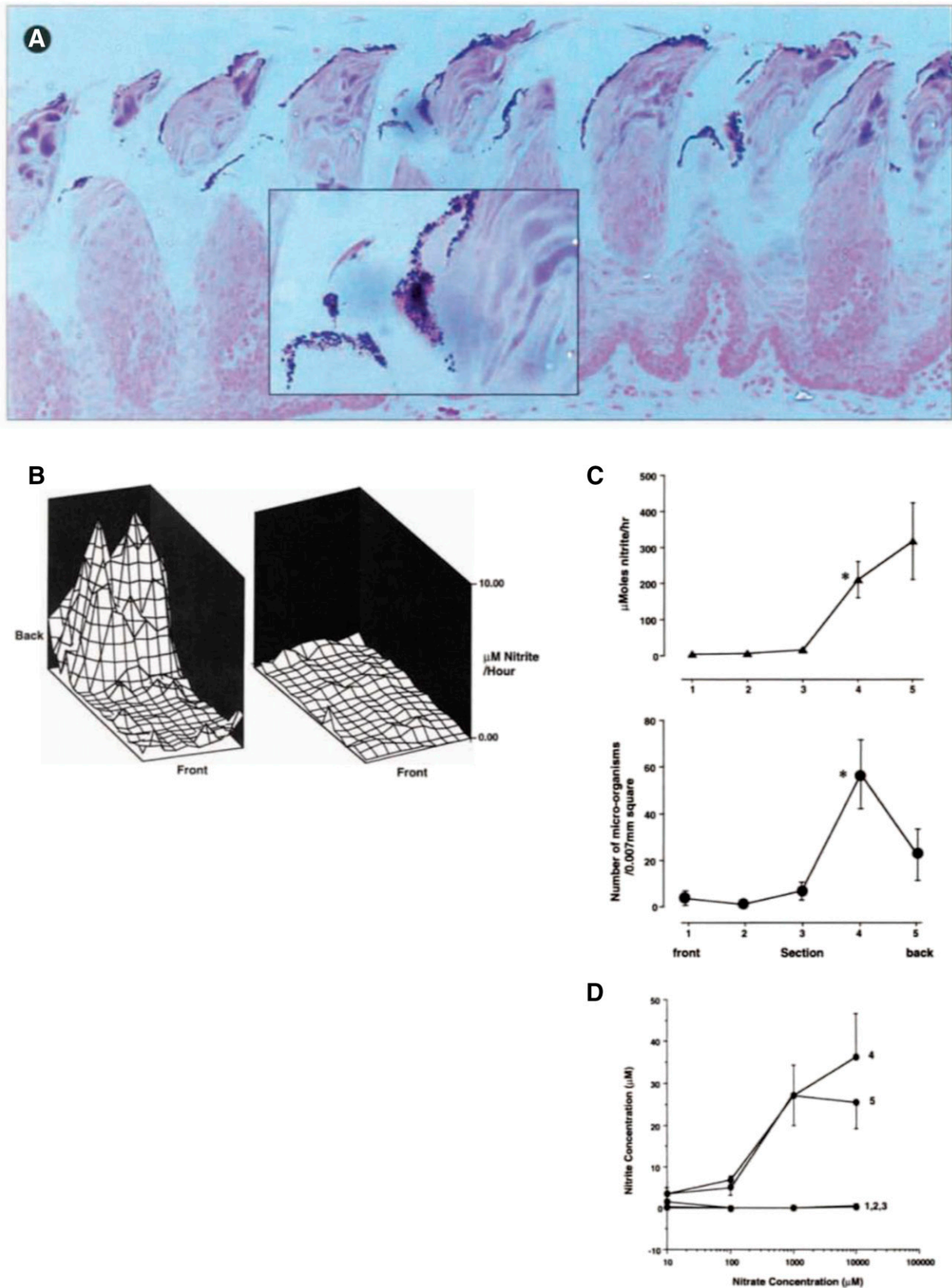
### *D. Enterosalivary Circulation and Oral Reduction of Nitrate*

The remaining (~25%) of nitrate that is not excreted by the kidneys is selectively taken up by the salivary glands (Spiegelhalter et al., 1976; Tannenbaum et al., 1976; Kortboyer et al., 1994) via the two-nitrate/one-proton electrogenic cotransporter sialin (Qin et al., 2012). This reuptake of nitrate is often described as the enterosalivary circulation of nitrate (Duncan et al., 1995). Salivary nitrate levels at baseline are >10-fold higher than circulating plasma nitrate levels (Spiegelhalter et al., 1976; Lundberg and Govoni, 2004), indicating a concentration of nitrate within the oral cavity. This circuit of nitrate is a relatively quick process; within 20–60 minutes post-inorganic nitrate load, an elevation in salivary nitrate levels is detectable (Harada et al., 1974; Ishiwata, 1976b; McKnight et al., 1997).

These early studies demonstrated that in addition to these rises in salivary nitrate with oral nitrate dosing, rises in the chemically related but distinct anion nitrite occur hand in hand. It is noteworthy that the primary aim of these investigations was to determine whether nitrate ingestion might result in sufficient nitrite generation to lead to formation of *N*-nitroso compounds linked to carcinogenesis (Harada et al., 1974; Tannenbaum et al., 1974, 1976; Eisenbrand et al., 1980). Studies have shown that there is no nitrite in saliva taken directly from salivary gland ducts, as opposed to mixed saliva in the oral cavity, indicating that conversion of nitrate to nitrite within the oral cavity, and not within the salivary glands, was responsible for salivary nitrite levels. It was in 1975 that it was first suggested that the appearance of nitrite within the saliva may be due to the activity of nitrate-reducing bacteria within the oral cavity (Ishiwata et al., 1975d). It was known at that time that bacteria existing in the lower GI tract (Salen, 1925; Zobell, 1932; Stieglitz and Palmer, 1936) could use nitrate as a terminal electron donor in respiration instead of oxygen and, thereby, reduce nitrate to nitrite (Moreno-Vivián et al., 1999; Lundberg et al., 2004). In addition, consumption of nitrate and corresponding formation of nitrite in human saliva *in vitro* at 37°C had been shown to be prevented by heating the saliva to 100°C or by passing the saliva first through a filter (Goaz and Biswell, 1961; Ishiwata et al., 1975a). If the filter residue was returned to the saliva filtrate, the changes in salivary nitrate and nitrite levels were restored, suggesting a denaturable, biologic element in the residue fraction that was necessary for nitrate reduction (Goaz and Biswell, 1961; Ishiwata et al., 1975a). Further studies using commercially available antibacterial mouthwash or systemic antibiotics resulted in up to 90% reduction in salivary nitrite levels after an inorganic dietary load, supporting the view that bacterial conversion of nitrate was responsible for salivary nitrite (Tannenbaum et al., 1976; Dougall et al., 1995; Duncan et al., 1995).

Sasaki and Matano (1979) used a filter-paper technique to identify particular areas of the oral cavity that were responsible for nitrate reduction. They impregnated small 1.5-cm<sup>2</sup> pieces of filter paper with potassium nitrate and placed them on particular areas of the oral cavity for 90 seconds. On removal, they agitated the paper squares with distilled water and determined nitrite accumulation colorimetrically. Their investigations revealed that significant nitrate reduction was localized to only the posterior, dorsal aspect of the tongue, an observation that has since been confirmed in rodents (Duncan et al., 1995). Incubation of rat tongue sections with nitrate solution *ex vivo* revealed abundant nitrate reduction completely attenuated by prior boiling (Duncan et al., 1995; Li et al., 1997). In addition, oral nitrate-reduction activity was found to be completely absent in any part of the oral cavity of rats raised in a germ-free environment (Duncan et al., 1995). Histologic examinations of these sections demonstrated the presence of abundant bacteria in the deep, interpapillary sulci (crypts) in the posterior third of the tongue and relatively less elsewhere, which coincided with the distribution of nitrate reductase activity across the tongue (Fig. 4) (Sasaki and Matano, 1979; Sasaki et al., 1981; Duncan et al., 1995; Li et al., 1997).

The first bacterial species identified capable of facilitating oral nitrate reduction, *Bacillus coagulans*, was found by incubating saliva samples on nitrate-containing blood agar under anaerobic conditions (Maruyuma et al., 1976). Studies with samples of rat tongue or human saliva, using standard culture and colony isolation, coupled predominantly with species identification by biochemical means, have implicated *Veillonella*, *Lactobacillus*, *Micrococcus*, *Corynebacterium*, *Propionibacterium*, *Neisseria*, *Actinomyces*, commensal *Staphylococcus*, and *Rothia* spp. as playing significant roles in oral nitrate reduction (Murumatsu et al., 1979; Doel et al., 2005). Importantly, these observations have been limited to only those bacteria that can be cultured. More recent use of biofilm modeling has revealed two additional species with nitrate reductase activity (Hyde et al., 2014); however, even this model only supported culture of ~100 different species-level taxa. Importantly, nonculturable organisms, detected using culture-independent molecular analyses, make up a significant proportion of the total oral microbiome (Dewhirst et al., 2010). The human microbiome project, utilizing next-generation sequencing methods, has shown that culture-independent analyses of the bacterial genome targeting the 16S rRNA gene identify 700 bacterial species resident in the human oral cavity, with a predicted total species richness of ~1000 (Dewhirst et al., 2010). With the advent of these next-generation sequencing techniques and the human oral microbiome project, further novel nitrate reductase-containing species are being identified, although the relative importance of any of these to overall oral nitrate reduction has been difficult



**Fig. 4.** Oral microbiome and nitrate reduction. Reprinted by permission from Springer: (Nature), Nature Medicine, Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate Duncan et al. (1995). (A) Micrograph showing the dorsal epithelial surface of the posterior third of the rat tongue. A convoluted surface with deep interpapillary clefts is seen and large numbers of microorganisms are present (dark areas). (B) Three-dimensional plot of nitrate reduction on the surface of (left) conventional ( $n = 7$ ) and (right) germ-free ( $n = 2$ ) rat tongues. Nitrate reduction (measured as micromoles nitrite per hour) is mainly confined to the posterior third of the tongue in conventional rats but is absent in those raised in a germ-free environment. (C and D) Sections 1 to 3 correspond to the anterior two-thirds of the tongue and 4 to 5 to the posterior third. Comparison between nitrate reduction and the microbial population over the surface of the rat tongue ( $n = 5$ ). The distribution of nitrate reduction (micromoles nitrite per hour) relates to the distribution of microorganisms.

to determine given the interdependency of microbial ecology (Hajishengallis et al., 2012).

Observations have also suggested that within the oral cavity, nitrate reduction is not, as previously thought, restricted to the anaerobic crypt environment at the dorsal surface of the tongue. Recent demonstrations show that bacteria residing on the teeth, on the cheek surface, and even in human dental plaque are capable of nitrate reduction (Schreiber et al., 2010). However, again, the relative contribution from this site versus the others is uncertain. In addition, there is some evidence that long-term dietary nitrate supplementation actually changes oral microbial ecology and may lead to further improvement in the processing of nitrate to nitrite (Hyde et al., 2014; Velmurugan et al., 2016; Vanhatalo et al., 2018; Burleigh et al., 2019). Feeding healthy pigs nitrate led to a change in the diversity of the oral microbiota (Trevisi et al., 2011), decreasing the Shannon index (a measure of the number of different types of bacteria and their relative abundance), suggesting that persistent (2-week) dietary nitrate treatment causes either a reduction in the richness of the community or triggers numbers of certain species to rise, thus reducing evenness. This effect was associated with acute increases in oral nitrate reduction followed by decreases with high doses of nitrate, although the identity of the species involved was not determined. In addition, in patients with hypercholesterolemia randomized to dietary nitrate for 6 weeks or control, there was a significant shift in 78 oral microbial taxa only after nitrate treatment, with *Rothia* and *Neisseria* spp. representing the greatest change (Velmurugan et al., 2016). Further studies have confirmed these increases in *Rothia* and *Neisseria* spp. in a healthy volunteer cohort after dietary nitrate supplementation for 10 days and have also noted reductions in *Prevotella* spp. and *Veillonella* spp. (Vanhatalo et al., 2018). However, whether these changes in oral microbiome in response to inorganic nitrate supplementation lead to further beneficial functional changes is not clear, as there is conflicting evidence of whether abundance of nitrate-reducing bacteria in the oral microbiome correlates to baseline plasma nitrite (Burleigh et al., 2018; Vanhatalo et al., 2018). Additionally, vascular responses to an acute dietary nitrate load were not different in healthy volunteers with increased nitrate-reducing bacteria identified in their oral microbiome (Burleigh et al., 2019).

Interestingly, there appear to be sex differences in the nitrate-nitrite-NO pathway, at least in part relating to oral microbial conversion of salivary nitrate to nitrite. The first hint of such an effect was demonstrated in a post hoc analysis of one of our first nitrate supplementation studies in healthy volunteers. Healthy women, in comparison with healthy men, had higher baseline plasma nitrite levels despite similar plasma nitrate levels and also greaterfold increases in plasma nitrite after a fixed dose of inorganic nitrate (Kapil et al., 2010).

Prospective evaluation confirms these baseline differences in plasma nitrite levels despite similar plasma nitrate levels and that this is represented in other biologic matrices additionally, namely in saliva and urine (Kapil et al., 2018). In addition, investigation of the enterosalivary pathway reveals that women have greater capacity for oral nitrate reduction compared with men. That is, for a fixed concentration of nitrate in the oral cavity, there is greater molar conversion to nitrite (Kapil et al., 2018). Despite these robust differences, there were no clear differences in oral microbial community diversity or structure (by 16s RNA profiling) overall or when restricted to taxa known to contain nitrate reductase genes (Kapil et al., 2018). A possible explanation is that bacterial nitrate reductase expression is influenced by sex hormones, although this has not been evaluated to date. Importantly, these differences in processing of nitrate to nitrite do not lead to divergence in response to inorganic nitrate supplementation, at least in CV responses such as BP and endothelial function (Kapil et al., 2018).

### *E. Nitrate Reductases*

**1. Prokaryotic Nitrate Reductases.** The nitrogen cycle details the chemical transformations that recycle nitrogen between the atmosphere, biosphere, and hydrosphere. The most reduced form of nitrogen is ammonia, the oxidized form of nitrogen, whereas the most oxidized form is nitrate. Microbes drive the nitrogen cycle, as they use an assortment of redox reactions to metabolize nitrogen for energy transduction, detoxification, or assimilation (Stein and Klotz, 2016). These microbial reactions are essential to most Terran life, as they are essential to maintain bioavailability of nitrogen.

Many bacteria readily reduce nitrate to nitrite via nitrate reductases. All prokaryotic nitrate reductases are molybdenum-dependent enzymes. They are traditionally separated into three major groups: membrane-bound periplasmic nitrate reductase (Nap), membrane-bound respiratory nitrate reductase (Nar), and the cytoplasmic, assimilatory nitrate reductase (Nas) (Sparacino-Watkins et al., 2014). Nitrate is retained in soils, sediments, and water, providing a biologically useful pool for many bacteria. Enzymatic reduction to nitrite generates enormous energy (Moreno-Vivián et al., 1999), which the organisms use to maintain homeostatic processes and life.

**2. Mammalian Nitrate Reductase Activity.** Despite the fact that it is accepted that mammals, in the main, lack the enzymes capable of undertaking nitrate reduction to nitrite and are therefore reliant on functioning oral microflora, there is some evidence that mammalian nitrate reductases may have a role. Although antibacterial mouthwash treatment in humans totally abrogated ex vivo nitrate reduction in saliva incubated with 1 mmol/l nitrate solution and prevented any increase in saliva nitrite levels in vivo, there was still a small increase in plasma nitrite level after an inorganic

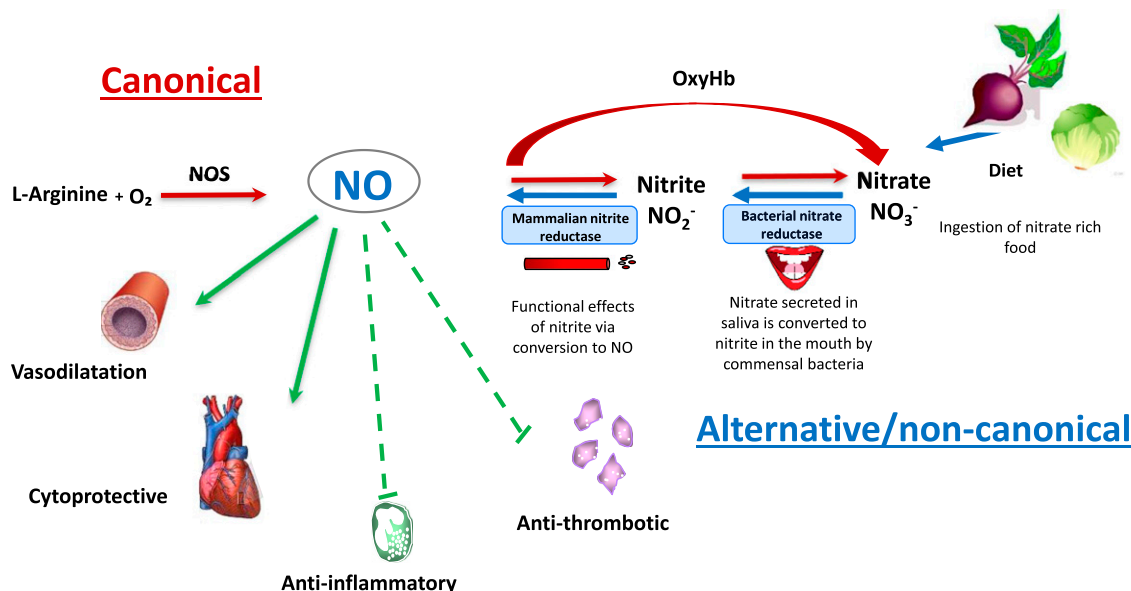


Fig. 5. NO cycle in humans. Colored arrows indicate oxidation and reduction.

nitrate load (Govoni et al., 2008). Although there could be nitrate reduction from lower GI commensals, an alternative explanation of mammalian nitrate reduction has also been postulated that is particularly focused on XOR.

XOR demonstrates nitrate-reduction activity in vitro under anoxic conditions that can be abrogated by XOR inhibition (Li et al., 2003). Furthermore, in normoxic wild-type rats, increases in plasma nitrite are partially abrogated after nitrate supplementation by XOR inhibition (Jansson et al., 2008). Similar results were demonstrated in endothelial  $\cdot$ NO synthase (eNOS)-deficient and germ-free mice, thereby excluding bacterial nitrate reduction and vascular  $\cdot$ NO synthase (NOS) activation as the source of nitrite (Jansson et al., 2008). Germ-free mice exhibited greater tissue levels of XOR, suggesting that this may represent a functional compensatory response to uphold nitrate production in the absence of a commensal microflora (Huang et al., 2010). These studies seem to confirm much earlier studies on ex vivo mammalian liver/muscle homogenates suggesting that XOR may exhibit nitrate-reducing capability (Bernheim and Dixon, 1928; Ward et al., 1985, 1986). In contrast, very recently, Moretti et al. (2019) have published findings suggesting a lack of mammalian nitrate reductase activity in germ-free mice in a separate study, indicating some heterogeneity in studies with animals rendered “germ-free.” Irrespective, the presence and importance of putative nitrate reductases in human physiology is yet to be firmly established.

#### IV. Sources and Pharmacokinetics of Nitrite

Plasma nitrite levels in the circulation are much lower than nitrate, with measurements from several different research groups falling in the 0.2–0.5  $\mu$ mol/l range under basal, fasting conditions (Grau et al., 2007). Nitrite also has a much shorter half-life than nitrate, estimated to be

between ~15 and 45 minutes in the circulation (Dejam et al., 2007; Hunault et al., 2009). In addition, nitrite levels measured in the plasma are likely to reflect multiple different sources. Nitrite that is generated from the metabolism of  $\cdot$ NO, in the oral cavity or from dietary sources, undergoes absorption and both enzymatic and nonenzymatic conversion to  $\cdot$ NO (Fig. 5).

##### A. Endogenously Derived Nitrite from the Enterosalivary Circuit

Elevation of systemic nitrite levels after ingestion of inorganic nitrate has been demonstrated to occur across a range of species as well as in humans. For example, provision of 1 g/l (16.2 mmol/l) inorganic nitrate in the drinking water to C57BL6 mice increases steady-state plasma nitrite levels by ~50% (from ~0.8 to 1.2  $\mu$ mol/l) and increases tissue levels (for example, heart tissue) by 500% (from ~3 to 18  $\mu$ mol/l), highlighting that plasma nitrite levels may not be indicative of tissue-specific levels or changes in levels (Bryan et al., 2007). Interestingly, supplementation of drinking water in Sprague-Dawley rats with much lower amounts of nitrate (to provide total daily nitrate intake of 0.1 and 1 mmol/kg) for 7 days increased plasma nitrite levels from 0.4 to 0.6 and 2.6  $\mu$ mol/l, respectively (Jansson et al., 2007). The differences reflected in these two studies reveal that the pharmacokinetics and handling of nitrate may be species-dependent.

Thus, studies in humans have been important to determine the impact of nitrate supplementation on systemic nitrite levels. In these studies, systemic nitrite levels have largely been examined in saliva, plasma, and urine only because, understandably, it is difficult to get tissue samples in healthy-subject studies. Although Stieglitz had shown more than 70 years ago that inorganic nitrate supplementation acutely (within 1 hour)

and chronically (up to 14 days) elevated blood nitrite levels (Stieglitz and Palmer, 1936), it is only more recently that researchers have studied systemic nitrite elevations after inorganic nitrate supplementation in depth, trying to elucidate whether sufficient nitrate-derived nitrite from the oral cavity reaches the circulation in humans. One of the first explorations of this phenomenon was conducted in healthy subjects who were supplemented with  $\sim 120 \mu\text{mol/kg}$   $\text{NaNO}_3$  (equivalent to  $\sim 8 \text{ mmol}$   $\text{NaNO}_3$  and 496 mg of nitrate for a 70-kg person) in a single dose (Lundberg and Govoni, 2004). Salivary, plasma, and urinary  $\text{NO}_x$  levels were assayed for up to 3 hours postingestion. In contrast to the very rapid appearance of nitrate in the plasma (within 15 minutes, as previously discussed above) and early plateau phase (within 1 hour), plasma nitrite levels were only significantly detected above basal values after 30 minutes and were still rising at 90 minutes. In this experiment, some subjects were asked to refrain from swallowing their saliva (by spitting all saliva out) to interrupt the enterosalivary circulation. In these subjects, although there was no difference in plasma nitrate levels compared with control (swallowing saliva), plasma nitrite levels did not rise. However, following resumption of swallowing saliva after 1 hour, plasma nitrite levels started to rise again (Lundberg and Govoni, 2004).

In 2006, Weitzberg and Lundberg also demonstrated that plasma nitrite levels were significantly higher ( $219 \pm 105 \text{ nmol/l}$ ) after 3 days of supplementation with  $0.1 \text{ mmol/kg}$   $\text{NaNO}_3$  ( $\sim 7 \text{ mmol}$  and 434 mg nitrate) compared with after 3 days of matched placebo ( $0.1 \text{ mmol/kg}$  sodium chloride,  $\text{NaCl}$ ) supplementation ( $138 \pm 38 \text{ nmol/l}$ ) (Larsen et al., 2006). The  $\sim 1.5$ -fold increases in plasma nitrite levels from baseline after supplementation with  $0.1 \text{ mmol/kg}$  in humans was similar to that observed in rats described above (Jansson et al., 2007). Similarly, we have demonstrated dose-dependent effects after the ingestion of nitrate in both inorganic salt and the dietary form of beetroot juice (Webb et al., 2008b; Kapil et al., 2010). Although beetroot is purple-red, it is a green leafy vegetable and contains significant nitrate levels (Santamaria, 2006). Ingestion of 500 ml of beetroot juice [mean (nitrate) =  $45.0 \text{ mmol/l}$ ] provided  $\sim 22.5 \text{ mmol}$  ( $\sim 1395 \text{ mg}$  nitrate) and was associated with an elevation of plasma nitrite levels from  $0.4$  to  $0.6 \mu\text{mol/l}$ . Plasma nitrite levels were slower to rise than plasma nitrate levels, with peak plasma nitrite levels apparent 3 hours after ingestion, compared with peak plasma nitrate levels at 60–90 minutes (Webb et al., 2008b). There were similar effects when a matched dose of nitrate salts ( $24 \text{ mmol}$  potassium nitrate) was used (Kapil et al., 2010). In addition, interruption of the enterosalivary circulation by avoidance of swallowing saliva prevented the rises in plasma nitrite but not plasma nitrate levels, confirming the critical importance of oral nitrate reduction in modulating systemic nitrite levels after inorganic nitrate ingestion (Webb et al., 2008b).

Further evidence for the importance of the oral microflora in facilitating this process is taken from studies that have sought to suppress the bacteria responsible for nitrate reduction. Prior treatment with a chlorhexidine antiseptic mouthwash in animals and humans has been shown to prevent the increases in plasma nitrite levels associated with nitrate ingestion (Govoni et al., 2008; Petersson et al., 2009; Jädert et al., 2012).

In summary,  $\sim 1$  hour after an inorganic nitrate load, plasma nitrite levels rise in a slow and sustained manner in humans, peaking in the circulation after 2.5–3 hours, reflecting the ingestion and enterosalivary processing of inorganic nitrate (Lundberg and Govoni, 2004; Webb et al., 2008b; Kapil et al., 2010).

### *B. Endogenously Derived Nitrite from NOS Activity*

Nitrite is formed by oxidation of  $\cdot\text{NO}$  as previously described above and has been suggested to be the most sensitive reflection of acute eNOS activity. In the human forearm circulation, stimulation of eNOS with acetylcholine or inhibition of NOS activity with NG-monomethyl-L-arginine acetate rapidly altered venous plasma nitrite levels up or down, respectively, but without changes in plasma nitrate levels (Lauer et al., 2001).

Furthermore, basal plasma nitrite levels in several species were found to be similar, suggesting similar levels of constitutive NOS activity, and application of several different NOS inhibitors demonstrated cross-species increases in vascular resistance that correlated to decreases in plasma nitrite levels but not plasma nitrate levels (Kleinbongard et al., 2003). Transgenic mice with genetic deficiency in eNOS have 70% lower plasma nitrite levels than wild-type C57BL6 littermate controls, which was similar to the levels measured in wild-type mice treated with an NOS inhibitor (Kleinbongard et al., 2003). These observations suggest that 70% of plasma nitrite is likely derived from eNOS. Similarly, in humans with CVD risk factors, it has been shown that plasma nitrite concentration inversely correlates to increasing number of CV risk factors and correlates directly to brachial artery flow-mediated dilation (FMD), suggesting that plasma nitrite levels could be used as a surrogate for endothelial function in those with CVD risk factors (Kleinbongard et al., 2006).

### *C. Exogenous Sources of Nitrite*

Plasma nitrite levels are also determined by dietary nitrite and nitrate consumption. Dietary nitrite intake levels in humans are not as substantial as dietary nitrate, with minimal intake occurring from drinking water. Nitrite levels in drinking water are usually below  $0.1 \text{ mg/l}$  (World Health Organization, 2011). Daily nitrite intake is similar across Europe and North America, with estimates suggesting ingestion of  $0.3$ – $0.9 \text{ mg/day}$ , which equates to  $6$ – $20 \mu\text{mol}$  nitrite ingested per day (Schuddeboom, 1993). The primary sources of dietary nitrite consumption are cured, processed meats.

Nitrite is added to meat as a preservative, as it prevents oxidation of fatty acids, preventing rancidity and controlling *Clostridium botulinum* (Hustad et al., 1973; Honikel, 2008). Meat products contain up to 6.4 mg of nitrite per kilogram (European Centre for Ecotoxicology and Toxicology of Chemicals, 1988), whereas vegetables contain very little nitrite (Hord et al., 2009).

In many studies exploring nitrite bioactivity, nitrite salts have been used, with the most commonly used being the sodium salt. The ~30-minute half-life mentioned above comes from work using NaNO<sub>2</sub> in healthy volunteers. In patients with diabetes, similar findings have been observed. Kevil and colleagues demonstrated that peak levels of circulating nitrite were achieved within 30 minutes after ingestion of an 80-mg dose of NaNO<sub>2</sub>, returning back to baseline within the 6-hour measurement period (Greenway et al., 2012). This group also assessed the impact of enteric coating upon circulating levels of nitrite in the same patient group. They showed that a statistically significant rise in circulating levels was not achieved with the enteric-coated NaNO<sub>2</sub> over the 6 hours; however, there was a trend for a rise that began to appear at around 1.5 hours after ingestion, peaking at 2.5 hours and maintained until 4.5 hours postingestion. Although, as noted, this rise was not statistically different from baseline (Greenway et al., 2012), the authors suggested that with doses higher than 80 mg, it may be possible to provide longer-lasting elevations of circulating nitrite that might prove useful in therapeutics.

However, mean salivary volume swallowed is estimated to be ~1.5 l/day (Dawes, 1972; Lagerlöf and Dawes, 1984), and mean basal salivary nitrite levels are ~100–300 μmol/l (Lundberg and Govoni, 2004; Govoni et al., 2008; Bahra et al., 2012). Thus, swallowing of saliva could be responsible for total amounts of intra-gastric nitrite in the order of 150–300 μmol daily. Mean plasma nitrite levels are 0.2–0.5 μmol/l, suggesting total circulatory nitrite levels at any one point of 1–2.5 μmol. Thus, the same total amount of nitrite would enter the stomach every 30 minutes. Although not all of swallowed nitrite survives to appear in the circulation, even if a fraction entered the circulation, it would make an important contribution to plasma nitrite levels under basal conditions. Some evidence suggests that salivary nitrite might account for the 30% of plasma nitrite concentration that cannot be accounted for by eNOS activity. Under basal conditions, chronic use of antibacterial mouthwash, which totally attenuated oral nitrate reduction, reduced salivary nitrite concentration by 90% and plasma nitrite levels by 25% (Kapil et al., 2013).

#### D. Systemic Nitrite Absorption

The nitrite that is formed in the oral cavity after bacterial conversion from nitrate is swallowed and may be directly acidified to increase levels of ·NO in the

stomach (Benjamin et al., 1994; Lundberg et al., 1994). However, a significant proportion of swallowed nitrite appears to survive passage into the stomach and, after transfer across the gut wall, enters the systemic circulation. The disappearance of nitrite from the stomach is a rapid process, with decline of nitrite levels by half within 10 minutes, as a consequence of absorption rather than chemical reactions of nitrite or gastric emptying (Licht et al., 1986).

Similar to the lack of knowledge relating to nitrate absorption in the GI tract, exactly how nitrite enters the circulation is uncertain. As a charged anion, an active uptake mechanism is likely to be involved, but this is yet to be proven. Although little work has been published describing active uptake mechanisms in gastric environs specifically, there have been some studies attempting to discern uptake mechanisms. In erythrocyte ghosts, nitrite exchange with bicarbonate (HCO<sub>3</sub><sup>-</sup>) is inhibited by the nonspecific anion-exchange inhibitor diisothiocyanatostilbene disulfonate, suggesting that at least for erythrocytes, there may be a role for the anion-exchange transporter-1 for the movement of nitrite across the membrane (Shingles et al., 1997; Vitturi et al., 2009; Jensen and Rohde, 2010). A similar anion-exchange transporter-1 dependence for nitrite transport has been demonstrated in pancreatic acini (Zhao et al., 1994). However, other researchers have failed to demonstrate the same effect of diisothiocyanatostilbene disulfonate (Zavodnik et al., 1999; May et al., 2000; Jensen, 2005). Additionally, May et al. (2000) demonstrated that nitrite uptake was dependent upon sodium and phosphate levels and therefore suggested that the sodium-dependent phosphate transporter was important.

However, in the gastric lumen, pH is between 1.0 and 2.5. The  $pK_a$  of nitrous acid (HNO<sub>2</sub>) in aqueous solutions is 3.3–3.4 (Butler and Ridd, 2004), and therefore, most of the nitrite will be in the neutral, lipophilic HNO<sub>2</sub> form. It has been hypothesized that the passive movement of HNO<sub>2</sub> from the acidic gastric environment into the neutral circulation may underlie the apparent transport of nitrite across the gastric wall (eq. 1.4), a possibility demonstrated to occur across the erythrocyte membrane (Samouilov et al., 2007; Webb and Ahluwalia, 2010). Arguing against the above proposal are studies in rats assessing the importance of gastric pH in circulating nitrite concentrations (Pinheiro et al., 2012). Treatment of rats with large doses (30 mg/kg, equivalent to 150 times the usual clinical dose) of the proton pump inhibitor omeprazole (clinical daily doses are usually 10–20 mg/day) followed by 15 or 45 mg/kg NaNO<sub>2</sub> orally, although increasing gastric pH from 4 to 6, did not impact upon circulating nitrite levels. Similar observations have been seen in healthy volunteers (Montenegro et al., 2017) in which treatment with 40 mg of esomeprazole did not lower circulating nitrite

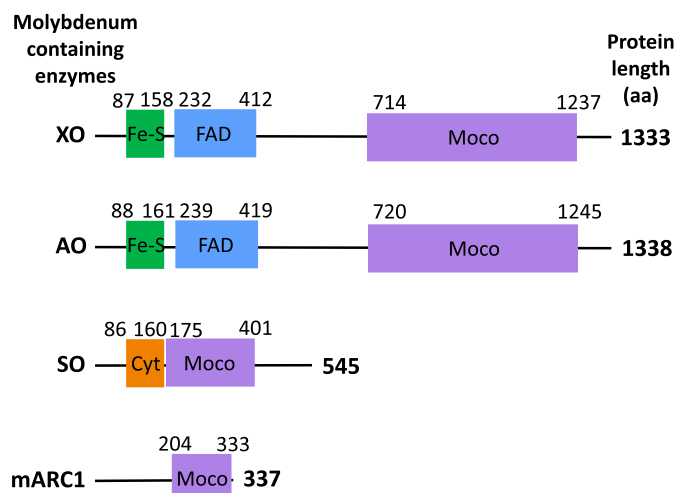
levels after an oral dose of 0.3 mg/kg (~0.3 mmol) of nitrite. The exact contribution of passive diffusion of  $\text{HNO}_2$  and/or active uptake of nitrite is still to be fully determined and quantified. However, what is apparent is that ingestion of inorganic nitrate, via the enterosalivary circulation and bacterial nitrate reduction, eventually leads to increases in plasma and tissue nitrite levels. Interestingly, it is now apparent that tissue levels of nitrite can be widely different and that plasma nitrite levels are, in general, the lowest in vivo, with potentially most of the “blood” nitrite carried inside the erythrocyte and much higher nitrite levels in certain tissues of the body, particularly the blood vessel wall (Bryan et al., 2005; Dejam et al., 2005).

## V. Mechanisms of Nitrite Bioactivation

It is recognized that there are two distinct pathways for nitrite bioactivation: either nitrite can be reduced to  $\cdot\text{NO}$  through acidification, as in the gut, or it can be reduced via a host of mammalian enzymes, which we describe below.

### A. Nonenzymatic Nitrite Reduction

The acidic environment of the stomach lends itself to the bioactivation of nitrite via the chemical reactions outlined above and shown in eqs. 1.4–1.6 (Benjamin et al., 1994). Firstly, nitrite is protonated, leading to the formation of nitrous acid, and at low pH,  $\text{HNO}_2$  decomposes to form  $\text{N}_2\text{O}_3$ , which in turn can dissociate to  $\cdot\text{NO}$  and  $\text{NO}_2$ . It was proposed that this acidification of nitrite to generate  $\cdot\text{NO}$  acts as a defense mechanism against swallowed pathogenic microorganisms (Benjamin et al., 1994) and that this effect was responsible for the increases in  $\cdot\text{NO}$  measured in response to dietary nitrate supplementation (Benjamin et al., 1994; Lundberg et al., 1994). Whereas Benjamin and colleagues used potassium nitrate to elevate stomach nitrite levels, Lundberg and colleagues demonstrated that ingestion of 50 g of iceberg lettuce (1.3 mg/kg nitrate content) led to a 4-fold elevation in exhaled  $\cdot\text{NO}$  5 minutes after ingestion. This response was abolished by pretreatment with 240 mg of omeprazole over a 24-hour period before lettuce ingestion (Lundberg et al., 1994). A year later, it was demonstrated using EPR spectroscopy and  $\cdot\text{NO}$  spin traps that in rat hearts, negligible  $^{15}\text{NO}$  is observed despite being treated with 1 mmol/l  $^{15}\text{N}$ -nitrite; however, in the ischemic rat heart, a 10-fold increase in the EPR signal was observed (Zweier et al., 1995). The authors suggested that the ischemia-induced acidosis provides the protons required for the acidic disproportionation of nitrite to  $\cdot\text{NO}$  without the need of a catalyst/enzyme. However, although the chemistry is correct, it is now clear that the reaction is facilitated by mammalian enzymatic activity within tissues and that this catalytic activity enables nitrite reduction under physiologic as



**Fig. 6.** The primary sequence structure of the four known mammalian molybdenum-containing enzymes. Numbers denote the first and last residues of the domains highlighted (data obtained from NCBI/Protein). aa, amino acid; Cyt, cytochrome  $b_5$ ; FE-S, iron-sulfur; Moco, molybdenum cofactor; NCBI, National Center for Biotechnology Information; XO, xanthine oxidase.

well as pathologic conditions, but the lower the  $\text{O}_2$  tension and pH, the greater the nitrite reduction. Below, we summarize the evidence supporting the key mammalian nitrite reductases that have been identified.

### B. Mammalian Nitrite Reductases

#### 1. Molybdenum-Containing Oxidase Enzymes.

In mammals, there are four known molybdenum-containing enzymes: XOR, sulfite oxidase (SO), aldehyde oxidase (AO), and mitochondrial amidoxime reductase (mARC) (Fig. 6). Although all have been shown to function as a nitrite reductase to various degrees, XOR has been identified as perhaps the major mammalian nitrite reductase and certainly is the most prominent molybdenum-containing enzyme responsible for nitrite bioactivation.

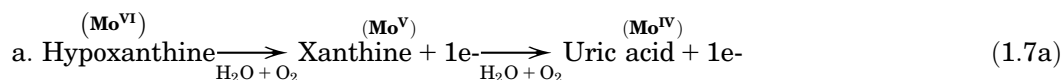
*a. Xanthine oxidoreductase.* XOR is widely distributed throughout the body, with the highest levels of expression in the liver (Linder et al., 1999). XOR is synthesized primarily in the liver and the lung, reflecting the high expression found within these tissues, with much lower levels of XOR expressed in most organs, including the brain, gut, heart, and in blood vessels (Abadeh et al., 1992, 1993). XOR has been localized within the cytoplasm of numerous cell types (Jarasch et al., 1981, 1986; Moriwaki et al., 1993; Radi et al., 1997; Houston et al., 1999) but has also been localized bound to the outer surface of cells through binding to heparin sulfate glycosaminoglycans (GAGs). This expression on the cell surface via GAGs has been suggested to account for substantial XOR expression identified on the endothelial surface in patients with coronary artery disease (Spiekermann et al., 2003) and that observed in erythrocytes from individuals undergoing coronary artery bypass grafting (CABG) (Webb

et al., 2008a), hypertensive rats, and patients with hypertension (Ghosh et al., 2013). More recently, we also identified XOR activity in erythrocyte preparations of apolipoprotein E (ApoE) knockout (KO) mice (Khambata et al., 2017), an atherosclerotic-prone mouse model in which an upregulation of XOR activity versus wild-type (WT) C57BL6 littermates was observed.

XOR has a FAD domain, two iron-sulfur domains, and a molybdenum-binding domain, the latter being the site for purine catabolism. The classically accepted function of XOR is the oxidation of hypoxanthine to xanthine and xanthine to uric acid, which occurs at the molybdenum site (eq. 1.7a), and the reduction of O<sub>2</sub>, occurring at the FAD site. However, in addition to these, it has been known for ~20 years that nitrite is reduced to ·NO at the molybdenum site (Millar et al., 1998; Zhang et al., 1998). The mechanism by which this reaction was proposed to occur was through NADH acting as a

under anaerobic conditions, i.e., <1% oxygen, XOR catalyzed this reaction. In this study, the authors confirmed that XOR drove nitrite reduction at the molybdenum-binding site with NADH as the reducing substrate, since both allopurinol and diphenyleneiodinium, the molybdenum and FAD-directed inhibitors, respectively, blocked this reaction. Further studies by Zweier's group demonstrated that XOR could also catalyze nitrite reduction to ·NO in both aerobic and anaerobic conditions (Li et al., 2004). Using substrates of xanthine or aldehyde, which act at the molybdenum site as electron donors, it was shown that O<sub>2</sub> functions as a competitive inhibitor of nitrite reduction, whereas using NADH as the electron donor at the FAD site, nitrite reduction still occurs even in the presence of oxygen at ~70% of the level observed in anoxia.

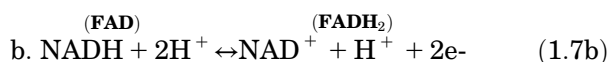
These observations have led us to suggest that a potentially useful approach for therapeutics may be to



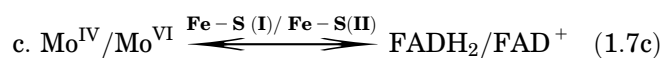
reducing substrate, donating electrons to the FAD site to form FADH<sub>2</sub> (eq. 1.7b); these electrons were then proposed to be shuttled from the FAD site to the molybdenum site via the iron-sulfur clusters (eq. 1.7c). The authors suggested that Mo (VI) is reduced to Mo (IV), and the O<sup>-</sup> that is donated from nitrite results in Mo (V) and the release of ·NO (eq. 1.7d). At the end of the reaction, for each reducing substrate oxidized, two molecules of ·NO were proposed to be formed in a one-electron-reducing process resulting in Mo (VI). Equation 1.7 shows reactions of XOR.

Reaction at the molybdenum-binding site is as follows:

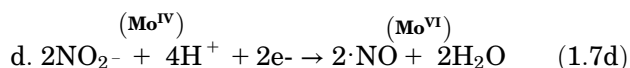
Reaction at the FAD site is as follows:



Internal electron shuttling is as follows:



Nitrite reduction at the Mo-Co site is as follows:



Millar et al. (1998) demonstrated that in the presence of O<sub>2</sub>, purified bovine XOR was unable to catalyze the reduction of nitrite (1–30 mmol/l) to ·NO; however,

take advantage of this balance of reactions. We have suggested that under optimal nitrite-reducing conditions, XOR may be “switched” from a detrimental ROS-generating enzyme to a protective one through preferential generation of ·NO. We propose that in conditions of excess nitrite and in the presence of the reducing substrate NADH, electrons will be shuttled from the FAD site to the molybdenum site via iron-sulfur domains, resulting in a decrease in the availability of the electrons needed for O<sub>2</sub> reduction. This would result in generation of ·NO and a concomitant reduction of superoxide and H<sub>2</sub>O<sub>2</sub>, which are thought to be major contributors to the oxidative stress that characterizes many chronic disease states, in particular CVD (Sugamura and Keaney, 2011), and to which XOR contributes (Berry and Hare, 2004).

This latter point is particularly important in disease, since both XOR expression and activity are substantially enhanced, and this possibly offers an opportunity for enhanced nitrite reduction too. An important observation, particularly relevant when discussing the potential utilization of XOR-mediated nitrite reduction in therapeutics, is that XOR activity is enhanced in scenarios of hypoxia and acidosis, conditions that are both prevalent in the setting of ischemia/reperfusion (I/R) injury. Using purified XOR under aerobic conditions, nitrite reduction was enhanced with acidic pH, with ·NO generation 3 times greater at pH 6 compared with pH 7.4 and a further 3 times greater from pH 6.4 to pH 5.5 when incubated with 1 mmol/l sodium nitrite



(Li et al., 2004). In isolated rat heart preparations, pH plummeted from 7.4 to 5.5 within 20 minutes of ischemia (Zweier et al., 1999), providing the ideal environment for XOR-dependent nitrite reduction. Our own observations have supported these findings. Using homogenates of rat and human heart, we demonstrated an elevation of nitrite-derived  $\cdot\text{NO}$  production not only with increasing concentrations of  $\text{NaNO}_2$  but, importantly, also with decreasing pH (6.0–5.0) (Webb et al., 2004). Additionally, Zweier and colleagues demonstrated that rat heart homogenates incubated with  $10 \mu\text{mol/l}$   $\text{NaNO}_2$  gassed with 2%  $\text{O}_2$  demonstrated 50% greater nitrite reduction than when under 5%  $\text{O}_2$ , an effect that was substantially inhibited by the XOR inhibitor oxypurinol (Li et al., 2004). These observations suggest that XOR-mediated nitrite reduction is perhaps a phenomenon particularly relevant under ischemia, as opposed to normal physiologic conditions of pH 7.4 and normoxia. Although it is worth noting that under physiologic conditions,  $\text{O}_2$  tension within organs can vary enormously, with partial pressure of oxygen ( $\text{PaO}_2$ ) of 90–100 mm Hg in arterial blood, 40 mm Hg in the venous return, and 35 mm Hg in the heart and brain, thus raising the possibility that, although within the arterial side of the circulation in normoxia, XOR may play little role as a nitrite reductase, this may not be the case in regions that normally exist at lower  $\text{O}_2$  tensions.

Accordingly, evidence for a role for XOR in mediating nitrite reduction under physiologic conditions (i.e., normoxia and pH 7.4) has grown in the last few years. We reported that bolus doses of  $\text{NaNO}_2$  (1–10,000 nmol/kg) reduce mean arterial pressure (MAP) in the spontaneously hypertensive rat (SHR) model, effects abolished by allopurinol (50 mg/kg) (Ghosh et al., 2013). We also demonstrated enhanced XOR levels of erythrocytes (measured using Western blotting) in the SHR model compared with the normotensive Wistar Kyoto strain control associated with elevated allopurinol-sensitive nitrite reductase activity, measured by gas-phase chemiluminescence. There was a very similar profile of activity in purified erythrocytes collected from normotensive and hypertensive humans. However, a recent study assessing erythrocytic nitrite reduction of blood collected from both normotensive and hypertensive volunteers demonstrated no effect of allopurinol upon nitrite reductase activity, measured using EPR detection of nitrosyl Hb (Liu et al., 2015). The authors highlighted a number of possible reasons for the differences between the two studies, including: concentrations of nitrite used (Ghosh et al., 10–100  $\mu\text{mol/l}$ ; Liu et al., 10 mmol/l); differences in the buffer that may have led to differing levels of XOR; potentially xanthine dehydrogenase expression versus xanthine oxidase expression; and that erythrocytes were collected from drug-naïve hypertensives in the previous study (Ghosh et al., 2013) as opposed to treated hypertensives. However, another likely explanation is that in the study

by Kim-Shapiro and colleagues, blood was collected with heparin as the anticoagulant, and heparin will cleave any XOR bound via GAGs on the erythrocytes (Adachi et al., 1993; Spiekermann et al., 2003). This fact underlies why in all studies in our laboratory, when assessing the nitrite reductase activity of blood elements, sodium citrate is used as the anticoagulant. In contrast to the above, Rosenbaek et al. (2018) pretreated healthy individuals with placebo or allopurinol, infused  $\text{NaNO}_2$  at  $3.5 \mu\text{mol/kg}$  per hour, and found no differences in brachial BP. However, the dose of  $\text{NaNO}_2$  used did not cause substantial BP lowering itself. Furthermore, allopurinol may cause opposing effects in reducing  $\cdot\text{NO}$  production but also potentially reducing superoxide production, thereby mitigating measurable vascular effects.

There is some evidence that nitrate and nitrite interfere with each other with respect to XOR activity. Damacena-Angelis et al. (2017), using high concentrations of purified XOR (concentrations unlikely to occur in vivo even in the most extreme of conditions), demonstrated that 30 mmol/l  $\text{NaNO}_3$  attenuated  $\text{NaNO}_2$  (1 mmol/l) reduction to  $\cdot\text{NO}$  by  $\sim 60\%$ . Although these findings initially suggest that there is competition between the two anions, the use of such high concentrations to evidence these effects creates uncertainty regarding potential physiologic relevance.

*b. Aldehyde oxidase.* Similar to XOR, AO consists of an FAD binding site, iron-sulfur domains, and a molybdenum-binding center and is highly expressed in the liver, but it is also found in other tissues, such as blood vessels, heart, lung, and kidney (Moriwaki et al., 1998, 2001). AO has multiple functions, including catalyzing the oxidation of aldehydes and heterocyclic compounds to produce carboxylic acid. The first demonstration of the nitrite reductase capacity of AO came from studies led by Jay Zweier. Using  $\text{NaNO}_2$  (100  $\mu\text{mol/l}$ ) and raloxifene (50 nmol/l), an AO inhibitor,  $\cdot\text{NO}$  production was reduced by  $\sim 40\%$  in 1 g of rat liver or heart homogenate (Li et al., 2008). Subsequent studies by Zweier's group demonstrated that the mechanism by which AO reduces nitrite is similar to that of XOR in that nitrite reduction is enhanced in hypoxic conditions and occurs at the molybdenum site (Li et al., 2009). Similar to XOR, NADH acts as a reducing substrate, donating electrons to the FAD site, an effect inhibited by diphenyliodonium. Aldehydes can also act as the electron donor at the molybdenum site of AO to facilitate nitrite reduction (Li et al., 2009). Again, similar to XOR, there is enhanced nitrite reduction under acidic pH, with the greatest activity occurring at pH 6 and decreasing at lower pHs (Li et al., 2009). In contrast, however, recent testing of raloxifene in healthy volunteers demonstrated that, if anything, the activity of  $\text{NaNO}_2$  was enhanced with treatment, intimating that pathways other than AO were involved in the effects seen (Omar et al., 2015). Thus, at present, the

physiologic significance of the nitrite reductase activity of AO is uncertain.

*c. Sulfite oxidase.* SO is located within the intermembrane space of mitochondria and exists as a homodimer, with each monomer containing a molybdenum-binding, an N-terminal cytochrome  $b_5$ -type heme, and a C-terminal domain. The physiological function of SO is to oxidize sulfite ( $\text{SO}_3^{2-}$ ) to sulfate ( $\text{SO}_4^{2-}$ ), which concomitantly reduces molybdenum from +6 to +4. It has recently been demonstrated that SO is also capable of reducing nitrite to  $\cdot\text{NO}$  under anaerobic conditions (Wang et al., 2010), a process enhanced under acidic conditions (Wang et al., 2015). Similar to XOR and AO, reduction of nitrite to  $\cdot\text{NO}$  occurs at the molybdenum domain; however, SO does this by the one-electron oxidation of Mo+6 to Mo+5, which was demonstrated using sulfite, a two-electron donor, and phenosafranine, a one- or two-electron donor in which nitrite reduction did not occur in the presence of the former (Wang et al., 2015). The authors also demonstrated that human fibroblasts collected from molybdenum cofactor-deficient and SO-deficient individuals have significantly reduced cGMP levels with the addition of 50  $\mu\text{mol/l}$  nitrite in 1% and 20% oxygen, in comparison with WT controls, demonstrating the importance of SO-dependent nitrite reduction in vivo (Wang et al., 2015).

*d. Mitochondrial amidoxime reducing component.* Although mARC, a molybdenum-containing enzyme, was described over a decade ago, its physiologic role is unknown (Havemayer et al., 2006). Humans possess two genes that encode for mARC-1 and -2, both of which have been shown to reduce nitrite to  $\cdot\text{NO}$ , albeit with suprapharmacological levels ( $\text{NaNO}_2$  1 mmol/l) (Sparacino-Watkins et al., 2014). The authors demonstrated that for nitrite reduction to occur, cytochrome  $b_5$  and cytochrome  $b_5$  reductase need to be present to facilitate electron transport from the FAD site to the molybdenum site within mARC, so NADH is also required (Sparacino-Watkins et al., 2014). Using site-directed mutagenesis to switch the cysteine of the active molybdenum site to alanine (C273A), nitrite reduction to  $\cdot\text{NO}$  was completely abolished. Similar to other molybdenum-containing enzymes, the rate of nitrite reduction is enhanced under acidic (3-fold, from pH 7.5 to pH 6.5) and hypoxic conditions (Sparacino-Watkins et al., 2014). The physiologic role of mARC facilitating nitrite to  $\cdot\text{NO}$  reduction remains uncertain.

*2. Mitochondrial Respiratory Chain Enzymes.* mARC is not the only mitochondrial component capable of reducing nitrite to  $\cdot\text{NO}$ . Experiments with rat liver homogenates demonstrate that inhibition of complex I or II leads to an attenuation of nitrite-derived  $\cdot\text{NO}$  ( $\text{NaNO}_2$  50  $\mu\text{mol/l}$ ) at pH 7.25 and under anoxic conditions (Kozlov et al., 1999). It was suggested that inhibition of complex I or II will prevent electron flow to complex III, which has been implicated as the site of

mitochondrial nitrite reduction. As such, inhibition of complex III (also known as cytochrome  $bc_1$  complex/cytochrome c reductase) completely abolished nitrite reduction (Kozlov et al., 1999; Nohl et al., 2000). There is also evidence to suggest that complex IV (cytochrome c oxidase) may reduce nitrite to  $\cdot\text{NO}$ . Treatment of rat liver homogenate with either antimycin A or myxothiazol (both complex III inhibitors) in the presence of the electron donor  $N,N,N',N'$ -tetramethyl-*p*-phenylenediamine dihydrochloride, which feeds electrons downstream of complex III of the respiratory chain, did not block nitrite reduction (Castello et al., 2006). Furthermore, carbon monoxide, a complex IV inhibitor, inhibited nitrite reduction (Castello et al., 2006). However,  $\text{NaNO}_2$  (1 mmol/l) reduction in this in vitro setting was only evident when experiments were conducted at  $\text{O}_2$  concentrations below 2% being maximal under anoxic conditions (Castello et al., 2006). The physiologic relevance of this phenomenon likely pertains only to the ischemic setting, and further investigation assessing the impact of this pathway upon biologic function is needed to better understand significance.

*3. Globins.* The globins are a family of globular heme-containing proteins that are involved in the transport of  $\text{O}_2$ ; however, an abundance of literature supports a role for these proteins in facilitating nitrite reduction. In particular, we summarize below the evidence for Hb, Mb, neuroglobin, and cytoglobin as nitrite reductases.

*a. Hemoglobin.* It was first discovered in the 1930s by Brooks and further elucidated by Doyle and colleagues in the 1980s that deoxyHb ( $\text{HbFe}^{2+}$ ) in the presence of nitrite has the ability to produce  $\cdot\text{NO}$ , metHB ( $\text{HbFe}^{3+}$ ), and hydroxide (Brooks, 1937; Doyle et al., 1981) (see eq. 1.2). Despite these observations from many years ago, it was not until 2003 that Gladwin and colleagues proposed the biologic significance of this reaction in terms of vasodilation. Gladwin's studies indicated that in the presence of deoxyHb, nitrite generates  $\cdot\text{NO}$ , a phenomenon much reduced in the presence of oxyHb (Cosby et al., 2003), the latter being as one would suspect due to the rapid  $\cdot\text{NO}$  scavenging characteristics of oxyHb. This opposing activity of different Hb states has led to inevitable uncertainty regarding the functional significance of Hb as a nitrite reductase.

Further studies by Gladwin and colleagues investigating the kinetics of these reactions has led to the proposal that the extent of nitrite reduction and the rate of reaction is dependent upon the state of the Hb tetramer. Hb can exist in two states, the tense state (T-state), in which Hb lacks oxygen, termed deoxyHb, and the relaxed state (R-state), in which Hb is oxygenated, termed oxyHb. It has been demonstrated that the R-state reacts with nitrite  $\sim 100$ -fold faster than the T-state (Huang et al., 2005a,b). This results in the peak rate of  $\cdot\text{NO}$  production occurring when Hb is 50%

saturated ( $p_{50}$ ) with oxygen. The physiologic relevance of these observations has been demonstrated using organ bath pharmacology. Precontracted rat aortic rings subject to increasing concentrations of  $\text{NaNO}_2$  (10 nmol/l to 300  $\mu\text{mol/l}$ ) under decreasing  $\text{O}_2$  tensions (60, 25, 15 mm Hg) relaxed the greatest under low  $\text{O}_2$  tension, with significant relaxant activity evident at concentrations of 200 nmol/l  $\text{NaNO}_2$  (Crawford et al., 2006), i.e., concentrations well within the physiologic range of plasma nitrite concentration.

However, the issue still remains, considering the nitrite chemistry proposed above, how can the  $\cdot\text{NO}$  produced from nitrite reduction by Hb escape scavenging by oxyHb, particularly since maximal nitrite reduction occurs at  $p_{50}$ , so considerable and sufficient oxyHb is present. There have been various hypotheses explaining how this might happen, including those mentioned earlier relating to the cell-free layer at the blood vessel wall created by flowing blood. However, there is also some chemistry that has been proposed to explain how this might occur. It has been suggested that the reaction of nitrite and deoxyHb forms an intermediate, such as  $\text{N}_2\text{O}_3$ , which is less reactive than  $\cdot\text{NO}$  and so is able to escape the erythrocyte and, once outside, can dissociate to release  $\cdot\text{NO}$  (Robinson and Lancaster, 2005; Basu et al., 2007; Hopmann et al., 2011). Similarly, a second hypothesis is that S-nitrosohemoglobin is generated, which allows escape from the erythrocyte and subsequent dissociation to release  $\cdot\text{NO}$  (Stamler et al., 1997; Pawloski et al., 2001; Nagababu et al., 2006; Salgado et al., 2009). Although it is clear that Hb does express nitrite reductase capacity, the continued uncertainty regarding the issues highlighted above make it difficult to be certain of the exact role of Hb in mediating the functional effects that have been attributed to nitrite in vivo.

*b. Myoglobin.* Deoxy Mb (deoxyMb) was first demonstrated to act as a nitrite reductase by Gladwin and colleagues, who discovered that nitrite bioactivation is  $\sim 36$  times faster with deoxyMb compared with deoxyHb: an effect that was attributed to its lower heme redox potential (Shiva et al., 2007a). The physiologic relevance of Mb-mediated nitrite reduction has been demonstrated in hearts excised from Mb KO mice, in which there was  $\sim 50\%$  reduction in  $\text{NaNO}_2$  (100  $\mu\text{mol/l}$ )-derived  $\cdot\text{NO}$ , compared with hearts of WT mice production after 30 minutes at pH 7.4 (Hendgen-Cotta et al., 2008). These transgenics have also helped to confirm the biologic importance of this chemistry: WT mice subjected to left anterior coronary artery occlusion and treated with 48 nmol/l  $\text{NaNO}_2$  had 61% smaller infarcts compared with those with no treatment, whereas there was no difference between  $\text{NaNO}_2$ -treated and untreated Mb KO mice (Hendgen-Cotta et al., 2008). It has also been suggested that this chemistry of Mb is not simply restricted to the heart, since Mb is also present in vascular smooth muscle. Studies with mice and

under systemic hypoxia, induced by ventilation with 10%  $\text{O}_2/90\%$   $\text{N}_2$ , facilitated  $\text{NaNO}_2$  (10  $\mu\text{mol/l}$ ) reduction that was associated with an  $\sim 3$ -fold and 2-fold elevation in plasma and aortic cGMP, respectively (Totzeck et al., 2012).

*c. Neuroglobin.* Neuroglobin is found in the brain of humans and mice and shares 21% and 25% homology with Mb and Hb, respectively (Burmester et al., 2000). Neuroglobin has been shown to express cytoprotective effects in vivo (Sun et al., 2003; Khan et al., 2006), although whether this relates to its ability to reduce nitrite to  $\cdot\text{NO}$  is unclear. The mechanism of nitrite reduction proposed is similar to that of the other globins; however, neuroglobin contains a hexa-coordinate heme atom, whereas Mb and Hb contain penta-coordinate heme atoms (Tiso et al., 2011). Recombinant murine and human neuroglobin elicit nitrite reductase activity when in the ferrous form under hypoxic conditions at pH 7.4 (Petersen et al., 2008; Tiso et al., 2011); however, the rate is  $\sim 2000\times$  faster in the penta-coordinate state as opposed to the hexa-coordinate state (Jayaraman et al., 2011; Tiso et al., 2011). One of the key differences between neuroglobin and the penta-coordinate globins, such as Mb, is that a distal histidine residue is bound to the heme, whether in the ferrous or ferric form, and for the heme to react with nitrite, the histidine must dissociate from the heme. This issue is particularly relevant because mutations in the histidine of neuroglobin profoundly influence rates of nitrite reduction, i.e., removal of His64 (E7) in neuroglobin results in a  $2500\times$  increased rate of nitrite reduction compared with the WT (Tiso et al., 2011). Further studies exploring the nitrite reductase activity of neuroglobin more closely, by expressing the zebrafish protein (which has high homology with the human protein) in *Escherichia coli*, indicate that subtle alterations in the distal heme binding pocket can result in a profound change in nitrite reductase capacity, hinting at possibilities of engineering proteins with improved nitrite reductase activity (Tejero et al., 2015).

*d. Cytoglobin.* Cytoglobin, although originally thought to be predominantly a liver protein, is now known to be widely expressed (Burmester et al., 2000) across species and has been found in human, mouse, and zebrafish, sharing  $\sim 30\%$  homology with Mb and Hb (Burmester et al., 2000). Similar to neuroglobin, the heme atom is in a hexa-coordinate state, and a penta-coordinate state is required for nitrite reduction. Under hypoxic conditions, which favor nitrite reduction, cytoglobin expression is elevated (Fordel et al., 2004). However, initially it was suggested that recombinant mouse ferrous cytoglobin had no nitrite reductase activity at pH 7.4, with 7.4  $\mu\text{mol/l}$  nitrite (Petersen et al., 2008). In contrast, a subsequent study has shown that although oxygenated cytoglobin, similarly to Hb, will react with  $\cdot\text{NO}$  to form nitrate and so act to scavenge  $\cdot\text{NO}$ , under anaerobic conditions and at pH

7.4 or 8, ferrous cytoglobin reduces  $\text{NaNO}_2$  (100  $\mu\text{mol/l}$ ) to form  $\cdot\text{NO}$  as measured by EPR (Li et al., 2012a). In addition to this reaction occurring under physiologic pH, nitrite reduction by cytoglobin was found to increase with decreasing pH (Li et al., 2012a). The discrepancy between the two studies may be due to the differing concentrations of nitrite used.

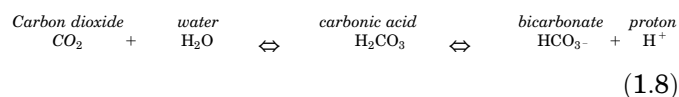
That the nitrite reductase activity is catalytic and thus potentially important in vivo (because of the widespread expression of cytoglobin in tissues) was recently elegantly demonstrated in experiments with both human and fish cytoglobin. Gladwin and colleagues demonstrated that cytochrome  $b_5$  and cytochrome  $b_5$  reductase reduce cytoglobin at rates that are up to 250 $\times$  faster than for either Hb or Mb. The authors suggest that this enhanced rate of activity with physiologic levels of the reactants intimates that cytoglobin is the primary substrate for these reductants in vivo and that, because of its relatively ubiquitous expression in tissues, cytoglobin may be a critical mechanism for  $\cdot\text{NO}$  generation from nitrite in physiology (Amdahl et al., 2017).

**4. Indoleamine 2,3-Dioxygenase 1.** Very recently, it has been demonstrated that indoleamine 2,3-dioxygenase 1 (IDO1), a cytosolic heme enzyme involved in the initial and rate-limiting step of L-tryptophan metabolism in the kynurenine pathway, is capable of reducing nitrite to  $\cdot\text{NO}$  under anaerobic conditions (Lim et al., 2019). Similar to Mb and Hb, the heme in IDO1 is in a penta-coordinate state, which may account for the increased rates, as discussed above. A contrasting feature, in comparison with other heme-containing nitrite reductases, is that the nitrite reductase activity of IDO1 is enhanced in alkaline conditions (pH 8), which Lim and colleagues speculate may be due to the close proximity of histidine residues potentially capable of donating a proton (Lim et al., 2019). These initial observations have been made in recombinant human IDO1, and further studies are required to assess the in vivo relevance of these findings.

**5. Nitric Oxide Synthase.** Under anoxic conditions, it has been demonstrated that endothelial NOS can function as a nitrite reductase (Gautier et al., 2006; Vanin et al., 2007). Recombinant eNOS in the presence of anoxia, with NADPH (100  $\mu\text{mol/l}$ ) and  $\text{NaNO}_2$  (500  $\mu\text{mol/l}$ ) at pH 7.6, resulted in ferrous-nitrosyl eNOS formation and then, consequently,  $\cdot\text{NO}$  generation (Gautier et al., 2006). This proposal was supported by studies using  $^{15}\text{N}$ -labeled  $\text{NaNO}_2$  with EPR measurements of  $^{15}\text{N}$ - $\cdot\text{NO}$  (Gautier et al., 2006). Subsequently, studies with recombinant iNOS and neuronal NOS have suggested that this activity is unique to the eNOS isoform, since neither isoform generated  $\cdot\text{NO}$  from relatively high concentrations of  $\text{NaNO}_2$  (500  $\mu\text{mol/l}$ ) under anoxic conditions generated with argon (Mikula et al., 2009). In addition, we have shown that erythrocytes, collected from healthy individuals, when incubated with physiologic concentrations of  $\text{NaNO}_2$

(10–100  $\mu\text{mol/l}$ ) under hypoxic conditions, generate  $\cdot\text{NO}$ ; a response that is attenuated by the eNOS inhibitors  $N(\gamma)$ -nitro-L-arginine methyl ester (L-NAME) and NG-monomethyl-L-arginine acetate (300  $\mu\text{mol/l}$ ) (Webb et al., 2008a). The identification of eNOS as a nitrite reductase under hypoxia and anoxia is an interesting finding and suggests that this enzyme, utilizing distinct substrates dependent upon the environmental conditions, may be equipped to produce  $\cdot\text{NO}$  under the full spectrum of oxygen tension that is experienced in vivo.

**6. Carbonic Anhydrase.** More recently it has been suggested that carbonic anhydrase, a key component involved in acid-base regulation in the body through the hydration of  $\text{CO}_2$  to produce protons and thus  $\text{H}_2\text{O}$  and bicarbonate ( $\text{HCO}_3^-$ ) (eq. 1.8), also acts as a nitrite reductase, particularly in metabolically active tissue. Hydration of  $\text{CO}_2$  is as follows:



In 2004 work from Innocenti et al. (2004) demonstrated that anions, including nitrite, could bind to the active site of carbonic anhydrase and, in this way, inhibit  $\text{CO}_2$  hydration (Innocenti et al., 2004). However, it was not until 2009 that the possibility that this interaction of nitrite with the enzyme might actually yield  $\cdot\text{NO}$  too was considered. Addition of  $\text{NaNO}_2$  (100  $\mu\text{mol/l}$ ) to purified carbonic anhydrase at pH 7.2 resulted in  $\cdot\text{NO}$  generation, an effect that was potentiated at pH 5.9 (Aamand et al., 2009). Addition of dorzolamide (250  $\mu\text{mol/l}$ ), an inhibitor of the hydration of  $\text{CO}_2$ , resulted in a significant elevation of  $\cdot\text{NO}$  generation (Aamand et al., 2009). The explanation for the inhibitory activity for  $\text{CO}_2$  hydration but not nitrite reduction was suggested by the authors to imply that distinct binding sites for the two exist, with the inhibitors only binding to the active  $\text{Zn}^{2+}$  binding site for  $\text{CO}_2$ . The reactions proposed to underlie this are shown previously (eqs. 1.4–1.6) with a theoretical  $\text{N}_2\text{O}_3$  intermediate formed (the levels of which are increased with reducing pH) that rapidly dissociates to give  $\cdot\text{NO}$  (along with an equivalent of  $\text{NO}_2$ ).

This nitrite-reducing activity of carbonic anhydrase was tested for functional importance using rat aorta segments treated with  $\text{NaNO}_2$  (10  $\mu\text{mol/l}$ ) with and without dorzolamide. The studies showed, as with the purified enzyme, an increase ( $\sim 50\%$ ) in the functional relaxation response to  $\text{NaNO}_2$  (Aamand et al., 2009). However, using EPR and measurement of nitrosyl Hb as a readout, inhibition of carbonic anhydrase using dorzolamide did not affect erythrocyte nitrite reduction (Liu et al., 2015). Recently, it has also been shown that human platelets treated with  $\text{NaNO}_2$  (100  $\mu\text{mol/l}$ ) showed no change in cGMP or phospho-vasodilator-stimulated phosphoprotein (phospho-VASP)<sup>S239</sup>, a marker

of protein kinase G activity; however, with the addition of recombinant bovine carbonic anhydrase, there was a significant elevation in both markers (Hanff et al., 2016). In healthy volunteers, treatment with dorzolamide enhanced  $\text{NaNO}_2$ -induced vasodilation of the radial artery (Omar et al., 2015) and a similar BP-lowering effect was seen in healthy volunteers treated with acetazolamide and  $\text{NaNO}_2$  (Rosenbaek et al., 2018). In contrast, more recently, a re-evaluation of the nitrite reductase activity of carbonic anhydrase has suggested little activity. Incubation of purified bovine carbonic anhydrase at pH 5.9 with 100  $\mu\text{mol/l}$   $\text{NaNO}_2$  demonstrated neither  $\text{N}_2\text{O}_3$  nor  $\cdot\text{NO}$  generation (Andring et al., 2018).

Although it is clear that there are numerous mammalian nitrite reductases that have been identified in cell-free and cellular preparations, in WT and transgenic animals, and in humans, the importance of these discoveries lies within the translational potential for enhanced  $\cdot\text{NO}$  delivery into real benefits in health and disease. Below we discuss the major therapeutic areas in which targeting and translation of the noncanonical pathway for  $\cdot\text{NO}$  in humans and patients has been investigated.

## VI. Functional Effects of the Noncanonical $\cdot\text{NO}$ Pathway and Clinical Translation

Nitrite has been used as a cyanide antidote for more than 75 years (Butler and Feelisch, 2008) and is used in food preservation to prevent oxidation of fatty acids and to prevent rancidity and malodor formation, as well as critically controlling *C. botulinum* growth and toxin production (Hustad et al., 1973; Honikel, 2008). In addition, inorganic nitrite and nitrate have been used as therapeutics in the CV system for over 85 years (Reichert and Mitchell, 1880; Stieglitz, 1927), but it is only through the relatively recent renewed interest in these anions that potential translational opportunities more broadly have been assessed.

The overwhelming evidence supporting the existence and functional efficacy of this noncanonical pathway for  $\cdot\text{NO}$  generation has raised interest, particularly to “rescue” levels of  $\cdot\text{NO}$  in diseases in which reduced bioavailability plays a prominent role in pathogenesis. This is of interest particularly in CVD, in which a deficiency of bioavailable  $\cdot\text{NO}$  driven by decreased L-arginine-derived  $\cdot\text{NO}$  generation is thought to contribute to pathology.

Although there is limited data on the beneficial effects of nitrite elevation directly or via oral conversion of inorganic nitrate in a miscellany of unrelated conditions, such as periodontitis (Jockel-Schneider et al., 2016) and urea cycle disorders (Erez et al., 2011; Nagamani et al., 2012), there is a wealth of published preclinical and clinical studies demonstrating efficacy in disease areas in which known  $\cdot\text{NO}$

deficiency contributes to, or  $\cdot\text{NO}$  supplementation slows, disease progression.

### A. Nitrite, Nitrate, and the CV System

Because of the classic role of eNOS-derived  $\cdot\text{NO}$ , and lack thereof, in CV health and disease (Moncada and Higgs, 2006), nitrite and nitrate as therapeutics are most advanced in the realm of CVD.

*1. Vasodilation.* Although it had been long since demonstrated that once-acidified supraphysiologic concentrations of inorganic nitrite exerted significant vasodilator responses (Furchgott and Bhadrakom, 1953), it was only more recently in 2001 that the view that nitrite was inert at physiologic levels within the CV system was finally eroded. In rat aortic rings, application of low micromoles per liter  $\text{NaNO}_2$  (2.5  $\mu\text{mol/l}$ ), although inactive under physiologic pH, was shown to cause significant relaxation of precontracted rat aorta under acidic (pH 6.6) conditions (Modin et al., 2001). The importance of this observation was finally appreciated with demonstration by Gladwin and colleagues that infusion of  $\text{NaNO}_2$  into the forearm of healthy volunteers causes vasodilatation with concomitant increased forearm blood flow, a phenomenon augmented when the volunteers were asked to exercise by conducting handgrips (Cosby et al., 2003). The authors proposed that this effect of exercise to increase nitrite-induced vasodilation related to the fact that conversion of nitrite to  $\cdot\text{NO}$  is enhanced under reduced  $\text{O}_2$  conditions. This observation simply and elegantly indicated that nitrite exerts significant biologic effects in the relative hypoxia created by conditions relevant to the normal and typical ranges in  $\text{O}_2$  tension that occur in day-to-day activity. These findings intimated a potentially important role of this anion in sustaining/regulating normal vasodilator tone in the healthy individual, particularly since, in the normal passage of blood through the circulation,  $\text{O}_2$  tension will drop from 100 mm Hg as it exits the heart to 40 mm Hg when it returns via the venous circulation.

The thesis that  $\text{O}_2$  tension determines the contribution that nitrite plays in regulating vascular tone was further developed by studies conducted by Frenneaux and colleagues, who used radiolabeled, autologous blood together with standard forearm plethysmographic techniques, thus enabling the separate determination of arterial and venous vasodilation. Vascular reactivity was measured over a 20-minute infusion period. Under normoxic conditions, intra-arterial delivery of increasing doses of  $\text{NaNO}_2$  (314 nmol/min to 7.84  $\mu\text{mol/min}$ ) decreased venous tone in a dose-dependent manner by up to 20%–35% (Maher et al., 2008). However, in contrast to Gladwin and colleagues’ earlier works (Cosby et al., 2003), low-dose  $\text{NaNO}_2$  infusions (314 nmol/min), associated with ~30% increases in forearm blood flow, did not dilate the arterial side of the circulation (Maher et al., 2008). Indeed, arterial dilation was only apparent (increasing forearm blood flow by 60%–80%) at much higher

doses (3.14–7.84  $\mu\text{mol}/\text{min}$ ) (Maher et al., 2008). These findings replicate much earlier work that used detailed tilt-table testing to elucidate the role of dilation of the venous circulation (and not arterial circulation) as the cause of nitrite-induced CV collapse (Weiss et al., 1937; Wilkins et al., 1937). However, to make the arterial side of the circulation “hypoxic,” subjects were put through the protocol again, but only after arterial oxygen saturations were maintained at 83%–88% by breathing 12%  $\text{O}_2$ . In this situation, although there was no change in the magnitude or duration of the effects on the venous side of the circulation, infusion of 314  $\text{nmol}/\text{min}$   $\text{NaNO}_2$  (which had no effect in normoxic conditions) increased forearm blood flow by  $\sim 40\%$  (Maher et al., 2008). Such a finding is consistent with the concept that the vasodilator potential of nitrite is not solely limited to anoxia or extreme ischemia but is proportional to the extent of “hypoxia” in the tissues and blood as it deoxygenates from arterial to venous sides.

More recently, the effect of nitrite on muscular, conduit blood vessels has been shown. Infusion of a single dose of  $\text{NaNO}_2$  (8.7  $\mu\text{mol}/\text{min}$ ) for 60 minutes into the brachial artery caused an  $\sim 30\%$  increase in radial artery diameter (Omar et al., 2015). Comparison of the dose-response relationship of  $\text{NaNO}_2$  to GTN demonstrated comparable effects. For example, with an infusion of  $\text{NaNO}_2$  of 26  $\mu\text{mol}/\text{min}$  (1.8  $\text{mg}/\text{min}$ ) for 20 minutes, a statistically significant  $\sim 30\%$  increase in diameter was evident, a magnitude of response similar to that produced in the same healthy volunteers by GTN 1.3  $\text{nmol}/\text{min}$ , which is equivalent to 0.3  $\mu\text{g}/\text{min}$  (Omar et al., 2015). Furthermore, similar effects have been seen in the coronary circulation (O’Gallagher et al., 2018). Paradoxically the effect in the peripheral circulation was most pronounced in normoxia and inhibited by hypoxia or hyperoxia (Omar et al., 2015), an observation that conflicts with the findings previously discussed. Exactly why this is the case is uncertain.

Physiologically, the role of nitrite as an endocrine storage form of  $\cdot\text{NO}$  that is released after a decrease in  $\text{O}_2$  tension and is thus responsible for hypoxic vasodilatation has been investigated. Hypoxic vasodilation is responsible for matching  $\text{O}_2$  demand from respiring tissues to  $\text{O}_2$  delivery; thus, when metabolic demand rises,  $\text{O}_2$  consumption increases, and with this, a mild hypoxia develops that then triggers vasodilation to increase  $\text{O}_2$  availability to the organ. Erythrocytes have been identified as a key hypoxic sensor acting as a source of  $\cdot\text{NO}$  delivery that underlies the biologic effect (Segal and Duling, 1986; Stamler et al., 1997). However, two competing theories for the form of  $\cdot\text{NO}$  that is transported and stored ready to deliver  $\cdot\text{NO}$  on demand have been proposed: nitrite and *S*-nitrosothiols. Arterial-venous gradients of nitrite (Gladwin et al., 2000) and nitrosothiols (Stamler et al., 1992), and the formation of iron-nitrosylHb or *S*-nitroso-Hb as markers of consumption and  $\cdot\text{NO}$  delivery have been measured

in the peripheral and cerebral circulation, with exercise and under hypoxia. These studies demonstrate clear evidence of arterial-venous gradients for nitrite and formation of iron-nitrosylHb only, suggesting a role for nitrite as an endocrine reservoir of  $\cdot\text{NO}$  activity (Gladwin et al., 2000; Bailey et al., 2017).

It is likely that nitrite-induced vasodilatation is dependent upon activation of the canonical  $\cdot\text{NO}$ -dependent GC-1 pathway, since the GC-1 inhibitor 1H-[1,2,4]-oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) attenuates responses to nitrite in blood vessels of rodents and pigs (Botden et al., 2012; Ghosh et al., 2013). It is noteworthy that these observations recapitulate much earlier studies using drugs such as methylene blue to block GC-1 activity (Mittal et al., 1978; Ignarro and Gruetter, 1980; Gruetter et al., 1981); however, such observations have not been observed in all vascular beds tested. Recent studies in the renal microvasculature of the mouse demonstrate nitrite is two orders of magnitude more potent than in other vascular beds (Gao et al., 2015). Interestingly,  $\cdot\text{NO}$  scavenging abolished nitrite-induced vasodilatation, but treatment with ODQ did not, indicating a GC-1-independent effect. In the same study, inorganic nitrate supplementation reduced NADPH oxidase activity, a prime source of oxidative stress, and the actions of NADPH oxidase inhibition were synonymous and not additive. These results suggest that NADPH oxidase, at least in the renal microvasculature, could be a mechanistic target for nitrite-derived  $\cdot\text{NO}$  and associated BP lowering through reduction in oxidative stress (Gao et al., 2015).

Furthermore, there may be additional effects on vascular tone that are affected by nitrite-derived  $\cdot\text{NO}$  that are centrally driven. Elevation of systemic nitrite levels reduces renal sympathetic nerve signaling in rats under L-NAME or angiotensin II-driven hypertension. This treatment also reduces muscle sympathetic nerve activity in humans, a gold-standard measure of efferent sympathetic signaling (Notay et al., 2017; Guimarães et al., 2019). The precise mechanism of nitrite-derived  $\cdot\text{NO}$  sympatholysis is not known but may relate to alterations in expression of angiotensin II receptors in the rostral ventral lateral medulla, the brainstem sympathetic control center (Guimarães et al., 2019), or to changes in peripheral chemoreflex activity. The peripheral chemoreflex is primarily responsible for augmenting ventilation in response to hypoxia but is known to be augmented in human hypertension in the absence of hypoxia and responsible for elevated BP through efferent central sympathetic signaling (Marshall, 1994), and this mechanism is attenuated after inorganic nitrate supplementation in older adult humans (Bock et al., 2018b).

Irrespective of the precise mechanism, these reported effects of nitrite on vascular tone raise the possibility that systemic nitrite administration or dietary nitrate

supplementation, as a means to elevate circulating levels of nitrite, may be useful in the treatment of hypertension.

2. *Blood Pressure.* These demonstrations of the vasodilator potential of nitrite are also concordant with the effects of nitrite supplementation in normotensive animals, as well as in animal models of hypertension. In anesthetized rats, administration of  $\text{NaNO}_2$  (10–1000  $\mu\text{mol/kg}$ , i.v.) was associated with dose-dependent reductions in MAP for up to 30 minutes (Vleeming et al., 1997), observations we ourselves have reproduced in conscious tethered rats (Ghosh et al., 2013). In free-moving rats, supplementation of drinking water with extremely high concentrations of  $\text{NaNO}_2$  (36 mmol/l equivalent to 2.48 g/l) reduced BP measured by telemetry (Vleeming et al., 1997). If we consider that a typical Sprague-Dawley or Wistar averaged-sized adult rat ( $\sim 250$  g) will drink 30–50 ml of water daily, this would give a maximum daily dose of  $\text{NaNO}_2$  of 124 mg, which in turn, would approximate to 500 mg/kg, a level exceeding the ADI by 135 times. Nevertheless, these early studies clearly demonstrate BP-lowering potential.

Similarly, in spontaneously hypertensive rats, acute bolus doses of  $\text{KNO}_2$  (1–30,000 nmol/kg, equivalent to  $\sim 10$  nmol to 0.3 mmol/l circulating concentrations) (Ghosh et al., 2013), as well as prolonged oral administration (up to 1 year) of extremely high concentrations of  $\text{NaNO}_2$  (50–100 mmol/l equating to 3.5–6.9 g/l in drinking water), were associated with dose-dependent reductions in BP (Beier et al., 1995; Haas et al., 1999). These observations indicate two important issues: firstly, that the efficacy of nitrite was still evident in the setting of raised BP [indeed, our studies indicated a substantially enhanced potency of nitrite in this setting (Ghosh et al., 2013)], and secondly, that significant BP lowering was evident even after 1 year of ingestion. This latter observation hints at a key characteristic of nitrite, and potentially nitrate, that might endow these anions with a superior potential for therapeutics over the organic nitrates, i.e., no development of tolerance, which is the key limiting factor in the use of organic nitrates clinically (Münzel et al., 2005). This was tested more formally by Dejam et al. (2007), in which  $\text{NaNO}_2$  (12.5  $\mu\text{g/kg}$  per minute) was infused in primates continuously over a 2-week period and was also followed by a daily bolus of  $\text{NaNO}_2$  at a dose of 12,000  $\mu\text{g/kg}$  per minute. This bolus dose lowered MAP by 18 mm Hg, with no diminution of effect over time, indicating lack of tolerance. The utility of nitrite as an antihypertensive either by acute or chronic daily oral gavage or supplementation in water has been confirmed in other models of hypertension, such as the deoxycorticosterone acetate (DOCA)-salt, two-kidney/one-clip, and L-NAME-induced hypertension (Tsuchiya et al., 2005; Montenegro et al., 2011, 2012; Amaral et al., 2015).

There is also evidence that nitrite lowers BP in humans. Infusion of  $\text{NaNO}_2$  into the brachial artery was associated with local nitrite concentrations of  $\sim 200$   $\mu\text{mol/l}$  and systemic elevation of nitrite to 16  $\mu\text{mol/l}$  and was associated with a reduction in MAP of 7 mm Hg (Cosby et al., 2003). Further studies from the same group using stepped brachial infusions of  $\text{NaNO}_2$  achieving local venous plasma nitrite concentrations of 30  $\mu\text{mol/l}$  were associated with a drop in MAP of 10 mm Hg, an effect that persisted for up to 3 hours (Dejam et al., 2007). Studies assessing the feasibility of long-term infusion of  $\text{NaNO}_2$  in humans has been assessed in healthy volunteers. Pluta et al. (2011) examined the duration of action of increasing doses of  $\text{NaNO}_2$  (4.2–445.7  $\mu\text{g/kg}$  per hour) in healthy volunteers, measuring circulating levels of nitrite, together with assessment of methHB levels and BP. This approach was taken because nitrite has a short half-life ( $\sim 30$  minutes), and the authors wished to identify whether continuous infusion might provide a mechanism to exert persistent effects upon blood flow, particularly focusing upon the potential in conditions such as cerebral vasospasm. In this study, they showed that the BP-lowering effects of  $\text{NaNO}_2$  were maintained over 48 hours and were dose-dependent but that ceasing of infusion resulted in a prompt restoration of BP to baseline levels in association with circulating levels of nitrite (Pluta et al., 2011).

The authors of some of the earlier animal studies assessing responses to  $\text{NaNO}_2$  hypothesized that elevation of systemic nitrite levels from nitrate via bacterial reduction would reduce BP, although they did not explicitly test this (Classen et al., 1990; Vleeming et al., 1997). However, there is now a substantial body of evidence in both animal models and humans to support this proposal.

In rats supplemented with 10 mmol/l  $\text{NaNO}_3$  in drinking water over a week, BP was reduced when measured by cannulation under anesthesia or in free-moving animals using telemetric methods compared with matched control. This effect of  $\text{NaNO}_3$  was abolished by use of antibacterial mouthwash twice daily. Notably, this oral treatment did not affect BP lowering in response to  $\text{NaNO}_2$  supplementation (Pettersson et al., 2009). In uninephrectomized rats fed a high-salt diet as a model of hypertension, supplementation with  $\text{NaNO}_3$  (0.1–1 mmol/kg per day) lowered BP and ameliorated cardiac and renal fibrosis, suggesting beneficial effects beyond BP lowering (Carlström et al., 2011).

A key feature in human hypertension and CVD is that eNOS is dysfunctional, resulting in a generalized  $\cdot\text{NO}$  deficiency. Several research groups have sought to explore the potential utility of nitrite and/or nitrate<sup>-</sup> to restore  $\cdot\text{NO}$  homeostasis and lower BP in hypertensive animal models driven by reduced eNOS activity. Using the eNOS inhibitor L-NAME to induce hypertension, administration of  $\text{NaNO}_2$  in drinking water

(20–200  $\mu\text{mol/l}$ ) or by oral gavage (0.2 mmol/kg per day) for 3 to 4 weeks prevented the development of hypertension (Tsuchiya et al., 2005; Montenegro et al., 2014). Similarly, oral gavage with 0.1 mmol/kg per day  $\text{NaNO}_3$  reduced BP in both eNOS KO mice and L-NAME-treated rats (Carlström et al., 2010, 2015). These results suggest that elevating systemic nitrite levels, whether directly or via dietary nitrate supplementation, can compensate for diminished eNOS-derived  $\cdot\text{NO}$ , whether it is provided as a pretreatment or used to reverse pre-existing hypertension driven by reduced  $\cdot\text{NO}$  levels.

The above studies and concepts have been translated into a range of clinical studies. Although much of the preclinical data suggests that inorganic nitrite and nitrate are efficacious approaches to lower BP, whatever the cause of the hypertension, in the clinical setting, the majority of the studies to date have tested dietary nitrate rather than nitrite, and this relates to a number of reasons that include issues related to half-life and potential toxicity (discussed later).

Given the pharmacokinetic disadvantages of nitrite as a long-term therapy, only one major human study has explored inorganic nitrite therapy and BP control specifically. In this study, individual adults aged 50–79, who were free of all cardiometabolic disease and were normotensive at the start of the study (but with diminished endothelial function at baseline), were randomized in three parallel groups to receive either 40 mg (0.6 mmol) or 80 mg (1.2 mmol)  $\text{NaNO}_2$  or placebo, all twice daily for 10 weeks ( $n = 10\text{--}11$ ). Trough plasma nitrite levels were significantly elevated ( $\sim 2$ -fold) in both intervention groups after 10 weeks, but this was not associated with any change in BP (DeVan et al., 2016). The lack of any BP lowering in subjects with optimal/normal BP is not surprising and may be considered ideal, identifying an approach that does not lead to symptomatic hypotension and/or hypotension-related syncope. The lack of any BP effect likely reflects the accepted dogma that the magnitude of BP reduction expected from any single dose of antihypertensive agent is proportional to basal BP level (Law et al., 2003). However, this result is at odds with the majority of studies that demonstrate BP lowering in normotensive persons after systemic nitrite elevation after inorganic nitrate supplementation (Table 1) (Larsen et al., 2006; Webb et al., 2008b; Bailey et al., 2009, 2010; Kapil et al., 2010; Sobko et al., 2010; Vanhatalo et al., 2010; Lansley et al., 2011a,b; Bahra et al., 2012; Bondonno et al., 2012, 2014b; Cermak et al., 2012a; Coles and Clifton, 2012; Bond et al., 2013, 2014; Joris and Mensink, 2013; Kelly et al., 2013; Liu et al., 2013; Wylie et al., 2013; Jajja et al., 2014; Rammos et al., 2014; Ashor et al., 2015; Ashworth et al., 2015; Bourdillon et al., 2015; Jovanovski et al., 2015; Lee et al., 2015; Choi et al., 2016; Flueck et al., 2016; Jonvik et al., 2016; Raubenheimer et al., 2017; Jones et al., 2019). The first contemporary demonstration

of BP-lowering effect of inorganic nitrate via conversion to nitrite used  $\text{NaNO}_3$  [0.1 mmol/kg (6.2 mg/kg)] daily for 3 days compared with matched NaCl control in 17 healthy subjects. Plasma nitrite levels increased  $\sim 1.5$ -fold after nitrate supplementation only, together with a significant reduction in diastolic BP (DBP;  $\Delta 3.7$  mm Hg) (Larsen et al., 2006).

Nitrate is also available in dietary form from vegetables, so the equivalency of providing nitrate in salt form (as  $\text{KNO}_3$ ) and dietary form (as beetroot juice) was established by us in 2008. Acute, single administration of 22.5 mmol (1395 mg) dietary nitrate as beetroot juice (compared with water control) and an approximate equivalent of  $\text{KNO}_3$  (24 mmol; 1488 mg nitrate) compared with matched KCl control in separate cohorts of healthy subjects demonstrated similar time courses of systemic nitrite elevation and BP reduction over 24 hours. Peak nitrite levels were contemporaneous to peak BP reduction at 2.5–3 hours post-inorganic nitrate supplementation. Additionally, peak BP reduction from baseline was similar for both forms of inorganic nitrate supplementation ( $\sim 10/7$  mm Hg), with no changes in either placebo group (Webb et al., 2008b; Kapil et al., 2010) (Fig. 7A). Furthermore, change in SBP was significantly inversely correlated to changes in plasma nitrite and the determining effect on BP (Webb et al., 2008b; Kapil et al., 2010). These studies additionally demonstrated a dose-dependent effect of inorganic nitrate supplementation on BP reduction and provided the first evidence of bioactive  $\cdot\text{NO}$  production after nitrate supplementation by determining significant elevation of circulating cGMP concentrations (Kapil et al., 2010); cGMP is a sensitive marker that confirms bioactive  $\cdot\text{NO}$  generation (Batchelor et al., 2010). An unintended but positive outcome of our work has been the introduction of beetroot juice, with known and measurable amounts of nitrate, as a mechanism to explore the biologic efficacy of the enterosalivary circuit of nitrate. The impact of this is shown in a PubMed search demonstrating the uptake of this approach after the 2008 publication (Fig. 8).

The importance of enterosalivary generation of nitrite was confirmed in our studies by asking subjects to refrain from swallowing saliva post-nitrate ingestion, thereby interrupting oral nitrate reduction but not interfering with nitrate absorption itself (Webb et al., 2008b). Elevation of circulating nitrate levels were unaffected by this intervention, but it did prevent the rise in circulating plasma nitrite level as had been demonstrated by others previously (Lundberg and Govoni, 2004). However, in addition to this, we demonstrated that this effect was associated with an absence of the BP reduction effect, thus confirming nitrite as the bioactive moiety after nitrate supplementation (Webb et al., 2008b).

Although the acute (i.e.,  $<24$  hour) effects of inorganic and dietary nitrate supplementation on BP are



TABLE 1  
Randomized, controlled studies evaluating nitrate supplementation on blood pressure in healthy volunteers

Reference	Cohort	Design	Intervention	Treatment Duration	Baseline BP (mm Hg)	Max ΔBP (mm Hg)
Larsen et al., 2006	HV Age = 24	Crossover <i>n</i> = 17	NaNO <sub>3</sub> , 0.1 mmol/kg per day NaCl, 0.1 mmol/kg per day placebo	3 days	Clinic BP not stated	N.S./-3.7
Webb et al., 2008b	HV Age = 26	Crossover <i>n</i> = 14	Beetroot juice (500 ml), 22.5 mmol nitrate Matched-volume water control	1 day	Clinic 108/70	-10.4/-8.0
Bailey et al., 2009	HV Age = 26	Crossover <i>n</i> = 8	Beetroot juice, 11.2 mmol nitrate Matched-volume black currant juice control	6 days	Clinic BP not stated	-6/ N.S.
Kapil et al., 2010	HV Age = 23	Crossover <i>n</i> = 20	KNO <sub>3</sub> , 24 mmol KCl, 24 mmol placebo	1 day	Clinic 110/70	-9.4/-6.0
Kapil et al., 2010	HV Age = 25	Crossover <i>n</i> = 9	Beetroot juice (250 ml), 5.6 mmol nitrate Matched-volume water control	3 h	Clinic 121/71	-5.4/ N.S.
Bailey et al., 2010	HV Age = 28	Crossover <i>n</i> = 7	Beetroot juice, 5.1 mmol nitrate Matched-volume black currant juice control	6 days	Clinic 125/73	-5/-2
Vanhatalo et al., 2010	HV Age = 29	Crossover <i>n</i> = 8	Beetroot juice, 5.2 mmol nitrate Matched-volume black currant juice control	15 days	Clinic 127/72	-7/ N.S.
Sobko et al., 2010	HV Age = 36	Crossover <i>n</i> = 25	Japanese diet, 0.3 mmol/kg per day nitrate Low-nitrate diet (nitrate content not stated)	10 days	Clinic BP not stated	N.S./-4.5
Lansley et al., 2011b	HV Age = 22	Crossover <i>n</i> = 9	Beetroot juice, 6.2 mmol nitrate Nitrate-deplete beetroot juice placebo	6 days	Clinic 129/66	-5/ N.S.
Lansley et al., 2011a	HV Age = 21	Crossover <i>n</i> = 9	Beetroot juice, 6.2 mmol nitrate Nitrate-deplete beetroot juice placebo	2.5 h	Clinic 131/72	-6/ N.S.
Cermak et al., 2012a	HV Age = 31	Crossover <i>n</i> = 12	Beetroot juice, 8 mmol nitrate Nitrate-deplete beetroot juice placebo	6 days	Clinic 119/74	N.S.
Coles and Clifton 2012	HV Age = 43	Crossover <i>n</i> = 30	Beetroot juice, 7.5 mmol nitrate Matched-volume apple juice (colored)	1 day	Clinic 132/81 24 h ABP not stated	N.S. N.S.
Bahra et al., 2012	HV Age = 28	Crossover <i>n</i> = 14	8 mmol KNO <sub>3</sub> 8 mmol KCl placebo	3 h	116/67	-4.9/ N.S.
Bondonno et al., 2012	HV Age = 47	Crossover <i>n</i> = 30	High-nitrate meal (spinach), 2.9 mmol nitrate Low-nitrate meal (apple) or drink (rice milk), each < 0.1 mmol	3.3 h	Clinic 112/68	-2.7/ N.S.
Kelly et al., 2013	HV Age = 64	Crossover <i>n</i> = 12	Beetroot juice, 9.6 mmol nitrate Nitrate-deplete beetroot juice placebo	3 days	Clinic 125/74	-5/-3
Liu et al., 2013	HV Age = 59	Crossover <i>n</i> = 26	High-nitrate meal (spinach), 3.5 mmol nitrate Low-nitrate meal + rice milk (nitrate content not stated)	3.5 h	Clinic 119/71	-7.5/ N.S. (peak 2 h)
Joris and Mensink, 2013	HV Age = 61	Crossover <i>n</i> = 20	Beetroot juice, 8.0 mmol nitrate Control drink (not specified)	4 h	Clinic 135/93	N.S./-4
Wylie et al., 2013	HV Age = 23	Crossover (four limbs) <i>n</i> = 10	Beetroot juice, 4.2, 8.4, and 16.8 mmol nitrate 140 ml water control	1 day	Clinic 119/68	16.8 mmol: -9/-4; 8.4 mmol: -10/-3; 4.2 mmol: -5/ N.S.
Bond et al., 2013	HV Age = 21	Crossover <i>n</i> = 12	Beetroot juice, 12.1 mmol nitrate Matched-volume orange juice	2 h	Clinic BP not stated	-5/ N.S.
Bondonno et al., 2014b	HV Age = 47	Crossover <i>n</i> = 38	High-nitrate diet, estimated >4.8 mmol nitrate daily Low-nitrate diet, estimated <1.6 mmol nitrate daily	7 days	Clinic 130/76	N.S.
Rammos et al., 2014	HV, moderate CV risk Age = 63	Parallel (two-arm) <i>n</i> = 10 to 11	NaNO <sub>3</sub> , 0.15 μmol/kg per day Matched-molar weight NaCl placebo	28 days	Clinic 137/80	-8/ N.S.
Jajja et al., 2014	HV, overweight Age = 62	Parallel (two-arm) <i>n</i> = 10 to 11	Beetroot juice, 2.6-6.5 mmol nitrate daily Matched-volume black currant juice control	21 days	Home 130/77 Clinic 135/77 24 h	Home: -7.2/ N.S.; clinic: N.S.; 24 h ABP: N.S.
Ashor et al., 2015	HV Age = 25	Crossover <i>n</i> = 27	High-nitrate soup (spinach), 13.6 mmol nitrate daily Low-nitrate soup, 0.01 mmol daily control	7 days	Clinic 116/69	-4.1/-4.4
Ashworth et al., 2015	HV Age = 20	crossover <i>n</i> = 19	High-nitrate food, estimated 5.5 mmol nitrate daily Low-nitrate food, estimated 0.13 mmol nitrate daily	7 days	Clinic 107/63	-4.0/ N.S.
Bourdillon et al., 2015	HV Age = 31	Crossover <i>n</i> = 12	NaNO <sub>3</sub> , 0.1 mmol/kg per day NaCl, 0.1 mmol/kg per day placebo	3 days	Plethysmographic finger BP 125/75	N.S.
Lee et al., 2015	HV Age = 22	Crossover <i>n</i> = 14	Beetroot juice, 6.4 mmol nitrate Nitrate-deplete beetroot juice placebo	15 days	Clinic 116/77	-4/-4
Jonvik et al., 2016	HV Age = 28	Crossover (four-limb) <i>n</i> = 18	NaNO <sub>3</sub> , 12.9 mmol Rocket, beetroot, and spinach juice, all 12.9 mmol nitrate	5 h	Clinic BP not stated	NaNO <sub>3</sub> : N.S./-4; rocket: -6/-6 Beet: -5/-7.5; spinach: -7/-5

(continued)

TABLE 1—Continued

Reference	Cohort	Design	Intervention	Treatment Duration	Baseline BP (mm Hg)	Max ΔBP (mm Hg)
Choi et al., 2016	HV Age = 23	Crossover (two-limb) <i>n</i> = 12	Beetroot juice, 5.6 mmol nitrate Colored water control	15 days	132/86	Both SBP and DBP reduced
Raubenheimer et al., 2017	HV Age = 64	Crossover (two-limb) <i>n</i> = 12	Beetroot juice, 12.9 mmol nitrate	3 h	133/89	−7.9/−5.7
Jones et al., 2019	HV Age = 63	Parallel (two-arm) <i>n</i> = 7–11	Nitrate-deplete beetroot juice placebo Beetroot juice, 6.5 mmol nitrate Prune juice control	4 wk	129/75	N.S.

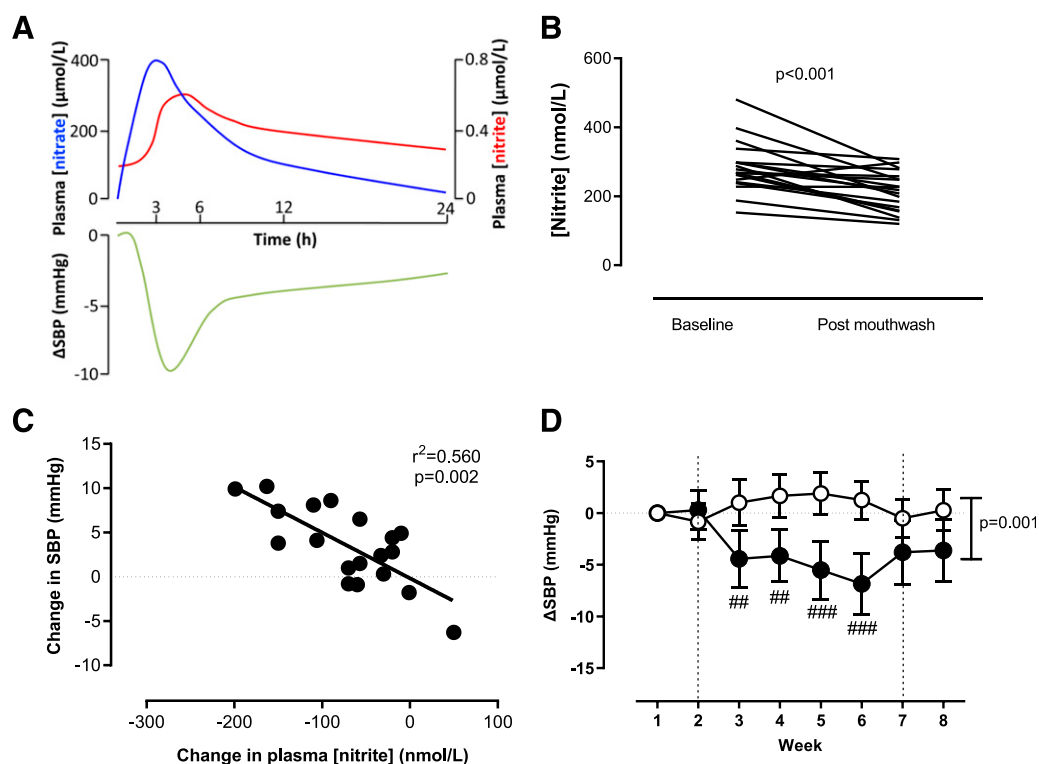
HV, healthy volunteers.

important, for translation to patients with hypertension, interventions need to provide sustained BP reduction in the long term. Therefore, studies over prolonged time periods (>7 days) in healthy subjects were required. This was first tested in humans indirectly by making a comparison of the effects of a traditional, nitrate-rich Japanese diet [0.3 mmol/kg (18.6 mg/kg) nitrate daily], with a low-nitrate-containing control diet in a crossover study (*n* = 25, 10 days per dietary intervention). In this study, the high-nitrate diet was associated with significantly lower BP (DBP −4.5 mm Hg) (Sobko et al., 2010). Since this observation, several studies have tested dietary nitrate supplementation over longer periods. Using beetroot juice to deliver dietary nitrate [5.2 (322 mg) or 6.4 (397 mg) mmol/day nitrate] compared with low-nitrate control juice (*n* = 8–14 for 15 days per intervention), sustained lowering of DBP (~5–6.5 mm Hg) was evident in healthy volunteers (Vanhatalo et al., 2010; Lee et al., 2015). In healthy older persons with prehypertension (baseline BP 137/80 mm Hg, *n* = 10 to 11), 4 weeks of daily 150 μmol/kg (9.3 mg nitrate/kg) NaNO<sub>3</sub> (~10 mmol nitrate for a 70-kg subject) lowered SBP by 8 mm Hg compared with a matched NaCl control (Ramos et al., 2014).

The first study in patients with hypertension followed a single dose over 24 hours and used water as a control in a crossover design. In all, 15 drug-naïve patients with hypertension (baseline daytime ambulatory BP; ABP 142/85 mm Hg) were given a single dose of dietary nitrate [3.3 mmol (205 mg) as beetroot juice]. Plasma nitrate and nitrite profiles were similar to responses seen in normotensive persons, confirming that the enterosalivary circulation was intact in hypertension. Additionally, a peak BP reduction of ~11/10 mm Hg (Ghosh et al., 2013) was observed, similar to the peak reduction in healthy volunteers given 22.5 mmol despite having a fraction of the nitrate dose (Webb et al., 2008b; Kapil et al., 2010). Importantly, in healthy persons given a comparable 4 mmol (248 mg) nitrate dose, BP reduction was not significant (Kapil et al., 2010). The enhanced BP response in subjects with hypertension after nitrate supplementation could simply reflect higher baseline BP evident in the hypertensive cohort,

as BP lowering is proportional to pretreatment BP (Law et al., 2003). However, it could also reflect increased potency of nitrate/nitrite. In Wistar Kyoto rats treated with phenylephrine to titrate BP to match the levels evident in the strain-matched SHR, there is a diminished BP response to acute bolus doses of KNO<sub>2</sub>, suggesting pretreatment BP is not solely responsible for the augmented response seen in hypertension (Ghosh et al., 2013). There is some evidence to suggest that, in the scenario of reduced ·NO levels, the downstream signaling target GC-1 or the components of the pathway further downstream from GC-1 become sensitized to ·NO. This has been demonstrated best with respect to vasoactivity, in which an enhanced vasorelaxant response to ·NO donors was shown in mice treated with NOS inhibitors (Moncada et al., 1991) and in eNOS KO mice (Brandes et al., 2000). However, whether this is similar in human hypertension, a situation in which eNOS activity is similarly reduced (Linder et al., 1990; Panza et al., 1993), is not known.

In our single dietary dosing study in patients with hypertension (Ghosh et al., 2013), SBP was reduced 24 hours postsupplementation by ~8 mm Hg, which is at a level comparable to the expected BP-lowering effect of antihypertensive medicines when used at standard dose in mild hypertension (~9.1 mm Hg) (Law et al., 2009). This dose also represented a peak-to-trough ratio of 60% for dietary nitrate in this study and is a profile of activity consistent with an effect size suitable (>50%) to consider as a once-daily dosage for an antihypertensive medication (Lipicky, 1994; Meredith, 1994). This initial study was taken forward into a prolonged study assessing whether the acute effects are sustained in the longer term (Kapil et al., 2015). In this double blind, randomized, placebo-controlled, parallel study (*n* = 32 in each limb) of drug-naïve and treated patients with hypertension and uncontrolled BP, 4 weeks of daily dietary nitrate [6.4 mmol (397 mg) as beetroot juice] was compared with a nitrate-free beetroot juice [originally developed to provide a suitable placebo (Gilchrist et al., 2014)] as a control intervention to maintain patient and investigator blindness to achieve optimal study design. BP, measured by three separate

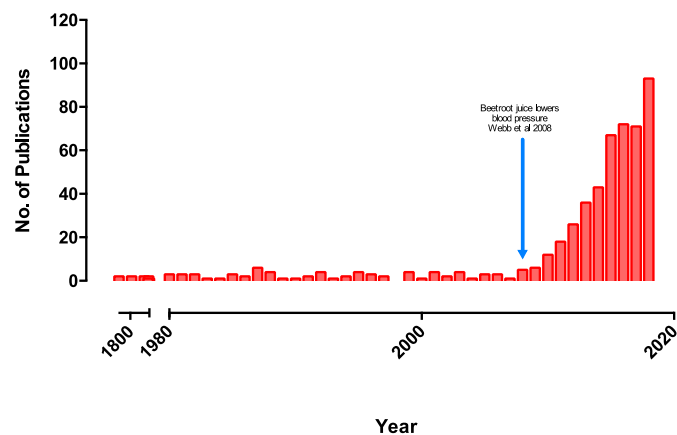


**Fig. 7.** Effects of changing nitrite concentration on BP in humans. (A) Changes in plasma nitrite (red) and nitrate (blue) and BP (green) after acute nitrate supplementation. (B) Effect of antiseptic mouthwash on plasma nitrite levels. (C) Correlation of changes in BP with changes in plasma nitrite levels after antiseptic mouthwash. (D) Changes in home BP after 4 weeks of dietary nitrate (black circles) or placebo (open circles) ingestion in patients with hypertension.  $^{##}P < 0.01$  and  $^{###}P < 0.001$  for Bonferroni post hoc test, following 2-way ANOVA.

methods, was significantly reduced over 4 weeks in the active intervention limb only: clinic ( $-8/2$  mm Hg), home ( $-8/4$  mm Hg), and 24-hour ABP ( $-8/5$  mm Hg), with no pharmacodynamic tolerance demonstrated by weekly home BP (Fig. 7D) (Kapil et al., 2015).

There are now several studies that have assessed the effects of dietary nitrate in patients with hypertension. The individuals in these studies in general have baseline BPs above 130/70 mm Hg (as some have controlled BP on medication) and on balance indicate a lowering of BP with dietary nitrate in patients with uncontrolled BP at baseline (see Table 2) (Ghosh et al., 2013; Kapil et al., 2015; Kerley et al., 2018; Broxterman et al., 2019; Zafeiridis et al., 2019). Conversely, there are studies that show BP-lowering benefits in treated, controlled patients on medication. As an example, in a smaller, shorter duration ( $n = 27$ , 1 week) in treated, controlled patients with hypertension (baseline BP 133/76 mm Hg), 7 mmol (434 mg) dietary nitrate daily as beetroot juice compared with control demonstrated no effect on home or ambulatory BP, with means in low- versus high-nitrate groups being 127–128/73–74 (Bondonno et al., 2015b). In a similar study to the Kapil et al. (2015) study, in a mixed population of both patients with prehypertension and untreated hypertension, 2.5 mmol/day nitrate compared with  $<0.5$  mmol/day nitrate, both as blended vegetable juice (4 weeks,  $n = 30$ ), in a crossover study showed no difference

in ABP (Blekkenhorst et al., 2018). The reasons for these differing data are uncertain; however, it is possible that these studies were underpowered ( $n = 27$ – $30$ ) and, also, that at least some of the volunteers had BP in the normotensive range and certainly substantially lower than the screening BPs; this fact may underlie the neutral effects. In addition, biochemical analyses suggest that at baseline, this group of participants had unusual and very high circulating levels of nitrite of  $2 \mu\text{mol/l}$  (Bondonno et al., 2015b; Blekkenhorst et al., 2018). Although the authors suggested that this was



**Fig. 8.** Number of publications using beetroot juice.

TABLE 2  
Randomized, controlled studies evaluating nitrate supplementation on blood pressure in hypertensive patients

Reference	Cohort	Design	Intervention	Treatment Duration	Baseline BP (mm Hg)	Max $\Delta$ BP (mm Hg)
Ghosh et al., 2013	Uncontrolled HTN Age = 53	Crossover $n = 15$	Beetroot juice, 3.3 mmol nitrate Matched-volume water control	1 day	140/86	-8.5/N.S.
Kapil et al., 2015	Uncontrolled HTN Age = 57	Parallel $n = 64$	Beetroot juice, 6.4 mmol nitrate Nitrate-deplete beetroot juice placebo	4 wk	Clinic 138/84 Home 139/82 ABP 139/82	Clinic -8/-2 Home -8/-4 Abp -8/-5 N.S.
Bondonno et al., 2015b	Controlled HTN Age = 63	Crossover $n = 30$	Beetroot juice, 7 mmol nitrate Nitrate-deplete beetroot juice placebo	1 wk	133/76	N.S.
Kerley et al., 2018	Uncontrolled HTN Age = 63	Crossover $n = 20$	Beetroot juice, 12.9 mmol nitrate Nitrate-deplete beetroot juice placebo	1 wk	ABP 137/80	-8/-4
Broxterman et al., 2019	Controlled HTN Age = 53	Crossover $n = 13$	Beetroot juice, 6.2 mmol nitrate Nitrate-deplete beetroot juice placebo	3 days	132/78	N.S.
Broxterman et al., 2019	Uncontrolled HTN Age = 49	Crossover $n = 14$	Beetroot juice, 6.2 mmol nitrate Nitrate-deplete beetroot juice placebo	3 days	141/88	-5/-4
Zafeiridis et al., 2019	Uncontrolled HTN Age = 44	Crossover $n = 18$	Beetroot juice, 8.1 mmol nitrate Nitrate-deplete beetroot juice placebo	2.5 h	144/95	-6/-2

HTN, hypertensive patients.

likely due to contamination of samples through the analytical procedures (although no measure of this was made), it is also possible that these levels relate to dietary intake. Dietary habits were recorded in this study; however, they were not reported, and thus it is not possible to assess this. Finally, 63% of the recruited patients in the first study were women (Bondonno et al., 2015b). Recent evidence from our laboratory has shown that although women appear to have greater baseline oral nitrate reductase activity and thus greater circulating nitrite levels, this seems to result in a reduced response to additional dietary nitrate loading, possibly due to the fact that the pathway is already being maximally activated (Kapil et al., 2018). It is possible that the high number of women in this cohort have therefore skewed the effect.

Although all these studies measured peripheral BP, recent evidence suggests that elevation of systemic nitrite levels may have a greater effect on central hemodynamics. Systemic intravenous  $\text{NaNO}_2$  nitrite (8.7  $\mu\text{mol}/\text{min}$ ) in healthy volunteers selectively reduced central BP rather than peripheral BP (Omar et al., 2015), and 6 months of dietary nitrate in patients with or at risk of diabetes had no effect on peripheral BP compared with placebo but did have a small, yet significant, effect on central BP (Mills et al., 2017). The explanation for the lack of peripheral BP reductions in these studies in contrast to the large body of evidence above is not clear, but it is worthy of further exploration. Certainly, previous studies have failed to demonstrate BP lowering in type 2 diabetes after increases in systemic nitrite levels (Gilchrist et al., 2013; Mohler et al., 2014; Shepherd et al., 2015a).

Overall, two recent independent meta-analyses of BP-lowering studies suggest a meaningful pooled effect size of  $\sim 5/2$  mm Hg reduction on resting clinic BP (Ashor et al., 2017; Jackson et al., 2018), although the studies included ranges in duration from a few hours to a few weeks, as indicated in Tables 1 and 2. For a

BP-lowering intervention to be adopted clinically, support for sustained efficacy over several months is clearly needed. Further studies are underway, including a randomized, double blind, placebo-controlled evaluation of 4 months of dietary nitrate in patients with hypertensive target organ damage to assess possible reversal (clinicaltrials.gov: NCT03088514).

3. *Enterosalivary Generation of Nitrite Regulates Basal BP.* Nitrite is bioactive in the circulation, leading to vasodilatation and BP lowering (Cosby et al., 2003; Dejam et al., 2007), and further, the inverse correlation between baseline plasma nitrite levels and BP in healthy subjects (Kapil et al., 2010) intimates that basal plasma nitrite levels (arising from endogenous sources, i.e., eNOS) regulate BP of healthy subjects. It was thus hypothesized that interruption of the enterosalivary circulation of nitrate to nitrite under basal conditions would lower basal plasma nitrite levels and increase BP. To test this hypothesis, oral microbiome-related nitrate reduction (i.e., nitrite synthesis) was disrupted using a validated antiseptic mouthwash protocol (Govoni et al., 2008). In a 2-week crossover study ( $n = 19$ ), twice daily use of antiseptic mouthwash for 7 days nearly abolished oral nitrate reduction to nitrite. More importantly, this effect was accompanied by a decrease in plasma nitrite levels by  $\sim 25\%$  (Fig. 7B), similar to that predicted by studies using eNOS inhibition in humans (Lauer et al., 2001; Kleinbongard et al., 2003). These studies indicate that, when one excludes the influence of diet, 25% of circulating nitrite levels arise from the enterosalivary circuit of nitrate derived from the oxidative metabolism of endogenously generated  $\cdot\text{NO}$ . In addition, this effect was associated with a concomitant increase in BP (2–3.5 mm Hg) that was measured by three distinct methods: clinic, home, and ambulatory BP (Kapil et al., 2013). Evaluation of home BP in this study revealed that the BP effect was apparent within 1 day of mouthwash use, with no evidence of tachyphylaxis over the week-long period of intervention (Kapil et al., 2013). This effect of increasing

BP with disruption of the enterosalivary circulation has been confirmed in further studies, including in healthy volunteers ( $n = 27$ ) with confirmed disease-free oral health status at baseline using chlorhexidine twice daily for 7 days (Tribble et al., 2019). Importantly, this effect is also seen in patients with CVD. In treated, controlled patients with hypertension ( $n = 15$ , baseline home BP  $\sim 134/79$  mm Hg) randomized to receive antiseptic mouthwash as above or water control, mouthwash use led to significant increase in home SBP (2.3 mm Hg) compared with control (Bondonno et al., 2015a). More recently, however, in 17 healthy females randomized to 3 days of antiseptic mouthwash or non-antibacterial placebo mouthwash, despite abrogation during the intervention period of both oral nitrate reduction and salivary nitrite levels, there was no change in plasma nitrite levels, and thus, ambulatory BP was not different (Sundqvist et al., 2016).

Further studies have shown that other oral hygiene treatments, in addition to chlorhexidine mouthwashes, impact nitrate reduction and that this effect is dependent upon the severity of the impact on oral microbiome function (McDonagh et al., 2015; Woessner et al., 2016). Support for the hypothesis that the changes in BP were related to lesser nitrite generated from the oral microbiome comes from robust correlation of changes in plasma nitrite concentration and changes in BP after 7 days of antiseptic mouthwash intervention (Fig. 7C) (Kapil et al., 2013). It is unlikely that these effects relate to other mechanisms, such as increased stress due to instillation of mouthwash, since the effects on SBP were evident in the averaged nighttime BP mean as well (Kapil et al., 2013). Although these increases in BP were small ( $\sim \Delta$ SBP 2–3.5 mm Hg), such effects at a population level are known to lead to a significantly increased risk of stroke and ischemic heart disease over a life course (Lewington et al., 2002). Importantly, it appears that recovery from acute use of antibacterial mouthwash (twice daily for 1 week) is apparent both in microbiological ecology and in vascular responses within 3–7 days (Tribble et al., 2019).

**4. Endothelial Dysfunction.** Endothelial dysfunction is synonymous with reduced bioavailable  $\cdot$ NO, is an early marker common to all CVD risk factors, and is predictive of future CVD events (Brunner et al., 2005). Long-term dietary nitrite and nitrate deficiency worsens ex vivo–assessed endothelial function in C57BL6 mice and was associated with CV death (Kina-Tanada et al., 2017). Additionally, nitrite supplementation improves ex vivo endothelial function in several different models associated with cardiometabolic disease (Sindler et al., 2015; Ling et al., 2016), whereas dietary nitrate has been shown to improve endothelial function in models of atherosclerosis (Bakker et al., 2016) as well as to prevent the endothelial dysfunction that occurs in mice subjected to long-term low dietary nitrate and

nitrite (Kina-Tanada et al., 2017) or aging (Ramos et al., 2015).

There are several studies that have explored whether such findings in mice translate to humans. With twice daily  $\text{NaNO}_2$  (80 or 160 mg total daily for 10 weeks) in older adults, there was a trend toward improved FMD (DeVan et al., 2016). However, conflicting results have been published for supplementation with inorganic nitrate in healthy persons with normal baseline endothelial function. Some studies have shown a positive effect of dietary nitrate (Bondonno et al., 2012; Heiss et al., 2012; Ramos et al., 2014), whereas others did not (Bahra et al., 2012). In addition, in healthy volunteers in which a transient endothelial dysfunction is induced by a brief exposure to an ischemic insult in the forearm, prior (3 hour) acute nitrate supplementation in the form of beetroot juice or  $\text{KNO}_3$  capsules prevented this dysfunction (Webb et al., 2008b; Kapil et al., 2010). In patients with pre-existing endothelial dysfunction, a somewhat mixed picture has developed. In scenarios in which endothelial function is impaired, and this impairment is associated with risk factors for CVD, chronic oral nitrate supplementation improves FMD, i.e., in patients with hypertension and hypercholesterolemia (Kapil et al., 2015; Velmurugan et al., 2016). However, FMD was not improved after chronic supplementation in patients with diabetes and after acute supplementation in patients with peripheral arterial disease (PAD) (Kenjale et al., 2011; Gilchrist et al., 2013).

**5. Arterial Stiffness.** Central, large-artery stiffening caused by aging-related arteriosclerosis is an important independent predictive marker of CV risk (Vlachopoulos et al., 2010b), with the gold-standard measurement of this phenomenon being aortic pulse wave velocity (PWV) (Laurent et al., 2006). The evidence indicates that in those with stiffened central arteries, a rapid reflection of incident pressure waves leads to greater augmentation of central SBP (Safar, 2010), which is more predictive of CV events than brachial SBP (Vlachopoulos et al., 2010a). It has been demonstrated that increased arterial stiffness precedes incident hypertension in large, prospective cohorts (Kaess et al., 2012; Zheng et al., 2015). Moreover, the importance of stiffening in driving hypertension comes from observations that large-artery stiffening alone explains age-related increases in BP and failure of normal renal and baroreflex-mediated regulatory mechanisms to prevent this (Pettersen et al., 2014).

Studies in healthy volunteers have shown that acute inhibition of eNOS activity increases measures of elastic artery stiffness in vivo (Wilkinson et al., 2002; Schmitt et al., 2005; Bellien et al., 2010). Hence, increasing bioavailable  $\cdot$ NO, via increment of systemic nitrite, should decrease PWV. In this respect, nitrite supplementation in aged rodents (0.8 mmol/l  $\text{NaNO}_2$  in drinking water for 3 weeks) and adults (1.2 or 2.4 mmol  $\text{NaNO}_2$  orally for 10 weeks) reduced PWV (Sindler et al.,

2011; Fleenor et al., 2012; DeVan et al., 2016). Infusion of  $\text{NaNO}_2$  ( $8.7 \mu\text{mol}/\text{min}$  i.v.) for 60 minutes into the brachial artery resulted in significant, large reductions in central SBP (Omar et al., 2015), with no change in brachial SBP, similar to the effects of oral nitrite dosing (DeVan et al., 2016). In healthy subjects, acute (8 mmol  $\text{KNO}_3$ ) or chronic [4 weeks of daily  $150 \mu\text{mol}/\text{kg}$   $\text{NaNO}_3$  ( $\sim 10$  mmol nitrate for a 70-kg subject)] inorganic nitrate supplementation reduced PWV (Bahra et al., 2012; Rammos et al., 2014), with elevation of plasma nitrite and cGMP levels, confirming the production of bioactive  $\cdot\text{NO}$  (Bahra et al., 2012).

In patients with hypertension, acute (3.3 mmol nitrate as beetroot juice) or chronic (daily 6.4 mmol nitrate as beetroot juice for 4 weeks) inorganic nitrate supplementation also resulted in significant reductions in PWV (Ghosh et al., 2013; Kapil et al., 2015). This result intimate, therefore, that benefits accrued from nitrate supplementation observed in hypertensive cohorts are not only due to reductions of elevated arterial tone but likely also reflect modulation of elastic distensibility of the arterial tree by nitrite<sup>-</sup>-derived  $\cdot\text{NO}$ . More recently, despite reduction of central BP after 6 months of dietary nitrate supplementation in patients with or at risk of diabetes, there was no effect on PWV (Mills et al., 2017). Hutchinson-Gilford progeria syndrome is a rare genetic disease associated with multiple hallmarks of accelerated aging, including arterial stiffness and premature CVD despite a relative lack of traditional CV risk factors, leading to an average age of death  $<15$  years old (Hennekam, 2006). Mutant LMNA (the gene encoding lamin A that is defective in the syndrome) mice supplemented with  $\text{NaNO}_2$  (500 mg/l in drinking water) had improved aortic stiffness parameters and remodeling, suggesting a potential therapeutic approach in this orphan disease (Del Campo et al., 2019).

Further studies are now needed to determine the relative contributions of the reduction of distending BP in the aorta and other elastic arteries, and specific direct effects on arterial destiffening in mediating the effects of nitrite-derived  $\cdot\text{NO}$ .

**6. Cerebral Blood Flow.** The cerebral vasculature is a vascular bed in which the potential effects of the noncanonical pathway have been particularly assessed. In part, this is due to the fact that  $\cdot\text{NO}$  is known to regulate cerebral blood flow (Buchanan and Phillis, 1993) and critically regulates neurovascular coupling of neural activity to blood flow (Attwell et al., 2010). Since elevation of systemic nitrite concentration delivers  $\cdot\text{NO}$  and improves blood flow in the peripheral circulation, whether similar effects can be seen within the cerebral circulation, which is normally tightly autoregulated for flow in health, is particularly intriguing.

Nitrite infusion ( $1 \mu\text{mol}/\text{kg}$  per minute) in rats has been shown to reverse L-NAME-induced cerebral vasoconstriction and the consequent reduction in cerebral blood flow (Rifkind et al., 2007). Raising systemic levels

of nitrite via acute dietary inorganic nitrate supplementation in older adults is also associated with improved blood flow to the frontal cortex, which is implicated in cognitive function and may be particularly vulnerable during aging (Presley et al., 2011), and reduces cerebral vascular resistance in the middle cerebral artery (Bond et al., 2013). The effect of inorganic nitrate supplementation on cognitive function has also been studied, although results are equivocal, with some smaller studies showing neutral results (Kelly et al., 2013; Bondonno et al., 2014; Thompson et al., 2014), and one study demonstrating improvement on some tasks that activate the frontal cortex in young adults (Wightman et al., 2015).

Recently, there has been some controversial work suggesting that migraneurs express an oral microbiome that is likely to lead to increased generation of  $\cdot\text{NO}$ , which might contribute to the cerebral vasodilatation that is thought to underlie headaches in this setting (Gonzalez et al., 2016). The authors used analyses of the 16S rRNA Illumina sequencing of a cohort of migraneurs and nonmigraneurs from The American Gut Microbiome Project, matching to known genomes for their analyses. The authors made predictions regarding the expression of nitrate, nitrite, and  $\cdot\text{NO}$ -reducing genes and suggested that there were higher levels of bacteria expressing these genes in the migraneurs (Gonzalez et al., 2016). However, the authors did not actually measure these genes, and unfortunately, detail regarding the individuals from whom the samples were collected was not made available. Further studies are needed to determine the importance, or not, of such a pathway in migraine.

Subarachnoid hemorrhage (SAH) is associated with significant morbidity and mortality related to delayed cerebral vasospasm that has been linked to diminished  $\cdot\text{NO}$  levels (Pluta et al., 2001; Sadamitsu et al., 2001). Infusion of  $\text{NaNO}_2$ , whether at the time of experimental SAH formation or delayed by 7 days, was associated with reduced arteriographic vasospasm in primates (Pluta et al., 2005; Fathi et al., 2011), suggesting nitrite could be a useful adjunct to existing therapies, and a small pilot study has recruited six patients for a 7-day nitrite infusion after a diagnosis of SAH with vasospasm, although results have not been published to date (clinicaltrials.gov NCT02176837). Interestingly, electroencephalographic responses to intravenous nitrite infusion ( $\text{NaNO}_2$   $10 \mu\text{g}/\text{kg}$  per minute for 1 hour) predicted the occurrence of delayed cerebral ischemia, suggesting such a test could be used to prognosticate and stratify treatment (Garry et al., 2016).

### *B. Nitrite and Nitrate in Exercise and Muscle Biology*

The intramyocyte and muscle pH and microvascular  $\text{O}_2$  tension decline in the contracting skeletal muscles (Richardson et al., 1995; Ferguson et al., 2015; Tanaka et al., 2016). Since it is well established that nitrite

reduction is potentiated in an acidic and hypoxic environment (Modin et al., 2001; Castello et al., 2006), there has been considerable interest in the possibility that this anion might offer a novel approach to deliver ·NO to enhance skeletal muscle function. ·NO has been demonstrated to influence a number of physiologic processes that are relevant to skeletal muscle function, including metabolism, perfusion, and contractility (Stamler and Meissner, 2001; Suhr et al., 2013). In support of the view that nitrite reduction is likely to occur within the exercised muscle are the findings published now some time ago demonstrating the negative difference in the arterial-venous plasma nitrite concentration across the contracting skeletal muscle bed (Abadeh et al., 1992). The potential benefits of the noncanonical pathway in improving muscle function have now been extensively investigated in different cohorts of individuals, in terms of exercise capacity, from the severely exercise tolerance impaired to the highly trained elite endurance athlete.

*1. Nitrate and Exercise Performance on Moderately Trained Athletes.* The first study to observe benefits of dietary nitrate ingestion on exercise was a randomized, double blind, placebo-controlled crossover study involving nine well trained healthy men of ~28 years (Larsen et al., 2007). In this study, the subjects consumed 0.1 mmol/kg per day of NaNO<sub>3</sub> or a NaCl placebo for 3 days before completing a continuous incremental cycle ergometer test. Nitrate supplementation caused a significant decrease in maximal oxygen consumption (VO<sub>2max</sub>) at submaximal work rates, with a mean reduction in VO<sub>2max</sub> of 5%. This reduction was surprisingly associated with significant improvements in muscle efficiency, from 19.7% ± 1.6% to 21.1% ± 1.3% (calculated as the work output per unit energy expended), and occurred without changes in blood (lactate), intimating that there was no increase in energy production. In addition, there were no differences in heart rate, ventilation, or respiratory exchange ratio between nitrate and placebo for any of the submaximal work rates (corresponding to 45%–80% VO<sub>2max</sub>). These results were particularly surprising since a basic principle of human exercise physiology is that the O<sub>2</sub> cost of submaximal exercise at a given work rate is fixed, irrespective of other factors such as health, fitness status, and age and indifferent to known nutritional, physical, or pharmacological interventions (Suhr et al., 2013). From these observations, the authors suggested that endurance exercise performance is a function of VO<sub>2max</sub>, the fractional utilization of VO<sub>2max</sub>, and exercise efficiency and that if all other factors are constant, an improvement in muscle efficiency would be expected to enable a greater work output for the same energy cost. This, in turn, translates into improved exercise performance (Larsen et al., 2007). These seminal findings caught the imagination of the sports and exercise world and led to a surge of interest to assess whether inorganic nitrate

may be useful in terms of placing the body in an optimal condition to use O<sub>2</sub> most efficiently to gain advantage in numerous forms of exercise, particularly for the elite endurance athlete.

The first independent study to corroborate the work of the Karolinska group came from Jones and coworkers in the UK (Bailey et al., 2009). This group took advantage of the recent discovery at that time that beetroot juice could be used as a simple and effective nitrate delivery method (Webb et al., 2008b). As per the study of Webb and colleagues, they gave 500 ml of beetroot juice (containing 11.2 ± 0.6 mmol/l nitrate) in a placebo-controlled crossover study of eight men (aged 19–38 years) in which black currant cordial (containing negligible nitrate) was used as the placebo for six consecutive days. The study was described as double blind; however, it is likely that the differences in the two liquids was evident to all. In this study the volunteers, as in the 2007 study of Larsen et al., completed a series of “step” moderate-intensity and severe-intensity exercise tests on the last 3 days (Bailey et al., 2009). As expected, plasma nitrate concentration was significantly elevated compared with placebo (beetroot juice: 273 ± 44 nmol/l vs. placebo: 140 ± 50 nmol/l), and this was associated with statistically significant decreases in SBP, demonstrating efficacy of the intervention. However, the key observation documented was that, in agreement with the Karolinska group, dietary nitrate reduced the “O<sub>2</sub> cost” of cycling at a fixed submaximal work rate. The authors show that muscle fractional O<sub>2</sub> extraction during modest exercise and gain in pulmonary O<sub>2</sub> uptake after onset of moderate exercise were reduced by 19%. However, in addition to this benefit, the authors found that during severe exercise, the O<sub>2</sub> uptake “slow component” was reduced. This slow component is thought to represent a progressive loss of muscle efficiency as high-intensity exercise continues (Jones et al., 2011). The peak oxygen uptake (VO<sub>2</sub>) attained during high-intensity exercise was not different between treatments, but attainment of the peak VO<sub>2</sub> was delayed with nitrate treatment, resulting in a longer time to exhaustion. These observations were repeated by the group in another study, which used a different form of exercise (i.e., knee extensors) (Bailey et al., 2010) and was again reproduced by Larsen et al. (2010) using combined arm and leg cranking as the form of exercise. Interestingly, in this study, the group found that the peak VO<sub>2</sub> was reduced with dietary nitrate (Larsen et al., 2010) during maximal exercise using a large active muscle mass and that it was associated with a trend toward increased time to exhaustion. The authors suggested that this implied involvement of two separate mechanisms in the benefits of inorganic nitrate: one that reduces VO<sub>2max</sub> and another that improves the energetic function of the working muscles.

Although these early observations have fueled interest in assessing the mechanisms and whether the

effects can be sustained in the long term, there has been some controversy regarding effective dose. Using a similar exercise test of submaximal cycle ergometry,  $\text{VO}_{2\text{max}}$  was shown to be reduced (by  $\sim 3\%$ – $5\%$ ) after the acute ingestion of 0.033 mol/kg nitrate, equating to  $\sim 2.5$  mmol nitrate in a 70-kg individual (Larsen et al., 2010), 5.2 (Vanhatalo et al., 2010), 6 (Wylie et al., 2016), and 16.8 mmol nitrate (Wylie et al., 2013), but conversely not after the acute ingestion of 3 (Wylie et al., 2016), 4.2 (Wylie et al., 2013) and  $\sim 8$  mmol nitrate (Betteridge et al., 2016) with no effect upon exercise tolerance (Vanhatalo et al., 2010; Wylie et al., 2013). However, in the study of Wylie and colleagues, increasing the dose substantially (8.4 and 16.8 mmol) did result in significant improvements (Wylie et al., 2013). These results seem to suggest that the acute ingestion of nitrate from 2.5 to 6 mmol of nitrate may be sufficient to enhance exercise economy during submaximal cycling but that higher doses are required to demonstrate any improvement in exercise tolerance.

Importantly, these effects on cycling economy after short-term nitrate supplementation have been shown to be maintained when dosing is extended over 15 days (although not at 5 days in this study) (Vanhatalo et al., 2010) or 6 mmol/day for 28 days (Wylie et al., 2016). These findings indicate that, as with the effects upon BP, there is no development of tolerance as occurs with organic nitrates (Münzel et al., 2005). It is also worth noting that there are dissenting studies in which, despite elevation in plasma nitrite concentration after supplementation with nitrate in both recreationally active and moderately trained endurance athletes, no improvement in cycling exercise economy or performance was evidenced (Breese et al., 2013; Kelly et al., 2014).

Similar improvements in exercise economy and tolerance after nitrate supplementation have been shown during running (Lansley et al., 2011b; Murphy et al., 2012; Porcelli et al., 2015), knee extension (Bailey et al., 2009), walking (Lansley et al., 2011b), desert marching (Kuennen et al., 2015), kayaking (Muggeridge et al., 2013; Peeling et al., 2015), and rowing (Bond et al., 2012; Hoon et al., 2014). However, whether improved exercise tolerance (i.e., time to exhaustion in a constant work rate task) in turn then translates into an associated improvement in exercise performance (i.e., a set distance or amount of work is completed in a shorter time frame) is controversial. In one study, no such benefit is seen with acute administration of nitrate (Cermak et al., 2012b), but chronic (up to 6 days) nitrate supplementation did enhance cycling performance (Cermak et al., 2012a) in moderately trained endurance, suggesting that chronic nitrate supplementation may have more possibilities as an ergogenic aid than acute nitrate ingestion.

*a. Mechanisms for enhanced exercise performance after nitrate supplementation.* A number of possible mechanisms have been proposed to underlie the

lower  $\text{O}_2$  cost of exercise. Bailey et al. (2010), using  $^{31}\text{P}$ -magnetic resonance spectroscopy, demonstrated a blunting of increases of ADP and inorganic phosphate (Pi) concentration and sparing of intramuscular phosphocreatine concentration (PCr) with nitrate treatment. These effects were apparent without increased contribution of anaerobic glycolysis to energy turnover, excluding the possibility that nitrate or nitrite had resulted in an inhibition of respiration (Brown and Cooper, 1994; Cleeter et al., 1994). The authors suggested that by reducing the ATP cost of force production, this facilitated a sparing of the finite PCr stores and a reduction in the  $\text{O}_2$  cost of exercise, resulting in an improved tolerance of intense exercise. The authors suggested that this effect likely relates to the activity of  $\cdot\text{NO}$  on the sarcoplasmic reticulum calcium ATPase or the actin-myosin ATPase (Ishii et al., 1998; Viner et al., 2000; Evangelista et al., 2010) and reason that a reduced ATP cost would blunt the changes in intramuscular substrates and metabolites that stimulate mitochondrial respiration (e.g., PCr, ADP, Pi) (Mahler, 1985; Meyer, 1989). Intriguingly, the depletion of muscle PCr and the buildup of Pi and ADP have been associated with the development of muscle fatigue during high-intensity exercise (Allen et al., 2008).

Other suggestions have been that nitrate supplementation improves muscle oxygenation and, therefore, mitochondrial efficiency (therefore lowering  $\text{VO}_2$ ) and muscle oxygenation (therefore sparing muscle PCr) (Wilson, 1994). Indeed, assessment of muscle biopsies postexercise and nitrate treatment shows a reduction in the expression of adenine nucleotide translocase (which is a protein involved in mitochondrial proton conduction) associated with a 19% improvement in the mitochondrial phosphate/oxygen ratio (which is a ratio of the amount of  $\text{O}_2$  consumed per ATP produced). This observation suggests that nitrate impacts upon leakage of protons across the inner mitochondrial membrane. The authors speculate that since  $\cdot\text{NO}$  has been shown to inhibit cytochrome C oxidase (Brown and Cooper, 1994; Cleeter et al., 1994) in the setting of hypoxia, nitrite reduction is facilitated, delivering increased  $\cdot\text{NO}$ , which results in the effects seen. Interestingly, acute supplementation of nitrite to isolated mitochondria in vitro did not affect phosphate/oxygen ratio, suggesting that several days of persistently elevated nitrite levels are needed for the induction of changes in the expression of mitochondrial proteins (i.e., adenine nucleotide translocase) and accords with observations suggesting improved benefits with dietary nitrate and prolonged daily ingestion.

There is also some suggestion that the mitochondria-independent effects of nitrate/nitrite relate to changes in muscle  $\text{Ca}^{2+}$  handling and, hence, contractile function. In mice fed  $\text{NaNO}_3$  (1 mmol/l in the drinking water) for 7 days, intracellular  $\text{Ca}^{2+}$  levels were increased and



were associated with enhanced contractions in fast-twitch muscle fibers, but not slow twitch, at stimulation frequencies from 20 to 150 Hz (Hernandez et al., 2012). Expression analyses demonstrated that within the fast-twitch muscle cells, there was an increase in both calsequestrin 1 and dihydropyridine receptor expression that resulted in enhanced  $\text{Ca}^{2+}$  store and, thus, availability of  $\text{Ca}^{2+}$  upon store release, thus causing a faster rate of force development and increased contractile force, particularly at low-frequency stimulation; although the exact molecular pathway responsible for this change in expression was not determined. The authors suggested, therefore, that improved performance with inorganic nitrate/nitrite, at least in part, related to increased muscle function during normal movement independent of changes in mitochondrial function (Hernandez et al., 2012).

Another potential contributory mechanism for improved muscle performance is the possibility that dietary nitrate increases blood flow, particularly considering the prominent vasodilator properties of nitrite, as discussed earlier. Indeed, in rats, dietary nitrate (in the form of beetroot juice fed to rats for 5 days) (Ferguson et al., 2013a) lowered exercising (treadmill) BP in tandem with lower blood lactate concentrations and substantial increases in hindlimb muscle blood flow. Using microspheres injected into the rats during the exercise, the authors were able to discriminate exactly which muscles were experiencing elevated blood flow and demonstrated that this occurred only in the exercising muscles and particularly in the type II muscle fibers [invoked in moderate to severe exercise intensity (Henneman et al., 1965)], thus providing improved  $\text{O}_2$  delivery where and when it was most needed. In a subsequent study, this view was supported by observations reporting much slower falls in microvascular  $\text{O}_2$  pressure after the onset of electrically evoked contractions of the spinotrapezius muscle of rats fed beetroot juice compared with those fed water (Ferguson et al., 2013b), implying increased muscle  $\text{O}_2$  delivery and, thus, a resistance to muscle fatigue, particularly of type II muscle fibers. These findings have been tested in humans. Breese et al. (2013) measured the effects of 4–6 days of nitrate supplementation on  $\text{VO}_2$  and muscle deoxyHb [which would reflect the balance between muscle  $\text{O}_2$  utilization and muscle  $\text{O}_2$  delivery kinetics (Koga et al., 2012)]. Nine healthy, physically active subjects were assigned in a randomized, double blind, crossover design to receive nitrate-containing beetroot juice (140 ml/day, containing ~8 mmol nitrate) or nitrate-deplete juice (140 ml/day) for 6 days. On days 4, 5, and 6 of the supplementation periods, subjects completed a double-step exercise protocol that included transitions from unloaded to moderate-intensity exercise (in which predominately type I muscle fibers are recruited), followed immediately by moderate- to severe-intensity exercise (in which the additional force

production would be predominately gained through the recruitment of type II muscle fibers). The study found that nitrate supplementation accelerated the  $\text{VO}_2$  and muscle deoxyHb kinetics in the moderate- to severe-intensity work rate increment but not the low- to moderate-intensity work rate increment (Breese et al., 2013).

*2. Nitrate and Exercise Performance in Patient Populations.* The above evidence supports the view that during the hypoxemia created by exercise in which type II muscle fibers are recruited to drive muscle contraction, inorganic nitrate/nitrite acts to improve function. Thus, in individuals with a greater proportion of type II muscle (Hernandez et al., 2012; Ferguson et al., 2013a; Kelly et al., 2014), such as that found in patients with metabolic, respiratory, and CVD (Schaufelberger et al., 1995; Gosker et al., 2000; Mador and Bozkanat, 2001; Raguso et al., 2004; Askew et al., 2005; Oberbach et al., 2006), inorganic nitrate/nitrite may be therapeutically useful. Indeed, several studies have now shown improved exercise capacity in patients with chronic obstructive pulmonary disease (COPD) (Berry et al., 2015; Kerley et al., 2015; Leong et al., 2015; Shepherd et al., 2015b), PAD (Kenjale et al., 2011) and heart failure (HF) (Coggan et al., 2015; Zamani et al., 2015; Eggebeen et al., 2016). However, healthy older adults (Kelly et al., 2013) or patients with type II diabetes did not benefit from dietary nitrate intervention (Shepherd et al., 2015a). These studies are discussed more fully below. Finally, in addition to improving exercise performance, there is also preliminary evidence to suggest that nitrate supplementation can aid recovery postexercise (Clifford et al., 2016).

There are now numerous studies that have explored the potential advantage that inorganic nitrate might provide to endurance athletes. The evidence to date is mixed, with some reports identifying small but competitively important benefits, and others showing no effect (see Table 3). This discord has in part been attributed to the fact that in well trained endurance athletes, physiologic remodeling of the skeletal muscle occurs during chronic endurance training. Moreover, this remodeling is associated with an increase in NOS expression in both skeletal muscle [neuronal NOS (McConnell et al., 2007)] and the vasculature supplying the muscles [eNOS (Green et al., 2004)], and also to an increase in the content of  $\text{Ca}^{2+}$ -handling proteins in type II muscle (Kinnunen and Manttari, 2012) to promote both a lower percentage of type II skeletal muscle (Wilson et al., 2012) and lower mitochondrial UCP3 content (Fernstrom et al., 2004), which is an important determinant of exercise efficiency (Mogensen et al., 2006). Hence, the differing vascular physiology and skeletal muscle of well trained endurance athletes compared with lesser trained athletes may explain a diminished advantage of nitrate supplementation in well trained endurance athletes.

Since there is a greater proportion of type II muscle used during high-intensity exercise (Krustrup et al., 2004), several research groups have focused on the possibility that this form of exercise may preferentially benefit from nitrate supplementation. Again, there are studies demonstrating benefit, whereas others show no advantage (see Table 4). This apparent discord may be due to the enormous heterogeneity in the studies with respect to the exercise modality, training status of the athletes/participants, the nitrate supplementation protocol, and/or the intermittent nature of the exercise regimes. On balance, however, it does seem that nitrate supplementation has ergogenic potential for athletes participating in sports in which speed, power, and repeated sprint ability are important determinants of success.

**3. Cardiac Muscle Function and Heart Failure.** Dysfunction of the classic pathways that underlie  $\cdot\text{NO}$  production are thought to play a major role in the pathogenesis of both HF with reduced ejection fraction (HFrEF, commonly referred to as systolic HF) and preserved ejection fraction (HFpEF, commonly referred to as diastolic HF) (Drexler, 1999; Cai and Harrison, 2000; van Heerebeek et al., 2012). Left ventricular ejection fraction (LVEF) for humans with HFrEF is typically  $<40\%$ , HFpEF LVEF  $>50\%$ , with an intermediate group of HF with mildly reduced ejection fraction for those with LVEF  $40\%–49\%$  (Ponikowski et al., 2016). Patients are high risk for HF as the cause of their symptoms with elevated NT-pro-brain natriuretic peptide (NT-proBNP) (sinus rhythm  $>1000$  pg/ml and atrial fibrillation  $>1600$  pg/ml) and brain natriuretic peptide (BNP) (sinus rhythm  $>300$  pg/ml and atrial fibrillation  $>500$  pg/ml) levels, although lower levels remain compatible with the diagnosis depending on the acuity of the clinical setting (Ponikowski et al., 2016). Standard prognostic therapy in HF with angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, statins, and the novel  $\beta$ -adrenoceptor antagonists all increase  $\cdot\text{NO}$  bioavailability through reduced oxidative stress and increased NOS activity (Omar et al., 2016). Although the organic nitrates provide an efficacious treatment option in the setting of acute HF and have shown some benefit in chronic HF (Cohn et al., 1986; Taylor et al., 2004), they have proven difficult to use as a long-term option because of the rapid development of tolerance, reflex tachycardia, and the induction of endothelial dysfunction (Münzel et al., 2011, 2013). As the global prevalence of HF increases, with the associated high levels of morbidity and mortality (Meta-analysis Global Group in Chronic Heart Failure (MAGGIC), 2012; Ponikowski et al., 2016), it has become increasingly important to identify and exploit novel methods by which to treat the HF syndrome. The role of inorganic nitrite and nitrate to restore diminished  $\cdot\text{NO}$  is one such possibility (Paulus and Tschöpe, 2013).

*a.  $\cdot\text{NO}$  as an inotrope.*  $\cdot\text{NO}$  has both positive (Sarkar et al., 2000; Paolucci et al., 2003) and negative (Sandirasegarane and Diamond, 1999) inotropic effects in the failing heart, with many observations suggesting that these effects are cGMP-dependent (e.g., Balligand et al., 1993; Kojda et al., 1996), but there is also clear evidence of independence of GC-1 and cGMP signaling (Sarkar et al., 2000) and stimulation of  $\beta$ -adrenoceptors (Paolucci et al., 2003). These GC-1-independent effects have largely been attributed to the formation of nitroxyl anion and recruitment of ATP-sensitive  $\text{K}^+$  ( $\text{K}_{\text{ATP}}$ ) channels and/or possibly the neuropeptide calcitonin gene-related peptide (Paolucci et al., 2001a,b; Zhu et al., 2015). There is also a growing body of evidence implicating *S*-nitrosation as the pathway for  $\cdot\text{NO}$ -mediated cardiac effects. In particular, with respect to cardiac hypertrophy, studies with mice show a dependence of  $\beta$ -adrenoceptor signaling upon  $\cdot\text{NO}$  generation that is thought to trigger consequent *S*-nitrosation of key cardiac proteins, including phospholamban and troponin C, to regulate cardiac function accordingly (Irie et al., 2015). Interestingly, there is growing support for the view that *S*-nitrosation, rather than being the protein modification responsible for the effects of  $\cdot\text{NO}$ , represents a transient chemical step on the path to protein oxidation and disulfide bond formation and that the oxidation of proteins underlies changes in protein function in the setting of nitrosative stress (Wolhuter et al., 2018).

However, many studies have shown the importance of  $\cdot\text{NO}$ -mediated GC-1 signaling using phosphodiesterase (PDE)-V inhibition to enhance cGMP availability. In a pressure-overload-induced model of HF using thoracic aortic constriction (TAC), C57BL6J mice received 6 weeks of treatment with 100 mg/kg per day of sildenafil in soft diet (vs. placebo). Sildenafil prevented further cardiac chamber dilatation, dysfunction, fibrosis, and molecular remodeling, all with beneficial increased myocardial PKG activity (Nagayama et al., 2009). This was coupled with *in vitro* evidence of improved cardiac muscle contractility, relaxation, and enhanced calcium handling. A further study demonstrated the beneficial effects of PDE-V inhibition using vardenafil in a rabbit model of I/R injury via  $\cdot\text{NO}$ -dependent opening of the mitochondrial  $\text{K}_{\text{ATP}}$  channel (Salloum et al., 2006), as well as with sildenafil, albeit over a shorter time frame (Salloum et al., 2003). Finally, there is also evidence of the  $\cdot\text{NO}$ -mediated protective effect of sildenafil in doxorubicin-induced cardiotoxicity secondary to generation of ROS formation in cardiac mitochondria and ROS-induced cardiomyocyte apoptosis (Fisher et al., 2005). Cardiotoxicity as a result of anticancer treatment is an increasingly recognized clinical area of major unmet need (Bloom et al., 2016; Zamorano et al., 2016). In their study, Fisher et al. (2005) demonstrated this protective effect in the male ICR strain of mice, in which sildenafil attenuated the

TABLE 3  
Clinical studies assessing the effect of nitrate supplementation upon exercise economy and/or endurance performance

Reference	Cohort	Intervention	Design	Outcome
Wilkerson et al., 2012	Eight well trained male cyclists	Nitrate-replete (~6 mmol nitrate) or -deplete beetroot juice as placebo	Randomized, single blind, crossover study Measurements performed on two laboratory-based 50-mile time trial 2.5 h after consuming juice intervention	Nitrate supplementation did not improve 50-mile time trial performance in well trained cyclists.
MacLeod et al., 2015	11 trained male cyclists	Nitrate-replete or -deplete beetroot juice as placebo	Randomized, double blind study Measurements 2 h after consuming 70 ml of intervention and prior to a 15-min self-selected cycling warm-up, then a fixed 15 min of steady-state exercise at 50% maximum power output, and a 10-km time trial in either normoxic or hypoxic (altitude) conditions	Nitrate supplementation did not lower the O <sub>2</sub> cost of steady-state exercise or improve exercise performance in normoxia or hypoxia in a small sample of well trained male cyclists.
Peacock et al., 2012	10 male junior cross-country skiers	Nitrate-replete or -deplete beetroot juice as placebo	Two trials both randomized, double blind Measurements 2.5 h prior to two 5-min submaximal tests on a treadmill at 10 km/h (~55% of VO <sub>2</sub> max) and 14 km/h (~75% of VO <sub>2</sub> max), followed by a 5-km running time trial	Nitrate supplementation did not reduce O <sub>2</sub> cost of submaximal exercise or enhance endurance exercise performance in highly trained cross-country skiers.
Boorsma et al., 2014	Eight male 1500 m elite distance runners	210 ml of nitrate-replete (19.5 mmol) or -deplete beetroot juice as placebo	Randomized, double blind, crossover study Volunteers supplemented for 8 days with >1 wk between interventions. On days 1 (acute) and 8 (chronic), subjects completed a submaximal treadmill run and 1500-m time trial.	Acute and chronic nitrate supplementation did not reduce running VO <sub>2</sub> or improve time trial performance.
Bescos et al., 2012	13 well trained athletes	Sodium nitrate (10 mg/kg) or sodium chloride (10 mg/kg) placebo	Randomized, double blind, crossover study. Measurements after a 40-min cycle ergometer distance-trial test after two 3-day periods of dietary intervention	Sodium nitrate supplementation did not improve trial performance in endurance athletes.
Christensen et al., 2013	10 highly trained cyclists	0.5 l nitrate-replete beetroot juice or 0.5 l black currant juice as a placebo	Randomized, double blind, crossover study Measurements of VO <sub>2</sub> kinetics (3 × 6 min at 298 ± 28 W), endurance (120-min preload followed by a 400-kcal time trial), and repeated sprint capacity (6 × 20-s sprints, recovery 100 s) during two 6-day dietary intervention intervals	Nitrate supplementation did not affect VO <sub>2</sub> kinetics and performance in elite cyclists.
Porcelli et al., 2015	21 subjects (mean age, 22.7 ± 1.8 yr) with different aerobic fitness level	Supplementation with either 0.5 l/day of nitrate (5.5 mmol)-containing water or nitrate-free water as placebo	Crossover, double blind, placebo-controlled study 6-day treatment. Participants performed an incremental running test until exhaustion and four repetitions of 6-min submaximal (approximately 80% of gas-exchange threshold) constant load exercise on a motorized treadmill. Moreover, subjects performed a 3-km running time trial on the field.	Individual aerobic fitness level influenced ergogenic benefits induced by nitrate.
Muggeridge et al., 2013	Eight male kayakers	70 ml of nitrate-replete (5 mmol) concentrated beetroot juice or tomato juice as placebo	Participants completed four performance trials of 15 min of paddling at 60% of maximum work rate, five 10-s all-out sprints, and a 1-km time trial. The second and third trials were preceded by supplementation 3 h prior to measurements	Nitrate supplementation had no effect on repeated supramaximal sprint or 1-km time trial kayaking performance.
Peeling et al., 2015	Male ( <i>n</i> = 6) athletes (Study A) and international-level female ( <i>n</i> = 5) athletes (Study B) Study A: a 70-ml beetroot juice shot containing ~4.8 mmol of nitrate or placebo Study B: two 70-ml beetroot juice shots containing ~9.6 mmol of nitrate or placebo	Nitrate-replete or -deplete beetroot juice as placebo	Study A: participants completed three laboratory-based sessions on a kayak ergometer of 7 × 4-min step test, two 4-min maximal effort performance trials. At 2.5 h prior to the warm-up of each test, the athletes received intervention. Study B: participants completed two field-based 500-m time trials, separated by 4 days. At 2 h prior to each test, athletes received intervention.	In national-level male and international-level female kayak athletes, nitrate supplementation improved exercise economy in tasks predominantly reliant on the aerobic energy system and time trial performance, respectively.

(continued)

TABLE 3—Continued

Reference	Cohort	Intervention	Design	Outcome
Hoon et al., 2014	10 highly trained male rowers	Nitrate-replete or -deplete beetroot juice as placebo Nitrate-replete juice administered as a single shot containing 4.2 mmol or a double shot containing 8.4 mmol nitrate	Placebo-controlled, double blind, crossover study Intervention was consumed 2 h prior to undertaking a 2000-m rowing-ergometer test.	Compared with placebo, a high (8.4 mmol nitrate) but not moderate (4.2 mmol nitrate) dose improved rowing performance.
Bond et al., 2012	14 well trained junior male rowers	Beetroot juice made from raw vegetables delivering 5.5 mmol/day of nitrate for 6 days A commercially available black currant juice with negligible nitrate content was used as placebo	Randomized, double blind, crossover study After supplementation, rowers completed six maximal 500-m ergometer. A 7-day washout period followed repeat of the trial. Blood pressure, oxygen saturation, maximum heart rate, urine, and lactates were measured.	Nitrate supplementation improved maximal rowing-ergometer repetitions.

toxic effects of doxorubicin, with loss of that protection after treatment with the NOS inhibitors L-NAME and 5-hydroxydecanoate.

*b. Cardiac hypertrophy and ·NO.* Cardiac hypertrophy in response to increased afterload (e.g., systemic hypertension) is initially an adaptive response to increase wall stress. Subsequently, this develops into a maladaptive response with progressive left ventricular (LV) dilatation and reduction of ejection fraction. Left ventricular hypertrophy is an important risk factor for developing HFpEF, HFpEF, and atrial fibrillation, as well as for sudden cardiac death in patients (Katholi and Couri, 2011). The importance of ·NO in LV remodeling has been demonstrated in several experimental works—for instance, in transgenic mice with cardiomyocyte-restricted overexpression of eNOS (NOS3-TG) versus WT littermates after myocardial infarction (MI) by left anterior descending artery ligation (Janssens et al., 2004). Pre-MI, a 30-fold increase in cardiac eNOS expression led to attenuation of the inotropic response to  $\beta$ -adrenoceptor agonist isoproterenol. Although infarct size was similar in both groups, NOS3-TG mice had significantly less cardiac dilatation and dysfunction (by LV end systolic diameter and fractional shortening), and enhanced contractile performance and ventricular relaxation (by rate of change of pressure (dP/dt)<sub>max</sub> and dP/dt<sub>min</sub>). There was no protective effect of enhanced eNOS expression on either remote cardiac fibrosis or survival.

ZSF1-HFpEF (leptin-resistant, obese, hypertensive Zucker diabetic fatty/spontaneously hypertensive HF F1 hybrid) rats develop a HFpEF phenotype after 20 weeks, with elevated LV filling pressures, preserved LVEF, increased lung weight with pulmonary congestion, and increased myocardial stiffness (Hamdani et al., 2013). Importantly, there was evidence to suggest reduced ·NO bioavailability (by 3-nitrotyrosine expression) in the HFpEF mice, with a significant reduction in PKG activity and cGMP concentration, confirming abnormalities in canonical ·NO/GC-1/cGMP/PKG signaling (Hamdani et al., 2013).

This lack of bioavailable ·NO and subsequent GC-1 signaling (Paulus and Tschöpe, 2013) has important

effects and not only correlates with the level of diastolic dysfunction (Tschöpe et al., 2005) but also plays a key role in the reduced systemic vasodilator response underlying impaired exercise capacity in patients with HFpEF (Borlaug et al., 2010; Edelmann et al., 2011; Haykowsky et al., 2012).

*c. Positive inotropic and antihypertrophic effects of nitrite and nitrate.* Subsequently, studies have shown that nitrite and nitrate treatment also produces a profile of activity reminiscent of ·NO. Pellegrino et al. (2009) used the Langendorff rat heart model to demonstrate that even at low concentrations, NaNO<sub>2</sub> (1 nmol/l) significantly improves contractility, with reductions in LV pressure and improved relaxation. These effects were shown to be ·NO-mediated and to act via the ·NO/cGMP/PKG pathway, but independent of NOS activity. The positive inotropic activity of inorganic nitrite is broadly expressed across a range of species, as demonstrated by the same group in fish, amphibian, and mammalian hearts, in which exogenously applied nitrite improved the Frank-Starling response, with increases in stroke volume, stroke work, LV pressure, and LV relaxation (LV dP/dt<sub>max</sub>) (Angelone et al., 2012). Using male Wistar rats, Ashmore et al. (2014) demonstrated similar benefits with dietary nitrate (0.7 mmol/l vs. equimolar NaCl) treatment, which prevented hypoxia-induced mitochondrial dysfunction and reduced oxidative stress, while increasing available circulating ·NO through increased tissue L-arginine and reduced suppression of cardiac arginase expression. Similarly, in a mouse model of doxorubicin-induced cardiac dysfunction, provision of NaNO<sub>3</sub> (1 g/l, equivalent to 12 mmol/l) for 7 days prior to administration of doxorubicin improved both LV systolic, end-diastolic pressures, and ejection fraction (Zhu et al., 2011). These positive effects of ·NO and the evidence indicating deficiencies of ·NO in the HF setting have spurred researchers on to determine whether the noncanonical pathway for ·NO delivery may be useful for improving outcome in this setting.

*d. Preclinical studies: cardiac dysfunction, nitrite, and nitrate.* In WT rats given L-NAME to induce

TABLE 4  
Clinical studies assessing nitrate supplementation on exercise performance during high-intensity exercise

Reference	Cohort	Intervention	Design	Results	Outcome
Wylie et al., 2013	14 male recreational team-sport players	Nitrate-replete or -deplete beetroot juice as placebo (490 ml)	Double blind, randomized, crossover study with juice delivered over ~30 h preceding the completion of a Yo-Yo intermittent recovery level 1 test	Performance in the Yo-Yo IR1 was 4.2% greater with nitrate compared with the placebo.	Acute nitrate supplementation improved performance during intense intermittent exercise in team sports players.
Thompson et al., 2014	16 male team-sport players	Intervention of nitrate-replete (140 ml/day; 12.8 mmol of nitrate <sup>-</sup> ) or -deplete beetroot juice as placebo (140 ml/day; 0.08 mmol nitrate)	Double blind, randomized, crossover study. On day 7 of supplementation, subjects completed tests on a cycle ergometer during which cognitive tasks were simultaneously performed.	Total work done during the sprints was greater with nitrate treatment (123 ± 19 kJ) compared with the placebo (119 ± 17 kJ; <i>P</i> < 0.05). Reaction time of response to the cognitive tasks was also improved. There was no difference in response accuracy.	Nitrate supplementation enhanced repeated sprint performance and attenuated the decline in cognitive function (and specifically reaction time) that may occur during prolonged intermittent exercise.
Aucouturier et al., 2015	12 male subjects	Intervention of beetroot juice, 500 ml with 680 mg/l of nitrate  Placebo of apple-black currant juice with nitrate content <5 mg/l	Randomized crossover design single blinded to the subjects with a 3-day supplementation	The number of repetitions completed before reaching volitional exhaustion was significantly higher in the nitrate group than in the placebo (26.1 ± 10.7 vs. 21.8 ± 8.0 respectively, <i>P</i> < 0.05). In contrast during exercise performed at intensity below the peak oxygen uptake (VO <sub>2</sub> peak), oxygen uptake (VO <sub>2</sub> ) was unaffected. AUC for microvascular total hemoglobin (AUC-THb) in the vastus lateralis muscle assessed by near infrared spectroscopy during exercise was increased with nitrate.	Nitrate supplementation enhanced tolerance to exercise at supramaximal intensity, with increased microvascular total RBC concentration in the working muscle, in the absence of effect on contractile function and resting hemodynamic parameters.
Muggeridge et al., 2013	Eight male kayakers	Intervention of 70 ml nitrate-replete (5 mmol) concentrated beetroot juice or tomato juice as placebo	Randomized crossover study measuring work rate during performance trials composed of 15 min of paddling at 60% of maximum work rate, five 10-s all-out sprints, and a 1-km time trial. The second and third trials were preceded by ingestion of intervention 3 h before exercise.	VO <sub>2</sub> during steady-state exercise was lower in the nitrate-treated than in the PL treated with no differences in either peak or time trial performance.	Nitrate supplementation had no effect on performance.
Martin et al., 2014	Team-sport athletes (9 male, age 22.3 ± 2.1 yr; 7 female, age 20.7 ± 1.3 yr)	Intervention of 70 ml of nitrate-replete (0.3 g nitrate) concentrated beetroot juice or nitrate-deplete beetroot juice as placebo	Double blind, randomized, crossover study with participants consuming interventions 2 h prior to tests	There was no difference in overall mean power output or the mean power output for each individual sprint test.	Nitrate supplementation did not improve performance.
Wylie et al., 2016	34 healthy subjects	Intervention of nitrate-replete (3 or 6 mmol nitrate/day) concentrated beetroot juice or nitrate-deplete (0.08 mmol nitrate/day) beetroot juice as placebo	Randomized counterbalanced design. Two moderate-intensity step exercise tests 2 h after the first ingestion and after 7, 28, and 30 days of supplementation, subjects completed.	Compared with pre-treatment baseline, 6 mmol nitrate reduced the steady-state VO <sub>2</sub> during moderate-intensity exercise by 3% at 2 h ( <i>P</i> = 0.06), 7 days and at 28–30 days (both <i>P</i> < 0.05) but was unaffected by 3 mmol nitrate at all measurement points.	Up to ~4 wk supplementation with nitrate at 6 but not 3 mmol reduced submaximal exercise VO <sub>2</sub> .
Bescos et al., 2012	13 well trained endurance athletes	Intervention of NaNO <sub>3</sub> , 10 mg/kg of body mass Placebo: sodium chloride, 10 mg/kg of body mass	Randomized, double blind, crossover study with a 40 min cycle ergometer distance-trial test after two 3 day periods of dietary supplementation	There were no differences in either the mean distance or mean power output between treatments.	Nitrate supplementation did not improve a 40-min distance-trial performance.
Coggan et al., 2015	Healthy men and women ( <i>n</i> = 12; age = 22–50 yr)	Nitrate-replete (140 ml, 11.2 mmol) or -deplete beetroot juice as placebo	Double blind, placebo-controlled, randomized trial  Assessments at 2 h after intervention	Nitrate treatment increased breath NO by 61%. This was accompanied by a 4% ( <i>P</i> < 0.01; effect size = 0.74) increase in peak knee extensor power at the highest angular velocity tested (i.e., 6.28 rad/s). Calculated maximal knee extensor power was greater after dietary nitrate.	Acute nitrate supplementation increased whole-body NO production and muscle speed and power in healthy men and women.

(continued)

TABLE 4—Continued

Reference	Cohort	Intervention	Design	Results	Outcome
Sandbakk et al., 2015	Nine male elite cross-country skiers [age 18 ± 0 y, VO <sub>2</sub> max 69.3 ± 5.8 ml · min <sup>-1</sup> · kg <sup>-1</sup> ]	Combined supplementation with 6 g L-arginine and 614 mg nitrate against 614 mg nitrate alone and placebo	Randomized, crossover, double blind study with 48 h pretreatment	There were no differences in physiologic responses during submaximal running or in 5-km performance between treatments.	There were no effects of nitrate supplementation on exercise economy or endurance running performance in endurance-trained cross-country skiers.
Haider and Folland, 2014	19 healthy untrained men (21 ± 3 yr)	Nitrate-replete (9.7 mmol/day) or -deplete beetroot juice as placebo	Double blind, randomized, crossover study, with intervention given for seven consecutive days. After the last supplementation dose, force was recorded while participants completed a series of voluntary involuntary (electrically evoked) unilateral isometric contractions of the knee extensors.	Nitrate enhanced peak force response to low-frequency electrical stimulation. Explosive force production during the first 50 ms of evoked maximal twitch and octet contractions (eight electrical impulses at 300 Hz) was 3%–15% greater after nitrate compared with placebo. Maximum voluntary force was unchanged nitrate.	Nitrate supplementation enhanced the contractile properties of human skeletal muscle.
Rimer et al., 2016	13 trained athletes	Nitrate-replete (~11.2 mmol nitrate) or -deplete beetroot juice as placebo (0.004 mmol)	Double blind crossover study, with maximal inertial-load cycling trials (3 to 4 s) immediately before and after consuming intervention. Participants also performed maximal isokinetic cycling (30 s) to assess performance differences after supplementation.	PMAX was increased after nitrate treatment. RP Mopt was increased with nitrate treatment.	Acute nitrate supplementation enhanced maximal muscle power in trained athletes.
Lane et al., 2014	12 male and 12 female competitive cyclists	Trials were undertaken with a caffeinated gum (3 mg/kg body mass, 40 min prior to the TT), concentrated beetroot juice supplementation (8.4 mmol of nitrate, 2 h prior to the TT), caffeine plus beetroot juice, or a control.	Cyclists each completed four experimental trials in a double blind random Latin square design.	There was no effect of nitrate supplementation when used or when combined with caffeine.	Nitrate supplementation was not ergogenic under the conditions of this study.
Lansley et al., 2011	Nine club-level competitive male cyclists	Nitrate-replete (BR) or -deplete beetroot juice (PL) BR containing ~6.2 mmol of nitrate PL containing ~0.0047 mmol of nitrate	Randomized, double blind, crossover study with intervention delivered 2.5 h before the completion of a 4- and a 16.1-km time trial	VO <sub>2</sub> during time trial were not different between groups but nitrate treatment significantly increased mean PO during the 4- and 16.1-km time trials.	Acute nitrate supplementation improved cycling economy.
Flueck et al., 2016	12 healthy, well trained men	Dosages were 3, 6, and 12 mmol nitrate as concentrated beetroot juice or sodium nitrate dissolved in water vs. water placebo.	Placebo-controlled, single blind, crossover, randomized study Measurements 3 h after ingestion. Participants cycled for 5 min at moderate intensity and a further 8 min at severe intensity. End-exercise O <sub>2</sub> consumption at moderate intensity.	At severe-intensity exercise, end-exercise oxygen consumption was ~4% lower in the 6 mmol nitrate juice group compared with the 6 mmol salt ingestion or placebo.	Nitrate supplementation with juice reduced O <sub>2</sub> consumption to a greater extent compared with nitrate salt supplementation.

BR, beetroot juice; IRL, intermittent recovery test level 1; PL, placebo; RBC, red blood cell; TT, time-trial.

hypertension leading to cardiac enlargement and fibrosis, supplementation in drinking water with NaNO<sub>2</sub> (100 mg/l) attenuated cardiac enlargement and fibrosis to a similar degree as captopril (100 mg/l), an angiotensin-converting enzyme inhibitor and first-line treatment of hypertension and HF (Sonoda et al., 2017). In a murine

model of pressure overload by TAC, inducing hypertrophy and subsequent HF, NaNO<sub>2</sub> (50 mg/l, ~9–12 mg/kg per day in drinking water) versus vehicle for 9 weeks significantly reduced BNP levels, cardiac hypertrophy, and pulmonary edema at 9 weeks (Bhushan et al., 2014). Furthermore, nitrite pretreatment significantly

prevented LV dilatation in both systole and diastole, as well as attenuating LV dysfunction (as measured by LVEF) at 9 weeks. These findings were replicated in a reversal model more representative of the clinical scenario in which patients might present, in which nitrite treatment was commenced 3 weeks after TAC.

More recently, the view that perhaps dietary nitrate might improve outcome by improving mitochondrial function has been tested in healthy rats. Interestingly, NaNO<sub>3</sub> in the drinking water (1 g/l) for 7 days resulted in elevation of circulating nitrate concentrations with reduction in both SBP and LV end diastolic volumes but absolutely no change in mitochondrial bioenergetics, suggesting that at least in healthy animals the effects of nitrate were not due to improved mitochondrial efficiency or oxidative capacity as has been suggested in other studies. However, what the authors did show was that nitrate treatment was associated with an increase in mitochondrial H<sub>2</sub>O<sub>2</sub> emissions in LV homogenates without any change in redox state (Monaco et al., 2018).

The ability of nitrite and nitrate to enhance skeletal muscle performance is discussed elsewhere in this review. However, there is increasing evidence for a specific role of this particular characteristic of the anions in patients with HF (Coggan and Peterson, 2016). A recent study in rats that develop HF after induction of MI by coronary artery ligation (Ferguson et al., 2016) has demonstrated the benefits of dietary nitrate treatment. At 21 days after coronary artery occlusion, rats were randomized to 5 days of nitrate-rich (1 mmol/kg per day nitrate) versus nitrate-deplete beetroot juice and underwent investigations of skeletal muscle blood flow and vascular conductance. Blood flow and vascular conductance at rest were 22% and 20% higher, respectively, and significantly greater during exercise in the nitrate-fed rats.

Perhaps one of the key aspects of the nitrate-nitrite-NO pathway that has led to so much interest in its therapeutic utility has been the fact that one can easily and safely test its potential in the clinical setting through dietary administration. HF is no exception, with very rapid translation to the clinical setting after these early preclinical observations.

*e. Clinical translation.* In the first-in-human study by Ormerod et al. (2015), 25 patients with HF<sub>rEF</sub> established on best-tolerated HF therapy, undergoing elective right heart catheter during the workup for cardiac transplantation, were subjected to an infusion of NaNO<sub>2</sub> for 5 minutes at 10 μg/kg per minute (group 1, *n* = 8) and 50 μg/kg per minute (group 2, *n* = 17), and a Swann-Ganz catheter used to measure cardiac and pulmonary hemodynamic parameters. Only the latter dose caused significant increases in plasma nitrite levels, but this rise was associated with a drop in SBP of 4 mm Hg over the course of the infusion, as well as decreases in pulmonary and systemic vascular

resistance. With this higher dose of nitrite, a significant decrease in right atrial pressure (40%) and increases in trans-septal gradient (3 mm Hg), cardiac output (13%), and stroke volume (14%) were evident. In addition, in a subgroup analysis of patients with high pulmonary capillary wedge pressure (>15 mm Hg), stroke volume was increased by 20%, whereas in those with wedge pressures below 15 mm Hg, no change was observed. This study therefore demonstrated therapeutic potential for stable patients with HF<sub>rEF</sub> with clinically marked disease, suggesting that the worse the symptoms, the greater the potential benefit (Ormerod et al., 2015).

A number of small studies have been undertaken investigating the role of dietary nitrate in patients with or at risk of HF. Type 2 diabetes mellitus is a strong risk factor for both HF<sub>rEF</sub> and HF<sub>pEF</sub> (Cavender et al., 2015). In patients with or at risk of developing type 2 diabetes mellitus, 6 months of dietary nitrate supplementation (4.5 mmol/day) was associated with a reduction of LV volumes assessed by standard transthoracic echocardiography, but not in other parameters of improved cardiac performance, or with BP reduction in comparison with placebo (Faconti et al., 2019). This disappointing result may reflect issues with coexisting drug therapy (i.e., metformin) or microbial bioactivation of nitrate (Cabreiro et al., 2013; Forslund et al., 2015; Sun et al., 2018), as similar neutral results have been seen in other studies with dietary nitrate and diabetes (Gilchrist et al., 2013).

Hirai et al. (2017) in a double blind, randomized, placebo-controlled crossover study in 13 patients with LVEF ≤ 40%, supplemented with 12.9 mmol daily of dietary nitrate for 9 days, investigated the impact of treatment upon time to exercise intolerance. Ten patients completed low- and high-intensity cardiopulmonary exercise testing with noninvasive measures of central hemodynamics, arterial BP, pulmonary oxygen uptake, quadriceps muscle oxygenation, and serum lactate. Over the short period of intervention, they demonstrate that the enterosalivary circuit is intact, with significantly higher plasma nitrite concentrations after intervention versus placebo (240 ± 48 vs. 56 ± 8 nmol/l, respectively). However, they were not able to demonstrate significant benefit in their primary outcome of time to exercise intolerance (495 ± 53 vs. 489 ± 58 s, *P* > 0.05) or in their other quoted measures over the short period of intervention. In contrast, in a separate randomized, placebo-controlled, double blind crossover study of nine patients with HF<sub>rEF</sub> (LVEF 28% ± 11%, NYHA II/III) assessing the potential of an 11.2 mmol dose of dietary nitrate as beetroot juice (Coggan et al., 2015), significant improvements in muscle power using isokinetic dynamometry compared with placebo in both peak knee extensor power and calculated maximal velocity of knee extension were evidenced.

Interestingly, these effects were made 2 hours after treatment, and although circulating nitrate levels were increased at this time point, nitrite concentrations were not.

This apparent dissonance between the effects of a single acute dose and daily dosing over several days was assessed in a single study in patients with HFpEF. Twenty older ( $69 \pm 7$  years) hypertensive patients with HFpEF received a once-daily dose of 6.1 mmol nitrate as beetroot juice (vs. nitrate-deplete placebo juice) in a phase II randomized double blind crossover study over 7 days (Eggebeen et al., 2016). The first dose of juice had no impact on submaximal exercise capacity  $\sim 1.5$  hours after ingestion; however, over 1 week of intervention, there was a 24% increase in the time to volitional exhaustion compared with placebo. There was a further benefit of a significant reduction in SBP, at rest and during unloaded exercise, with a trend at volitional exhaustion. This is in line with previous findings using approximately double the dose, with 12.9 mmol dietary nitrate, with improvements in exercise duration, work done, and peak  $\text{VO}_2$  in addition to increased cardiac output and a fall in systemic vascular resistance (Zamani et al., 2015), and concurs with two recent pilot studies in patients with hypertension and HFpEF in which time to volitional exhaustion was again increased after dietary nitrate supplementation (Shaltout et al., 2017). Shaltout et al. (2017) report the results of their pilot studies in older patients with hypertension and older patients with HFpEF, in which no additional benefit of dietary nitrate (8 and 6.1 mmol) above and beyond exercise training compared with placebo was evident. However, within-group comparisons in terms of peak  $\text{O}_2$  consumption, reductions in SBP, and improved arterial compliance were observed. In this study, the authors suggest that escalating doses of dietary nitrate might confer additional benefit, but these studies also suggest enhanced benefits of prolonged over acute dosing in patients with HF similar to observations in healthy volunteer studies (Vanhatalo et al., 2010; Hoon et al., 2013). More recently, a multicenter, placebo-controlled, cross-over, randomized trial has investigated the effects of nebulised sodium nitrite (1.2 mmol nitrite thrice daily for 4 weeks in 105 patients) in patients with HFpEF (Borlaug et al., 2018). Despite significant resting BP reduction with inhaled nitrite (difference in MAP  $\sim 5$  mmHg) suggesting sufficient nitrite dosing/elevation to cause a hemodynamic effect (there were no direct measures of plasma nitrite concentrations), there was no effect on  $\text{VO}_{2\text{MAX}}$  (primary outcome there) or other important secondary outcomes measures used in HF trials (daily activity levels, symptom and functional class scores, NTproBNP levels, echocardiographic indices of diastolic dysfunction) (Borlaug et al., 2018). The reasons for the discrepancy between this and previous studies is not immediately clear but may relate to the half-life of nitrite and the dose/route used.

Despite the variable results noted to date, the general excitement regarding such a simple intervention for this patient cohort is reflected by the fact that there are a number of further studies registered across the globe that continue to investigate the potential therapeutic role of inorganic nitrate/nitrite (summarized in Table 5). The outcome of these studies is eagerly awaited.

### C. Nitrite and Nitrate in Inflammation

$\cdot\text{NO}$  is a potent anti-inflammatory mediator. Perhaps the primary characteristic that underlies this particular feature of  $\cdot\text{NO}$  bioactivity is that it potently inhibits leukocyte recruitment (Kubes et al., 1991), a key feature of inflammatory responses (Kolaczowska and Kubes, 2013). The mechanism of this action of  $\cdot\text{NO}$  has been attributed primarily to the canonical pathway, i.e., GC-1 activation, to elevate cGMP levels, which then results in a suppression in expression of the adhesion molecule CD62P (P-selectin), which plays a key role in the first step, rolling, of the leukocyte recruitment process (Ahluwalia et al., 2004). Despite this, relatively few studies have investigated whether nitrate/nitrite-derived  $\cdot\text{NO}$  has the potential to reduce inflammatory responses in humans. In contrast, there are now several studies in mouse models demonstrating important anti-inflammatory effects of the anions.

Bryan and his coworkers were the first to explore this particular phenomenon in a mouse model of hypercholesterolemia-induced microvascular inflammation. In this study, healthy C57BL6 mice were fed a high-cholesterol diet for 3 weeks, increasing cholesterol levels from  $\sim 70$  mg/dl in controls to  $\sim 115$  mg/dl, elevating circulating leukocyte numbers from 4.8 to  $6.8 \times 10^6$  cells/ml, and resulting in a 4-fold increase in leukocytes adhering to the venular endothelium and an  $\sim 70$ -fold increase in emigrated cells measured by intravital microscopy in the cremaster tissue (Stokes et al., 2009). In mice treated concomitantly with 50 or 150 mg/l  $\text{NaNO}_2$  in the drinking water, a similar reduction in both leukocyte adhesion and emigration was demonstrated. The authors suggested that this effect upon leukocyte recruitment related to an improvement in endothelial function due to elevations in tetrahydrobiopterin levels and, in this way, improves endothelium-derived  $\cdot\text{NO}$  activity rather than acting as a delivery method of  $\cdot\text{NO}$  per se.

Similar effects have been observed in another study, in which leukocyte recruitment was induced by an acute (90-minute) superfusion of the cremaster muscle with the neutrophil-specific chemokine (C-X-C motif) ligand 2 (CXCL2) (Macrophage Inflammatory Protein 2). This resulted in elevations of leukocyte adhesion and emigration, which were attenuated by a bolus of  $\text{NaNO}_2$  (1.3 mg/kg) given intravenously 1 hour prior to CXCL2 superfusion (Jädert et al., 2012). This effect of nitrite upon leukocyte adherence was shown to be dependent



upon the activation of GC-1 and elevation of cGMP since the guanylyl cyclase inhibitor, ODQ, administered simultaneously with NaNO<sub>2</sub>, significantly attenuated leukocyte adhesion but, curiously, had no effect on leukocyte emigration. The authors suggested that this differential effect was likely due to an important role for cGMP in leukocyte adhesion but not margination, although if adherence of leukocytes is attenuated, one would expect some knockon effect upon emigration since the former is a prerequisite for the latter. The authors also demonstrated that after 1 week of treatment with NaNO<sub>3</sub> (10 mmol/l) added to the drinking water, a similar reduction in baseline leukocyte rolling was evident, although adhesion or emigration were unaffected, and that leukocyte adhesion and emigration were also reduced in response to CXCL2 (Jädert et al., 2012).

It is now reasonably certain that systemic treatment with nitrite (predominantly NaNO<sub>2</sub>) results in a reduction of the numbers of proinflammatory leukocyte at sites of inflammation. This has been demonstrated in a wide array of distinct animal models in several different species, including in dextran sodium sulfate-induced colonic inflammation as a model of colitis in rats (Ohtake et al., 2010; Jädert et al., 2013); a mouse model of postoperative ileus, a frequent outcome after abdominal surgery (Cosyns et al., 2015); chlorine gas exposure as a model of environmental toxicity and potential chemical warfare in mice, rats, and rabbits (Samal et al., 2012; Honavar et al., 2014, 2017); elastase-induced pulmonary emphysema in a mouse model (Sonoda et al., 2018); and lung transplantation in rats (Sugimoto et al., 2012). In all of these models, reduced inflammatory responses correlate with reduced numbers of cells, and in many instances, this relates specifically to reduced numbers of neutrophils (Ohtake et al., 2010; Samal et al., 2012; Sugimoto et al., 2012; Cosyns et al., 2015).

This activity of nitrite against neutrophil recruitment has also been demonstrated with dietary nitrate. In a mouse model of atherosclerosis, the ApoE KO mouse, fed 15 mmol/l KNO<sub>3</sub> in the drinking water for 12 weeks, exhibited reduced leukocyte rolling and adhesion measured using intravital microscopy (Khambata et al., 2017). In addition, after only 2 weeks of dietary nitrate, neutrophil recruitment into the peritoneum in response to zymosan or tumor necrosis factor alpha (TNF $\alpha$ ) in the C57BL/6 or littermate ApoE KO mice were also attenuated. Jädert et al. (2012, 2013) demonstrated similar effects of dietary nitrate (10 mmol/l NaNO<sub>3</sub> for up to 7 days in the drinking water) against both Dextran sulfate sodium and nonsteroidal anti-inflammatory drug (NSAID)-induced colitis.

The studies above demonstrate a clear impact of nitrite or nitrate treatment on neutrophil recruitment and activation; however, this effect has been suggested to be responsible for the associated reductions in monocyte/macrophage recruitment. In ApoE

KO mice fed a high-fat Western diet with 15 mmol/l KNO<sub>3</sub> for 12 weeks, although plaque size was not altered, macrophage accumulation within the atherosclerotic plaque was significantly attenuated (Khambata et al., 2017). A similar outcome was evident with nitrite treatment and lung grafting (Sugimoto et al., 2012).

A possible mechanism for these antileukocyte effects of nitrate and nitrite is the selective inhibition of key adhesion molecules involved in the leukocyte recruitment process that enable the passage of the leukocyte from the centerline of blood flow to the endothelium and then across the blood vessel wall. As mentioned above, there is good evidence that  $\cdot$ NO targets CD62P on endothelial cells to attenuate leukocyte rolling. In a rat model of NSAID-induced inflammation of the small intestine, endothelial CD62P expression was profoundly suppressed by treatment of rats prior with NaNO<sub>3</sub> (10 mmol/l) in the drinking water—interestingly, an effect that was lost when the rats were pretreated with chlorhexidine mouthwash to suppress the oral nitrate reductase microbiome (Jädert et al., 2012). Further support for such an effect comes from the reported sensitivity of the anti-inflammatory effects of nitrate or nitrite to agents that scavenge  $\cdot$ NO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO)] or block rises in cGMP through inhibition of GC-1 activity, namely by ODQ treatment (Sugimoto et al., 2012; Cosyns et al., 2015), since it is known that cGMP mediates  $\cdot$ NO-induced suppression of CD62P expression (Ahluwalia et al., 2004).

However, there are a number of observations demonstrating that other endothelial adhesion molecules and key molecules expressed on the leukocytes themselves may also be targets for nitrite-derived  $\cdot$ NO. In TNF $\alpha$ -treated human dermal microvascular endothelial cells, NaNO<sub>2</sub> (1–100  $\mu$ mol/l) caused a concentration-dependent attenuation of intercellular adhesion molecule-1 (ICAM-1) expression, which is a ligand for several  $\beta_2$ -integrins (Jädert et al., 2012). Our own studies in ApoE mice have shown that treatment with KNO<sub>3</sub> in the water (15 and 45 mmol/l) causes a selective reduction in TNF $\alpha$ -induced neutrophil CD11b expression (an  $\alpha$ -integrin, a component of CD11b/CD18, also known as macrophage-1 antigen), with no effects observed on either CD62L (L-selectin) or CD162 (P-selectin glycoprotein ligand-1) expression (Khambata et al., 2017). Unfortunately, in this study, the potential impact upon endothelial CD62P expression was not assessed.

There is also some indication that treatment with these anions likely impacts upon mediator generation at the site of inflammation. Several of the above studies demonstrate reduced cytokine expression after treatment with the anions, particularly with respect to interleukin (IL)-1, IL-6, and TNF $\alpha$  (Ohtake et al., 2010; Sugimoto et al., 2012; Cosyns et al., 2015; Justice et al., 2015; Kautza et al., 2015). The exact

TABLE 5  
Currently recruiting trials investigating the role of nitrate and nitrite in Heart Failure

Identifier	Trial Name	Lead Investigators	Design	No. of Patients	Key Inclusion Criteria	Agent and Dose	Delivery	Treatment Duration	Primary/Powered Outcomes	Key Additional Measures
NCT02713126	Inorganic Nitrite to Amplify the Benefits and Tolerability of Exercise Training in Heart Failure with Preserved Ejection Fraction (HF <sup>p</sup> EF) (INABLE-Training)	Barry Borlaug MD	Phase II Randomized Double blind Placebo-controlled	100	HF <sup>p</sup> EF (LVEF >50%) NYHA II-IV, age >40 Enrolled in cardiac rehabilitation (exercise training)	Sodium nitrite, 40 mg TDS	Oral Capsules	12 wk	• Peak VO <sub>2</sub> on CPEX	• Change in daily activity levels (accelerometer) • Change in cardiac hemodynamics by Echo • QoL assessment
ACTRN12615000906550	Effects of Dietary Inorganic Nitrate Supplementation on Exercise Performance in Heart Failure	Prof. Jason D. Allen	Phase I/II Randomized Placebo-controlled Crossover (minimum 2-wk washout)	15 HF <sup>p</sup> EF + 15 HF <sup>r</sup> EF	HF <sup>p</sup> EF and HF <sup>r</sup> EF, age 18–85	Sodium nitrite, 20–~16 mmol OD	Beetroot juice	7 days	• Peak VO <sub>2</sub> on CPEX • Time to exhaustion on CPEX • Plasma nitrite levels	• Change in tissue oxygenation • Plasma NOx levels  • Change in mitochondrial function (muscle biopsy) • Brachial artery flow-mediated dilatation • Vascular stiffness (PWA and PWV) • Hemodynamic parameters on Echo • Perceived fatiguability during exercise • Change in daily activity levels (accelerometer) • Measures of pulmonary hemodynamics (by right heart catheter) • Skeletal muscle bioenergetics • Plasma nitrate • BNP
NCT02918552	Oral Nitrite for Older Heart Failure with Preserved Ejection Fraction (ONOH)	Mark Gladwin MD	Phase II Randomized Double blind Placebo-controlled	16	HF <sup>p</sup> EF (LVEF >40%), age >70	Sodium nitrite, 20–40 mg TDS	Oral capsules	8 wk active treatment	• Peak VO <sub>2</sub> on CPEX	• Change in daily activity levels (accelerometer) • Measures of pulmonary hemodynamics (by right heart catheter) • Skeletal muscle bioenergetics • Plasma nitrate • BNP
HF <sup>r</sup> EF NCT01682356	Dietary Nitrates for Heart Failure	Linda Peterson MD + Andrew Coggan MD	Phase II Randomized Double blind Placebo-controlled Crossover (7-day washout)	126	HF <sup>r</sup> EF in transplant workup (NYHA III/IV) or pulmonary hypertension, age >18	Dietary nitrate, 5 to 6 mmol OD	Beetroot juice	4 wk	• Peak power with neuromuscular power test • Change in cardiac mass after LVAD	• Peak VO <sub>2</sub> on CPEX  • Pulmonary artery pressure by right heart catheter (if clinically indicated) • Cardiac function by Echo or cardiac MRI (if clinically indicated) • Submaximal exercise tolerance (6MWT) • Sub-maximal exercise capacity (6MWT)
NCT03511248	Investigation of Dietary Nitrate Optimisation by Hyperuricaemia Stratification in Heart Failure (DiNOmo-HF)	Dr. C.P. and A.A.	Phase II Randomized Double blind Placebo-controlled	92	HF <sup>r</sup> EF (LVEF <40%) NYHA II/III, age >18 Elevated BNP/NT-proBNP Stable medical therapy for 3 months	Dietary nitrate, 5 to 6 mmol OD	Beetroot juice	12 wk	• Change in uric acid levels between strata (< and ≥ 9.8 mg/dl) • LVEF • NOx levels	• QoL questionnaire • NT-proBNP and BNP • Measures of oxidative stress • Erythrocyte XOR activity

(continued)

TABLE 5—Continued

Identifier	Trial Name	Lead Investigators	Design	No. of Patients	Key Inclusion Criteria	Agent and Dose	Delivery	Treatment Duration	Primary/Powered Outcomes	Key Additional Measures
ACTRN12616000951459	Feasibility and Effects of Inorganic Nitrate in Acute Decompensated Heart Failure (FINO-ADHF)	Prof. Christopher Neil	Phase I/II Randomized Double blind Placebo-controlled	40	Acute decompensated HF, age >18	Sodium nitrate, 8.4 mmol over four 12-h intervals	Beetroot juice + capsules	48 h	<ul style="list-style-type: none"> <li>Plasma NOx levels</li> <li>A change in vasorelaxation as assessed by systemic vascular resistance index</li> </ul>	<ul style="list-style-type: none"> <li>Vascular stiffness (PWA and PWV)</li> <li>Change in renal function by cystatin C levels</li> <li>Change in oxygenation using pulse oximetry</li> <li>Dyspnea relief</li> <li>Cardiac troponin T</li> <li>Change in natriuresis measured by urinary sodium</li> <li>Changes in cardiac function by Echo</li> <li>Peak muscle power (quadriceps)</li> <li>Plasma NOx levels</li> <li>Breath NO level</li> </ul>
NCT02797184	Inorganic Nitrate and Exercise Performance in Heart Failure (iNIX-HF): Dose Response	Linda Peterson MD + Andrew Coggan MD	Phase II Double blind Dose crossover (7-day washout)	25	HFrEF (LVEF <45%) NYHA II–IV, age 18–74 Stable medical therapy for 30 days	Potassium nitrate, 10 and 20 mmol once	Oral capsules	7 days between acute dosing	<ul style="list-style-type: none"> <li>Peak VO2 on CPEX (acute: 2.5 h postdose)</li> </ul>	<ul style="list-style-type: none"> <li>Changes in cardiac function by Echo</li> <li>Peak muscle power (quadriceps)</li> <li>Plasma NOx levels</li> <li>Breath NO level</li> </ul>
ACTRN12613000689774 (withdrawn)	Randomized double blind controlled pilot trial investigating the effect dietary nitrate in the treatment of acute decompensated heart failure	Prof. David Kaye	Phase II Randomized Double blind Placebo-controlled	20	Acute decompensated HF (LVEF <40%) NYHA III/IV, age 18–75	Dietary nitrate, 5 to 6 mmol	Beetroot juice	5 days	<ul style="list-style-type: none"> <li>Change in endothelial function by EndoPAT</li> <li>QoL</li> <li>Treatment success: a composite of patient-reported improvement in dyspnea with no criteria for failure</li> </ul>	<ul style="list-style-type: none"> <li>Systolic and diastolic function by Echo</li> <li>Peak VO2 on CPEX</li> <li>QoL questionnaire</li> <li>Treatment success: a composite of patient-reported improvement in dyspnea with no criteria for failure</li> </ul>
NCT03658174	Upregulating the Nitric Oxide Pathway to Restore Autonomic Phenotype (UNTRAP)	Dr. Zakiryya Vali and Dr. Andre Ng	Double blind Placebo-controlled Crossover (unspecified washout)	20	HFrEF (LVEF <40%) NYHA II/III, age >18 Sinus rhythm with ICD in situ	Dietary nitrate, 5 to 6 mmol	Beetroot juice	4 wk with each intervention (unspecified washout)	<ul style="list-style-type: none"> <li>heart rate variability</li> <li>Q/T variability index</li> <li>Regional Restitution Instability Index (marker of ventricular arrhythmia and SCD)</li> <li>Peak ECG Restitution Slope (marker of ventricular arrhythmia and SCD)</li> </ul>	<ul style="list-style-type: none"> <li>Systolic and diastolic function by Echo</li> <li>Peak VO2 on CPEX</li> <li>QoL questionnaire</li> </ul>

CPEX, cardiopulmonary exercise test; Echo, echocardiography; ICD, implantable cardioverter-defibrillator; LVAD, left ventricular assist device; 6MWT, 6-minute walk test; OD, once daily; PWA, pulse wave analysis; QoL, quality of life; SCD, sudden cardiac death; TDS, three times daily; VO2, maximal oxygen consumption.

mechanisms of this effect are uncertain but may relate to  $\cdot\text{NO}$ -induced suppression of nuclear factor kappa B expression and thus block of transcription of these cytokines (Sugimoto et al., 2012; Jädert et al., 2013). In addition, there has been some attention to the possibility that attenuation of leukocyte recruitment might relate to suppression of local chemokine generation and, again, there is some evidence to support this contention, including reduced circulating neutrophil chemokine levels (CXCL1 and CXCL2) and reduced monocyte chemokine (C-C motif) ligand 2 (CCL2) in ApoE KO mice fed dietary nitrate (Khambata et al., 2017). However, others have found no change in the levels of these cytokines in other inflammatory scenarios, including chlorine toxicity in lungs (Honavar et al., 2014), or in the inflammatory setting of aging. In the latter, the Karolinska group demonstrated no impact upon CCL2 and a number of key inflammatory cytokines after 17 months of treatment with  $\text{NaNO}_3$  (1 mmol/l) in their drinking water (Hezel et al., 2015).

Finally, there is some evidence to suggest that, in addition to suppressing proinflammatory pathways, the nitrate/nitrite anions may also induce anti-inflammatory pathways. Interestingly, levels of mRNA for IL-10 levels, an anti-inflammatory cytokine, were increased within the plaque region in ApoE KO mice fed dietary  $\text{KNO}_3$  compared with KCl-treated controls (Khambata et al., 2017). In addition, in  $\text{TNF}\alpha$ -induced peritonitis, 2 weeks of pretreatment with 15 mmol/l  $\text{KNO}_3$  resulted in elevated IL-10 peritoneal levels at 4 hours (Khambata et al., 2017). However, again, there is data contradicting these observations. In the extended studies of aging and chronic treatment with dietary nitrate for 17 months, circulating IL-10 levels were reduced (Hezel et al., 2015).

In sum, there is strong evidence that inorganic nitrate and nitrite treatment reduces the inflammatory response in a variety of preclinical inflammatory disease models. Whether these effects translate to humans and in the clinical setting has not yet been extensively tested prospectively. In two studies, the effect of dietary nitrate supplementation on proinflammatory monocyte-platelet aggregates has been assessed. In both, an acute supplementation study in healthy older volunteers ( $n = 12$ , 13 mmol nitrate) and our own work in a medium-term study in patients with hypercholesterolemia ( $n = 67$ , 6 mmol nitrate/day for 6 weeks), a reduction in proinflammatory monocyte-platelet aggregates was observed that was not apparent after placebo (Velmurugan et al., 2016; Raubenheimer et al., 2017). Furthermore, acute dietary nitrate supplementation was associated with a modest reduction in the activation state of circulating neutrophils (measured by CD11b expression) 3 hours postsupplementation, a time when nitrite levels peak post-dietary nitrate supplementation (see earlier) (Raubenheimer et al., 2017). Two further studies in healthy volunteers are underway specifically to look

at whether dietary nitrate is able to abrogate both the inflammatory process and the resulting vascular dysfunction associated with this, utilizing two different acute experimental models of inflammation, and the results are eagerly awaited (clinical trials NCT: NCT03183830, NCT02715635).

*1. Thrombosis.* Platelets are critically important in the formation of clinically relevant thrombosis (Davi and Patrono, 2007) and are exquisitely sensitive to local generation of  $\cdot\text{NO}$  (Azuma et al., 1986), so much so that in the early days of  $\cdot\text{NO}$  biology, this response was used as a bioassay for  $\cdot\text{NO}$  production (Radomski et al., 1990).  $\cdot\text{NO}$  has numerous effects on platelet biology, including reduction in adhesion, aggregation, recruitment, and formation of platelet-leukocyte aggregates (Radomski et al., 1987, 1990; Freedman et al., 1997; Chung et al., 2004). Accordingly, a deficiency of  $\cdot\text{NO}$  is associated with a state of platelet activation in relation to the actions above (Cadwgan and Benjamin, 1993; Stagliano et al., 1997; Freedman et al., 1998, 1999; Gkaliagkousi et al., 2009), and organic nitrovasodilators are known to have modest effects on platelet function (Hampton et al., 1967; Schafer et al., 1980).

Platelet inhibition by  $\cdot\text{NO}$  is mediated by GC-1/cGMP/PKG canonical signaling (Massberg et al., 1999), and this PKG activation, in turn, through phosphorylation of specific targets, leads to attenuation of platelet reactivity by triggering downstream inhibition of P-selectin expression (Murohara et al., 1995), phosphorylation of the thromboxane receptor (Wang et al., 1998), and VASP (Massberg et al., 2004), which further inhibits glycoprotein IIb/IIIa. Whatever the specific molecular pathways involved, it is clear that  $\cdot\text{NO}$  influences all of the major components of platelet function, including  $\text{Ca}^{2+}$  mobilization, shape change, secretion, and both integrin activation and outside-in signaling and thus, in this way, plays a key role in ensuring a coordinated regulation of platelet activity (Naseem and Roberts, 2011; Makhoul et al., 2018). However, the exact cellular source and localization of  $\cdot\text{NO}$  generation that reduces platelet activity is more controversial. There is evidence that supports the endothelium (Moore et al., 2010); however, there are also several observations identifying the platelet itself as the site for NOS-derived  $\cdot\text{NO}$  (Radomski et al., 1990), particularly eNOS (Freedman et al., 1999; Webb et al., 2008a; Radziwon-Balicka et al., 2017), although others have failed to localize eNOS within platelets (Gambaryan et al., 2008; Gambaryan and Tsikas, 2015). Irrespective of localization, it is clear that basal  $\cdot\text{NO}$  generation plays a critical role in repressing platelet reactivity under normal conditions. Thus, in scenarios in which there is a deficiency of bioavailable  $\cdot\text{NO}$ , restoration of  $\cdot\text{NO}$  levels, through  $\cdot\text{NO}$  delivery, should recover normal platelet function. Several research groups have assessed whether provision of inorganic nitrate and nitrite might achieve this outcome.

Ex vivo incubation of platelet-rich plasma, collected from healthy volunteers with supraphysiologic nitrite concentrations (60  $\mu\text{mol/l}$   $\text{NaNO}_2$ ) was shown many years ago to reduce aggregatory responses to a wide range of platelet stimuli (ADP, arachidonic acid, and collagen), whereas similar concentrations of  $\text{NaNO}_3$  had no effects (Schafer et al., 1980). This finding has been supported more recently, when physiologically relevant nitrite concentrations (no more than 1  $\mu\text{mol/l}$ ) achieved through in vivo treatment with dietary inorganic nitrate (Webb et al., 2008b) resulted in a suppression of ex vivo–assessed platelet aggregation responses. However, further work has shown convincingly that ex vivo treatment with nitrite salts does not directly alter platelet reactivity at physiologic concentrations. Rather, the evidence suggests that to reveal the antiplatelet effects of nitrite, reactivity must be assessed in the whole blood environment wherein platelets remain in close contact with the circulating elements of the blood.

In platelet-rich plasma, 0.1  $\mu\text{mol/l}$   $\text{NaNO}_2$  inhibited stimulus-induced platelet aggregation only when in the presence of erythrocytes (Srihirun et al., 2012). Similar findings were separately published by Velmurugan et al. (2013), in which  $\text{KNO}_2$  (0.1–0.3  $\mu\text{mol/l}$ ) caused concentration-dependent suppression of platelet aggregation only when incubated with whole blood and not when incubated with platelet-rich plasma alone. Interestingly, the antiplatelet effects of nitrite are enhanced by deoxygenation of erythrocytes and blocked by  $\cdot\text{NO}$  scavenging (Srihirun et al., 2012), as well as being associated with elevations in cGMP (Velmurugan et al., 2013). These findings fit well with the view that nitrite reduction in blood is in part dependent upon the reductase activity of erythrocytic Hb and requires partial deoxygenation (Cosby et al., 2003).

As one would suspect, the elevation of cGMP in the platelet with nitrite treatment results in the antiplatelet effects typical of  $\cdot\text{NO}$ -induced GC-1 activation and is associated with suppression not only of P-selectin expression (Akrawinhawong et al., 2014) but also gpIIb/IIIa expression, both key pathways regulating platelet activity and known to be influenced by  $\cdot\text{NO}$  (Murohara et al., 1995; Keh et al., 1996). Further studies with inhaled  $\text{NaNO}_2$  (40 mg) have also demonstrated increased platelet phospho-VASP, confirming NO/GC-1/cGMP/PKG signaling and diminution of stimulus-induced platelet aggregation (Parakaw et al., 2017). These results together suggest that nitrite reduction does not occur at the level of the platelet itself but rather occurs at the level of the erythrocyte and that the  $\cdot\text{NO}$  generated acts on the neighboring platelet to elevate cGMP, resulting in downstream inhibition of key platelet activating pathways.

Platelets themselves contain nitrite (Apostoli et al., 2014), the levels of which are substantially reduced by treatment with ascorbic acid ex vivo [a nonspecific reducing agent, as per previous evidence shown in

erythrocytes (Sibmooh et al., 2008)], which also simultaneously results in inhibition of platelet aggregation that is associated with elevations in cGMP and phospho-VASP and effects that are enhanced by inhibition of PDE-V and inhibited if treated with ODQ or C-PTIO. However, in this paper, the authors also show nitrate in platelets, the levels of which likewise are decreased with ascorbate. This observation is curious, since ascorbate provides a one-electron reduction; however, for nitrate reduction, two electrons are needed. The authors also show that treatment with  $\text{NaNO}_2$  causes inhibition of platelet aggregation that is also enhanced by treatment with ascorbate or PDE-V inhibitors; and that is prevented by treatment with ODQ or C-PTIO. The authors propose a number of controversial explanations for their observations. Since coincubation with NOS inhibitors had no impact upon any of the responses and since the authors previously have failed to show eNOS expression in platelets, they reason that the source of the widely reported capacity of platelets to generate  $\cdot\text{NO}$  is in fact due to nitrite reduction and not NOS catalytic activity. Moreover, unlike several other publications, since the authors conducted all of their ex vivo experiments in the absence of erythrocytes, they suggest that the platelet itself has the capacity to reduce nitrite directly (Apostoli et al., 2014), although the exact nitrite reductase was not identified.

As mentioned briefly, the beneficial effect of nitrite upon platelet reactivity can be exposed after elevation of circulating nitrite levels through oral administration of inorganic nitrate. Oral supplementation of healthy volunteers with either 2–8 mmol  $\text{KNO}_2$  (Richardson et al., 2002; Velmurugan et al., 2013) or 3.1–22.5 mmol dietary nitrate (192–1395 mg nitrate as beetroot juice) (Webb et al., 2008b; Velmurugan et al., 2013) prior to venepuncture, followed by examination of ex vivo platelet aggregation, results in a modest reduction in the platelet aggregatory response to stimulatory agonists such as ADP and collagen, with associated increases in platelet cGMP and suppression of P-selectin expression (Velmurugan et al., 2013). The modulating effects of dietary nitrate supplementation are additionally seen in healthy older adults after acute nitrate supplementation (13 mmol nitrate,  $n = 12$ ) with reduction in platelet P-selectin expression and clotting time in whole blood, relating to both intrinsic and extrinsic pathways in whole blood but not plasma coagulation tests post-dietary nitrate supplementation (Raubenheimer et al., 2017).

Interruption of the enterosalivary circulation by spitting negated the anti-platelet effects of dietary nitrate, reconfirming the requirement for bioactivation of nitrate to nitrite within the oral cavity (Webb et al., 2008b). Similar effects are seen in mice—whilst treating mice for 1 week with  $\text{NaNO}_2$  (0.1 g/l) or  $\text{NaNO}_3$  (1 g/l) reduced ex vivo platelet aggregation and prolonged bleeding time, when mice were fed with a low- $\text{NO}_x$  diet, the opposite occurred, with enhanced

platelet aggregation and shorter bleeding time (Park et al., 2013). Finally, this beneficial profile of activity of nitrite and nitrate upon platelets evidenced in these acute healthy volunteer and preclinical studies does translate into the patient setting. In patients with endothelial dysfunction and modest hypercholesterolemia, 6 weeks of a once-per-day dietary nitrate supplementation (~6 mmol) was associated with lower basal and stimulus-induced platelet P-selectin expression and leukocyte-platelet aggregates (Velmurugan et al., 2016). Again, these studies show a modest but significant effect. Such a profile of activity, we argue, lends itself as an alternative approach in primary and secondary prevention. Perhaps one of the key difficulties associated with antiplatelet therapy in the secondary prevention setting is the negative side effect profile of bleeding complications. Similarly, it is these very same bleeding complications that have resulted in cessation of the use of aspirin as a primary prevention strategy. We speculate that with such a modest but important antiplatelet profile, dietary nitrate offers a relatively safe and easy-to-administer option.

**2. Peripheral Arterial Disease.** The positive effects of inorganic nitrate/nitrite against inflammation and platelet function, in addition to the benefits in terms of skeletal muscle function, suggest that in diseases with chronic tissue, ischemia such as PAD associated with impaired blood flow, enhanced inflammation, and platelet activation, inorganic nitrate may be of value. In addition, since  $\cdot\text{NO}$  has a positive impact upon angiogenesis (Papapetropoulos et al., 1997; Cooke and Losordo, 2002), effective  $\cdot\text{NO}$  delivery through inorganic nitrate/nitrite might offer a mechanism for therapeutic angiogenesis.

In a mouse hindlimb ischemia model, both chronic nitrite and nitrate therapy increase ischemic tissue blood flow with associated endothelial cell proliferation and angiogenesis (Kumar et al., 2008; Hendgen-Cotta et al., 2012). In addition, evidence in humans suggests that dietary nitrate supplementation is associated with mobilization of circulating angiogenic cells (Heiss et al., 2012), strongly supporting the suggestion that nitrate therapeutics may work in PAD. However, again there is controversy. Major optimism was generated by the first study in patients with PAD demonstrating benefit. After a 3-month supervised exercise program in patients with PAD, significant improvements in exercise capacity were seen and correlated to increases in plasma nitrite concentration (Allen et al., 2010). But more importantly, acute dietary nitrate supplementation (18 mmol nitrate in beetroot) was associated with ~20% improvement in claudication onset time and peak walk time (Kenjale et al., 2011). However, divergent results have been apparent with chronic administration. After 10 weeks of twice-daily supplementation with  $\text{NaNO}_2$  (40 or 80 mg) versus placebo in diabetic

patients with PAD, there was an improvement in endothelial function in these patients with nitrite treatment, but there was no effect on 6-minute walk time (Mohler et al., 2014). Conversely, 8 weeks of  $\text{NaNO}_3$  (8.5 mmol/day nitrate) was associated with a ~40 m improvement in the 6-minute walk test (Bock et al., 2018a), although there was only a trend for similar effect (~30 m) after 12 weeks of 4.2 mmol nitrate with a structured exercise program compared with exercise and placebo (Woessner et al., 2018). However, these effects may have been explained, despite randomization and blinding, by a slightly worse baseline in the nitrate-treated group and thus greater apparent improvement. Further definitive studies are awaited, but at present, whether dietary nitrate might be useful for PAD is uncertain.

**3. Atherosclerosis.** There is a wealth of evidence demonstrating that vascular  $\cdot\text{NO}$  is critical in sustaining vascular patency and that a deficiency in bioavailable  $\cdot\text{NO}$  is a pivotal phenomenon involved in triggering atherogenesis (Böger et al., 1997; Antoniadou et al., 2007). As discussed above, we know that  $\cdot\text{NO}$  reduces leukocyte adhesion and transmigration, reduces low density lipoprotein cholesterol oxidation, and inhibits vascular smooth muscle proliferation [for review, see Moncada and Higgs (1993)]. Moreover, from studies using NOS inhibitors and eNOS KO mice, we know that eNOS is the primary source of the vascular  $\cdot\text{NO}$  that underlies protection against atherogenesis in health (Kausar et al., 2000; Kuhlencordt et al., 2001). Furthermore, the uncoupling of eNOS in the disease setting is thought to be responsible for the reduced bioavailable  $\cdot\text{NO}$  and endothelial dysfunction, which is implicated in the atherosclerotic process (Libby, 2002; Napoli et al., 2006). As a result, numerous studies have sought to restore  $\cdot\text{NO}$  levels utilizing  $\cdot\text{NO}$  donors, particularly the organic nitrates, L-arginine (the substrate for conventional  $\cdot\text{NO}$  synthesis), or the essential cofactor for NOS, tetrahydrobiopterin. Many of these studies have shown positive outcomes, including improvements in vascular function, systemic inflammatory profile, and local inflammation at lesional sites and positive effects upon platelet reactivity (Clarkson et al., 1996; Theilmeyer et al., 1997; Wolf et al., 1997). However, translation of these findings to the clinical setting has not gone as well as hoped (e.g., Nakamura et al., 1999; Blum et al., 2000; Cunnington et al., 2012). The reasons for these discordant findings are variously attributed to eNOS uncoupling for L-arginine (Förstermann and Sessa, 2012), tolerance and induction per se of vascular dysfunction with the organic nitrates (Münzel et al., 2013), and/or oxidation of tetrahydrobiopterin (Cunnington et al., 2012). Although all of these approaches are distinct, they all aim to provide  $\cdot\text{NO}$  delivery to the atherogenic blood vessel. The failure of translation of all of these approaches supports the contention that alternative, but more effective, strategies to improve bioavailable  $\cdot\text{NO}$  levels are warranted. It is this

need that underlies the testing of inorganic nitrate and nitrite in atherosclerosis.

As mentioned earlier in the review, there have been some studies that have begun to assess the potential of nitrate/nitrite as an  $\cdot\text{NO}$  delivery method in atherosclerosis. Inorganic nitrite and nitrate supplementation has been shown to reduce leukocyte-endothelial cell interactions in hypercholesterolemia (Stokes et al., 2009), and a 6-week, once-per-day dietary nitrate treatment in patients with hypercholesterolemia improved endothelial function together with some evidence (although the study was not powered for some of these indices) suggesting that this improved vascular function was associated with a reduced systemic inflammation and reduced platelet reactivity (Velmurugan et al., 2016).

These positive mechanistic findings have been followed up by testing of the impact of inorganic nitrate salt supplementation upon plaque formation and size in mouse models of atherosclerosis. The first study assessing this reported in 2016 and, disappointingly, was negative. In this study, low density lipoprotein cholesterol receptor KO mice were fed a Western diet for 14 weeks and treated concomitantly with  $\text{NaNO}_3$  (1 g/l) or equimolar  $\text{NaCl}$  in drinking water. At the end of this treatment period, there were no differences between the two groups in plaque size, histologic plaque scores, and macrophage, collagen, and smooth muscle content. It is noteworthy, however, that although there have been numerous studies demonstrating efficacy of such a dosing schedule, in this study, nitrate supplementation was not associated with an increase in systemic nitrite levels, and there was no change in BP. These results suggest that, at least in these animals, this dosing regimen was insufficient to evoke modulation of the nitrate-nitrite- $\cdot\text{NO}$  pathway (Marsch et al., 2016). This failure may underlie the absence of any effects seen.

Interestingly, in ApoE KO mice, supplementation with 15 mmol/l  $\text{KNO}_3$  for 12 weeks was associated with increases in systemic and organ nitrite levels in comparison with mice treated with equimolar  $\text{KCl}$ . This rise was associated with reduced leukocyte rolling and adherence, assessed using intravital microscopy of the cremaster microcirculation, reduced circulating neutrophil numbers and monocyte activation, and upregulation of IL-10-dependent anti-inflammatory pathways. This multitude of anti-inflammatory effects resulted in a reduction in the inflammatory load within the plaque, while having no effect upon plaque size (Khambata et al., 2017). Intriguingly, in this study, we showed that tissues of the ApoE KO expressed greater nitrite reductase activity assessed *ex vivo* and that this activity, at least in part, was driven by elevated XOR activity, observations fitting with studies in humans indicating that individuals with coronary artery disease have increased vascular XOR expression and activity (Spiekermann et al., 2003). In a separate study, low to

moderate dosing with  $\text{KNO}_3$  (0.1 and 1 mmol/kg per day) but not high-dose (10 mmol/kg per day) supplementation for 10 weeks in ApoE KO mice was associated with increased collagen expression and reduced lipid deposition and also, similarly, no differences in plaque size (Bakker et al., 2016). These two latter studies support the contention that reduced inflammation within the atherosclerotic plaques coupled with increased smooth muscle and collagen content, although not influencing plaque size, likely provides a more stable plaque phenotype (Stefanadis et al., 2017).

Interestingly, and perhaps contradicting the above work, is evidence suggesting that nitrite has important activity limiting intimal hyperplasia. Rats pretreated with  $\text{NaNO}_2$  (administered intraperitoneally, orally, or nebulized) inhibited pathologic intimal hyperplasia, caused in response to vascular wall injury through insertion of a balloon and inflation in the carotid artery. Importantly, treatment of rats after injury (15 days later) also resulted in a statistically significant reduction in hyperplasia assessed at 28 days. In addition, the authors demonstrated that this beneficial effect of nitrite was attenuated if rats were pretreated allopurinol, implicating XOR as the nitrite reductase in this setting (Alef et al., 2011).

Although there does seem to be some contrast in pathways, the evidence does support the view that the nitrate-nitrite- $\cdot\text{NO}$  pathway may offer opportunities for limiting atherosclerosis and that such an approach may have utility not only in terms of dietary lifestyle interventions that limit disease from occurring in the first place but also as an additional strategy that may prove useful in the secondary prevention setting.

#### *D. Nitrite and Nitrate in the Respiratory System*

In the respiratory system,  $\cdot\text{NO}$  can be produced under physiologic conditions by heterogeneous cell types, including pulmonary vascular endothelial cells, immune cells, respiratory epithelial cells, and intrinsic respiratory nerves (Barnes and Belvisi, 1993). Exhaled  $\cdot\text{NO}$  can be detected in the range of 20–60 ppb, although it may be that the majority of this is derived from the upper respiratory tract and that the distal airways in the lung parenchyma are associated with much lower levels (1–6 ppb) (Dillon et al., 1996). The physiologic role of  $\cdot\text{NO}$  in the respiratory system is complex to understand in any specific area given the many different sources of  $\cdot\text{NO}$ . The activity of  $\cdot\text{NO}$  will naturally depend on other local factors, including levels of oxidant stress (in a high-oxygen environment).

In early studies, nitrovasodilators were shown in isolated airway smooth muscle to activate the canonical GC-1/cGMP pathway to produce muscular relaxation (Gruetter et al., 1989). Furthermore, inhaled  $\cdot\text{NO}$  was found to have small effects on bronchodilation in

patients with asthma and to prevent methacholine-induced bronchoconstriction in a concentration-dependent manner in rodents. (Dupuy et al., 1992; Högman et al., 1993). There is also a contribution to physiologic bronchial tone by inhibitory nitrenergic signaling that counteracts the excitatory cholinergic pathway (Belvisi et al., 1992; Ward et al., 1993). Perhaps therapeutically more important than effects on bronchial tone,  $\cdot\text{NO}$  is a key signaling molecule regulating basal pulmonary vascular tone to counteract pulmonary hypoxic vasoconstriction (Crawley et al., 1990; Persson et al., 1990; Liu et al., 1991). In addition to its actions mediated via elevation of cGMP, there are some  $\cdot\text{NO}$  effects in the respiratory system that are largely independent of this pathway. In particular, high levels of  $\cdot\text{NO}$  that can be generated by iNOS may be antimicrobial and tumoricidal by DNA deamination and inhibiting DNA synthesis (Kwon et al., 1991; Wink et al., 1991). Given the wide-ranging actions of  $\cdot\text{NO}$  in the pulmonary system, it is not surprising that strategies to harness  $\cdot\text{NO}$  activity have been explored in various respiratory-related diseases.

**1. Pulmonary Hypertension.** Pulmonary arterial hypertension (PAH) is a rare condition characterized by increased pulmonary vascular resistance and associated elevated pulmonary arterial pressure (PAP), leading to right HF. Patients with PAH experience significant dyspnea, fatigue, and accelerated mortality. Pathophysiological alterations in PAH include abnormal pulmonary vasoconstriction and endothelial dysfunction, smooth muscle cell proliferation, and adverse remodeling of the pulmonary circulation. It has been known for some time that defects in  $\cdot\text{NO}$ -mediated vasodilatation is one of the hallmarks of disease (Adnot et al., 1991), and this has led to the exploration of drugs that target the  $\cdot\text{NO}$ -GC-1-cGMP pathway for the reduction of PAP.

Inhaled NO (iNO), by virtue of its scavenging in the circulation by Hb, results in selective delivery of  $\cdot\text{NO}$  to the pulmonary circulation without the potential adverse effects of systemic  $\cdot\text{NO}$  delivery, such as systemic arterial hypotension. Inhaled  $\cdot\text{NO}$  was demonstrated to be a selective pulmonary vasodilator more than 30 years ago (Frostell et al., 1991; Pepke-Zaba et al., 1991), but the need for continuous nebulization restricts its utility in ambulant patients with PAH. This limitation has, however, been overcome by taking the strategy of using selective inhibitors of cGMP breakdown by blocking the activity of PDE-V, thus augmenting the pulmonary vasodilatory effects of endogenously produced  $\cdot\text{NO}$ . This approach is associated with clear improvements in patient-related and hemodynamic outcomes (Palmieri et al., 2004; Galiè et al., 2005, 2009; Pepke-Zaba et al., 2008) and is now commonly used clinically for this indication. Since it is widely accepted that there is a reduction in  $\cdot\text{NO}$  bioavailability in PAH (Hu et al., 2010), there has been considerable focus on bypassing this step of the pathway through generation of direct activators/stimulators of GC-1. Riociguat is the first in

class of these drugs to have received widespread marketing authorization for PAH after the positive results of the PATENT studies, demonstrating improvements in the 6-minute walking test (the most commonly used intermediate endpoint) and dyspnea scores in patients with PAH (Ghofrani et al., 2013).

Given the recent evidence of the ability of nitrate-nitrite- $\cdot\text{NO}$  reduction to produce bioactive  $\cdot\text{NO}$  and the fact that this pathway appears to be augmented in hypoxia, this knowledge has spurred interest in whether inorganic nitrite and nitrate could ameliorate pulmonary hypertension. Recent preclinical studies have explored the utility of this pathway in pulmonary hypertension. In ovine models of PAH induced by hypoxia, thromboxane, and hemolysis, both iNO and inhaled  $\text{NaNO}_2$  reduced pulmonary vasoconstriction and ameliorated PAP. An important distinction between the two treatment forms was that nitrite therapy was associated with less systemic hypotensive effects (Hunter et al., 2004; Blood et al., 2011). The effects of inhaled nitrite were kinetically slower than those for iNO and associated with less rebound vasoconstriction on termination of intervention (Hunter et al., 2004). Similarly, in both monocrotaline and hypoxia-induced PAH in rats, repeated inhaled nitrite application prevents but also reverses established effects of PAH on PAP, vascular smooth muscle proliferation, and right ventricular function (Zuckerbraun et al., 2010). Assessment of nitrite reductase *ex vivo* suggested that this effect was largely XOR-mediated, and *in vivo*, the effects of nitrite were abrogated by both XOR inhibition and dietary tungsten replacement of molybdenum in XOR (Zuckerbraun et al., 2010).

The potential beneficial effects of elevation of systemic levels of nitrite have been explored by both injection of inorganic nitrite and through dietary nitrate (via the enterosalivary circulation) and nitrite provision in drinking water. Acute intravenous and chronic intraperitoneal inorganic nitrite application are associated with improvements in PAP and right ventricular hypertrophy in monocrotaline- and hypoxia-induced PAH in rats (Casey et al., 2009; Pankey et al., 2012). However, for effective translation to human therapeutics, a more convenient form of systemic nitrite elevation would be desirable. In mice exposed to 3 weeks of hypoxia to induce typical pathologic changes of PAH, both dietary nitrite (0.6 mmol/l) and nitrate (45 mmol/l) supplementation via drinking water reversed elevation of right ventricular pressure and hypertrophy. Dietary nitrate supplementation was also effective in a pathologically distinct model of PAH induced using bleomycin (Baliga et al., 2012) and monocrotaline (Tawa et al., 2019). Further work utilizing eNOS KO mice and XOR inhibitors suggested that both enzyme systems were important for the beneficial effects of elevated nitrite levels in these models, with evidence also implicating  $\cdot\text{NO}$ -



mediated activation of GC-1-cGMP pathways (Baliga et al., 2012).

Whether local or systemic elevation of nitrite will translate to effective treatments in human disease is currently unknown. Earlier clinical studies in healthy volunteers given i.v. NaNO<sub>2</sub> (1 μmol/min) suggested acute improvements in hypoxia (12%)-induced pulmonary artery pressures (measured by transthoracic echocardiography) (Ingram et al., 2010). Furthermore, a series of dose-ranging phase I pharmacokinetic studies of inhaled NaNO<sub>2</sub> in healthy persons have suggested suitable safety parameters supporting a thrice daily maximal tolerated dose of 90 mg of NaNO<sub>2</sub> (Rix et al., 2015) to allow further exploration of inhaled nitrite in patients with PAH. In patients with PAH due to HFpEF and chronic lung disease, acute inhaled nitrite lowered PAP, pulmonary capillary wedge pressures, and pulmonary vascular resistance (Simon et al., 2016). Recently, a report in five patients with echocardiographic evidence of PAH associated with β-thalassemia has suggested that acute inhaled nitrite rapidly decreases PAP, and this reverts shortly after inhalation terminates (Yingchoncharoen et al., 2018). In a pilot crossover study in 15 patients with PAH, randomized to two 7-day treatment periods (16 mmol/day nitrate vs. placebo), ingestion of a fixed dose of dietary nitrate was associated with increases in exhaled ·NO and plasma and salivary NO<sub>x</sub> and was not associated with any adverse systemic hemodynamic changes. There were no significant changes in pulmonary hemodynamic parameters assessed by echocardiography or cardiopulmonary exercise testing, or of function tests, such as the 6-minute walk test, although there were trends for improvements in right ventricular function and peak power per liter oxygen consumed, particularly in those patients in whom plasma nitrite flux was >30% post-nitrate ingestion (Henrohn et al., 2018). Although these data are encouraging, it remains for larger, adequately powered trials to determine whether inorganic nitrate supplementation has benefit in PAH, with or without other drugs that modulate the ·NO-GC-1-cGMP system.

**2. Chronic Obstructive Pulmonary Disease.** COPD describes a progressive respiratory disease affecting the airways and lung parenchyma, resulting in irreversible airway obstruction. Patients with COPD describe dyspnea and reduced exercise tolerance due to a combination of ventilatory/gas-exchange impairment leading to hypoxia and skeletal muscle deconditioning. Exercise and pharmacological management improve the exercise capacity and physical function of patients with COPD (Barnes et al., 2015).

Given the effects of inorganic nitrate supplementation, via elevation of systemic nitrite levels, on various measures of exercise capacity in healthy persons described above and hypoxia-related augmentation of the nitrite-derived ·NO production, the utility of inorganic nitrate supplementation in COPD has been studied.

Two separate studies have shown that acute supplementation of dietary nitrate (7.6–12.9 mmol nitrate) improves 75% submaximal exercise time by 30 seconds and incremental shuttle walk test by 40 m compared with placebo (Berry et al., 2015; Kerley et al., 2015); in contrast, however, further studies using 13.5 mmol nitrate daily for 2.5 days (Shepherd et al., 2015b) or 20 mmol nitrate for 6 days (Friis et al., 2017) showed no benefit on the O<sub>2</sub> cost of exercise or 6-minute walk test (Shepherd et al., 2015b; Friis et al., 2017). Importantly, in these studies, plasma nitrite levels were increased, and BP was robustly reduced, suggesting that both the chemical and functional effects of the nitrate-nitrite-·NO pathway are present in patients with COPD. The difference between the responses in the two sets of studies is uncertain but may relate to differences in dosing schedules and warrants further investigation. Further evaluation of dietary nitrate supplementation (6.5 mmol/day nitrate for 8 days) in patients with COPD showed no improvement in gas diffusion or peak exercise capacity, despite robust increases in exhaled ·NO, but was associated with statistically significant (but small in magnitude) improvements in respiratory-focused quality of life (St. George's Respiratory Questionnaire) scores (Behnia et al., 2018). Similar to the position with PAH, large adequately powered studies are awaited to definitively explore the therapeutic potential of inorganic nitrate in COPD.

**3. Sleep-Disordered Breathing.** Obstructive sleep apnea syndrome (OSA) is the most common form of sleep-disordered breathing problem and results from recurrent occlusion of the upper airway during sleep. Numerous epidemiologic studies have implicated OSA as a causative, independent factor for CVD (Arzt et al., 2005; Yaggi et al., 2005; Somers et al., 2008). The pathophysiological links between OSA and CVD are not fully understood, but several mechanisms, such as elevated sympathetic drive, increased oxidative stress, and vascular inflammation resulting from repeated nocturnal hypoxia/reoxygenation cycles, have been suggested (Somers et al., 2008). Importantly, ·NO levels are diminished in OSA and are partially restored with the most common, effective form of therapy, continuous positive airway pressure (Ip et al., 2000; Schulz et al., 2000; Alonso-Fernández et al., 2009), suggesting that ·NO delivery in this setting may be of value.

The only study exploring the nitrate-nitrite-·NO pathway in patients with OSA is restricted to a small ( $n = 3$ ) 14-day, controlled crossover study. Nitrate supplementation (12.9 mmol nightly) was associated with increased plasma nitrate levels (nitrite was not measured) and with improvement in fatigue and visual attention scores, although no change in sleep quality or Epworth sleepiness scores, and changes in apnea/hypopnea index were not assessed. All patients had controlled BP levels on ambulatory monitoring at index, but there was a trend toward reduced nocturnal

BP after nitrate supplementation (Kerley et al., 2016), providing confidence in the dosing schedule efficacy in this study.

Sleep-disordered breathing is also an occurrence in the acclimatization to hypobaric hypoxia at altitude. Given the reductions in O<sub>2</sub> consumption associated with nitrate supplementation seen at sea level (see earlier), Patrician et al. (2018) investigated the acute effects of acute nocturnal dietary nitrate supplementation (10 mmol/day nitrate) in lowlanders after ascent to 3700–4900 m, in comparison with a peri-sea level (300 m) on cardiorespiratory responses during sleep. Acute dietary nitrate supplementation had no effect on various measures of sleep-disordered breathing. Similar longer-term studies in lowland volunteers ascending to high altitude (4600 m) demonstrated that 7 days of dietary nitrate supplementation (3.7 mmol nitrate three times daily) had no effect on respiratory parameters, such as O<sub>2</sub> saturation, or acute mountain sickness, despite robust increase in fractional exhaled ·NO (Cumpstej et al., 2017).

Further studies are required to delineate the potential benefit of nitrite-derived ·NO in patients with sleep-disordered breathing, not only on the respiratory abnormalities but also on the elevated CV risk profile that such patients exhibit.

**4. Toxic Lung Injury.** Chlorine gas has been implicated in mass casualties due to accidental or deliberate exposure in civilian and military settings. The pathophysiology of chlorine gas-related lung injury includes pulmonary and systemic increased oxidative and nitrosative stress and hypoxemia, with persistence of airway remodeling and airway hyperreactivity over time (White and Martin, 2010). Since nitrite-derived ·NO has beneficial effects in I/R lung injury (Sugimoto et al., 2012; Okamoto et al., 2013), this has spurred interest as to whether it may be beneficial in other forms of lung injury. Rats exposed to 400 ppm chlorine gas for 30 minutes were given NaNO<sub>2</sub> (1 mg/g, i.p.) repeatedly for up to 6 hours and then euthanized. Treatment with nitrite was associated with reduced bronchoalveolar lavage protein levels and reduced epithelial cell apoptosis (Yadav et al., 2011), with some evidence that intramuscular delivery was more effective than intraperitoneal delivery (Samal et al., 2012), which is more translatable to the management of mass casualty disaster scenarios by first responders. In an adaptation of this methodology, in rats exposed to a higher exposure of chlorine (600 ppm for 30 minutes) that induces 80% fatality, a single intramuscular injection of nitrite (10 mg/kg) 30–60 minutes postexposure reduced mortality to 50%.

These findings have been repeated in a nonrodent model using New Zealand white rabbits. Chlorine gas (600 ppm, 45 minutes) is 35% lethal after 18 hours and is associated with airway protein and neutrophil accumulation. Nitrite (1 or 10 mg/kg, via i.m. injection)

administered 30 minutes post-chlorine gas exposure prevented death in all rabbits, and this was associated with attenuated indices of acute lung injury (Honavar et al., 2017). Further mechanistic evaluation revealed that neutrophil depletion reduced the lethality of chlorine exposure but also abrogated any further improvement by nitrite, suggesting a neutrophil-dependent mechanism for nitrite-derived protection in this model (Honavar et al., 2014).

Taken together, these data provide further rationale for developing nitrite as a postexposure treatment to ameliorate chlorine gas exposure-related lung injury, although the pathway for further development of this approach is awaited given the complexities of trying to perform a clinical trial in this area.

### *E. Nitrite and Nitrate in the GI System*

In normal GI tract physiology, ·NO modulates smooth muscle tone, thereby regulating intestinal peristalsis and gastric emptying, and maintains GI mucosa through regulation of acid and gastric mucus secretion and maintenance of mucosal blood flow (Salzman, 1995). Additionally, ·NO has roles in controlling pathogen growth (Fang, 1997), and from the first demonstrations of nitrite-derived ·NO production, the antimicrobial effects of this pathway particularly relevant to the gut were explored. Nitrite-derived ·NO controls both pathogenic fungal and bacterial growth in culture (Benjamin et al., 1994; Dykhuizen et al., 1996, 1998) through bactericidal activity (Björne et al., 2006).

In addition to antimicrobial activity, intragastric ·NO production, through acidification of nitrite, has local gastroprotective effects, increasing gastric mucosal blood flow and mucus thickness (Björne et al., 2004; Petersson et al., 2007). This pathway is thought to underlie ·NO-mediated reductions of gut injury from both NSAID and stress-induced ulcer formation (Miyoshi et al., 2003; Jansson et al., 2007). In inflammatory bowel disease, a common finding is disruption of the protective mucosal barrier and diminished mucus layer. In an experimental dextran sodium sulfate model of colitis, both pretreatment and post-treatment with inorganic nitrite (1 mmol/l in drinking water for 7 days) improved mucus thickness, disease activity score, and histologic scores and restored goblet cell abundance (Jädert et al., 2013). Further studies in cultured human colonic epithelial cells demonstrate that nitrite application (100 μM) improved wound closure after an incision into the cultured monolayer (Jädert et al., 2013), demonstrating that in addition to improvements in blood flow, nitrite treatment accelerates healing of wounds.

More recently, pretreatment with intravenous nitrite (48 nmol) before intestinal manipulation that induces a model of postoperative ileus showed an improvement in intestinal transit and restored intestinal contractility within 24 hour (Cosyns et al., 2015). This effect was

attributed to reductions in inflammatory cytokines and ROS and was dependent also upon elevations of cGMP (Cosyns et al., 2015). This approach is being assessed in a randomized clinical trial of pretreatment of 1 week with dietary nitrate prior to planned colorectal surgery ( $n = 30$ , NCT03772444). In contrast, a recent report using an acute supraphysiological dose of  $\text{NaNO}_2$  (20, 40, 60, and 75 mg/kg b.wt.) in rats demonstrated dose-dependent increase in abnormal structure and significant morphologic damage in the intestine in animals examined 24 hours postdose (Ansari et al., 2017), although the relevance of these acute doses to human gut physiology is unclear, and previous reports in rats using similar doses have not shown the same effect (Roediger et al., 1986).

Whether nitrite- or nitrate-based therapeutic interventions are pursued in additional clinical studies for GI disorders is not clear at the present time, and manual search of publicly available clinical trial databases did not discover additional planned studies at this time.

#### *F. Nitrite and Nitrate in Metabolic Diseases*

With increasing prevalence worldwide of obesity (Hruby and Hu, 2015), there has been a dramatic increase in the numbers of patients suffering from insulin resistance syndromes such as type II diabetes mellitus (Kharroubi and Darwish, 2015) and the related metabolic syndrome (Sorrentino, 2005), a clustering of cardiometabolic risk factors that are associated with increased risk of diabetes and CVD (Eckel et al., 2005). eNOS deficiency in animal models (Duplain et al., 2001; Cook et al., 2003) or long-term treatment with eNOS inhibitors leads to features of metabolic syndrome and diabetes, such as weight gain, insulin resistance, and dyslipidemia (Balon et al., 1999), a profile that is replicated in humans with eNOS polymorphisms (Monti et al., 2003; Fernandez et al., 2004). These data suggest that manipulating  $\cdot\text{NO}$  levels may have therapeutic utility in diabetes and metabolic disorders. Importantly, in C57BL6 mice on long-term (up to 18 months) dietary restriction of nitrite and nitrate, these abnormalities (increased visceral adiposity, dyslipidemia, glucose intolerance, insulin resistance) are replicated (Kina-Tanada et al., 2017). These results suggest not only that reduction in NO signaling is important irrespective of the source but also that augmenting bioactive NO through the nitrate-nitrite-NO pathway may be a useful therapeutic strategy.

Furthermore, intriguing epidemiologic data could also support this view: green leafy vegetable consumption (rich in dietary nitrate) is associated with reduced risk of developing type 2 diabetes (Carter et al., 2010), and habitual use of mouthwash [potentially interfering with enterosalivary conversion of nitrate to nitrite (c.f. effects on BP (Kapil et al., 2013))] is associated

with progression of dysglycemia in overweight adults (Joshiyura et al., 2017).

Several preclinical studies have explored elevating systemic nitrite levels in models of diabetes or metabolic syndrome, using both nitrite and nitrate. In adenosine  $\text{A}_{2\text{B}}$ -deficient mice (a model of metabolic syndrome on chow diet), acute nitrate loading (0.1 mmol/kg i.p.) was associated with improvement in insulin resistance (homeostatic model assessment of insulin resistance) and glucose clearance (Peleli et al., 2015). These acute effects are also established in longer-term experiments. In another model of metabolic syndrome (eNOS deficiency), mice supplemented for 8–10 weeks with  $\text{NaNO}_3$  at a dose expected to recapitulate normal endogenous nitrate production from oxidative  $\cdot\text{NO}$  metabolism (1 mmol/l in drinking water) had reductions in visceral fat and weight and improvements in glucose homeostasis (Carlström et al., 2010). Similar results have been seen with nitrite supplementation in genetic models of diabetes. Nitrite supplementation of drinking water (50 mg/l nitrite) to db/db mice, KKA(y) mice, and obese ZSF mice (Jiang et al., 2014; Ohtake et al., 2015; Lai et al., 2016) or 100 mg/kg nitrite via miniosmotic pump in  $\text{ob}^{\text{lep}}$  mice (Singamsetty et al., 2015) was associated with improvements in glucose tolerance and insulin sensitivity across all models.

Similar results have been seen for drug-induced models of diabetes using streptozotocin using nitrate supplementation (100 mg/l) for up to 2 months, resulting in improved glucose tolerance and insulin sensitivity (Khalifi et al., 2015; Gheibi et al., 2017). Lastly, in dietary models of metabolic syndrome using high-fructose diets (with or without high-fat diets), both dietary nitrate (by gavage, 60 mg/kg) (Li et al., 2016b) or nitrate salts (by gavage, 150 mg/kg) (Essawy et al., 2014) were similarly associated with improvements in glucose tolerance and insulin sensitivity.

The mechanisms of such benefits via elevation of nitrite may be linked to: increased glucose transporter type 4 translocation in skeletal muscle (Jiang et al., 2014; Ohtake et al., 2015); by nitrite-related sirtuin 3-AMP-activated protein kinase (AMPK) activation, which shares a common effect with metformin (Lai et al., 2016); from improved pancreatic blood flow and  $\beta$ -cell stimulation for insulin release (Nyström et al., 2012; Gheibi et al., 2017); or from browning of white adipose tissue (Roberts et al., 2015) that may have antimetabolic effects.

Taken together, these results are similar across genetic, dietary, and drug-induced models of dysglycemia—nitrite or nitrate supplementation is associated with improvements in glucose tolerance and insulin sensitivity and suggest an exciting possibility that nitrite- or nitrate-based therapeutics could be added to the armamentarium for type 2 diabetes and/or metabolic syndrome. Unfortunately, there has been scant translation of these effects in human studies to date. In patients with type 2 diabetes given 7.5 mmol of dietary nitrate daily for

2 weeks, despite significant elevations in systemic nitrite levels, there was no change in insulin sensitivity using the hyperinsulinemic euglycemic clamp method or improvements in BP or FMD (Gilchrist et al., 2013) that have been seen in numerous other patient groups (Rammos et al., 2014; Kapil et al., 2015; Velmurugan et al., 2016). Furthermore, in healthy persons, acute dietary nitrate supplementation with 11.9 mmol nitrate did not change plasma glucose, C-peptide levels, or incretin levels and did not change hepatic blood flow, measured using magnetic resonance imaging (MRI) (Shepherd et al., 2016). The explanations for the lack of effect in type II diabetes is puzzling, but it might be that sirtuin 3-AMPK activation by metformin renders further nitrite stimulation of this pathway (Lai et al., 2016) redundant or that glycated Hb has altered nitrite reductase activity (James et al., 2004), meaning that despite elevated nitrite levels, bioactive  $\cdot\text{NO}$  is not produced. There is also growing evidence that the biologic activity of metformin is dependent upon the metabolism of the microbiome. Although the focus of studies has revolved around the gut, it is possible that the bioactivation of nitrate is impaired by the impact of metformin on the oral microbiome community (Cabreiro et al., 2013; Forslund et al., 2015; Sun et al., 2018).

An additional avenue of therapeutic utility in this area may be in the context of the complications of hyperglycemia, namely painful diabetic peripheral neuropathy. The first study to suggest possible benefit was conducted in patients with PAD ( $n = 55$  total,  $\sim 2/3$  type 2 diabetes) randomized to 40 or 80 mg twice daily of sodium nitrite (or matching placebo) for 10 weeks followed by a further week of double-dosing—at the end of the study, there was a significant improvement in the pain domain of the physical component of the RAND 36 quality of life questionnaire despite no overall improvement in quality of life, although this was only apparent for the lower dose studied (40 mg twice daily of nitrite) (Mohler et al., 2014). This paper was recently followed by a pilot study using a novel sustained-release formulation of sodium nitrite in patients with a diagnosis of diabetic peripheral neuropathy of  $>3$  month's duration and significant pain using a numeric pain scale ( $n = 24$  total). Patients were similar, randomized to either 40 mg twice daily, 80 mg twice daily, or matched placebo. In this study, there were three questionnaire-based assessments of pain as an unpowered secondary endpoint, with a suggestion of improvement with nitrite dosing for one of three pain scores used, although the study was underpowered to produce meaningful effect sizes or statistical confidence (Soin et al., 2018). Further studies are to be planned with larger  $n$  numbers.

### G. Nitrite and Nitrate in I/R Injury

**1. Cytoprotection.** There is a significant body of evidence demonstrating that nitrite operates as a major storage form of  $\cdot\text{NO}$  in blood and tissues (Bryan et al.,

2005) that can be reduced readily to  $\cdot\text{NO}$  to initiate cytoprotective signaling during pathologic states, particularly during I/R injury (van Faassen et al., 2009; Calvert and Lefer, 2010). The mechanisms by which this process occurs have been extensively studied, with increasing evidence suggestive of changes in mitochondrial function and anti-inflammatory, antiplatelet, and vasodilatory effects.

**2. Potential Mechanisms for Nitrite-Induced Reduction in I/R Injury.** A number of distinct molecular pathways have been proposed to underlie the reduction of nitrite in the setting of I/R injury. There is strong evidence demonstrating that the benefits of nitrite in I/R injury are due, at least in part, to its conversion to  $\cdot\text{NO}$ . Several studies have shown that nitrite-induced protection is blocked by the  $\cdot\text{NO}$  scavenger C-PTIO (Webb et al., 2004; Jung et al., 2006; Tripatara et al., 2007). This effect is also independent of NOS activity, as cytoprotection induced by nitrite is not prevented by NOS inhibition (Webb et al., 2004; Lu et al., 2005) or affected in eNOS KO models (Duranski et al., 2005; Milsom et al., 2010). We now also understand that nitrite reduction during I/R is not just a phenomenon precipitated by acidic disproportionation, as suggested by Zweier et al. (1995), who identified nitrite-derived  $\cdot\text{NO}$  generation in the ischemic heart in their seminal findings in 1995. Over the past decade, a number of different nitrite reductases have been implicated in nitrite-derived  $\cdot\text{NO}$  formation during I/R injury, with the role of each catalytic pathway identified being influenced both by the site/organ of injury and the severity of hypoxia. Much of the research focused on identifying the key nitrite reductase involved in the I/R injury setting has been conducted within the heart, where it appears that the two key nitrite reductases are XOR and Mb.

XOR has been implicated in the reduction of nitrite to  $\cdot\text{NO}$  in the myocardium in both animal and human heart tissue, with the first studies being conducted in the isolated rat heart and atrial appendage collected from patients undergoing cardiac surgery, respectively. In tissue supernatants, nitrite-derived  $\cdot\text{NO}$  generation was profoundly attenuated (50%) after incubation with the XOR inhibitor allopurinol (Webb et al., 2004), observations that have been replicated by others (Baker et al., 2007; Li et al., 2008). Similarly, XOR has been implicated as the nitrite reductase in other organs, including when delivered topically in renal I/R injury in vivo in both rat and mouse models (Tripatara et al., 2007; Milsom et al., 2010). XOR also contributes to nitrite bioactivation in other hypoxic settings, including pulmonary hypertension (Baliga et al., 2012) and vascular remodeling after balloon injury (Alef et al., 2011), thus identifying a widespread function of XOR as a key nitrite reductase in hypoxic environments. This profile of activity is not unexpected, since studies with purified bovine enzyme demonstrate increasing XOR-dependent nitrite

reduction with reducing O<sub>2</sub> tension (Li et al., 2001). This bioactivity, as mentioned earlier, occurs at the molybdenum site of the enzyme and is a reaction facilitated by at least three different reducing substrates, including xanthine, NADH, and aldehydes (Khambata et al., 2015). Perhaps of particular note in this respect is NADH, the levels of which rise in ischemic environments (Sahlin, 1983), possibly suggesting why XOR-dependent nitrite reduction predominates in such reduced O<sub>2</sub> tension environments and plays a much lesser role under physiologic conditions (Dejam et al., 2007).

Mb represents the other key nitrite reductase implicated in the bioactivation of nitrite during I/R injury and, again, particularly in the heart (Rassaf et al., 2007; Shiva et al., 2007a). Mb must be partially deoxygenated to act as a nitrite reductase, only occurring when O<sub>2</sub> levels fall below the *p*<sub>50</sub> of Mb, as for Hb (Cosby et al., 2003; Crawford et al., 2006). Studies in the heart suggest that 60%–70% of nitrite-derived ·NO is due to deoxyMb-dependent conversion, with the balance due to XOR activity (Rassaf et al., 2007; Shiva et al., 2007a). Accordingly, nitrite reduction was abolished in the Mb KO mouse (Hendgen-Cotta et al., 2008), and the beneficial effects of nitrite after coronary artery occlusion were lost, including improved postischemic LV developed pressure. These studies suggest that Mb plays an important role in nitrite-induced regulation of cardiac energetics and oxygen utilization under conditions of hypoxia. To date, whether Mb is also critical in nitrite reduction in the human heart has not been elucidated.

**3. Potential Mechanisms for Nitrite-Induced Cytoprotection in I/R Injury.** The activity of nitrite in I/R injury has been attributed to both direct and ·NO-dependent actions as a consequence of chemical modifications of both cellular proteins or lipids, mitigating the injury occurring at reperfusion. Potential molecular mechanisms for cytoprotection of the former include the direct *S*-nitrosation of critical regulatory thiols on proteins such as caspase-3, a pivotal protein of the apoptotic pathway, leading to its inactivation and preventing apoptotic cell death (Maejima et al., 2005), or the L-type calcium channel, which results in reduced cytosolic Ca<sup>2+</sup> and subsequent I/R injury (Sun et al., 2006). There are several functional consequences of these chemical interactions that underlie the benefits of nitrite, with the key ones summarized below.

**a. Improved mitochondrial function.** ·NO, through the activation of GC-1 and ultimately the production of cGMP, is known to be a key regulator of mitochondrial function as well as a stimulator of mitochondrial biogenesis (Clementi and Nisoli, 2005; Nisoli et al., 2005). It is thus unsurprising that the mitochondrion has emerged as a major subcellular target of nitrite, with evidence demonstrating the specific targeting of key proteins within the mitochondria organelle [for review, see Murillo et al. (2011)]. Studies have convincingly

shown that nitrite directly inhibits complex 1 of the electron transport chain, causing a consequent decrease in ROS generation, a decrease in Ca<sup>2+</sup> influx, and a reduction in cytochrome c release (Shiva et al., 2007b) after I/R injury in both the heart and liver (Gladwin et al., 2005). This inhibition of complex I may also in turn prevent opening of the mitochondrial permeability transition pore (Fontaine et al., 1998; Nadtochiy et al., 2007), leading to an improved mitochondrial tolerance to Ca<sup>2+</sup> overload (Nadtochiy et al., 2007). Nitrite-induced elevations of ·NO activating cGMP, in turn, also activate PKG, resulting in stabilization of mitochondrial big conductance K (Frankenreiter et al., 2017) and K<sub>ATP</sub> channels (Sasaki et al., 2000), which is associated with decreased mitochondrial Ca<sup>2+</sup> accumulation, and prevention of mitochondrial permeability transition pore opening (Takuma et al., 2001; Brown and Borutaite, 2002), properties that are likewise exhibited by ·NO (Brookes et al., 2000; Heusch et al., 2008). A significant component of the action of nitrite has also been attributed to the reversible inhibition of cytochrome c oxidase (complex IV of the electron transport chain). ·NO binds to this enzyme, in competition with O<sub>2</sub>, resulting in a reversible inhibition of O<sub>2</sub> consumption (Poderoso et al., 1998). This effect in turn has consequences for the preservation of high-energy phosphate stores during I/R injury, and this effect in particular may play a role in the phenomenon in the heart of “hibernation,” in which the metabolic activity of the heart is temporarily shut down during ischemia to preserve high-energy phosphate reserves (Hendgen-Cotta et al., 2008; Murillo et al., 2011).

As mentioned, there is also some suggestion that the effects of nitrite are in part mediated by direct actions of the anion and not ·NO (Mo et al., 2012; Kamga Pride et al., 2014). Mo and colleagues, using a rat model of carotid injury, showed that nitrite treatment reduced the hyperproliferative smooth muscle cell response to mechanical injury. The authors suggested that this action was due to upregulation of peroxisome proliferator-activated receptor-γ coactivator-1 (the master regulator of mitochondrial biogenesis) and, thus, accordingly, an increase in the number of mitochondria in the injured artery. In cultured rat aortic smooth muscle cells, the authors showed that the nitrite-induced increase in mitochondrial number was unlikely to account for the beneficial effect profile, despite small increases in cGMP. The authors further demonstrated that nitrite augmented adenylate kinase activity, leading to phosphorylation of AMPK, the consequent downstream activation of sirtuin-1 and deacetylation of peroxisome proliferator-activated receptor-γ coactivator-1. More importantly, all of these changes occurred independently of cGMP (Mo et al., 2012). Kamga Pride et al. (2014) showed in cultured rat H9c2 cardiomyocytes subjected to hypoxia and reoxygenation that treatment with nitrite for only 30 minutes under normoxic conditions substantially reduced the cell

death after an ensuing hypoxic episode initiated 1 hour after the treatment. Since the treatment was conducted in normoxia, the authors suggested that conversion of nitrite to  $\cdot\text{NO}$  was unlikely. Using purportedly selective inhibitors, the authors provided evidence of protein kinase A-mediated phosphorylation, and thus inhibition, of dynamin-related protein 1, leading to enhanced mitochondrial fusion. Functionally, this augmented mitochondrial superoxide production, which oxidized and activated AMPK, and is an essential step in nitrite-mediated preconditioning.

*b. Antiplatelet effects.* The activation and consequent adhesion and aggregation of platelets is a critical event in the lead up to an acute MI (AMI) and other ischemic injury (Didisheim et al., 1974; Badimon et al., 2002; Gawaz, 2004; Vorchheimer and Becker, 2006). It is widely accepted that platelet activation is key in the formation of thrombosis that culminates in stroke, AMI, and other vascular events (Davì and Patrono, 2007). As well as the obvious physical implications of thrombus formation after an event, activated platelets release several mediators that participate in the damage caused by the second wave of injury, occurring as a consequence of reperfusion, but also to the long-term consequence of I/R injury contributing to the increased risk for secondary events in such patients (Badimon et al., 2002). It is therefore unsurprising that strategies repressing platelet reactivity play a key role in the treatment of organ ischemia and I/R injury as well as reducing risk for further events. It is accepted that  $\cdot\text{NO}$ , in health, acts as an important repressor of platelet activation and adhesion to intact vascular endothelium (Radomski et al., 1987; Dangel et al., 2010) as well as platelet aggregation itself (Radomski et al., 1990), as discussed earlier, and thus, there has been considerable interest in identifying  $\cdot\text{NO}$ -based strategies that might be useful in I/R injury. Since there is substantial evidence that nitrite in vivo modulates platelet function, it is possible that some of the benefits in the I/R setting are derived through actions on platelets. In particular, in our own trial, NITRITE-AMI, in which patients received an intracoronary dose of nitrite at the point of reperfusion after primary percutaneous coronary intervention (PCI), we demonstrated reductions in ex vivo assessments of platelet reactivity (aggregation and cell activation markers) acutely but also, interestingly, up to 6 months after the intervention. We speculated that the acute reduction of platelet reactivity likely contributed to the reduced microvascular obstruction that is thought to be pathogenic and a primary mechanism in worsening cardiac cell death postreperfusion, which has recently been highlighted as an important target for developing novel cardioprotection therapies in the AMI setting (Nazir et al., 2016; Niccoli et al., 2019).

*c. Anti-inflammatory effects.* A key phenomenon contributing to damage in I/R injury is inflammation, and since several observations recently have identified

anti-inflammatory properties of nitrite, it has been suggested that this effect may be contributing to the beneficial actions of nitrite in the I/R setting. The recruitment of leukocytes and their consequent proinflammatory effects plays a critical role in determining the magnitude of damage induced post I/R injury (Entman and Smith, 1994). Polymorphonuclear cells are the major leukocytes found in necrotic tissue immediately after ischemic injury, with neutrophils the early cellular mediators of local microvascular changes and parenchymal damage (Vinten-Johansen, 2004). Monocytes and macrophages infiltrate later and extend the early injury phase (Ysebaert et al., 2000). In addition, lymphocytes have been identified as contributing to the damage in the later stages in the response to I/R injury in a variety of organ systems (Linfert et al., 2009). Lymphocyte-related cytokines are upregulated in the postischemic heart (Squadrito et al., 1999), and leukocyte adhesion molecules and chemokines, such as P-selectin glycoprotein ligand-1, CD11/CD18, ICAM-1, CCL2 (Herskowitz et al., 1995; Birdsall et al., 1997), and lymphocyte adhesion, migration, and signaling (Loetscher et al., 1996) have been implicated in experimental myocardial I/R injury.

As mentioned,  $\cdot\text{NO}$  exerts an array of anti-inflammatory effects, and many of these have been demonstrated in experimental models of I/R injury in various vascular beds (Kubes et al., 1991; Palazzo et al., 1998; Ahluwalia et al., 2004). Leukocyte recruitment is a multistep process constituting leukocyte tethering, rolling, adhesion, and ultimately emigration from the microvasculature (Petri et al., 2008), and  $\cdot\text{NO}$  has been shown to influence each of these steps through the damping/repression of interactions of the leukocyte with endothelial cells (Kubes et al., 1991). Specifically, there is evidence showing that  $\cdot\text{NO}$  inhibits adhesion molecule expression and activation, including that of P-selectin, ICAM-1, vascular cell adhesion molecule-1, and CD11b (Gauthier et al., 1994; Lefer and Lefer, 1996; Leite et al., 2009). Accordingly, data in humans have emerged from a phase 2 study suggesting that nitrite, and presumably nitrite-derived  $\cdot\text{NO}$ , exerts anti-inflammatory effects when given during primary PCI for AMI (Jones et al., 2015a). In these patients, we demonstrated important reductions in neutrophil numbers and activation post-primary PCI (Jones et al., 2017). In the placebo-treated patients, we observed increases in the total circulating neutrophil numbers and levels of high-sensitivity C-reactive protein postreperfusion, which then decreased over time. In contrast, in nitrite-treated patients, these changes were suppressed up to 6 months post-primary PCI ( $P < 0.01$ ). These differences were also associated with reduced expression of neutrophil CD11b, plasma CXCL1, CXCL5, and CCL2 levels ( $P < 0.05$ ). Importantly, there were no differences in the number of any other leukocyte population measured (monocytes and lymphocytes) or activation markers expressed by these cells between the

treatment groups. In addition, these effects on inflammatory markers were associated with a reduction in both microvascular obstruction and infarct size, suggesting important effects of nitrite on neutrophil activation and recruitment (Jones et al., 2017). Further study is needed to examine these effects.

**4. Nitrite as a Mediator of Remote Ischemic Preconditioning.** In the early 1990s, Przyklenk et al. (1993) proposed the existence of signal transduction between the local site of remote ischemia and the myocardium. This link has been identified as underlying the efficacy of remote ischemic preconditioning (rIPC), although the exact nature of this endocrine communication has remained uncertain. In 2014, Rassaf and coworkers conducted a series of experiments suggesting that nitrite may be the elusive endocrine factor responsible for the effects of rIPC (Rassaf et al., 2014). Their studies demonstrated that plasma nitrite concentration increases during reactive hyperemia in the brachial artery of healthy volunteers as a consequence of shear-stress triggering eNOS activation (Rassaf et al., 2006). In mouse models of rIPC, the authors demonstrated a similar elevation in circulating nitrite levels and that the benefits of rIPC were blocked with C-PTIO and absent in eNOS KO mice. In the final experiment, the authors infused human plasma collected from individuals subjected to, or not, rIPC into isolated mouse heart Langendorff preparations. In these experiments, they demonstrated that nitrite accounted for the effects of rIPC, since treatment of the transferred plasma with acidified sulfanilamide eliminated the transfer of protection (Rassaf et al., 2014). Prior to this work, Elrod et al. (2008), who used a transgenic mouse with cardiac restricted overexpression of eNOS, also provided evidence in support of an endocrine action of nitrite. These mice are characterized by increased levels of nitrite and other ·NO metabolites in the circulation, resulting in subsequent transport and storage in organs throughout the body, including the liver. Elrod et al. (2008) demonstrated that this elevation of tissue nitrite provided protection against the damaging effects of a consequent hepatic I/R injury.

An important caveat of the above findings is that the levels of nitrite generated in these models are approximately 10-fold lower than those typically shown to be cytoprotective in preclinical models of I/R injury (Webb et al., 2004; Duranski et al., 2005). This discrepancy in efficacy implies potential differences in bioactivity/potency of endogenously derived versus exogenously delivered nitrite and is an issue that is worthy of further interrogation.

#### **5. Translational Aspects.**

**a. I/R injury.** The cytoprotective actions of nitrite have been reproduced in a variety of animal models by different investigators focusing on different target organs (Webb et al., 2004; Duranski et al., 2005; Pluta et al., 2005; Jung et al., 2006; Tripatara et al., 2007).

Our group provided the first evidence of the cytoprotective effects of nitrite (Webb et al., 2004). In this study, we demonstrated that administration of NaNO<sub>2</sub> (10 or 100 μmol/l) either prior to or at reperfusion in the isolated rat Langendorff heart preparation improved both LV function and coronary perfusion pressure as well as decreasing infarct size after an I/R insult. Many others have confirmed these effects in the heart using not only in vitro (Baker et al., 2007; Hendgen-Cotta et al., 2008) but also in vivo preclinical rodent models of AMI (Duranski et al., 2005; Bryan et al., 2007). Duranski et al. (2005) administered sodium nitrite via an intraventricular injection (2.4–1920 nmol) 5 minutes prior to reperfusion after 30 minutes of occlusion of the left main coronary artery occlusion in mice. This group observed a dose-dependent decrease in infarct size. The maximum benefit was provided with a dose of 48 nmol, although the higher dose of 1920 nmol nitrite failed to exert any significant cardioprotective effect. Bryan et al. (2007) used the same model and demonstrated that dietary nitrite supplementation (50 mg/l) for 7 days prior to ischemia caused a 48% reduction in infarct size.

In addition to rodents, the functional benefits of nitrite have been reproduced in larger species. In a canine model induced by ligation of the left anterior descending coronary artery in vivo, intravenous administration of NaNO<sub>2</sub> (0.2 μmol/min per kilogram for the first 20 minutes and then 0.17 μmol/min per kilogram for 40 minutes) for the last hour of a 2-hour period of ischemia reduced infarct size by ~50% compared with control (Gonzalez et al., 2008). The authors also considered intravenous administration of nitrite during the last 5 minutes (0.2 μmol/min per kilogram) of the 2-hour occlusion in a bid to identify an approach with greater chance of clinical translation and showed that, with this treatment, infarct size and apoptosis were reduced to a similar level as with treatment of 60 minutes prior to reperfusion. There is, however, some evidence contradicting the above findings. In the multicenter preclinical CAESAR cardioprotection initiative (Jones et al., 2015), a lack of efficacy of nitrite was observed (Lefer et al., 2014). However, this multicenter assessment used intravenous delivery of NaNO<sub>2</sub> and not local intracoronary administration (with higher local concentrations), as in many of the studies described above, and still remains unpublished in full form, thereby making it impossible to comment on methodological differences.

Similar protective effects of nitrite have also been evidenced in other organs, including the liver (Duranski et al., 2005), brain (Pluta et al., 2005), and kidney (Tripatara et al., 2007). In a murine model of hepatic I/R, intraperitoneal treatment with NaNO<sub>2</sub> (1.2–480 nmol) during the ischemic period caused dose-dependent cytoprotective effects that resulted in reductions in hepatocellular necrosis and apoptosis and reductions of serum elevations of liver transaminases (Duranski et al., 2005). In a rat model of cerebral I/R, 48 and 480 nmol of nitrite,

infused intravenously at the start of reperfusion, exerted dose-dependent neuroprotective effects, reduced cerebral infarct volume by 33% and 77%, respectively, and promoted neurologic functional recovery (Jung et al., 2006). Similarly, topical administration of  $\text{NaNO}_2$  (0.12 nmol/g) in a rat model of renal I/R injury 1 minute prior to reperfusion significantly attenuated renal dysfunction, reperfusion injury, glomerular dysfunction, and tubular injury (Tripatara et al., 2007). It is noteworthy that, in this study, intravenous administration of the same dose of  $\text{NaNO}_2$  at the same time point (1 minute before reperfusion) did not improve renal dysfunction or reperfusion injury (Tripatara et al., 2007). In all of these studies, the beneficial effects were shown to be due to the activity of  $\cdot\text{NO}$  and were most often specifically associated with the local application into or on the organ of interest.

Given this robust preclinical data regarding the cytoprotective effects of nitrite therapy, it is logical to consider the clinical translation of nitrite-based therapies for I/R injury. Clinical situations in which this may be important include the settings of AMI, cardiac surgery, angiogenesis, cardiac arrest, organ transplantation, and cerebrovascular accidents. This rapidly evolving field is summarized in the following sections.

*b. Humans models of I/R injury: transient endothelial dysfunction.* A number of studies conducted in healthy volunteers demonstrate that elevations in the levels of circulating nitrite levels (achieved through administration of a high dietary nitrate load) lead to a number of beneficial effects, including prevention of transient endothelial dysfunction in a model of I/R injury in the forearm (Webb et al., 2008b; Kapil et al., 2010). In this study it was shown that I/R injury significantly reduced FMD (by ~60%) of the brachial artery, a phenomenon that was prevented in the volunteers after ingestion of a dietary nitrate load (Webb et al., 2008b). Similar beneficial effects have been demonstrated with  $\text{NaNO}_2$  infused at a dose of 1.5  $\mu\text{mol}/\text{min}$  for 20 minutes prior to I/R injury in the forearm of healthy volunteers. In this study, the authors demonstrated that peak FMD decreased by 43% after I/R when subjects received saline ( $6.8\% \pm 0.7\%$  vs.  $3.9\% \pm 0.7\%$ ,  $P < 0.01$ ); however, nitrite treatment prior to ischemia prevented this decrease ( $5.9\% \pm 0.7\%$  vs.  $5.2\% \pm 0.5\%$ ,  $P = \text{N.S.}$ ). However, the protective effects of nitrite were abolished when the nitrite infusion was initiated during the ischemic event (Ingram et al., 2013).

*c. Organ-specific I/R injury.*

*i. Myocardial I/R Injury.* In adults with inducible myocardial ischemia, intravenous infusion of  $\text{NaNO}_2$  improved functional cardiac responses during dobutamine stress echocardiography. In this small study (Ingram et al., 2013), 10 patients with inducible myocardial ischemia were randomized to either low-dose  $\text{NaNO}_2$  (1.5  $\mu\text{mol}/\text{min}$  for 20 minutes) or placebo in a double blind fashion. Long-axis myocardial function was quantified by peak systolic velocity (Versus) and

strain rate (SR) responses. Comparing saline and nitrite infusions, Versus and SR at peak dobutamine increased in regions exhibiting ischemia (Versus from  $9.5 \pm 0.5$  to  $12.4 \pm 0.6$  cm/s, SR from  $-2.0 \pm 0.2$  to  $-2.8 \pm 0.3$  second<sup>-1</sup>), whereas they did not change in normally functioning regions (Versus from  $12.6 \pm 0.4$  to  $12.6 \pm 0.6$  cm/s, SR from  $-2.6 \pm 0.3$  to  $-2.3 \pm 0.1$  second<sup>-1</sup>) ( $P < 0.001$ ). With nitrite treatment, Versus improved only in those areas of the myocardium that were poorly functioning (+122%,  $P < 0.001$ ), implying that the effects of this low-dose  $\text{NaNO}_2$  occurred primarily in regions of ischemic myocardium, with no effect in normoxic regions (Ingram et al., 2013).

Recently, two phase II studies have been conducted investigating the effects of nitrite therapy for AMI. There were many similarities between the studies, including that both enrolled patients undergoing primary PCI for ST-elevated MI. However, a key difference between the studies was that the  $\text{NaNO}_2$  was delivered via different routes, i.e., intravenous and intracoronary. The first of these studies, by Siddiqi et al. (2014), was based on the intravenous administration protocol employed to good effect by Gonzalez et al. (2008) in dogs. Siddiqi and colleagues recruited a total of 229 patients presenting with AMI who were randomized to receive either an intravenous infusion of 70  $\mu\text{mol}$   $\text{NaNO}_2$  ( $n = 118$ ) or matching placebo ( $n = 111$ ) over 5 minutes immediately before primary PCI (Siddiqi et al., 2014). In this study, a prerequisite for inclusion was that patients must have been categorized as with thrombolysis in myocardial infarction (TIMI)  $\leq 1$  flow in the infarct-related artery at the time of reperfusion. The dose was chosen assuming a mean body weight of 70 kg, corresponding to 1  $\mu\text{mol}/\text{kg}$ . Sadly, the primary endpoint of MI size (assessed at 6–8 days by cardiac MRI) did not differ between the groups. After adjustment for the area at risk for infarct, diabetes, or the recruitment center (patients were recruited at four different centers), there was still no difference between the groups. A number of secondary outcome measures also showed no signal, including plasma troponin I or creatine kinase area under the curve (72-hour), LV volumes or LVEF measured at 6–8 days, and infarct size at 6 months. This study would suggest that despite the promising preclinical data, the intravenous route of nitrite administration does not decrease infarct size after primary PCI for AMI (Siddiqi et al., 2014).

The second study was conducted by our own group (Jones et al., 2015) and examined the effects of intracoronary nitrite given prior to balloon inflation in patients with ST-elevated MI undergoing primary PCI. Eighty patients in total were randomized to receive intracoronary 10 ml  $\text{NaNO}_2$  (1.8  $\mu\text{mol}$ ) or NaCl (placebo) through an over-the-wire balloon positioned distal to the occlusion in the culprit coronary artery prior to balloon inflation. In this study, there were no reported adverse effects of nitrite associated with a



significant increase in circulating nitrite that was evident 30 minutes after  $\text{NaNO}_2$  administration. Again, similarly to the above trial, the primary outcome of creatine kinase release ( $P = 0.92$ ) was not different between the groups, as neither was troponin T ( $P = 0.85$ ), or cardiac MRI-assessed infarct size ( $P = 0.254$ ) in the whole patient cohort. However, in contrast, there was an improvement in myocardial salvage index ( $P = 0.05$ ) and reduction in major adverse cardiac events (defined as all-cause mortality, hospitalization for HF, MI, or target vessel revascularization) at 1 year (2.6% vs. 15.8%;  $P = 0.04$ ) in the nitrite group. Additionally, in a 66-patient subgroup with TIMI  $\leq 1$  flow, there was a 20% reduction in serum creatine kinase ( $P = 0.030$ ), a 19% reduction in cardiac MRI-determined infarct size ( $P = 0.034$ ), a 48% reduction in microvascular obstruction ( $P = 0.015$ ), and a 30% increase in myocardial salvage index ( $P = 0.002$ ) with nitrite therapy. This would suggest that in patients with TIMI  $\leq 1$  flow at time of primary PCI, intracoronary nitrite may have benefit as an adjunctive therapy (Jones et al., 2015).

The reason for the discrepancy between the two studies is not clear; however, a number of potential factors could have played a role, including patient selection, doses of nitrite, and/or mode of administration. In terms of the dose, there is a large body of preclinical evidence indicating that nitrite is cytoprotective against I/R injury only when given at concentrations (2.5–10  $\mu\text{mol}$ ) that far exceed physiologic (0.2–0.5  $\mu\text{mol/l}$ ) levels of circulating nitrite. In the study by Siddiqi and colleagues, circulating nitrite with their intervention only reached 1.4  $\mu\text{mol/l}$ , a concentration that is lower than that reported in dogs, in which the same dosing regimen led to circulating levels of 5  $\mu\text{mol/l}$  (Gonzalez et al., 2008). However, the levels achieved were 7.8-fold higher than in the placebo group and consistent with levels achieved in a previous murine preconditioning study demonstrating at least some molecular effect of nitrite in mice at such concentrations (Duranski et al., 2005). Indeed, raising circulating levels of nitrite to those shown effective in most preclinical studies is likely to be associated with adverse effects, such as hypotension and methemoglobinemia, as demonstrated in healthy volunteers (Pluta et al., 2011). It is this possibility that underlies the advantage of intracoronary nitrite administration in which this route provides high local concentrations (2.5–10  $\mu\text{M}$ ) within the myocardium only, thus minimizing potential systemic side effects that may occur with systemic nitrite administration. In the study delivering intracoronary nitrite, although levels of systemic nitrite were elevated in the study patients, indicating a degree of systemic absorption, levels achieved were approximately 0.5  $\mu\text{mol/l}$ , at the upper level of physiologic levels, which would be in keeping with the low rates of side effects seen in the study.

The question of what is the optimal route (intravenous vs. intracoronary) for nitrite therapy is an important issue to address for future studies. The two routes have been compared directly in the kidney; in this study, the local administration of nitrite was effective at reducing infarct size, whereas the same dose given via the intravenous route was not, indicating a superiority of local administration (Tripatara et al., 2007). In contrast, the intravenous route was effective in myocardial I/R injury in dogs (Gonzalez et al., 2008), although as noted above, the intracoronary route does have several advantages. Additionally, if agents are to be delivered at reperfusion, i.e., after angiography to ensure TIMI flow  $\leq 1$ , the intracoronary route does not delay reperfusion. This is compared with intravenous administration, which may result in a delay in reperfusion while administered and might require the establishment of very high circulating levels to achieve sufficient levels of nitrite. Together, these observations suggest that nitrite may have use in the treatment of acute ST-elevated MI in which nitrite could be delivered locally (intracoronary) before balloon inflation at the time of primary PCI; further studies in this area are needed.

*ii. Cardiopulmonary Surgery.* The injury that occurs as a consequence of ischemia and reperfusion during cardiac surgery, such as CABG, is distinctly different from that occurring during spontaneous AMI. In this setting, ischemia is induced artificially by aortic cross-clamping, and myocardial preservation strategies are employed throughout this ischemic period. Once surgery has been completed, the clamp is released, and the heart is then suddenly and globally reperfused with anticoagulated blood. This critical process, although necessary, does result in the immunologic priming of leukocytes due to exposure to the cardiopulmonary bypass circuit, but it is also characterized by a very high partial pressure of  $\text{O}_2$ . Together, this restoration of the circuit delivers a substantial I/R stress. To date, assessing the role of nitrite in cardioprotection post-CABG has not been done. There is currently one clinical trial ongoing to investigate this (clinical trial NCT01098409); however, the start date for this study was 6 years ago, and few updates have occurred, suggesting that recruitment may be difficult. There has, however, been some reporting from a similar study from the same institution in patients. In this study, patients undergoing CABG were subjected to either placebo (saline) or  $\text{NaNO}_2$  treatment (10  $\mu\text{g/kg}$  per minute) either 30 minutes or 24 hours before surgery. In preischemia LV biopsies, 10  $\mu\text{g/kg}$  per minute nitrite administration 24 hours or 30 minutes before CABG surgery was associated with statistically significant increases in the expression of both total eNOS and phosphorylated eNOS at position Ser 1177, an effect similarly evident in LV biopsies taken at reperfusion. These results

intimate that some of the benefits of nitrite may relate to improved eNOS signaling (Bailey et al., 2014).

*iii. Skeletal Muscle I/R Injury (Crush Syndrome).* Skeletal muscle crush syndrome occurs after prolonged physical compression of limb muscles (after events such as earthquakes, road traffic accidents, etc.), followed by the reperfusion that accompanies decompression of the limbs. It is associated with significant morbidity and mortality, and the current standard of care is i.v. crystalloid fluid therapy. Using a rat model of crush syndrome, muscle nitrite levels were shown to be reduced by crush injury, suggesting some eNOS dysfunction (Murata et al., 2012). Both bolus (200–500  $\mu\text{mol/kg}$ ) and continuous infusions for 3 hours (200–500  $\mu\text{mol/kg}$ ) of  $\text{NaNO}_2$  reduced lethality of the model (Murata et al., 2012, 2017), with improvements in both local skeletal muscle and distant (lung, kidney) indices of damage (Murata et al., 2017), suggesting a possible therapeutic option for this catastrophic condition.

*iv. Transplantation.* Since I/R injury is thought to play a major role in graft failure in liver and lung transplantation (Calvert and Lefer, 2010), there has been interest in assessing the efficacy of nitrite in this. In preclinical studies assessing hepatic I/R injury in mice, nitrite (1.2–480 nmol) has been shown to exert profound dose-dependent protective effects on cellular necrosis and apoptosis, with protective effects observed at near-physiologic nitrite concentrations (Duranski et al., 2005). Nitrite-mediated protection of the liver was dependent on  $\cdot\text{NO}$  generation and independent of eNOS and hemoxygenase-1 enzyme activities (Duranski et al., 2005). In a preclinical murine model of liver transplantation, nitrite supplementation attenuated the release of liver enzymes, with the protective effects being even greater with longer cold preservation times. Separately, a reduction in apoptosis with preserved tissue morphology was evident with histologic analysis, and liver graft acute function post-transplantation was improved (Li et al., 2012). Despite this promising preclinical data for nitrite therapy, translation into clinical studies has been slow, and so far there are no published studies of nitrite in human organ transplantation. Interestingly, iNO has proven beneficial in both transplantation of the lung (Yerebakan et al., 2009) and of the liver (Lang et al., 2007). Yerebakan et al. (2009) evaluated the effects of treatment with iNO (starting dose 40 ppm) in nine patients (six female, three male; mean age  $42.9 \pm 15.8$ ) requiring lung transplantation. Significant reductions in mean PAP ( $36.8 \pm 15.8$  to  $22 \pm 6.8$  at 6–8 and  $22.8 \pm 7.96$  mm Hg at 12–14 hours) were evident with  $\cdot\text{NO}$  treatment in all patients except one, with improvements in overall respiratory function. In patients undergoing orthotopic liver transplantation, Lang et al. (2007) demonstrated that treatment with inhaled  $\cdot\text{NO}$  doubled plasma nitrite levels and that this was associated with improved liver function and reduced injury (i.e., transaminases and coagulation studies) compared

with the control group. Importantly, these positive effects were associated with hard outcomes for these patients, with reduced hospital length of stay by 1.24 days ( $P = 0.034$ ). Reperfusion resulted in increased apoptosis [indicated by terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine triphosphate nick-end labeling staining (TUNEL)] in both the placebo and iNO groups; however, the magnitude of increased TUNEL staining was significantly attenuated ( $\sim 75\%$ ) with  $\cdot\text{NO}$  treatment. Although the authors stated that not all effects of iNO may be mediated by nitrite, they suggested that those benefits related to I/R injury were likely due to the actions of nitrite. Currently, there are ongoing clinical trials assessing nitrite therapy in lung transplant recipients (NCT01715883).

*v. Global Ischemia (Cardiac Arrest).* Cardiac arrest results in significant mortality after initial resuscitation due in most cases to I/R-induced brain injury and, to a lesser degree, myocardial dysfunction (Nichol et al., 2008; Wachelder et al., 2009). Experimental models have suggested a protective role of intravenous nitrite or iNO both on neurologic and cardiac function after cardiac arrest (Dezfulian et al., 2009, 2012; Minamishima et al., 2011). Nitrite therapy after murine cardiac arrest improved 22-hour survival through improvements in myocardial contractility (Dezfulian et al., 2009). These improvements accompanied transient mitochondrial inhibition, which reduced oxidative injury to the heart; however, late brain injury related to cardiac arrest was still apparent and resulted in high rates of mortality. Further studies performed in a rat model of cardiac arrest with prolonged survival (7 days) have subsequently been performed (Dezfulian et al., 2012). In this study, cardiac arrest resulted in hippocampal CA1 delayed neuronal death, which has been well characterized in human cardiac arrest survivors (Horn and Schlote, 1992). Nitrite therapy resulted in increases in hippocampal nitrite and *S*-nitrosothiol levels, but not cGMP, shortly after therapy, with associated significant (75%) increases in CA1 neuron survival. Based on this promising data, one pilot clinical study has been performed, which has demonstrated the feasibility and safety of low-dose nitrite infusion in cardiac arrest survivors but has, to date, not reported any improvement in outcome (Dezfulian et al., 2012). In the initial phase of this study, four patients who suffered cardiac arrest (within 12 hours) and were successfully resuscitated and survived to intensive care unit admission have been randomized in a double blind manner—3:1 ratio to  $\text{NaNO}_2$  (1 mg in 100 ml normal saline) or 100 ml saline placebo infused i.v. over 5 minutes. This initial dose of 1 mg nitrite represents 0.2  $\mu\text{g/kg}$  (based on 70 kg b.wt.). Nitrite infusion at this dose did not appear to cause significant hypotension or tachycardia, and mean metHB levels were  $0.74\% \pm 0.14\%$  over time, with the highest recorded level of 1.1%. Nitrite infusion

increased plasma and blood levels in three of four patients (one received placebo), but the degree of increase was in all cases less than 5-fold from the patient's baseline. Further data from the same study have now been published in 11 patients (seven nitrite/four placebo) receiving 1 ( $n = 3$ ) or 9.6 mg ( $n = 4$ ) NaNO<sub>2</sub> after resuscitation from out-of-hospital cardiac arrest. Only the higher dose was associated with significant elevation in plasma nitrite levels, and neither dose was associated with worsening hemodynamics, although no hard outcomes were reported, and the study finished without completing its planned third dose (14.5 mg NaNO<sub>2</sub>) and was only able to recruit 11 patients into the study out of >450 out-of-hospital cardiac arrests in the 5 years that the study was open and recruiting (Dezfulian et al., 2018).

Perhaps a more pertinent time frame to start potentially protective nitrite therapy is at the time of resuscitation by emergency services personnel rather than in hospital. In this regard, a pilot study of paramedic-initiated intravenous nitrite therapy (over 30 seconds) immediately after standard advanced cardiac life support interventions were conducted (defibrillation, intubation, epinephrine) was performed in 120 patients. The initial dose chosen was 25 mg nitrite but after review of pharmacokinetic data midtrial, the dose was increased for subsequent patients to 60 mg (25 mg:  $n = 59$ ; 60 mg:  $n = 61$ ) to achieve the desired post-bolus nitrite concentration in venous blood of 10–20  $\mu\text{mol/l}$ , probably due to hypoxic consumption of nitrite in the arrested state. The control group was acquired from contemporaneous controls in the paramedic data base of all out-of-hospital cardiac arrests who had received standard of care and did not receive nitrite ( $n = 355$ ) during the period the study was open. Interestingly, there was no difference in outcomes (return of spontaneous circulation, rearrest, use of epinephrine, BP, survival to discharge, neurologically favorable survival) between the low- or high-dose nitrite groups or between patients receiving nitrite compared with control patients (Kim et al., 2018). A larger ( $n = 1500$ ) randomized placebo-controlled trial is underway comparing two doses of nitrite (45 or 60 mg) and placebo, which should provide a definitive answer as to whether it is useful or not, with primary outcomes of survival to hospital admission and rearrest/epinephrine use prehospital (NCT03452917).

Although there is a multitude of preclinical and early-phase clinical studies highlighting the diverse potential of nitrite-derived  $\cdot\text{NO}$  in human disease states, particularly in the CV system, almost 20 years of clinical studies have not yielded a positive phase III study in any condition, and neither inorganic nitrate nor nitrite is an established, guideline-recommended treatment as of yet. However, with the large number of adequately powered and mechanistically sound studies ongoing, it is likely that the medical community will have definitive answers as to the true therapeutic utility of these approaches within the next few years.

## VII. Concerns Regarding Nitrite- and Nitrate-Based Therapeutics

Over the past 30 years, there have been many attempts to manipulate the L-arginine: $\cdot\text{NO}$  pathway through provision of substrate or cofactors to the NOS system (Zhang et al., 2011) to facilitate greater  $\cdot\text{NO}$  production. The discovery of authentic  $\cdot\text{NO}$  production from nitrite reduction (Benjamin et al., 1994; Lundberg et al., 1994; Zweier et al., 1995) has provided a further avenue within which to explore  $\cdot\text{NO}$ -based therapeutics.

### A. Epidemiologic Links to Possible Benefits of Dietary Nitrate

Vegetables are a rich source of nitrate, with high amounts of nitrate found particularly in green leafy vegetables, which account for up to 85% of nitrate intake (Hord et al., 2009). Vegetarianism has long been associated with lower BP (Donaldson, 1926; Sacks et al., 1974; Armstrong et al., 1977, 1979; Ophir et al., 1983), and small-scale controlled clinical trials have demonstrated that increasing fruit and vegetable intakes are associated with lower BP (Rouse et al., 1983; Margetts et al., 1986; Appel et al., 1997). Epidemiologic studies have found consistent patterns demonstrating reduction in BP (Li et al., 2016a), CVD (Joshiyura et al., 1999, 2001; Bhupathiraju et al., 2013; Hu et al., 2014; Gan et al., 2015), and CV and non-CV death (Hung et al., 2004; Miller et al., 2017) associated with increased daily vegetable intake.

The precise constituent and mechanism of cardioprotection related to vegetable intake is elusive. There are many proposed vegetable constituents that could be responsible for beneficial CV effects, including soluble fiber, potassium, and antioxidants. Although there are issues with using epidemiologic data to try to identify micronutrient intake because of issues such as dietary recall (Naska et al., 2017) and, in the context of dietary nitrate, seasonal nitrate variation in vegetables (<https://www.food.gov.uk/research/research-projects/nitrate-monitoring-in-spinach-and-lettuce-surveillance-programme>), further analyses of such data have suggested green leafy vegetables, which are the largest source of dietary nitrate (Hord et al., 2009), appear to confer the greatest cardioprotective effects (Joshiyura et al., 1999, 2001). Such data have led to the proposal that dietary nitrate is responsible for the beneficial effects of vegetable-rich diets (Lundberg et al., 2006; Webb et al., 2008b; Ralt, 2009), given the recent discoveries of reductive pathways that can take nitrate in humans to  $\cdot\text{NO}$  via nitrite as an intermediate (Lundberg et al., 2008).

Putting questions of therapeutic efficacy in human outcome trials aside given the lack of currently published data, there have been additional concerns raised about the safety of such approaches. Nitrate content of drinking water in Europe and the US is regulated and kept at <50 mg/l. There is an ADI recommendation for

dietary nitrate as well, set at 3.7 mg/kg daily, which equates to ~4 mmol nitrate daily for an average 70-kg person (European Food Safety Authority, 2008). However, as can be seen in particular from the section on BP and nitrate, most of the clinical studies that have beneficial effects have used acute or chronic dosing that is far above this recommended level. Fruit and vegetable-rich diets that have beneficial effects on CVD (Joshiyura et al., 1999, 2001; Hung et al., 2004) and BP, such as the DASH diet (Appel et al., 1997), contain large amounts of nitrate naturally, perhaps up to 10–20 mmol daily (Hord et al., 2009), far in excess of the recommended ADI above. Despite this, concerns relating to nitrite- and nitrate-based therapeutics continue largely based on two main areas: metHB and carcinogenesis.

### B. Methemoglobinemia

Infants have very low nitrite levels, as their oral microbiome takes time to develop the right ecology for effective oral nitrate reduction (Jones et al., 2015b), but they are susceptible to preformed nitrite. Initial concerns regarding the etiological role of nitrate and methemoglobinemia were first reported in the early 1900s in children treated with bismuth subnitrate (Beck, 1909). It was recognized by these early clinician-scientists that many people could tolerate very large doses of inorganic nitrate with no ill effects, but that children, and those with intestinal infections, were particularly prone to methemoglobinemia (Beck, 1909). It was discovered that nitrate was metabolized to nitrite and that the problems of methemoglobinemia after nitrate ingestion were synonymous with that of nitrite ingestion, as first described by Gamgee (1868). As described earlier, nitrite reacts with oxyHb to form metHB, which is incapable of O<sub>2</sub> transport (Doyle et al., 1981), and the young are particularly susceptible to the ill effects (Roe, 1933) because of preponderant fetal Hb and immature metHB reductase system (Gupta et al., 1999; Greer and Shannon, 2005).

In relation to this, post-World War II well-water surveys in rural American districts with reported cases of infant methemoglobinemia (Comly, 1945; Walton, 1951) led to the establishment of regulatory frameworks to control the nitrate level in such water at levels <50 mg/l that are still in place (U.S. Public Health Service, 1962). Contemporary analysis of dietary nitrate consumption has re-evaluated this historical data and makes it less likely to be a significant concern (Fewtrell, 2004). The early reports of infantile methemoglobinemia were commonly in children that were unwell with presumed bacterial gastroenteritis (Cornblath and Hartmann, 1948), and therefore, the possibility of high systemic nitrite levels from intestinal bacterial conversion of nitrate to nitrite in vivo or in contaminated water supply has been suggested for apparent cases of nitrate-induced infantile methemoglobinemia (Hanukoglu and Danon, 1996; Avery, 1999). Indeed, recent data show no consistent association between drinking water nitrate levels and

either the risk of developing clinical methemoglobinemia or blood metHB levels themselves (Ward et al., 2005).

Importantly, in oral dietary nitrate intervention studies, especially with beetroot juice with nitrate concentrations far higher than those set as safety limits for drinking water described above, there is only a modest rise in plasma nitrite levels (commonly <1 μmol/l, within the physiologic realm) and chronic supplementation with dietary nitrate that leads to long-term systemic nitrite elevation is not associated with elevation of metHB measured by co-oximetry (Kapil et al., 2015; Velmurugan et al., 2016). In adults, there is no report of vegetable intake causing clinically relevant methemoglobinemia, and cases in children are largely thought to be related to preformed nitrite accumulation due to bacterial contamination and preingestion nitrate-to-nitrite conversion (Chan, 2011).

Furthermore, systemic application of nitrite in mechanistic studies has achieved much higher levels of nitrite without significant elevation of metHB. Intra-arterial infusion of NaNO<sub>2</sub> into the human forearm achieved systemic nitrite levels of 16 μmol/l (Cosby et al., 2003), far higher than that needed to achieve clinically meaningful reductions in BP (Ghosh et al., 2013; Kapil et al., 2015). In this study, metHB levels were pegged at ~1% (Cosby et al., 2003). A more detailed pharmacokinetic study aimed at establishing dose-limiting toxicity of intravenous sodium nitrite as a therapeutic agent has been conducted. Infusions of sodium nitrite for 3–9 hours at ~7 μmol/kg per hour produced large symptomatic reductions in BP associated to plasma nitrite levels of 1–5 μmol/l, which caused clinically insignificant, asymptomatic elevation of metHB to only 2%–5% (Pluta et al., 2011), well below the levels that are associated with symptomatic dyspnea (Ash-Bernal et al., 2004).

### C. Carcinogenesis

The potential for carcinogenesis has long been a concern in relation to nitrite and nitrate biology. Indeed, almost all of the works in the 1970s and 1980s looking at oral nitrate reduction were done for the purpose of exploring possible contributions to carcinogenesis via *N*-nitrosamines (Harada et al., 1974; Tannenbaum et al., 1974, 1976; Ishiwata et al., 1975a,b,c,d; Ishiwata, 1976a,b; Spiegelhalder et al., 1976; Eisenbrand et al., 1980; Tannenbaum and Correa, 1985), and more recently, concerns have been re-expressed relating to cancers in the upper GI tract (Iijima et al., 2003).

It is more than 60 years since the first appreciation that *N*-nitrosamines were carcinogenic. Six months of feeding rats with dimethylnitrosamine (50 ppm) produces large, necrotic hepatocellular carcinoma (Magee and Barnes, 1956), and long-term supplementation in rats of other related *N*-nitrosamines causes malignant tumors of other major organ systems, including esophagus, stomach, liver, and kidney as well (Magee and Barnes, 1967). These effects are not limited to rats but have been

found in ~40 other species in which *N*-nitrosamines are directly carcinogenic (Bogovski and Bogovski, 1981).

The relevance of *N*-nitrosamines to nitrite and nitrate biology is apparent on understanding that they can be formed *in vitro* by incubating gastric juice, nitrite, and secondary amines (Sen et al., 1969) and *in vivo* in humans after dietary ingestion of nitrite-containing foods (Fine et al., 1977). Endogenous nitrosation reactions can occur from swallowed nitrite, either from dietary sources or from oral nitrate reduction that can be protonated to nitrous acid, which can further yield the powerful nitrosating agent dinitrogen trioxide, which in turn can nitrosate secondary and tertiary amines via the addition of nitronium cation, eventually forming *N*-nitrosamines (Leaf et al., 1989; Tricker, 1997). Ascorbate (vitamin C) and other food-based micronutrients such as polyphenols and tocopherol (vitamin E) inhibit this formation (Bartsch and Frank, 1996), suggesting that any putative risk relating to dietary nitrite/nitrate ingestion may be potentially mitigated in persons consuming whole vegetables as a source rather than through water or nitrite-containing meat.

The US National Toxicology Program produced a technical report studying 2-year drinking water supplementation studies of high levels of nitrite (~16–65 mmol/l) in rodents and concluded that there was no evidence of carcinogenesis (National Toxicology Program, 2001). In contrast, the International Agency for Research on Cancer evaluated the effects of both nitrite and nitrate on human carcinogenesis and reported (International Agency for Research on Cancer, 2010) the following:

- inadequate evidence linking dietary and water sources of nitrate to cancer overall,
- limited evidence linking dietary nitrite to stomach cancer risk,
- inverse risk of dietary nitrate and stomach cancer, and
- nitrite and nitrate under conditions resulting in endogenous nitrosation are probably carcinogenic.

In addition, additional expert reports have been prepared that contend there is no effect. For example, the WHO Expert Committee on Food Additives concluded that there was no evidence that nitrate was carcinogenic to humans (Speijers and van den Brandt, 2003). In subsequent large cohorts, fruit and vegetable-rich diets that exceed the ADI severalfold are not associated with any increase in cancer or mortality (Hung et al., 2004) and may indeed offer protection against cancer (World Cancer Research Fund/American Institute for Cancer Research, 2007; Boffetta et al., 2010). Epidemiologic work in this area is naturally hamstrung by the methods for data collection in such works. Exposure estimates for dietary nitrite and nitrate across different populations are problematic because of the lack of

widely applicable dietary databases that include the nitrate and nitrite content of common foods.

Furthermore, there are agricultural issues that alter nitrate content found naturally in batches of plants. Plants accumulate nitrate through the roots and use it for novel synthesis of amino acids and proteins (Vogtmann and Biedermann, 1985). If the plant does not use the nitrate immediately, it is stored in vacuoles, and it remains in the vacuoles if the plants are supplied with more nitrate than they can use and therefore will be stored more readily in vegetables with a low rate of photosynthesis (Martinoia et al., 2007). Hence, vegetables harvested in winter have higher nitrate content than those harvested in the summer (<https://www.food.gov.uk/research/research-projects/nitrate-monitoring-in-spinach-and-lettuce-surveillance-programme>). Further variability comes from the amount of nitrate in irrigation water, potential use of nitrogen-containing fertilizers, and postharvest storage (Blom-Zandstra, 1989). This produces a problem when trying to estimate dietary nitrate intake using databases of mean nitrate content for epidemiologic purposes. These also produce a problem when trying to consider dietary nitrate as a therapeutic, though the use of inorganic nitrate salts in capsule or tablet form (Zand et al., 2011), although excluding the protective effects of coexistent micronutrients, or concentrated, fixed-dose dietary nitrate supplements, could avoid this (Muggeridge et al., 2014).

A recent National Heart, Lung, and Blood Institute working group reviewed all these epidemiologic data and made some recommendations for future research, including the addition of location-specific information relating to nitrate content of foodstuffs; use of urine and plasma nitrate measures as an index of exposure; addition of drinking water exposure to dietary databases and questionnaires; and a focus on subpopulations at risk of carcinogenesis, such as smokers or those taking supplements (Ahluwalia et al., 2016).

#### *D. Pharmacokinetic Considerations considering Nitrite and Nitrate as NO-Therapeutics*

Inorganic nitrate can be thought of as a prodrug for bioactive nitrite, as it is largely thought to be inert in mammalian systems unchanged. In almost all disease areas studied in preclinical and clinical studies to date, the effect of systemic nitrite elevation through nitrite or nitrate supplementation (the latter requiring the enterosalivary conversion to nitrite involving oral microbiota) are synonymous.

There are possible pharmacokinetic advantages to oral dosing with nitrate over nitrite, however. Nitrate has a much longer half-life in human plasma (~6 hours) (van Velzen et al., 2008) compared with oral or intravenous nitrite application (Dejam et al., 2007; Hunault et al., 2009) and, therefore, is appropriate for dosing with a once-daily regimen. With chronic oral nitrate dosing, there is sustained nitrite elevation at trough 24 hours postdosing (Kapil et al., 2015; Ahluwalia

et al., 2016) while nitrite is typically given two to three times daily (Mohler et al., 2014; DeVan et al., 2016). Adherence is improved in chronic medical conditions, such as systemic and pulmonary hypertension, that could be targeted by nitrite/nitrate therapeutics by once-daily compared with multiple-times-daily posology (Waxman et al., 2013; Vrijens et al., 2017). There is the additional benefit that dietary nitrate supplementation is applicable on a public health scale through campaigns to increase vegetable intake (<https://www.nhs.uk/live-well/eat-well/why-5-a-day>) and is potentially low-cost and therefore applicable to constrained emerging health economies.

### VIII. Conclusions

Until recently, the prevailing view of mammalian  $\cdot\text{NO}$  biology included the production of  $\cdot\text{NO}$  uniquely from the five-electron oxidation of the amino acid L-arginine by NOS (Stuehr, 1999). However, recent evidence has proved the existence of an alternative or noncanonical pathway that utilizes the sequential reduction of nitrate to nitrite, and thence  $\cdot\text{NO}$ , and involves a fundamental, symbiotic role for hitherto bystander oral microbiota (Lundberg et al., 2008). This alternative pathway for  $\cdot\text{NO}$  generation is more active in hypoxia and acidosis, a situation in which the classic L-arginine pathway is dysfunctional and therefore provides a backup system for  $\cdot\text{NO}$  generation by re-cycling the oxidative products of  $\cdot\text{NO}$  metabolism, with important contributions from the diet as well. This reveals that nitrite and nitrate are no longer end products of  $\cdot\text{NO}$  metabolism but exist in a balanced  $\cdot\text{NO}$  cycle (Reutov and Sorokina, 1998; Reutov, 2002).

Given the protean roles that have been discovered for  $\cdot\text{NO}$  in physiology and pathophysiology, it is not surprising that there has been a wide-ranging exploration of the potential roles of this new biology. Translational studies, predominantly in the realm of cardiorespiratory disease, have demonstrated beneficial effects with systemic or local elevation of nitrite levels, whether achieved directly or after nitrate supplementation and oral reduction of nitrate to nitrite. The next few years will see the publication of important outcome studies in patient populations in need of additional therapeutics and will determine whether this early evidence translates into truly beneficial outcomes and useful therapeutic medicines.

Or, as said almost 80 years ago by Stieglitz and Palmer (1937):

“Because of the rapid disappearance of nitrite from blood and the relative constancy of the level found in freshly drawn blood, one may tentatively assume that there is a constant production and destruction of the nitrite going on in the body.

The source of the nitrite of the blood may be from administration by any route, absorption of nitrites from

the bacterial reduction of food or drug nitrates in the lower portion of the bowel or absorption of nitrates and a subsequent reduction in the blood stream itself or a reduction of nitrates in the tissues. Any of these sources may be foci of a more or less constant production of nitrite...

Nitrite... has profound effects in very small amounts on a great many functions of the body directly by its action of relaxing smooth muscle, especially arteriolar muscle, and indirectly by its effects on the blood flow in secretory organs.

The exact physiologic significance of the blood nitrite is uncertain, but it may be that normally it aids in maintaining those functions which are stimulated by the administration of therapeutic doses. Clinical application of nitrite analysis of the blood may reveal some correlation between a disturbed nitrite metabolism and abnormalities of the arterial tension.”

#### Authorship Contributions

*Wrote or contributed to the writing of the manuscript:* Kapil, Khambata, Jones, Rathod, Primus, Massimo, Fukuto, Ahluwalia.

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