Local delivery of nitric oxide prevents endothelial dysfunction in periodontitis

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**A B S T R A C T**

**Aims:** Increased cardiovascular disease risk underlies elevated rates of mortality in individuals with periodontitis. A key characteristic of those with increased cardiovascular risk is endothelial dysfunction, a phenomenon synonymous with deficiencies of bioavailable nitric oxide (NO), and prominently expressed in patients with periodontitis. Also, inorganic nitrate can be reduced to NO in vivo to restore NO levels, leading us to hypothesise that its use may be beneficial in reducing periodontitis-associated endothelial dysfunction. Herein we sought to determine whether inorganic nitrate improves endothelial function in the setting of periodontitis and if so to determine the mechanisms underpinning any responses seen.

**Methods and results:** Periodontitis was induced in mice by placement of a ligature for 14 days around the second molar. Treatment in vivo with potassium nitrate, either prior to or following establishment of experimental periodontitis, attenuated endothelial dysfunction, as determined by assessment of acetylcholine-induced relaxation of aortic rings, compared to control (potassium chloride treatment). These beneficial effects were associated with a suppression of vascular wall inflammatory pathways (assessed by quantitative-PCR), increases in the anti-inflammatory cytokine interleukin (IL)-10 and reduced tissue oxidative stress due to attenuation of xanthine oxidoreductase-dependent superoxide generation. In patients with periodontitis, plasma nitrite levels were not associated with endothelial function indicating dysfunction.

**Conclusion:** Our results suggest that inorganic nitrate protects against, and can partially reverse pre-existing, periodontitis-induced endothelial dysfunction through restoration of nitrite and thus NO levels. This research highlights the potential of dietary nitrate as adjunct therapy to target the associated negative cardiovascular outcomes in patients with periodontitis.

**Abbreviations:** ABC, Alveolar bone crest; ACh, Acetylcholine; CEJ, Cemento-enamel junction; CVD, Cardiovascular Disease; DEA-NO, Diethylamine-nitrosoate; DPI, Diphenyliodonium; eNOS, endothelial nitric oxide synthase; FMD, Flow mediated dilation; IL, Interleukin; iNOS, Inducible nitric oxide synthase; KCl, Potassium Chloride; KNO2, Potassium Nitrate; L-NAME, N’-nitro-l-arginine methyl ester; NADH, Nicotinamide adenine dinucleotide; NADPH, β-Nicotinamide adenine dinucleotide 2’-phosphate; NFκB, Nuclear factor kappa-light-chain-enhancer of activated B cells; OTUs, Operational Taxonomic Units; TNFα, Tumour necrosis factors; XDH, Xanthine dehydrogenase; XOR, Xanthine oxidoreductase.

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1. Introduction

It is generally accepted that endothelial dysfunction is synonymous with a lack of bioavailable NO and this deficiency of NO is thought to underlie the consequences of endothelium dysfunction i.e. vasoconstriction, pro-inflammatory and pro-thrombotic state, [1] ultimately leading to an increased risk of cardiovascular disease (CVD). In this regard, increasing evidence shows that inorganic nitrate (NO₃⁻) offers an approach to restore NO levels to the cardiovascular system, that is independent of the conventional L-arginine-NO synthase pathway and easy to administer [2]. Inorganic nitrate is found in vegetables, particularly the green-leafy variety and beetroot. When ingested nitrate is rapidly absorbed across the upper intestine and enters the circulation. The majority of this absorbed nitrate is excreted in the urine, however up to 25% is actively extracted by the salivary glands, via the salian transporter, [3] and secreted into the saliva where it can be reduced to nitrite by commensal bacteria expressing nitrate reductases [4]. The nitrite-rich saliva is then swallowed and whilst a fraction of the nitrite is chemically reduced to NO in the acidic environment of the stomach a significant proportion of this nitrite enters the circulation [5]. Here, this nitrite is reduced to NO by vascular nitrite reductases [6]. Bioactivation of nitrate via this pathway is associated with a number of beneficial effects including blood pressure lowering, [5,7] repression of platelet activation [5,8] and inhibition of inflammatory pathways [9]; a profile of activity offering a potential route to overcoming the negative impact of endothelium dysfunction in CVD. Plasma nitrite levels have been proposed to indicate endothelial nitric oxide synthase (eNOS) function and correlate with vascular function; the latter a relationship thought to reflect endothelial health, [10,11] and due to shear stress-induced endothelial NO generation [12,13].

Periodontitis is a chronic inflammatory disease with a dysbiotic biofilm at the interface between gingiva and the teeth. The oral inflammation leads to alveolar bone destruction and loss of soft tissue attachment to the teeth and eventually tooth loss. Individuals with periodontitis during their life-course are 20–30% more likely to have a heart attack, stroke, or other serious cardiovascular event, [14,15] independent of confounding factors such as smoking [16]. Although the mechanisms accounting for the relationship between oral disease and CVD have not been fully defined, the generally accepted view is that periodontitis is associated with endothelium dysfunction; [17–19] a phenomenon occurring early in the disease process and thought to play a key role in CVD pathogenesis and progression [20]. Although, it should be noted that there is a population study in individuals in Germany indicating that brachial artery flow mediated dilation (FMD) increases with worsening of periodontitis [21]. Treatment of severe periodontitis has been consistently linked to improvement of endothelial function [17,22] and this beneficial effect seems to be mediated by a reduction of chronic inflammation.

A consequence of systemic inflammation is activation of an oxidative stress within the vascular wall driven in part by neutrophil and endothelium-derived free radical species generation [23]. These reactive oxygen species react with free NO leading to a loss of bioavailable NO and thus also a loss of positive downstream NO signalling (including activation of guanylyl cyclase), which further exacerbates endothelial dysfunction. Importantly, it has also been proposed that the inflammation and oxidative stress induced within the oral cavity during periodontitis transfers into the systemic system and is evident within cardiovascular tissues (for review see [24]). Recent evidence suggests that inorganic nitrate attenuates oxidative stress in models of hypertension; an effect linked to reduced expression of the key pro-oxidant enzyme NADPH oxidase [25,26]. In addition, we and others have proposed that superoxide generation from xanthine oxidoreductase (XOR) is suppressed by inorganic nitrite; an effect due to a shift in the biochemical activity of the enzyme resulting in enhanced NO levels at the expense of superoxide production [27]. Thus, in this manuscript we have sought to determine whether NO levels and endothelial function correlate in patients with periodontitis and, using an experimental model simulating oral disease, tested whether dietary supplementation with inorganic nitrate might offer an approach to combat vascular dysfunction in this setting. Furthermore, we have interrogated the mechanisms involved in any effects seen to highlight any novel approaches that might be exploited for therapeutics.

2. Materials and methods

For expanded methods see on-line supplement.

2.1. Animal experiments

All experiments were conducted according to the Animals (Scientific Procedures) Act 1986, UK, and approved by the UK Home Office. Animal studies are reported in compliance with the ARRIVE 2.0 guidelines [28]. Male C57BL/6 J (25–27 g, Charles River Ltd, Margate, UK) were used and housed in individually ventilated cages of 4–5 mice on a 12 h light/dark cycle and allowed food and water ad libitum. Mice were randomly allocated to surgical and treatment groups.

2.2. Procedure for ligature placement and sham surgery

Ligature placement was performed as previously described [29] with some modifications. Briefly, a 5/0 cotton ligature was placed around the cervix of the maxillary second molars on both the right and left sides to induce experimental periodontitis. Ligatures remained in place in mice throughout the experimental period for 14 days. For animals allocated to sham surgery, following anaesthesia ligatures were tied around the molar teeth, as described above, and then removed immediately.

2.3. Dietary treatment with KNO₃ (intervention) or KCl control

Mice were randomly assigned to receive drinking water supplemented with previously validated concentrations of potassium nitrate (KNO₃, 15 mmol/L) [9] or potassium chloride (KCl, 15 mmol/L, control) 1 week prior to ligature placement or sham procedure, and continued for the duration of the experiment (‘pre-treatment’ protocol). In a separate experiment, mice were randomly assigned to receive drinking water supplementation for 2 weeks, at 7 days following ligature implantation or sham surgery (‘reversal’ protocol).

2.4. In vivo cardiac functional assessment

In some experiments echocardiography (VEVO 3100) was conducted at 14 days post-ligature implantation.

2.5. Alveolar bone loss assessment

Maxilla were scanned by Micro-CT (VECTor®CT) with the following parameters: tube voltage 50 kv, tube current 0.21 µA, exposure time 75 ms and the bone volume fraction (BV/TV) calculated.

2.6. Blood analyses

Flow cytometry of whole blood was conducted following incubation with fluorochrome-conjugated antibodies. Plasma nitrite and nitrate levels were measured by ozone based chemiluminescence. Plasma IL-1β, TNFα, IL-6, IL-10, CXCL1 and CXCL12 levels were determined using DuoSet ELISAs (R&D Systems, UK) according to manufacturer’s guidelines. Plasma CCL2 and CCL5 levels were determined using the BD™ Cytokine Bead Array according to manufacturer’s guidelines (BD Biosciences, UK). Plasma endothelin levels were determined using Endo-LISA® (Hyglos, Germany) ELISA according to manufacturer’s guidelines.
2.7. Inflammatory cell profiling within the heart

Hearts were isolated and single cell suspensions prepared which were analysed using flow cytometry.

2.8. Organ bath pharmacology

Thoracic aortae were mounted in organ baths and vascular reactivity determined in response to acetylcholine, spermine NONOate or phenylephrine.

2.9. Langendorff isolated heart preparation

Hearts were excised and perfused retrogradely via the aorta and coronary perfusion pressure determined in response to N\textsuperscript{G}-nitro-\textit{l}-arginine methyl ester (L-NAME).

2.10. Assessment of inflammatory marker mRNA expression

Gingival, heart and aortic tissue were homogenised and RNA extracted for quantitative RT-PCR (qRT-PCR) analysis of inflammatory markers. These genes were selected as they have been shown to be elevated in patients with periodontitis.

2.11. Oral bacterial community profiling

DNA was extracted from implanted ligatures, and second molars using the GenElute Bacterial DNA Extraction Kit (Sigma-Aldrich, UK) according to the manufacturer’s instructions. Samples were then subjected to qPCR analysis for 16 S. In addition, 16 S rRNA genes were amplified using universal primers and next generation sequencing (NGS) performed using the MiSeq platform (Illumina).

2.12. Superoxide determination

Heart and aorta were homogenised and superoxide levels of the homogenates determined using a lucigenin-enhanced chemiluminescence assay.

2.13. Western blotting

Aorta were homogenised and the expression of eNOS and NF\textkappaB determined using Western blotting.


The modified ligature-induced mouse model of periodontitis was established in initial exploratory experiments with \( n = 5 \) and 6 in each of the sham and ligature implanted groups respectively, to assess bone loss and vascular function. In addition, a further exploratory experiment (\( n = 9 \)) to determine appropriate \( n \) values for mechanistic inflammatory pathway markers, including mRNA analysis of gingival and cardiovascular tissues was conducted. These data were utilised to power further experiments assessing the impact of inorganic nitrate on vascular function and inflammation. Our analyses demonstrated that \( n = 6 \) is sufficient to assess efficacy in vascular studies but that for a statistically significant inhibition of inflammation using the relative TNF\alpha mRNA levels as the primary outcome measure an \( n = 14 \) is required for an effect size of a difference of 1.13 with an average SD of 0.52 with an \( \alpha = 0.05 \) and a power of 0.8. In order to account for potential technical loss \( n \) value was increased to 15. All other assessments conducted to interrogate mechanisms were based upon sample availability.

2.15. Proof-of-concept: analysis of plasma samples in patients with periodontitis

To enable proof-of-concept we analysed a sub-group of plasma samples of a cohort of patients recruited into the on-going ‘periodontitis and vascular dysfunction study’ (ClinicalTrials.gov Identifier: NCT03072342). Following provision of informed consent from participants suffering from moderate to severe periodontitis (at least 30 periodontal pockets with probing pocket depth >4 mm and bleeding on probing) patients were enrolled into a randomised controlled trial on the effect of the treatment of periodontitis on cardiovascular outcomes. In this trial blood samples were collected and endothelial function assessed using flow mediated dilatation (see supplement for details). We collated the endothelial function data at baseline with measurement of plasma nitrite and nitrate levels measured using ozone chemiluminescence (see supplement for details). The study was approved by the London Queen Square Research Ethics Committee (06/Q0512/107) and all procedures conformed to the principles outlined in The Declaration of Helsinki.

2.16. Statistical and data analysis

Data are expressed as mean ± SEM of \( n \) mice. Statistical significance was determined using unpaired t-test, One-way ANOVA followed by Bonferroni’s or Dunnett’s post hoc t-test, or Two-way ANOVA, followed by Bonferroni’s post hoc t-test as appropriate and indicated in the legends. A P value < 0.05 was considered significant. Gaussian distribution and homogeneity of variances of datasets were determined by the Shapiro-Wilk test. Concentration-response curves were fitted using nonlinear regression. Agonist potencies and maximum responses were expressed as the logarithm of the molar concentration of agonist producing 50% of the maximum response (EC50) and the maximum effect elicited by agonist (Max), respectively. For all experiments \( n \) values were equal by design however where unequal \( n \) values within experiments are shown this relates to technical failure or from identification of outliers using the ROUT [30] exclusion test. Where any exclusions have occurred, this has been stated explicitly in the figure legends. All analysis was conducted using Graphpad Prism 8.0.

3. Results

3.1. Ligature implantation induces periodontitis and an associated ‘modest’ systemic inflammation

We first sought to confirm that an experimental model of oral inflammation in mice, simulating periodontitis, also displayed endothelial dysfunction (previously not shown). Implantation of ligatures resulted in an immediate and pronounced decrease in food and water consumption with associated weight loss; all indices recovered to baseline levels over the following 2 days. Identical changes were evidenced in the sham-operated mice (Fig. 1 B-E). Bone loss was accompanied by an increase in gingival expression of the mRNA of pro-inflammatory cytokines IL-1\beta, IL-6 and tumour necrosis factor (TNF\alpha) (Fig. 1 F-H), the reactive oxygen species (ROS) generator xanthine dehydrogenase (XDH, Fig. 1 I) and inducible nitric oxide synthase (iNOS, Fig. 1 J); as per previous observations [31–33]. These changes in markers of inflammation were associated with changes in gingival chemokine mRNA expression of CCL5, CCL2, CX3CL1 and CXCL2 (Fig. 52A-D) with no change in CXCL1, CXCL5 and the anti-inflammatory cytokine IL-10 (Fig. 52E-G). In addition, ligature-implantation led to an oral bacteremia (Fig. 1K) with bacterial community profiling indicating a pronounced (\( p < 0.001 \)) alteration in bacterial community structure with a mean coverage of the bacterial communities of 96% and a significant reduction in bacterial richness, diversity and composition (Fig. 1L-O) versus the sham operated mice. These differences were driven predominantly by changes in...
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the levels of a number of distinct Operational Taxonomic Units (OTUs) (Table S1) including particularly two unclassified strains of Enterobacteriaceae and Actinobacteria taxa.

This localised oral inflammation and oral bacteraemia did not lead to measurable endotoxin levels in the blood (Fig. 2A) or observable changes in total circulating leucocyte number (Table S2). However, assessment of leucocyte sub-populations with flow cytometry identified a doubling of circulating neutrophil numbers (Fig. 2B and Table S2). Further assessment of the activation state of these cell types showed no difference in the expression of a range of leucocyte activation markers CD62L, CD162, or CD11b (Table S3). Interestingly, despite clear evidence of a neutrophilia, albeit mild, there were measurable rises in the levels of various circulating cytokines and chemokines in ligature-implanted mice compared to sham including IL-1β, CXCL1, CXCL12, CCL2 and CCL5 (Table S4). Additionally, whilst we found no evidence of an endotoxaemia we observed increased bacterial DNA levels within cardiovascular tissues (Fig. 2C and D), suggesting that whilst a robust oral inflammation is induced in this model of periodontitis that the associated and consequent systemic inflammation is modest and likely associated with entry of bacteria within peripheral tissues; importantly these observations do fit well with the clinical scenario (for review see [34]).

In line with the above, the mRNA expression for IL-1β and TNFα (Fig. 2E and F) were raised in cardiac tissue, associated with a trend for suppression of IL-10 in ligature-implanted mice in comparison to sham-operated mice (Fig. 2G); no differences were found in all other cardiac mRNA assessed (Fig. S3). These findings indicating a pro-inflammatory state. To determine whether this low level of inflammation might result in changes in vascular function, particularly with respect to endothelial function, we assessed vascular responses to endothelium-dependent (Acetylcholine, ACh) and independent vasodilators (Spermine NONOate) in aortic rings. We show a selective endothelial dysfunction, reflected by reduced vasodilator responses to ACh in the ligature-implanted group compared to sham (Fig. 2H). In contrast, no
differences between the groups were observed in response to Spermine NONOate or the vasoconstrictor phenylephrine (Fig. 2I and J). These results demonstrate endothelial dysfunction, mimicking that observed in patients with periodontitis [17,18].

3.2. Dietary nitrate reduces gingival inflammation and prevents endothelial dysfunction

Since inorganic nitrate improves endothelial function in patients with CVD we reasoned that benefits might also be accrued in periodontitis. We used a previously validated dose for mice of 15 mM KNO₃ in the drinking water [9]. Levels of nitrate and nitrite were raised in both sham and ligature-implanted mice (Fig. 3A and B) indicating expected ingestion of the anion and conversion as expected via the enterosalivary circuit. There were no differences between the groups with respect to drinking water or food consumption and no difference between KNO₃-treated sham and ligature-implanted mice with respect to dose of nitrate received (Fig. S4A-C). Food ingestion was reduced, approximately 70%, immediately following sham or ligature procedure but all groups returned to pre-surgery levels within 24 h with no differences between KCl or KNO₃-treated mice (Fig. S4C). These findings indicate that the model provides a robust scenario for assessing the effects of dietary nitrate in this experimental model simulating periodontitis.

Treatment with KNO₃ had no significant effect on alveolar bone loss (Fig. 3C and S5A-B). However, there was some evidence of reduced gingival inflammation with a significant reduction in gingival IL-1β mRNA (Fig. 3D) expression, a trend to lower IL-6 (Fig. 3E) but no effect upon TNFα (Fig. 3F), and importantly an elevation of the mRNA levels of the anti-inflammatory IL-10 cytokine (Fig. 3G). We did not observe changes in gingival mRNA levels of any of the other pro-inflammatory mediators assessed (Fig. S5). NGS of the oral microbiome indicated no impact of KNO₃ treatment versus KCl treatment on the bacterial community (Fig. 3H-K). However, KNO₃ treatment completely reversed the endothelial dysfunction caused by ligature implantation (Fig. 4A and Table S5), whilst responses to spermine NONOate (Fig. 4B and Table S5) and phenylephrine (Fig. 4C and Table S5) were unchanged. To examine vascular function further and particularly with relevance for CVD we also assessed coronary vascular reactivity using the isolated Langendorff method. We show that whilst baseline CPP was not altered between groups (Sham KCl, 106.1 ± 10.5 mmHg; Sham KNO₃ 102.5 ± 7.8 mmHg; Ligature KCl 113.7 ± 7.3 mmHg; Ligature KNO₃ 125.8 ± 7.8 mmHg, n = 6–10), that the vasoconstriction to L-NAME was substantially impaired in control ligature implanted mice and that KNO₃ treatment restored impaired vascular responses to L-NAME (Fig. 4D) whilst responses to the NO donor sodium nitroprusside were unaltered (Fig. 4E). This improved endothelial function was associated with improved eNOS activation reflected by increased phospho-eNOS (p-eNOS at s1177) expression in the aorta (Fig. 4F). Unexpectedly, this increased phospho-eNOS expression was associated with increased in NFκB activation Fig. 4G.

Assessment of cardiac function using echocardiography identified modest non-statistically significant effects upon LV structure and

Fig. 3. Inorganic nitrate reduces gingival inflammation. Treatment of mice with nitrate resulted in elevated plasma levels of nitrate (A) and nitrite (B). Ligature implantation resulted in bone loss that was unaffected by inorganic nitrate (C). Inorganic nitrate attenuated gingival IL-1β (D) and IL-6 (E) with no effect on TNFα (F) and elevated IL-10 (G) mRNA expression. Ligature implanted mice fed KNO₃ or KCl (15 mmol/L) in drinking water showed similar species richness (H) and diversity (I). Principal coordinate plot based on ThetaYC metric (J) showed a similar bacterial community structure. Heatmap shows the relative abundance of bacterial genera (K) Ligature KCl n = 13; Ligature KNO₃ n = 12. For plasma NO₂ Sham KCl/KNO₃ n = 13; Ligature KCl n = 20; Ligature KNO₃ n = 19. For bone loss Sham KCl n = 12; Sham KNO₃ n = 13; Ligature KCl n = 14; Ligature KNO₃ n = 15. For qRT-PCR ligature KCl/KNO₃ n = 14. For Langendorff Sham KCl n = 6; Sham KNO₃ n = 5; Ligature KCl n = 9; Ligature KNO₃ n = 6. For plasma NO₂, bone loss, qRT-PCR and NGS, statistical significance was determined using unpaired t-test. 3 samples for qRT-PCR in KNO₃-treated mice lost due to technical failure.
function of ligature implantation with only a slight increase in LV mass evident (Table S6). However, in addition, we observed statistically significant differences in aortic arch flow parameters aligning with the in vitro organ bath pharmacology. Whilst no differences were evident between Sham KCl and KNO₃ treated animals, there were significant reductions in peak (977.3 ± 30.4 mm/s vs 1074.0 ± 27.0 mm/s in KNO₃-treated mice versus KCl-treated (P = 0.029)) and mean aortic arch blood flow velocity (550.0 ± 18.5 mm/s versus 603.2 ± 15.0 mm/s (p = 0.03)) respectively in mice implanted with ligatures. Thus, KNO₃ treatment causes a reduction in oral inflammation together with a protection against the associated vascular dysfunction consequent to the oral pathology in both the conduit and coronary vasculature.

Fig. 4. Inorganic nitrate protects against aortic and coronary endothelial dysfunction. In isolated aortic rings acetylcholine-induced relaxation responses were reduced in mice implanted with ligatures; an effect reversed by treatment with inorganic nitrate (A). There was no effect of ligature implantation or KNO₃ treatment upon Spermine NONOate (B) or phenylephrine-induced responses (C). In isolated Langendorff hearts, the response to L-NAME (300 μM) (D) was reduced in mice implanted with a ligature compared to sham control. Ligature implanted mice pre-treated with inorganic nitrate had a significantly elevated CPP in response to L-NAME. CPP response to SNP (10 nmol) was similar across the groups (D). In aortic homogenates, expression of phospho-eNOS (F) and phospho-NFκB (G) were elevated by treatment with KNO₃. For concentration-response curves statistical significance was determined using Two-way ANOVA followed by Bonferroni post hoc test represented by ***P < 0.0001. For Langendorff data statistical significance was determined using One-way ANOVA followed by Sidak’s post hoc test. For Western blotting aorta ligature comparisons were conducted using unpaired Students T test for KCl/KNO₃ n = 8.
3.3. Dietary nitrate reduces circulating neutrophil number and aortic superoxide production

To determine whether the improvement in vascular function might be secondary to a reduction in systemic inflammation, we assessed a wide panel of circulating inflammatory biomarkers. We observed no differences in total circulating leucocyte counts (Fig. S6A), resident or inflammatory monocyte numbers or activation state (Fig. S6B and C and Table S7) between the treatment groups. However, treatment with KNO$_3$ caused a reduction in the neutrophilia consequent to ligature-implantation (Fig. 5A), albeit no observed change in the activation state of the neutrophil (Table S7). This difference in neutrophil numbers was not due to differences in the circulating levels of IL-1β (key cytokine involved in neutrophil recruitment) (Fig. 5B) or to differences in the bone marrow neutrophil mobilisation chemokine CXCL1 (Fig. 5C). However, we did observe a reduction in plasma CXCL12 (Fig. 5D), with

![Graph A](imageA.png)

![Graph B](imageB.png)

![Graph C](imageC.png)

![Graph D](imageD.png)

![Graph E](imageE.png)

![Graph F](imageF.png)

**Fig. 5.** Inorganic nitrate reduces ligature induced neutrophilia. The development of oral inflammation was associated with a neutrophilia that was suppressed by pretreatment with inorganic nitrate (15 mmol/L KNO$_3$) in comparison to KCl (15 mmol/L) control (A). In contrast plasma levels of IL-1β (B) and CXCL1 (C) were similar between the groups. Plasma CXCL12 (D) levels were reduced by inorganic nitrate. Pretreatment of ligature-implanted mice with inorganic nitrate (15 mM KNO$_3$) resulted in an elevation of IL-10 (E) and CXCL5 (F) mRNA levels in comparison to mice treated with KCl (15 mM) control in homogenates of heart tissue. For neutrophil count Sham KCl n = 6; Sham KNO$_3$ n = 5; Ligature KCl n = 15; Ligature KNO$_3$ n = 13. For IL-1β n = 8; CXCL1 n = 25; CXCL12 KCl n = 9, KNO$_3$ n = 8; For qRT-PCR KCl n = 13; KNO$_3$ n = 12. Statistical significance was determined using unpaired t-test comparison of KCl treatment with KNO$_3$ treatment in either ligature implanted or sham operated mice. (CXCL1 5 KCl and 3 KNO$_3$ data points and CXCL5 1 KNO$_3$ excluded using Rout’s outlier test).
KNO$_3$ treatment, which was unexpected as CXCL12 functions to retain neutrophils within the bone marrow. Despite this, a significant elevation in IL-10 mRNA expression was evident in cardiac tissue (Fig. 5E), together with an increase in CXCL5 (Fig. 5F) with a trend to lower CCL5 cardiac mRNA expression (Fig. 5A). No differences were found in any of the cardiac mRNA levels of the other pro-inflammatory mediators assessed (Fig. 5B-K) and no change in the levels of bacteria, measured within cardiac tissue, were found (Fig. 5L). Additionally, in a small exploratory study of 8 mice in each group we assessed the numbers of resident macrophages or neutrophils. Whilst we found no statistically significant changes there was a trend towards a reduction in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O). No differences were observed in the numbers of inflammatory macrophages within the heart in mice treated with inorganic nitrate (Fig. 5M-O).

In sham and ligature implanted mice we assessed superoxide production in aortic and cardiac homogenates and found no difference in the basal superoxide generation (Fig. 6A and B). We also assessed xanthine, NADH and NADPH-driven superoxide production in these tissues and found no differences (Fig. 6C-E). In order to assess the role of xanthine oxidase or NADPH oxidase driven ROS generation in these tissues, aortic homogenates were preincubated with the XOR inhibitor febuxostat, or the non-selective FAD inhibitor, DPI. Xanthine driven superoxide production was inhibited by febuxostat but this inhibitor had no effect upon NADH or NADPH-driven superoxide generation. In contrast, DPI significantly attenuated the response to all 3 substrates (Fig. 6C-E), whilst there was no significant effects of the NOS inhibitor L-NAME on superoxide generation (Sham 480 ± 88; Sham + L-NAME 438 ± 74; Ligature 530 ± 101; Ligature + L-NAME 503 ± 87, n = 12). Whilst basal levels of superoxide generation were equivalent between KCl or KNO$_3$, in aortic or cardiac homogenates collected from ligature-implanted mice (Fig. 6F and G), xanthine (Fig. 6H and I), but not NADH (Fig. 6J and K) or NADPH (Fig. 6L and M)-driven superoxide production was reduced by KNO$_3$ treatment. Xanthine-driven superoxide generation in these tissues was inhibited by febuxostat or DPI treatment (Fig. 6N). These data suggest that XOR-derived superoxide may have a role in initiating endothelial dysfunction in this experimental model of periodontitis.

3.4. Effect of inorganic nitrate supplementation on established periodontitis and associated endothelial dysfunction

Since within the clinical setting treatment for periodontal disease is initiated only once disease is evident, we assessed whether dietary inorganic nitrate might improve vascular function if initiated only once gingival inflammation and bone loss were established. We show that whilst dietary nitrate treatment at 7 days post ligature implantation did not prevent alveolar bone loss (Fig. 7A) or gingival mRNA IL-1β expression (Fig. 7B), that it partially reversed endothelial dysfunction by restoring the magnitude of the maximum response to endothelial stimulation induced by AChs (Fig. 7C and Table S8). Responses to Spermine NONOate and phenylephrine were unaffected (Fig. 7D and E and Table S8), and there were no differences between the groups in the amount of nitrate, food or water consumed during the experiment (Fig. S9A-C).

3.5. Plasma nitrite and FMD do not correlate in patients with periodontitis

Finally, to determine whether the observations evident in this mouse experimental system may be of relevance to the clinical human setting we determined whether patients with periodontitis with endothelial dysfunction might also display reduced circulating nitrite levels. Fig. 8 demonstrates that unlike healthy volunteers circulating nitrite levels in patients with periodontitis (Table S9) were not correlated with FMD (Fig. 8); indicating a loss of the relationship between FMD and nitrite in healthy volunteers; [10,11]. We speculate that this lack of association relates to impaired endothelial function and deficiencies in vascular NO generation that could be rectified with dietary nitrate treatment as shown in this study in mice.

4. Discussion

Herein, we demonstrate that a simple dietary intervention based upon elevation of inorganic nitrate intake, resulting in consequent rises in circulating nitrate and nitrite, improves vascular function in a mouse model of periodontitis. Our findings suggest that this effect is, in part, due to direct targeting of the systemic vasculature to attenuate the pro-inflammatory and pro-oxidative effects associated with periodontal disease. More importantly, we highlight the potential benefits of treatment with dietary nitrate to improve vascular function in patients with periodontitis and further suggest that those individuals globally (primarily low or middle income countries) with limited access to state of the art dental therapy might benefit from access to a cheap dietary intervention of inorganic nitrate to protect against one of the main causes of death in such patients.

The nitrate-nitrite-NO pathway has been proposed as a complementary source of NO [2] in CVD where the conventional pathways for NO synthesis are compromised [35]. Here, we show that dietary inorganic nitrate treatment results in an elevation of circulating concentrations of both nitrate (~5.4-fold) and nitrite (~1.5-fold), indicating an intact enterosalivary circuit in ligature-induced periodontitis in mice. This occurred even though a substantial shift in the oral bacteria community occurred with oral disease development, indicating that oral bacterial nitrate reductase activity, a key step in the bioactivation of nitrate via the enterosalivary circuit, was unaltered in the disease setting.

Ligature implantation, as a method to induce oral inflammatory disease, resulted in a number of pathological outcomes typical of periodontitis. We found an elevation of IL-1β, TNFα and IL-6 in the gingiva; cytokines all found in elevated levels in gingival crevicular fluid in human periodontal disease [36]; observations recently confirmed in meta-analysis for IL-1β and IL-6, but not for TNFα [37]. Additionally, we observed elevations in CCL5, CX3CL1 and iNOS; inflammatory factors also elevated in patients [38-40]. As per human disease, the key debilitating consequence of this oral inflammation in mice is severe bone loss, another core feature of human periodontitis. These observations provide confidence that this model faithfully reproduces the key features of the human periodontal disease scenario.

Pre-treatment with inorganic nitrate had no effect on alveolar bone loss and did not alter the bacterial community despite the profound change in the community in the disease setting. These results indicate that any effects of dietary nitrate did not relate to changes in the oral bacterial community. This is in contrast to a recent study demonstrating a compositional change among oral bacteria and an increase in the abundance of nitrate reducing bacteria in subgingival samples taken from periodontal pockets, in which patients were treated for 14 days with nitrate-rich lettuce juice [41]. Furthermore, previous studies in both hypercholesterolaemic patients and in Wistar rats observed statistically significant shifts in the microbiome community structure following persistent/prolonged dietary nitrate treatment [8,42]. Why such a shift was not evident in this model of periodontitis is uncertain but may relate to the relatively short exposure, the site of collection of the oral sample or the species of the host investigated. Further long-term studies would be of value, although this is difficult in such a severe bone loss model.

Whilst no change was observed in alveolar bone loss a reduction in...
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(a) Aorta Baseline

(b) Heart Baseline

(c) Aorta Xanthine

(d) Aorta NADH

(e) Aorta NADPH

(f) Aorta Baseline

(g) Heart Baseline

(h) Aorta Xanthine

(i) Heart Xanthine

(j) Aorta NADH

(k) Heart NADH

(l) Aorta NADPH

(m) Heart NADPH

(n) Aorta Xanthine

(caption on next page)
key inflammatory mediators within the gingival tissue were evident coupled with a rise in the levels of the anti-inflammatory cytokine IL-10. This profile fits well with recent observations in the Apolipoprotein E knockout mouse model of atherosclerosis where dietary nitrate exerted an anti-inflammatory effect over the atheroma, stabilising the plaque, that was attributed to a rise in anti-inflammatory monocyte derived IL-10 [9]. This profile of change is similar to that caused by other effective treatments for periodontitis including oral hygiene instructions, scaling and root surface debridement and surgical periodontal treatment [43]. The importance of IL-10 in protection against oral disease has also recently been highlighted in IL-10 KO mice that express spontaneous bone loss [44] and in patients with periodontitis who have lower levels of IL-10 in gingival crevicular fluid versus healthy controls [45].

Over the last decade it has become increasingly evident that patients with periodontitis during their lifetime experience more CVD; a marker of this being endothelial dysfunction [18]. Importantly, effective treatment of periodontitis is associated with a likewise improvement in cardiovascular function by a reduction of systemic inflammation. Indeed, removal of the dental biofilm associated with an intensive reduction of periodontal inflammation improved endothelial function at 2 and 6 months post treatment, compared to patients receiving standard scaling and polishing of their teeth [17]. A recent large prospective study in Korea demonstrated that increased frequency of tooth brushing lowers the risk of cardiovascular events by 9% and professional cleaning by 14% [46]. Various explanations for this observation have been proposed including alterations in the oral microbiota, systemic inflammatory mediators and oxidative stress, [47,48] all of which we have sought to assess in the studies shown herein. We observed prevention of endothelial dysfunction with dietary inorganic nitrate pre-treatment, as well as partial reversal of the endothelial dysfunction with inorganic nitrate treatment in mice with established periodontitis. These effects were evidenced as improvements in ACh-induced relaxation of isolated aortic rings as well as increased coronary tone with L-NAMe treatment. Both of these responses are mediated by targeting of endothelium-derived NO, intimating improved endothelial NO bioavailability. Supporting this possibility is the observation demonstrating increased phospho-eNOS in aorta with dietary nitrate treatment [49]. This finding could in part explain the improved endothelial function seen in both tissues, although recent evidence has suggested a potential disconnect between eNOS phosphorylation and NO generation [50]. This observation is in direct contrast to previously published findings indicating that nitrate supplementation in healthy rats and mice decrease the levels of phospho-eNOS [51]. The reasons for the differences are uncertain, however it may relate to the fact that in the study of Carlstrom et al., the rodents were healthy and endothelial function intact whilst in the current study the effect of dietary nitrate was assessed in the setting of disease.

Whilst in the former the improvements in vascular function were associated additionally with reduced oral inflammation, in the reversal experiments no improvements in oral inflammation were seen. These findings indicate that the benefits of dietary nitrate upon the vascular system were in part direct and occurred independently of improvements in oral disease. This difference is important since it highlights the opportunities available from a simple dietary intervention to improve cardiovascular function in those with common chronic inflammatory diseases including periodontitis. This data accords with several observations in both animal models and in humans (healthy volunteers and patients) demonstrating that dietary inorganic nitrate improves endothelial function. These benefits have been evidenced in models of ischaemia-induced endothelial dysfunction [5] and hypertension/hypercholesterolaemia-associated endothelial dysfunction [6,52] and our observations herein now add endothelial dysfunction associated with periodontitis to the list.

Patients with periodontitis exhibit systemic inflammation reflected by an elevated number of circulating neutrophils [53,54] and similar to our findings others have shown that neutrophil CD11b and CD62L expression is unchanged in periodontitis patients vs healthy controls [55,56]. Conversely it has been shown that circulating monocytes are activated causing subsequent adherence to vascular endothelium and consequent endothelial dysfunction [57]. It is possible therefore, that rather than a change in leucocyte adhesion molecule expression that endothelial cell adhesion molecule expression, such a P-selectin or VCAM-1, was upregulated and that this drives the tissue inflammatory state. Further studies assessing vascular wall adhesion molecule expression would be of value.

We did not observe a change in circulating IL-1β and levels of IL-6, TNFα and IL-10 were undetectable suggesting that the systemic changes in vascular function were not due to alterations in the circulating levels of these pro/anti-inflammatory mediators. In a recent study of ligature induced periodontitis, no change was found in neutrophil numbers in the bone marrow [58]. In our study, we did not measure bone marrow cell numbers but we did observe raised circulating neutrophil numbers that was reduced by inorganic nitrate treatment. CXCL12, via interaction with CXCR4, is an important regulator of neutrophil mobilisation, particularly within the bone marrow, where it promotes neutrophil retention. We observed a reduction in plasma levels of CXCL12 with a concomitant reduction in circulating neutrophil numbers in dietary nitrate fed mice. CXCL12 is elevated in gingival crevicular fluid in patients with periodontitis [59]. Due to the very small volumes collectable it was not possible to assess this in mice. It is possible that the levels of CXCL12 measured in the blood simply represent spill-over from the bone marrow. Measurement of bone marrow CXCL12 levels is an issue that warrants further investigation. The mechanism by which NO inhibits the expression of various inflammatory markers has been shown to involve inhibition of the transcription factor NFκB; an effect thought to be due to induction and stabilisation of its inhibitor, IκB [60-62]. However, in aortic homogenates, we observed a clear elevation in phospho-NFκB with dietary inorganic nitrate treatment. Such an effect, whilst counterintuitive has been observed previously, both in macrophages and in vascular tissue. In RAW264.7 macrophages the NO donor, diethylamine-NONOate (DEA-NO) at lower concentrations (300 nM-3 μM) caused an elevation in NFκB levels/activity whilst higher concentrations inhibited NFκB activity [63]. Similarly, DETA NO also elevates NFκB activity in endothelium-denuded aortic homogenates, in the presence of lipopolysaccharide [64]. These data suggest that the effects of NO observed in our study are unlikely due to inhibition of NFκB activity.

Separately there is evidence demonstrating that NO regulates endothelial cell calcium concentration and hence activation. Recent studies assessing the vascular transcriptome in aortae from mice treated
with dietary nitrate identified “calcium signalling” pathways as those most enriched; with 30 differentially expressed genes related to calcium pathways altered [65]. Moreover, it is well accepted that reactive oxygen species interferes with endothelium calcium signalling to impair endothelial-mediated function (see [66] for review). It is likely that these pathways are involved in the effects evidenced herein. With respect to how NO drives IL-10 levels, this is less well understood and further studies identifying the exact mechanism for such an effect are required. IL-10 is synthesised by a myriad of immune cells including neutrophils and monocytes [67]. Further analysis of the impact of NO on IL-10 synthetic pathways is required to determine the exact molecular target for this effect.

Inorganic nitrate treatment elevated CXCL5 and IL-10 mRNA with a reduction in CCL5 in heart tissue. CXCL5 is protective in coronary artery disease patients [68] and is thought to be key in triggering changes in CXCL1 and CXCL2 and thus promoting neutrophil mobilisation to the
Importantly, in ligature-implanted mice, inorganic nitrate elevated cardiac CXCL5 levels. We speculate that this could lead to elevated levels of CXCL1 and CXCL2 within the heart promoting neutrophil accumulation in order to clear the infection. Although in our studies we did not find statistically significant elevations in these chemokines, nor did we observe an elevation in neutrophils within the heart. However, of note there was a trend to reduced inflammatory macrophage numbers within the heart. It is possible that the sample size was insufficient to demonstrate statistically significant changes in CXCL1 and CXCL2 and also macrophage numbers and we suggest that this warrants further investigation.

Our studies also provide some support for the suggestion that dietary inorganic nitrate reduced superoxide generation within cardiovascular tissues (Fig. 9). Within the vasculature there are multiple sources of ROS that scavenge and thus reduce NO bioavailability and in this way contribute to endothelium dysfunction [70,71]. In our study we utilised both aortic and cardiac homogenates to assess superoxide generation. Overall, our data supports the view that dietary nitrate treatment lowered levels of superoxide in these tissues through reductions of XOR-derived superoxide generation. These findings concur with our previous suggestions that nitrite reduction by XOR enzyme may occur at the expense of superoxide generated by this enzyme [27,72]. Such an effect suggests that delivery of dietary nitrate likely offers dual benefits through interference with XOR activity: i.e., increase in the provision of NO through elevation of circulating nitrite coupled with suppression of oxygen reduction at the flavin adenine dinucleotide site of the XOR enzyme and thus a reduction in local superoxide levels (for review see [27]). In this study we did not evidence substantial increases in either NADH or NADPH driven superoxide generation, indicating that at least in this model it is xanthine-driven superoxide that is primarily responsible for the superoxide generation linked to this low level dysfunction. Thus, we show novel data suggesting that at least one of the mechanisms by which dietary nitrate improves endothelial function is through the reduction of oxidative stress within the cardiovascular system and it is possible that this effect not only plays a key role in mediating the benefits of dietary nitrate in the setting of periodontal disease but also possibly in other CVD states characterised by endothelial dysfunction that is improved with inorganic nitrate such as hypertension [73], hypercholesterolaemia [8] and that seen in aging [52]. Furthermore, a recent meta analysis has suggested that changes in proportion and function of circulating inflammatory cells in patients with periodontitis is similar to that observed in other systemic inflammatory diseases such as type 2 diabetes and cardiovascular diseases [74].

Fig. 8. Plasma nitrite does not correlate with FMD in patients with periodontitis. Periodontitis patients showed no association between plasma nitrite (A) or nitrate (B) levels and FMD responses. Association was determined using Pearson’s correlation coefficient measurement. n = 38 (1 data point excluded using Rout’s exclusion test).

Fig. 9. Summary of the pathways targeted by NO in periodontitis and CVD. Periodontitis is associated with bacterial accumulation around the teeth and a local inflammatory response of which the latter is dampened by NO by reducing IL-1β and elevating IL-10 levels. Periodontitis induced neutrophilia and raised superoxide production resulting in endothelial dysfunction, which was reversed by NO. Similarly, cardiac tissue exhibited raised superoxide and pro-inflammatory mediators which was reduced by NO. Blue line indicates inhibition; green arrow indicates elevation.
as systemic lupus erythematosus, rheumatoid arthritis, and psoriasis [54]. It is therefore possible that the benefits observed in periodontitis can be extrapolated to other diseases associate with low grade inflammation, although this requires further investigation.

A limitation of our study is that we have relied on changes in specific mRNA levels in cardiovascular tissues to provide an indication of bacteremia, which may not correlate with protein expression levels. A further limitation is that whilst we observed changes in aortic and cardiac endothelial function in isolated preparations, we did not detect in vivo changes of cardiac functional parameters. This may be due to the fact that a mild systemic and cardiac inflammation was induced in this experimental model. It is also likely that 2 weeks duration of ligature implantation is insufficient duration to cause statistically significant changes in cardiac chamber structure and function, however animal ethical considerations preclude such extended studies due to the consequent tooth loss and thus excess distress to the animal. With the addition of comorbidities such as hypertension, type 2 diabetes etc, we may have seen structural/functional cardiac effects, which have recently been shown in clinical periodontitis. Patients with severe periodontitis and metabolic syndrome display concentric left ventricular remodelling [74], suggesting periodontitis may have functional effects on the heart. This observation also suggests that a double insult model may be needed to better mimic the patient scenario experimentally and warrants further investigation. Finally, we used the method of lucigenin-based luminescence to assess superoxide. This method has come under substantial criticism due to issues with specificity [75]. However, we specifically used a concentration of lucigenin known to provide selectivity for superoxide, we verified our findings using highly selective inhibitors of XOR (febuxostat) and we used distinct substrates for XOR providing confidence in the findings observed. The limited availability of sample meant that we used only this single method for analyses since further repeats were not ethically justified.

The current step-wise approach for the management of periodontitis is aimed at the removal of the periodontal biofilm combined with motivational interventions for improved dental hygiene habits. Whilst the professional physical intervention of cleaning and scaling is an enormously successful therapeutic option, universal accessibility of this professional physical intervention of cleaning and scaling is an enormous success therapeutic option, universal accessibility of this intervention requires delivery from specialised professionals that is not easily available to some of the communities that are in most need [76]. Thus, identification of alternative, easy to deliver, strategies that target the cardiovascular system in patients with periodontitis may offer therapeutic benefits that prevent the most serious of outcomes for these patients and thus improve quality of life whilst not necessarily treating the cause of the disease. A dietary approach may have higher compliance than traditional periodontal treatment as individuals receiving oral nutritional supplements for malnutrition have adherence rates of 78%, whilst individuals with chronic diseases have less than 50% adherence rates [77]. Moreover, a simple dietary approach is likely to offer a long term supportive treatment aimed at keeping the dental biofilm and gingival inflammation at a minimum level and could in fact be particularly useful in preventative programmes, as reflected by the efficacy of pre-treatment studies in this manuscript.

In the samples analysed from patients with periodontitis patient we found circulating nitrite levels were not correlated with FMD which we speculate is due to the endothelial dysfunction in these patients. These observations are distinct from those evident in healthy volunteers where a strong association between circulating nitrite levels and FMD exists which we speculate is due to the endothelial dysfunction in these patients. These observations are distinct from those evident in healthy volunteers where a strong association between circulating nitrite levels and FMD exists [78]. In support of our findings, patients with periodontitis given 3x100ml daily lettuce juice for 14 days containing 667 mg/L nitrate exhibited reduced gingival inflammation, however FMD was not measured in these patients [79]. Our pre-clinical study now provides an explanation for these benefits but also provides strong support for the assessment of the impact of dietary nitrate treatment as a preventative strategy for cardiovascular events in patients with common chronic inflammatory diseases including periodontitis.

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Conflict of Interest

Prof A Ahluwalia is a Director of Heartbeat Ltd; a start-up company established seeking to identify commercial opportunities for inorganic nitrate. A Ahluwalia has received consultancy fees from Palatin Inc.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2022.106616.

References
