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It is Rocket Science – Why Dietary Nitrate is Hard to Beet!

Part II: Further Mechanisms and Therapeutic Potential of the Nitrate-Nitrite-NO Pathway

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Abstract

Dietary nitrate (found in green leafy vegetables such as rocket and in beetroot) is now recognised to be an important source of nitric oxide, via the nitrate-nitrite-NO pathway. Dietary nitrate confers several cardiovascular beneficial effects on blood pressure, platelets, endothelial function, mitochondrial efficiency and exercise. Having described key twists and turns in the elucidation of the pathway and the underlying mechanisms in Part I, we explore the more recent developments which have served to confirm mechanisms, extend our understanding, and discover new properties and potential therapeutic uses of the Pathway in Part II. Even the established dependency on low oxygen states for bioactivation of nitrite has recently been challenged. Dietary nitrate appears to be an important component of 'healthy diets', such as the DASH diet to lower blood pressure and the Mediterranean diet, with its potential to lower cardiovascular risk, possibly through beneficial interactions with a range of other constituents. The World Cancer Research Foundation report strong evidence for vegetables including spinach and lettuce (high nitrate-containing) decreasing cancer risk (mouth, pharynx, larynx, oesophagus and stomach), summarised in a 'Nitrate-Cancer Risk Veg-Table'. The European Space Agency recommends beetroot, lettuce, spinach and rocket (high-nitrate vegetables) are grown to provide food for long term space missions. Nitrate, an ancient component of rocket fuel, could support sustainable crops for healthy humans.

Introduction

In Part I we described how the nitrate-nitrite-NO pathway was initially established. In Part II we address the more recent developments which have served to confirm mechanisms, extend our understanding, and discover new properties and potential therapeutic uses of the Pathway.

11. Is nitrate protective in IRI?

Having previously discovered the protective effects of inorganic nitrite in a model of ischaemia-reperfusion injury (IRI) in the isolated perfused rat heart (Langendorff preparation) [1], Webb *et al.* performed a translational study to determine whether beetroot juice, as a source of dietary nitrate-derived nitrite, could protect against transient endothelial stunning/dysfunction induced by a model of IRI in the forearm of healthy humans (20 minutes ischaemia and 20 minutes reperfusion), with endothelial function determined by flow-mediated dilatation (FMD) [2]. Whilst beetroot juice had no effect on FMD prior to the ischaemic insult, the nitrate source preserved endothelial function following IRI, compared to control, in which a 60% suppression of FMD response was observed. That the protective effect was due to inorganic nitrate-derived nitrite was confirmed by Kapil *et al.* using potassium nitrate instead of beetroot juice [3]. Therefore, protection against IRI, at least in terms of endothelial dysfunction is also conferred by readily administered sources of dietary or inorganic nitrate as a source of nitrite, besides directly-applied nitrite.

12. Does nitrite inhibit platelets?

Given that nitrate had previously been shown to inhibit platelet aggregation, and that dietary nitrate had now been shown to result in an increase in circulating nitrite levels, it was possible that the antiplatelet effects of nitrate were mediated by nitrite. As part of a wider study to explore the biological activity of nitrite in normal physiology, Bryan *et al.* measured

ex-vivo platelet aggregation to ADP from venous blood of rats following intraperitoneal administration of nitrite (0.1, 1 or 10 mg/kg) [4]. They found that platelet aggregability remained unchanged, or even increased, following nitrite administration. The authors proposed that this lack of inhibition of platelet aggregation, suggested that metabolism of nitrite to NO within the circulation was likely to be limited under a physiological oxygen gradient.

Webb *et al.* examined the effects of nitrite on adenosine diphosphate (ADP) or collagen-induced platelet aggregation by interrupting the enterosalivary circulation in the spit versus swallowing of saliva study, following beetroot juice consumption [2]. As expected, a significant reduction in aggregation was apparent when volunteers swallowed their saliva normally following the consumption of beetroot juice; an effect that was associated with a significant increase in plasma nitrite concentration. However, when volunteers spat out all their saliva for 3 hours, plasma nitrite concentration did not increase, and there was no change in either ADP- or collagen-induced platelet aggregation following beetroot juice consumption. Whilst the antiplatelet effects of dietary nitrate/beetroot juice were predictable from previous studies, that this was due to nitrite (as demonstrated by interrupting the enterosalivary circulation), was not. Indeed, a trend to the opposite effect of nitrite had been suggested by the study in rats [4]. The effect of nitrite on inhibition of platelet aggregation in humans was confirmed by Velmurugan *et al.* with direct spiking of *ex vivo* blood with potassium nitrite, and *in vivo* following acute nitrate consumption, an effect associated with a reduced platelet P-selectin expression [5]. However, this effect was non-significant in females, which concurs with the previous study by Kapil *et al.,* reflecting differences in baseline nitrite [3]. Velmurugan *et al*. have subsequently demonstrated the longer term effects of 6 weeks' ingestion of dietary nitrate (active beetroot juice) versus nitrate-depleted placebo beetroot juice in 67 patients with hypercholesterolaemia, resulting in reductions in monocyte platelet aggregates in addition to stimulated (*ex vivo*) P-selectin expression [6].

13. Does nitrate/nitrite exhibit tolerance?

Tolerance to organic nitrates such as GTN is a commonly observed phenomenon that occurs with their continuous application, resulting in a reduction in their therapeutic efficacy [7, 8]. It is therefore possible that inorganic nitrate and nitrite would have the same problem. However, Dejam et al., found no evidence of tolerance for acute blood pressure reductions with daily boluses of inorganic (sodium) nitrite on a background of a continuous infusion of nitrite over 14 days [9]. Similarly, several studies support an absence of tolerance issues with dietary nitrate. Sobko *et al*., (2010) showed a sustained reduction in diastolic BP of ~4.5 mmHg over 10 days, with a high-nitrate Japanese Traditional Diet, compared to a low-nitrate control diet [10]. In the same year, Vanhatalo *et al.* reported that the blood pressure-lowering effects of daily beetroot juice persisted over a two-week period in healthy volunteers [11]. Kapil *et al.* performed a randomised, double-blind, placebo-controlled parallel-group trial in which 68 patients, half of whom with treated hypertension, and the other half with untreated hypertension, received a dietary nitrate (250 ml beetroot juice) or placebo (nitrate-free beetroot juice) daily over a 4-week period [12]. They found a significant and sustained blood pressure-lowering response to dietary nitrate, which was similar across all 3 methods of measurement: clinic blood pressure: 7.7/2.4 mmHg, 24-hour ambulatory blood pressure: 7.7/5.2 mm Hg, and home BP 8.1/3.8 mm Hg. For home blood pressures, there was a trend to a greater blood pressure-lowering effect over time. This suggests that dietary nitrate does not exhibit tolerance following daily usage. Therefore supplementation with dietary nitrate provides an inexpensive and effective approach to reduce blood pressure.

14. Does nitrite alter mitochondrial metabolism?

NOS-derived NO has an important and established regulatory role in the mitochondria, and therefore it is possible that nitrite may also have an effect, given appropriate conditions. In normoxia, NOS-derived NO binds reversibly to the $copper_B/haem_a3$ binuclear centre of complex IV (Cytochrome C oxidase (CcOx)), inhibiting mitochondrial respiration by

competing with oxygen [13-15]. Complex IV is the terminal part of the mitochondrial chain which normally reduces 95% of inspired oxygen [16]. Therefore inhibition of complex IV by NO allows oxygen to be diverted away from the mitochondria to other targets, for example mediating cell signalling and oxidative stress through the generation of ROS. In hypoxia/ischaemia, NOS activity is inhibited in the absence of oxygen. However, nitrite can be reduced to NO by enzymes described above (including XOR, deoxymyoglobin) and also by the electron transport chain (ETC) itself, and thus can continue to regulate oxygen gradients via inhibition of respiration at complex IV. However, during IRI, nitrite may also directly inhibit complex I via S-nitrosation, decreasing ROS generation at reperfusion [17].

As Bailey *et al*., described, "a fundamental tenet of human exercise physiology is a predictable oxygen (O_2) cost for a given submaximal work rate" [18]. Therefore, the ultimate challenge is to make mitochondrial respiration more efficient, as this would be expected to improve exercise performance. To this end, Larsen *et al.* demonstrated that supplementation of healthy volunteers with inorganic nitrate reduced the oxygen cost during graded submaximal exercise, with a significant reduction in VO_2 compared to placebo [19]. Bailey *et al.* confirmed a reduction in O_2 cost with dietary nitrate (beetroot juice) and that this was coupled with a reduction in ATP turnover, as demonstrated by a reduction in phosphocreatine degradation and ADP concentration [20]. The mechanism was further elucidated by Larsen *et al.* who determined the amount of oxygen consumed per ATP produced (P/O ratio) through skeletal muscle biopsies in humans [21]. They found a significant improvement of 23% in the P/O ratio following nitrate ingestion compared to placebo. This improvement correlated with a reduction in oxygen cost during exercise. In addition to effects on Complex I and IV, dietary nitrate was found to reduce the expression of ATP/ADP translocase, the adenine nucleotide transporter (ANT), a protein involved in proton conductance. Nitrate reduced proton leak into the mitochondria. This improved mitochondial efficiency by reducing the amount of ATP required to expel the protons.

The studies described above were performed under normoxic conditions. However, given the enhanced reduction of nitrite to bioactive NO in hypoxia, Vanhatalo *et al*. studied the effects of 750 mL of beetroot juice (9.3 mmol nitrate) ingested 24 hours prior to exercise under conditions of hypoxia (with subjects breathing 14.5% O_2). Nitrate restored exercise tolerance and oxidative function to values observed in normoxia [22]. Masschelein *et al*. studied the more chronic effects of 6 days' of beetroot juice on cycle ergometry in a hypoxia facility breathing 11% O_2. The nitrate-containing juice reduced VO_2 at rest and at 45% of peak O_2 consumption and increased $\%SpO_2$ compared to placebo [23]. Whilst nitrate improved the tissue oxygenation index of muscle at rest and at 45% of peak O_2 consumption and maximal exercise, there was no effect on cerebral tissue oxygenation index or symptoms of acute mountain sickness.

In addition to studies performed under hypoxia in healthy volunteers, the effects of dietary nitrate on exercise performance have been studied in patients with important comorbidities associated with generalised or local hypoxia. For example, patients with peripheral arterial disease have impaired perfusion with local tissue hypoxia of the lower limb. Kenjale *et al*., (2011) showed that administration of a single dose of beetroot juice (500 mL) to patients with peripheral arterial disease increased their time before onset of claudication by 18%, and the total walking time by 17%, [24]. However, Mohler et al., found no effect of sodium nitrite (40 mg or 80 mg twice daily) taken by patients with peripheral artery disease (over half of whom also had type 2 diabetes) for 10 weeks on the 6-minute walk test or quality of life parameters [25].

Patients with chronic obstructive pulmonary disease (COPD) have skeletal muscle deconditioning and ventilatory and gas exchange impairments resulting in tissue hypoxia and lactic acidosis, and decreased exercise capacity. Therefore patients with COPD would appear to be a perfect group to study the potential benefits of dietary nitrate. Indeed, five such studies were published in 2015; however they present mixed findings. Berry *et al*.

showed that in 15 patients with COPD, beetroot juice increased the submaximal constant work rate exercise time at 75% of maximal work capacity [26]. The arterial oxyHb saturations remained ~95% throughout. Kerley *et al*., observed an increase in the incremental shuttle walk test (ISWT) distance of 25 m after 11 patients with COPD consumed the high nitrate juice compared with a reduction of 14 m after the low nitrate juice [27]. Here, the pre-ISWT arterial oxyHb saturations were ~95%, but decreased to ~89% post-ISWT. Using the 6-minute walk test (MWT), Shepherd *et al*., did not find any effect of beetroot juice on the oxygen cost of exercise or distance covered in 13 patients with COPD [28]. Leong *et al*. found no effect of 3 days' of beetroot juice (4.8 mmol twice a day) on the endurance shuttle walk test (ESWT) distance and time to fatigue at 85% VO_2max in 19 patients with COPD [29]. Whilst Curtis *et al*. did not find any effect on endurance time at 70% of maximal workload of beetroot juice (12.9 mmol nitrate) ingested 3 hours beforehand, isotime oxygen consumption (VO_2) was 3-4% lower following nitrate supplementation [30]. The arterial oxyHb saturations decreased from ~95-96% to ~93-94% with exercise. Therefore 3 of the 5 studies found an effect of dietary nitrate. Three of the studies reported oxyHb saturations, with only relatively small changes.

Pawlak-Chaouch *et al*. have very recently performed a systematic review and a meta-analysis on the effect of dietary nitrate supplementation on metabolic rate during rest and exercise in humans [31]. This found that dietary nitrate supplementation significantly decreased VO_2 during submaximal intensity exercise overall, and specifically that of moderate and heavy intensity exercise. However, a sub-analysis in patients with chronic disease did not reveal any significant effect in this group. In a further crossover study in 48 patients with type 2 diabetes mellitus, not included in this analysis, Shepherd *et al*. found that 4 days' supplementation with nitrate-rich beetroot juice did not reduce the O_2 cost of walking or the distance covered in a 6MWT compared to placebo [32]. There were also no effects seen with blood pressure. This lack of a blood pressure effect is consistent with a

previous study by Gilchrist et al., which also found no effect of dietary nitrate on endothelial function and insulin sensitivity in patients with type 2 diabetes [33].

15. Are nitrite's effects *all* hypoxia-dependent?

In small, resistance arterioles, the extraction of oxygen by skeletal muscle, particularly exercising muscle, facilitates the established hypoxia-dependent reduction of nitrite to vasodilating NO [34]. Thus in conduit vessels - where haemoglobin is fully saturated with oxygen, it was expected that nitrite would have minimal, if any, vasodilatory activity. However, Omar et al., recently found that infusion of sodium nitrite into the brachial artery, with mean haemoglobin oxygen saturations of 99%, resulted in selective dilatation (measured by ultrasound) of the radial artery (a medium-sized conduit vessel) over resistance vessels (measured by forearm blood flow); this selectivity to conduit vessels being similar that of GTN - the most selective large artery dilator currently studied [35]. Furthermore, rather than being enhanced by hypoxia, radial artery dilatation to nitrite was markedly *inhibited* when participants inhaled 12% oxygen ("hypoxia" - resulting in haemoglobin oxygen saturations of ~90%) compared to 21% (normoxia), this was in contrast to the confirmation of increased forearm blood flow in hypoxia. A potential mechanism may be via carbonic anhydrase, as the inhibitor acetazolamide increased radial artery dilatation to nitrite. Carbonic anhydrase lacks a redox centre and may act as a nitrite anhydrase rather than a nitrite reductase, an action which may be less dependent on hypoxic/acidic/reducing conditions. Whilst acetazolamide inhibits the CO_2 hydration reaction of carbonic anhydrase it may increase the nitrite anhydrase activity. The functional relevance of conduit artery dilatation may relate to improvement in central haemodynamics. Indeed, with intravenous nitrite Omar et al., found systemic conduit artery dilatation associated with selective lowering of central (aortic) systolic blood pressure, in addition to augmentation index and pulse wave velocity [35].

Such improvements in central haemodynamic indices may provide the explanation for the effect of nitrate supplementation via beetroot juice improving exercise duration by ~1 minute in patients with Heart Failure with Preserved Ejection Fraction (HFpEF) published in parallel by Zamani *et al* [36, 37]. Instead of finding an improvement in exercise efficiency as expected, oxygen consumption (VO_2max) was actually increased following active nitrate-containing beetroot juice (versus placebo) – as a result of the increased exercise capacity and increased cardiac output. This improved cardiac performance may have been due to a more favourable central haemodynamic profile and reduced after-load with nitrate as suggested by a reduced augmentation index. Indeed, Borlaug *et al*, have subsequently found that in a parallel-group study versus placebo, intravenous infusion of sodium nitrite in patients with HFpEF reversed the increase in pulmonary capillary wedge pressure (PCWP) during exercise to pre-exercise levels, measured during invasive cardiac catheterisation [38]. This was associated with an improvement in cardiac output reserve with exercise (due to increased stroke volume), an increase in VO_2, but also normalisation of the increase in cardiac output relative to oxygen consumption ($\Delta CO/\Delta VO_2$), suggesting an improvement in efficiency.

16. Is nitrate/nitrite harmful?

Humans are exposed to nitrate and nitrite from birth, with concentrations of nitrate and nitrite of ~30 µM and ~20 µM respectively in colostrum [39]. This may provide an important early anti-infective function. Whilst nitrite concentration falls to ~0.3 µM in transition and mature breast milk, nitrate concentration increases to ~85 µM in transition milk, before falling to ~45 µM in mature milk. Nitrate and nitrite were also present in the formula milks tested, although most with considerably lower concentrations than breast milk. From infancy onwards, exposure continues as nitrite (E249, E250) and nitrate (E251, E252) are widely-used EU-approved food additives for meat preservation, to reduce pathogenic growth, most notably Clostridium botulinum [40], with sodium nitrite added in concentrations up to 150 mg/kg.

However, the nitrite concentration has recently been shown to decrease by 85% within the first 24 hours of addition for chicken liver pate [41]. We speculate that this could be related to the high concentrations of xanthine oxidoreductase in liver, which will rapidly metabolise nitrite to NO. This contrasts with a lesser decline of ~50% in nitrite concentration for chicken sausage and ~65% for beef/pork sausage within the first 24 hours, although these concentrations were then fairly well maintained over the subsequent 2-3 weeks. Whilst processed meats were estimated to account for around a third of dietary nitrite exposure in a 4 year old child, the majority of nitrite, ~60% was estimated to be derived from endogenous conversion of nitrate from vegetables (and drinking water).

The key question for many years has been whether nitrate and nitrite cause cancer? The International Agency for Research on Cancer (IARC) evaluated the carcinogenic hazard to humans of ingested nitrate and nitrite for the first time in 2006 in the 94th volume of *IARC Monographs* [42]. It identified the reduced risks of gastric cancer from some epidemiological studies of high nitrate ingestion primarily from vegetables, as opposed to increased risks from other studies of nitrate/nitrite ingestion from nitrite-preserved meats. The mechanistic explanation proposed was that the formation of *N*-nitroso compounds is accelerated by the presence of nitrosatable compounds (found in meat), but inhibited by vitamin C and other antioxidants (abundant in vegetables). As the cancer hazard from nitrate/nitrite ingestion could not be determined without considering these other factors, the Working Group defined the agent not as “ingested nitrate or nitrite”, but as “ingested nitrate or nitrite under conditions that result in endogenous nitrosation”, which was categorised as probably carcinogenic to humans (Group 2A). This marked the first use of a mechanistic event (endogenous nitrosation) leading to carcinogenesis in the wording of an evaluation statement.

Bryan *et al*. conducted a review of the evidence from experimental animal studies and human epidemiological studies on cancer risk from ingested nitrate or nitrite, emphasising

studies not included in, or published subsequent to the 2006 IARC evaluation. They concluded that, in the absence of co-administration of a carcinogenic nitrosamine precursor, there was no evidence for carcinogenesis. It was thought that N-nitrosamines formed in the acidic environment of the stomach led to cancer formation. However, this review highlights how *N*-nitrosation is an essential part of physiological cell signalling. The kinetics of the stomach are such that *S*-nitrosation is favoured, leading to the formation of RSNOs rather than "potentially carcinogenic" substances. They found no association between nitrate/nitrite intake and risk of stomach cancer [43].

In fact, nitrate/nitrite may even protect individuals from cancer. Petersson *et al.* found nitrate to be gastroprotective in rats, by regulating gastric mucosal blood flow and protecting against NSAID-induced damage [44]. When rats were treated with antiseptic mouthwash, this beneficial effect and the associated rise in plasma nitrite concentration was inhibited, supporting the enterosalivary circulation of nitrate as a protective factor. Apart from any effects due to nitrate, red beetroot extract has been shown to have anti-cancer properties against several cancer cell lines, including oesophagus, lung, breast, liver, prostate and skin [45-48] attributed to the high content of the betacyanin, betanin, being protective, possessing strong antioxidant activity [49].

The 114th IARC Monograph recently classified processed meat as *carcinogenic to humans* (Group 1), based on *sufficient evidence* in humans that the consumption of processed meat causes colorectal cancer [50]. This key conclusion was based on a meta-analysis of colorectal cancer in ten cohort studies by [51]. This reported a statistically significant dose–response relationship, with an 18% increase (95% CI 1·10–1·28) per 50 g per day of processed meat. Whilst the meta-analysis had also reported a very similar 17% increased risk (95% CI 1·05–1·31) per 100 g per day of red meat, the Working Group concluded that there was limited evidence in human beings for the carcinogenicity of the consumption of red meat as no clear association was seen in several of the high quality studies. However, given

strong mechanistic evidence supporting a carcinogenic effect, the consumption of red meat was classified as *probably carcinogenic to humans* (Group 2A). In addition, positive associations were identified between the consumption of processed meat with stomach cancer and the consumption of red meat with pancreatic and with prostate cancer. Processed meats are also high in salt, an established risk factor for stomach cancer.

The World Cancer Research Fund Continuous Update Report (December 2015) reports strong evidence for non-starchy vegetables, such as broccoli, cabbage, spinach, kale, cauliflower, carrots, lettuce, tomatoes, leek, swede and turnip decrease the risk of cancer of the mouth, pharynx, larynx, oesophagus and stomach [52]. Therefore this includes several green leafy vegetables that are high in dietary nitrate and which appear to decrease the risk of cancer of the upper gastrointestinal tract – particularly the oesophagus and stomach, where the greatest concern is; see the 'Nitrate-Cancer Risk Veg-Table' (Table 1). Specifically, decreased oesophageal cancer is associated with foods containing vitamin C. Vitamin C/Ascorbic acid is known to inhibit *N*-nitrosamine formation [53]. Vermeer *et al*. showed that ascorbic acid (250 mg and 1 g) profoundly inhibited *N*-nitrosodimethylamine (NDMA) excretion in 25 women consuming a fish meal rich in amines as nitrosatable precursors, in combination with nitrate-containing drinking water at the Acceptable Daily Intake (ADI) over 7 consecutive days [54]. Chung *et al*. also showed that whole strawberries, garlic juice, and kale juice all independently inhibited NDMA excretion in 27 males and 13 females following nitrate (400 mg/day) in combination with an amine-rich diet [55]. Of note, kale itself contains a moderately high amount of nitrate [56] and therefore this result is despite the additional nitrate load. The effect of these fruits and vegetables is unlikely to be due solely to ascorbic acid. For example, Helser *et al*. found that ascorbic acid (46 mg in 100 ml water) only inhibited nitrosamine formation (as assessed by the N-nitrosoproline (NPRO) test) by 24% compared with 41–63% following ingestion of juices (100 ml) made of green pepper, pineapple, strawberry, or carrot containing an equal total amount of ascorbic acid [57]. Other compounds that may be involved include polyphenols [58]. Indeed, Gago *et al*.

have demonstrated an interesting role for red-wine polyphenols in enhancing nitrite reduction to NO [59].

17. Other therapeutic opportunities for nitrate-nitrite

The Review has mainly focused on the effects of nitrate and nitrite on IRI, blood pressure, platelet function, mitochondrial function and exercise as these findings were pivotal to defining the nitrate-nitrite-NO pathway. However, the pathway involves almost all organs and systems and there is a rapidly expanding range of mechanisms and effects being reported for nitrate and nitrite, with associated potential therapeutic opportunities for their use in health and disease. For example, Pluta et al., found that the intravenous infusion of sodium nitrite prevented delayed cerebral vasospasm in a primate model of acute subarachnoid haemorrhage (SAH) [60]. This finding has been translated into a phase IIa study, which demonstrated the safety of nitrite in patients following SAH, administering doses up to 64 nmol/kg/min; although patient numbers were too small to draw any conclusions about clinical efficacy [61]. Following beneficial effects of nebulised nitrite [62], and also dietary nitrate in models of pulmonary arterial hypertension [63], and favourable kinetics and safety of nebulised nitrite over 6 days in healthy humans [64], this therapeutic approach has been advanced into randomised trials in patients with pulmonary arterial hypertension.

Beyond the use of nitrite as a preservative in meat to reduce pathogenic growth as described above, nitrite has the potential to inhibit bacterial growth in humans, related to the antimicrobial activity of NO. For example, many opportunistic organisms that occupy the lower urinary tract (e.g. E. coli) possess nitrate reductase enzymes, forming the basis of urine dipstick detection of nitrites (see Figure 2 in Part I of the Review). In acidic environments, nitrite is reduced to NO and can be highly toxic to bacteria. Carlsson *et al.* found that the transfer of nitrite-rich urine containing E. coli to a more acidic environment (e.g. pH 5.5) effectively inhibited bacterial growth, in a dose dependent manner [65]. This

effect was potentiated in the presence of vitamin C. The antibacterial potency of this method was comparable to conventional antibiotics such as trimethoprim and nitrofurantoin.

Within the airways, in an animal model of cystic fibrosis (CF), acidified nitrite (pH <7) has been demonstrated to clear the deadly bacterium mucoid *Pseudomonas aeruginosa (P. aeruginosa)*, commonly found in patients with CF [66]. *P. aeruginosa* is often refractory to therapy and therefore, nitrite offers an alternative solution to treating this pathogen. More recently, Zemke et al., have shown that sodium nitrite prevents 99% of biofilm growth of P. aeruginosa on the apical surface of primary human airway epithelial cells [67]. This may represent an additional use for nebulised nitrite, possibly in combination with polymixins where there is evidence of an enhanced effect [67]. However, this was not the case with the gentamicin and tobramycin, where sodium nitrite was found to induce resistance to both aminoglycosides of P. aeruginosa grown in liquid culture, or as abiotic, or biotic biofilms [68].

18. Are there potentially beneficial dietary combinations?

We have primarily focused on the specific properties of nitrate and nitrite. However, their interactions with other nutrients are of importance when one considers them in the context of healthy diets such as the Mediterranean diet, where the beneficial effects are unlikely to relate to a single component; this is also relevant to approaches to minimise the risk of cancer. Such combinations are summarised in Table 2. For example, in Part I we described the importance of ascorbic acid as a reducing agent to enhance NO production from nitrite, and thus maximise nitrite's activity, in terms of vasorelaxation [69]. At least as important as this property, is ascorbic acid's effect in inhibiting *N*-nitrosamine formation [53], to decrease the risk of gastro-oesophageal cancer [52].

The Mediterranean diet is rich in unsaturated fatty acids, such as oleic acid, present in olive oil, and conjugated linoleic acid (CLA), produced in the ruminant stomach and found in dairy products such as milk and cheese. These fatty acids are nitrated by nitrous acid, derived

from nitrite in the acid environment of the stomach, forming the nitro-fatty acids (NO_2-FAs) nitro-oleic acid and nitro-linoleic acid, respectively [70]. Nitro-fatty acids are found in the circulation in nanomolar to low micromolar concentrations [71]. Nitro-fatty acids have important anti-inflammatory properties. For example, Borniquel et al., showed that orally administered nitro-oleic acid was effective in suppressing inflammation in experimental inflammatory bowel disease [72], with an action through peroxisome proliferator-activated receptor-γ (PPAR-γ) [72, 73]. Inhibition of macrophage cytokine and iNOS expression has been demonstrated through S-alkylation of nuclear factor κB [74]. Through a mechanism involving reduced foam cell formation and oxidised LDL-induced phosphorylation of signal transducer and activator of transcription-1 (STAT-1), Rudolph et al., demonstrated reduced atherosclerotic lesion formation following subcutaneous injection of nitro-oleic acid in apoliproprotein E-deficient mice [75]. In addition to their enhanced formation under conditions of hypoxia [76], and during IRI [77, 78], administration of exogenous nitro-fatty has protective properties in IRI, increasing expression of the antioxidant enzyme heme oxygenase-1 (HO-1), elevating MicroRNA-499 (miRNA-499) levels, and abolishing the expression of p53 and dynamin-related protein-1 (DRP-1) normally expressed in cardiac tissue post-IRI [79]. Charles et al., demonstated the inhibition of soluble epoxide hydrolase (sEH) with mice fed oral CLA and nitrite, and following the systemic infusion of nitro-oleic acid; the latter resulting in elevation of epoxyeicosatrienoic acid (EET) substrates, which lowered BP in an angiotensin II-induced hypertension model [80].

Bondonno *et al*. found no additive effects of the combination of flavonoid-rich apple and spinach on plasma nitrite or RXNO, endothelial function, or blood pressure in healthy volunteers [81]. Rodriguez-Mateos, *et al*. explored the combination of nitrate and cocoa flavanols [82]. They found an increase in FMD peaking at 1 hour with lower doses (3 mg/kg body weight of cocoa flavanols and 2.7 mg/kg nitrate), relative to the additive effect of each agent separately; however such an effect was not found at higher doses of each (10.9 mg/kg cocoa flavanols with 8.5 mg/kg nitrate). The improvement in FMD was coupled with an

increase in nitrate related gastric NO (compared to nitrate alone), but attenuation in plasma nitrite.

Dietary polyphenols, including those found in red wine, possess nitrite reductase activity, which may enhance NO production in the stomach, and potentially in the circulation; examples of such polyphenols are provided in Table 2 [59] [83] [84]. A further mechanism through which wine may result in a vasodilatory effect is through the formation of ethyl nitrite, from the nitrosation of ethanol [85]. Finally, other factors, such as a healthy outdoor lifestyle in a sunny climate may improve blood pressure through the mobilisation of nitrite from the skin and dermal vasculature into the circulation by UVA irradiation [86] [87]. Whilst the blood pressure lowering effect of UVA irradiation does not appear to be enhanced with dietary nitrate; Muggeridge et al have recently shown that the combination (of dietary nitrate with UVA irradiation) improves oxygen efficiency during steady-state exercise, and exercise performance in a time trial [88].

Conclusion

We have described important recent developments in the understanding of mechanisms relating to, and potential therapeutic uses of the nitrate-nitrite-NO pathway. These are summarised in Table 3, and include nitrite's and nitrate's effects on inhibiting platelets, minimising IRI, the lack of problems with tolerance, improving mitochondrial function and exercise performance, normoxia-dependent dilatation, the potential of dietary nitrate containing vegetables to reduce the risk of certain cancers, and potentially beneficial combinations of dietary nitrate with other components of a healthy diet, and lifestyle such as the Mediterranean diet. Further studies are needed to determine the long-term beneficial effects of dietary nitrate. By furthering the understanding the nitrate-nitrite-NO pathway, additional inexpensive dietary and therapeutic approaches could be developed for the treatment and prevention of disease.

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Table 1. 'Nitrate-Cancer Risk Veg-Table' showing the amount of nitrate (in mmol, or 'units') of various vegetables, per UK portion (80 g) and the associated strong evidence decreased risk of cancer of according to the World Cancer Research Foundation (WCRF).

Nitrate Units (mmol)	**Vegetables 1 UK Portion (80 g)**	**WCRF: Strong Evidence for Decreased Risk of Cancer of:**
High **~2** (>1000 mg/kg)	Spinach, lettuce	Mouth, pharynx, larynx, oesophagus, stomach
	Rocket, beetroot, celery, fennel, Chinese cabbage, radish	
Medium **~1/2** (100-1000 mg/kg)	Kale, turnip, cabbage, green beans, cucumber, carrot, leek, sweet pepper	Mouth, pharynx, larynx, oesophagus, stomach
	Spring onion, garlic	Stomach
Low **~1/10** (<100 mg/kg)	Onion	Stomach
	Tomato	Mouth, pharynx, larynx, oesophagus, stomach
Low **~1/10** (<100 mg/l)	Water (250 ml): tap water	
Very Low **~1/100** (<10 mg/l)	Mineral water	

Table 2. Nitrate-dietary/lifestyle Combinations. Evidence for potentially beneficial combinations of dietary nitrate with other common components of the Mediterranean diet/lifestyle.

Additional component	Effect of additional dietary/lifestyle component in combination with dietary nitrate/nitrite
Ascorbic acid (vitamin C)	• Reducing agent: enhance nitrite reduction to NO and vasorelaxation [69] • Inhibit N-nitrosamine formation [53] • Decreases gastro-oesophageal cancer risk [52]
Oleic acid **(Olive oil)** **&** **Conjugated Linoleic acid** **(e.g. milk from ruminants)**	• Form nitro-fatty acids: nitro-oleic acid and nitro-linoleic acid: • anti-inflammatory properties (inhibiting neutrophils, platelets and macrophages) – e.g. in model of colitis, [72] • S-alkylation of nuclear factor κB (resulting in inhibition of macrophage cytokine and iNOS expression) [74] • and peroxisome proliferator-activated receptor-γ (PPAR-γ) [72, 73], • reduced atherosclerotic lesion formation in apoliproprotein E-deficient mice (reduced foam cell formation/ oxidised LDL-induced phosphorylation of signal transducer and activator of transcription-1 (STAT-1) [75]; • inhibition of soluble epoxide hydrolase (sEH) with accumulation of epoxyeicosatrienoic acid (EET) substrates which lowered BP in angiotensin II-induced hypertension model [80]
Cocoa flavanols	• low dose cocoa (3 mg/kg) with nitrate (2.7 mg/kg) increased flow mediated dilatation [82]
Red wine polyphenols	• anthocyanin fraction and catechol (caffeic acid) enhance nitrite reduction to NO in the stomach [59]
Dietary phenols	• nitrite reductase activity: • epicatechin-3-O-gallate, quercetin, procyanidin B8 dimer, oleuropein, procyanidin B2 dimer, chlorogenic acid, epicatechin, catechin, procyanidin B5 dimer [83]; • Hawthorn berry extract [84]
Alcohol	• ethanol is nitrosated forming ethyl nitrite, a potent vasodilator [85]; • inhaled ethyl nitrite improves arterial oxygenation and haemodynamics in persistent pulmonary hypertension of the newborn [89]
Sunlight UVA irradiation	• UVA mobilises nitrite from the skin and dermal vasculature [86] • UVA increases circulating nitrite concentration and lowers blood pressure [87] • UVA + nitrate supplementation reduces VO_2 during steady-state exercise and improves performance in a time trial [88]

Table 3. Twists and turns in the realisation of the nitrate-nitrite-NO pathway. Complete Table including the earlier questions covered in Part I.

	Negative/Neutral	Positive
Background to nitrate's (NO_3^-) & nitrite's (NO_2^-) properties?	Physiologically inert metabolites of NO [90]	
1. Is nitrite a source of NO?	No – nitrite is a physiologically inert metabolite of NO [90]	Yes – in the ischaemic heart: Zweier et al.,1995 [91]
2. What effect does nitrite have in IRI?	Deleterious - Zweier et al., 1995 [91]	Protective - Webb et al., 2004
3. Does nitrite dilate blood vessels?	No – Lauer et al., 2001 [92]	Yes - Modin et al., 2001 [69], Cosby et al., 2003
4. Are there nitrite reductases?	Non-enzymatic/non-NOS Zweier et al.,1995 [91]	Hb [34], Mb [93], XOR [1], AO [94], eNOS [95]
5. Is nitrate a source of NO?		Benjamin et al., 1994 [96], Lundberg et al.,1994 [97]
6. Does nitrate inhibit platelets?		Yes – McKnight et al., 1999 [98]
7. Does nitrate inhibit platelets via SNOs?	No? – Richardson et al., 2002 [99]	
8. Does nitrate increase plasma nitrite?	No - Pannala et al., 2003 [100]	Yes – Lundberg et al., 2004 [101], Webb et al., 2008 [2]
9. Does nitrate lower BP?		Yes – Larsen et al., 2006 [102], Webb et al., 2008 [2]
10. Does nitrate lower BP via nitrite?		Yes – Webb et al., 2008 [2]
11. Is nitrate protective in IRI?		Yes – Webb et al., 2008 [2]
12. Does nitrite inhibit platelets?	No – Bryan et al., 2005 [4]	Yes – Webb et al., 2008 [2]
13. Does nitrate/nitrite exhibit tolerance?	No – Dejam et al., 2007 [9]; Vanhatalo et al., 2010 [11]; Sobko *et al*., (2010) [10]; Kapil et al., [12]	
14. Do nitrate/nitrite alter		Yes – exercise – Larsen 2007 [19], 2011 [21], Bailey

mitochondrial metabolism?		2009 [18]
15. Is nitrite bioactivation hypoxia dependent?	Yes – most of field [103]	Not always – Omar 2015 [35]
16. Is nitrate-derived nitrite harmful?	Yes – carcinogenic potential - early studies [104]	No definite association Bryan [43]
17. Other therapeutic opportunities for nitrate-nitrite		Subarachnoid haemorrhage [60]; pulmonary arterial hypertension [62]
18. Are there potentially beneficial dietary combinations?	Ascorbic acid, oleic acid, conjugated linoleic acid, cocoa flavanols, dietary and red wine polyphenols, alcohol, sunlight	