

## Dietary Nitrate Provides Sustained Blood Pressure Lowering in Hypertensive Patients

### A Randomized, Phase 2, Double-Blind, Placebo-Controlled Study

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**Abstract**—Single dose administration of dietary inorganic nitrate acutely reduces blood pressure (BP) in normotensive healthy volunteers, via bioconversion to the vasodilator nitric oxide. We assessed whether dietary nitrate might provide sustained BP lowering in patients with hypertension. We randomly assigned 68 patients with hypertension in a double-blind, placebo-controlled clinical trial to receive daily dietary supplementation for 4 weeks with either dietary nitrate (250 mL daily, as beetroot juice) or a placebo (250 mL daily, as nitrate-free beetroot juice) after a 2-week run-in period and followed by a 2-week washout. We performed stratified randomization of drug-naïve ( $n=34$ ) and treated ( $n=34$ ) patients with hypertension aged 18 to 85 years. The primary end point was change in clinic, ambulatory, and home BP compared with placebo. Daily supplementation with dietary nitrate was associated with reduction in BP measured by 3 different methods. Mean (95% confidence interval) reduction in clinic BP was 7.7/2.4 mm Hg (3.6–11.8/0.0–4.9,  $P<0.001$  and  $P=0.050$ ). Twenty-four-hour ambulatory BP was reduced by 7.7/5.2 mm Hg (4.1–11.2/2.7–7.7,  $P<0.001$  for both). Home BP was reduced by 8.1/3.8 mm Hg (3.8–12.4/0.7–6.9,  $P<0.001$  and  $P<0.01$ ) with no evidence of tachyphylaxis over the 4-week intervention period. Endothelial function improved by  $\approx 20\%$  ( $P<0.001$ ), and arterial stiffness was reduced by 0.59 m/s (0.24–0.93;  $P<0.01$ ) after dietary nitrate consumption with no change after placebo. The intervention was well tolerated. This is the first evidence of durable BP reduction with dietary nitrate supplementation in a relevant patient group. These findings suggest a role for dietary nitrate as an affordable, readily-available, adjunctive treatment in the management of patients with hypertension (funded by The British Heart Foundation).

**Clinical Trial Registration**—URL: <http://www.clinicaltrials.gov>. Unique identifier: NCT01405898. (*Hypertension*. 2015;65:320-327. DOI: 10.1161/HYPERTENSIONAHA.114.04675.) • [Online Data Supplement](#)

**Key Words:** blood pressure ■ nitric oxide ■ nitrate ■ nitrites

Systemic hypertension remains the largest attributable risk factor for mortality worldwide.<sup>1,2</sup> Worryingly, the scale of the problem is increasing, with the proportion of adults with hypertension predicted to increase to almost 1 in 3 (1.57 billion) by 2025.<sup>3</sup> Despite >60 years of innovation in the pharmacotherapy of hypertension,<sup>4</sup> only  $\approx$ half of hypertensives are treated for their blood pressure (BP) and of those only  $\approx$ half are controlled to guideline-driven targets.<sup>5,6</sup> Thus, novel therapeutic strategies including dietary approaches are of great interest.

An approach that has been explored in the treatment of hypertension is the delivery of the potent vasodilator nitric oxide (NO). Endothelial NO generation, achieved through the conventional L-arginine/NO synthase pathway, plays a critical role in sustaining vascular health. However, in most cardiovascular diseases (CVDs), including hypertension, the levels of endothelial NO are diminished.<sup>7,8</sup> Accordingly, therapeutic

strategies restoring NO levels in hypertension have been explored. However, supplementation with substrate (L-arginine) and other essential cofactors required for healthy NO generation from NO synthase have yielded equivocal results.<sup>9</sup> In addition, NO donors (organic nitrates), such as nitroglycerin, have also been tested but having problems of induced endothelial dysfunction<sup>10</sup> and tachyphylaxis<sup>11</sup> that have limited their clinical utility.<sup>12</sup>

However, NO production from the chemical reduction of inorganic nitrite ( $\text{NO}_2^-$ ), a phenomenon previously thought to occur only in extreme acidosis<sup>13–15</sup> has emerged as a potential pathway that might be exploited as a method for delivery of NO to the blood vessel. Evidence in healthy volunteers suggests nitrite reduction occurs readily within the circulation after elevation of plasma nitrite levels, by provision of dietary or oral inorganic nitrate salts.<sup>16,17</sup> It is now accepted that a

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significant proportion of orally ingested inorganic nitrate once absorbed across the upper intestine is extracted from the blood via the salivary glands and secreted into the oral cavity where it comes into contact with symbiotic bacteria that reduce inorganic nitrate ( $\text{NO}_3^-$ ) to nitrite.<sup>18</sup> On swallowing the saliva, the nitrite then enters the circulation,<sup>16,17,19</sup> where it meets mammalian nitrite reductases that convert it to NO resulting in vasodilation<sup>20,21</sup> and significant BP lowering.<sup>16,17,19</sup> Exploitation of this  $\text{NO}_3^-$ - $\text{NO}_2^-$ -NO (alternative) pathway by supplementation with dietary sources of  $\text{NO}_3^-$  (eg, beetroot juice) is associated with elevations in both plasma nitrite and cyclic guanosine monophosphate (cGMP, an exquisitely sensitive marker of bioactive NO production)<sup>22</sup> and significant BP reductions over 24 hours in healthy volunteers and drug-naïve stage 1 patients with hypertension.<sup>17,23</sup> We explored in this phase 2 clinical study whether a once daily dietary nitrate supplementation for 4 weeks would confer sustained BP reduction in both drug-naïve and treated patients with hypertension.

## Materials and Methods

### Study Design

This study was a prospective single-center, double-blind, randomized, placebo-controlled trial. Eligible patients recruited were those that satisfied a range of inclusion/exclusion criteria (see online-only Data Supplement) including aged between 18 and 85 years old, an estimated glomerular filtration rate  $>50$  mL/min, no manifest CVD, and uncontrolled BP on ambulatory BP (ABP) monitoring (day-time BP  $>130/85$  mm Hg).<sup>24</sup>

Ethics approval was granted by the East London Research Ethics Committee and the trial registered on clinicaltrials.gov. Patients were recruited between July 2011 and February 2013. Patients gave written informed consent, and the study conforms to the principles of the Declaration of Helsinki.

### Study Procedures

All tests were performed at the William Harvey Clinical Research Centre. Thirty-four drug-naïve and 34 treated patients with hypertension were randomized to receive either 4 weeks daily supplementation with dietary nitrate (250 mL beetroot juice, James White Drinks Ltd, Ipswich, UK) or placebo (250 mL nitrate-depleted beetroot juice,<sup>25</sup> James White Drinks Ltd, Ipswich, UK). Patients were asked not to alter their usual diet and to keep antihypertensive medication(s) constant (in treated patients with hypertension) over the course of the study. Patients recorded once daily home BP (using a validated oscillometric BP device:705IT, Omron Corp, Tokyo, Japan) over a 2-week run-in and then had a preintervention 24-hour ABP monitor (90207, Spacelabs Healthcare Ltd, Issaquah, WA) performed. After this, patients attended in the morning after an overnight fast for clinic BP, vascular function testing, and collection of saliva, urine, and blood samples (visit: pre). Patients were then instructed to consume 1 bottle of juice at the same time in the morning daily and continue to record home BP daily during the ensuing 4 weeks. One day before the end of this period, patients returned for ABP monitoring, and then returned the following day after an overnight fast for clinic BP, vascular function testing, and collection of saliva, urine, and blood samples (visit: W/O; Figure S1 in the online-only Data Supplement; for full details of BP measurements, vascular function tests, and biological sample collection, see online-only Data Supplement). Transcutaneous arterial methemoglobin concentrations were determined before venepuncture in a subset of patients (placebo

$n=12$ ; dietary nitrate  $n=7$ ) at all visits using a validated co-oximeter (Masimo Rad-57, Masimo Inc, Irvine, CA).

### Statistical Analyses

The data in the article and in the figures are presented as mean $\pm$ SD or 95% confidence intervals (CIs) for comparisons between treatment allocations, unless otherwise specified. All statistical analyses were performed using Graphpad Prism software v6. For full statistical methods, including power analyses, prespecified end points, and subgroups, see online-only Data Supplement.

## Results

Of the 151 patients screened for the study, 68 patients were enrolled, of whom 64 completed the study protocol and had complete data. Four patients withdrew after randomization but before the first study visit and returned no analyzable data (Figure S2). Both dietary nitrate and placebo interventions were well tolerated. No serious adverse events were reported. Common, expected findings were beeturia and fecal discoloration. All patients completed the dietary interventions for the duration of the study. The nitrate content of the active treatment juice was  $25.7\pm 5.3$  mmol/L, giving  $\approx 6.4$  mmol nitrate in a 250 mL daily dose. The nitrate content of the placebo

**Table. Baseline Characteristics Stratified by Treatment Allocation**

Treatment Allocation	Placebo	Dietary Nitrate	Significance
<b>Demographics</b>			
n (female)	32 (22)	32 (16)	0.14
Age, y	56.3 $\pm$ 16.4	57.6 $\pm$ 13.9	0.73
BMI, kg/m <sup>2</sup>	26.5 $\pm$ 4.0	26.8 $\pm$ 5.0	0.74
<b>Medications</b>			
Hypertension drugs	1.0 $\pm$ 1.2	1.0 $\pm$ 1.2	0.84
<b>Patients on (n)</b>			
ACE-i/ARB	10	11	
$\beta$ -Blocker	3	3	
CCB	14	10	
Diuretic	5	4	
$\alpha$ -Blocker	2	4	
Aldosterone antagonist	1	1	
Statins	3	4	
Antiplatelet drugs	0	0	
<b>Screening ABP, mm Hg</b>			
SBP	148.2 $\pm$ 10.0	149.0 $\pm$ 11.0	0.73
DBP	88.2 $\pm$ 8.0	88.9 $\pm$ 9.8	0.75
HR	70.6 $\pm$ 8.3	72.9 $\pm$ 10.7	0.32
<b>Biochemistry</b>			
eGFR, mL/min	79.1 $\pm$ 16.3	85.0 $\pm$ 16.6	0.17
Total cholesterol:	3.4 $\pm$ 1.3	3.1 $\pm$ 0.7	0.37
HDL-C ratio			

Data are presented as mean $\pm$ SD. Significance shown in the last column for unpaired Student *t* test, except for analysis of sex for which Fisher exact test was performed. ABP indicates ambulatory blood pressure; ACE-i, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; CCB, calcium channel blocker; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; HR, heart rate; and SBP, systolic blood pressure.

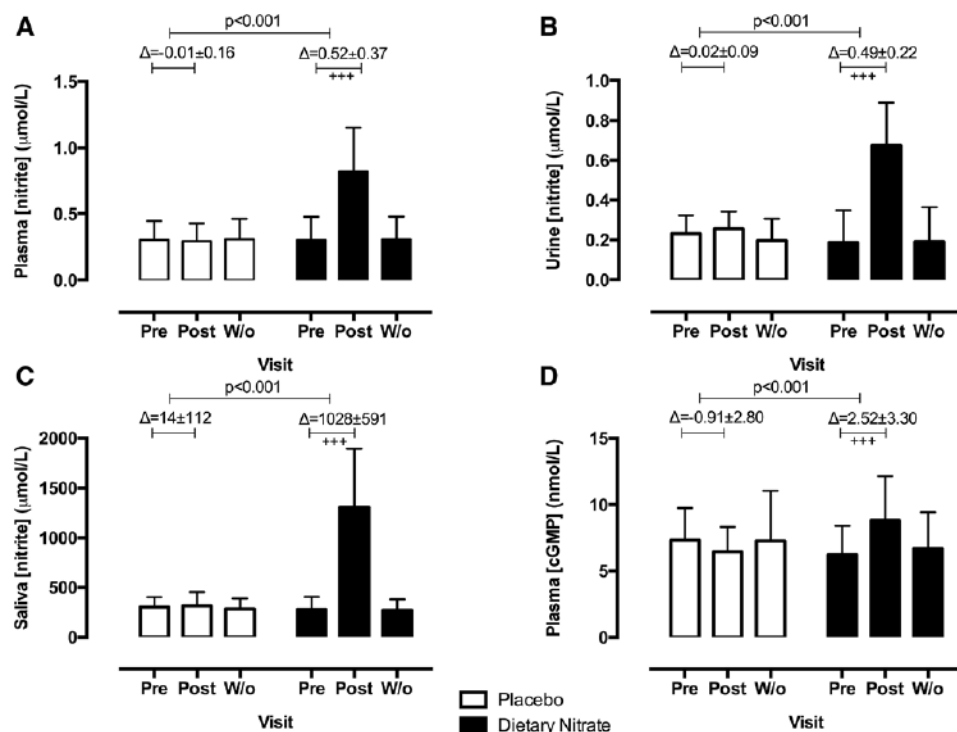
juice was  $0.028 \pm 0.008$  mmol/L, giving  $\approx 0.007$  mmol nitrate daily. The nitrite content was below the limits of detection in both interventions (ie,  $<50$  nmol/L). It has been shown previously that there are no significant differences in major cationic components (ie, sodium, potassium) of the active and placebo juices.<sup>25</sup> Demographics and baseline screening characteristics were similar in both treatment allocation groups (Table). All patients were confirmed to have significant hypertension by ABP monitoring at trial inception.<sup>24</sup>

Dietary nitrate ingestion was associated with elevations in both nitrate and nitrite concentrations in all biological compartments assessed. These levels returned to baseline after a 2-week washout period (Figure 1; Figure S3). Importantly, plasma nitrite concentrations were also elevated from baseline by  $\approx 2.7$ -fold with a mean  $\Delta 0.52$   $\mu\text{mol/L}$  (95% CI, 0.39–0.65) after consumption of dietary nitrate with no change in the placebo limb ( $P < 0.001$ ; Figure 1A). The change in circulating nitrite and nitrate levels in the dietary nitrate limb was also associated with an  $\approx 1.4$ -fold increase in plasma cGMP concentrations with no change in the placebo limb ( $P < 0.001$ , Figure 1D). There were no changes in transcutaneous arterial methemoglobin concentrations after either intervention (Figure S3D) and no changes from baseline or between groups in glycemic, serum biochemical, or hematological indices (Table S1).

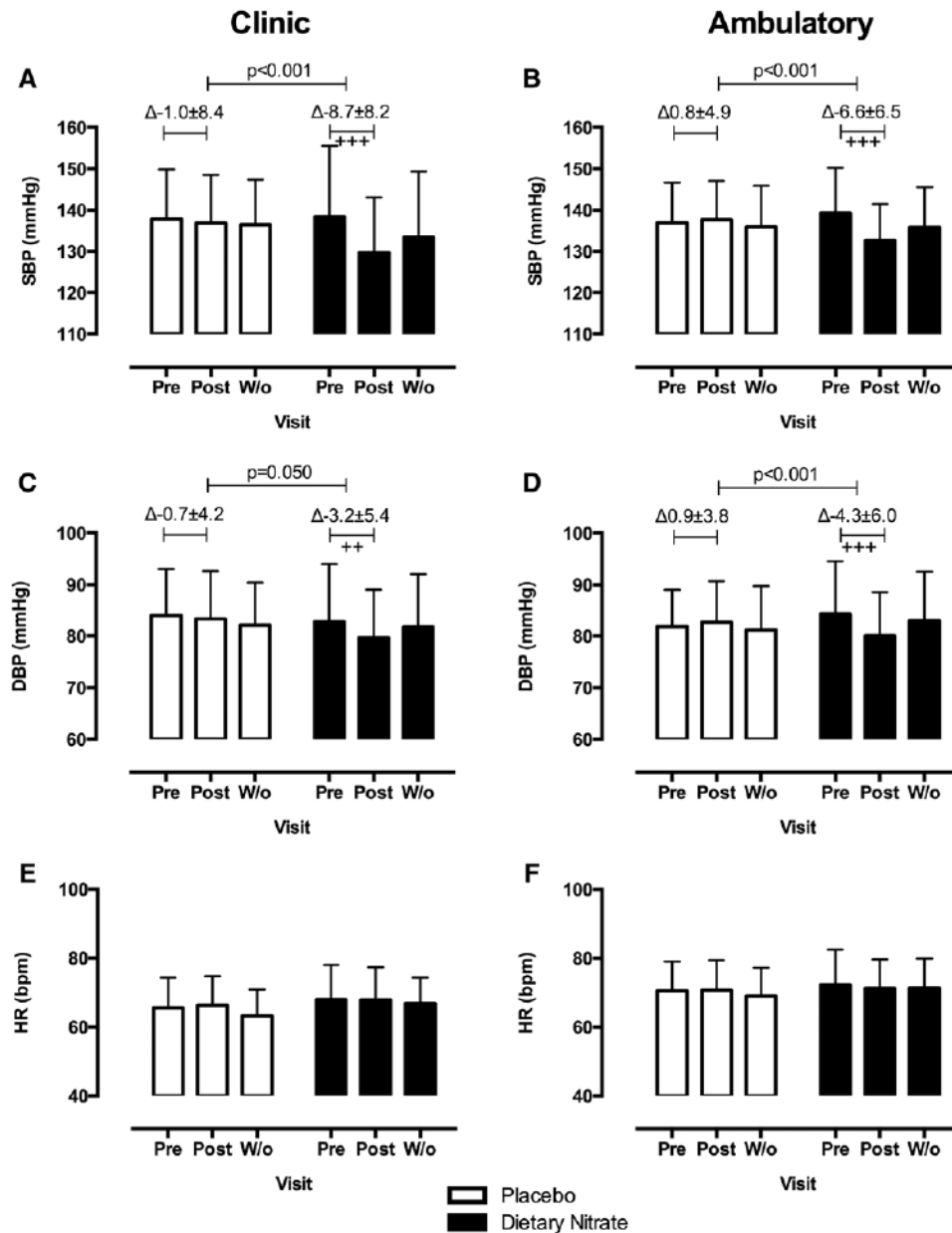
The 2 treatment allocation groups were well matched for baseline (preintervention) BP and heart rate measured by the methods utilized in this study (Table S2). Consumption of dietary nitrate was associated with decreases in clinic BP (Figure 2). Clinic systolic BP (SBP) and diastolic BP (DBP)

decreased compared with baseline by 7.7 mmHg (95% CI, 3.5–11.8;  $P < 0.001$ ) and 2.4 mmHg (95% CI, 0.0–4.9;  $P = 0.050$ ), respectively; changes not evident in the placebo group (Figure 2A and 2C). Similarly, 24-hour ABP measurements exhibited a similar pattern with a mean decrease in SBP and DBP compared with baseline of 7.7 mmHg (95% CI, 4.1–11.2,  $P < 0.001$ ) and 5.2 mmHg (95% CI, 2.7–7.7,  $P < 0.001$ ), respectively (Figure 2B and 2D). Observation of the hourly profile of the change in 24-hour ABP after intervention revealed that consumption of dietary nitrate was associated with reduction in BP over the entire 24-hour period for both SBP and DBP (Figure S4) compared with placebo. Splitting 24-hour ABP into day-time (07:00–23:00) and night-time (23:00–07:00) periods, dietary nitrate consumption was associated with decreases in BP in both time periods (Figure S5).

Home BP was reduced within 1 week of consumption of dietary nitrate, but not placebo, for both SBP and DBP and reduced over the entire 4-week intervention period (Figure 3A and 3B). Peak decreases in BP occurred at week 6 (ie, last week of dietary nitrate intervention), with decreases in SBP compared with placebo of 8.1 mmHg (95% CI, 3.8–12.4;  $P < 0.001$ ) and in DBP of 3.8 mmHg (95% CI, 0.7–6.8;  $P < 0.01$ ; Figure 3A and 3B). After washout, both SBP and DBP started to return to baseline (Figure 3). There were no changes relative to baseline or compared with placebo in heart rate after dietary nitrate intervention by any method used (Figures 2E, 2F, and 3C; Figure S5E and S5F). Change in plasma nitrite from baseline in the intervention arm correlated inversely with



**Figure 1.** Dietary nitrate consumption elevates nitrite concentration in biological compartments in patients with hypertension. The effects of 4 weeks dietary nitrate consumption (nitrate-rich juice 250 mL daily) or placebo (nitrate-depleted juice 250 mL daily) on nitrite concentrations in (A) plasma, (B) urine, (C) saliva, and (D) plasma cGMP concentrations. Data are expressed as mean  $\pm$  SD. Significance shown for comparisons between treatment allocations of the change between pre and post for unpaired Student *t* test; and as  $+++P < 0.001$  for Dunnett post hoc test comparison to baseline (pre) after 2-way ANOVA for changes within each treatment allocation cohort. Pre indicates first visit preintervention; post, second visit postintervention; and W/o, third visit washout.



**Figure 2.** Dietary nitrate consumption reduces clinic and 24-hour ambulatory blood pressure (ABP) in patients with hypertension. The effects of 4 weeks dietary nitrate consumption (beetroot juice 250 mL daily) or placebo (nitrate-depleted beetroot juice 250 mL daily) on clinic measures of (A) systolic blood pressure (SBP) and (C) diastolic blood pressure (DBP), and (E) heart rate (HR); and on 24-hour ABP measures of (B) SBP and (D) DBP and (F) HR. Data are expressed as mean  $\pm$  SD. Significance shown for comparisons between treatment allocations of the change between pre and post for unpaired Student *t* test; and as ++*P* < 0.01 and +++*P* < 0.001 for Dunnett post hoc test comparison to baseline (pre) after 2-way ANOVA for changes within each treatment allocation cohort. Pre indicates first visit preintervention; post, second visit postintervention; and W/o third visit washout.

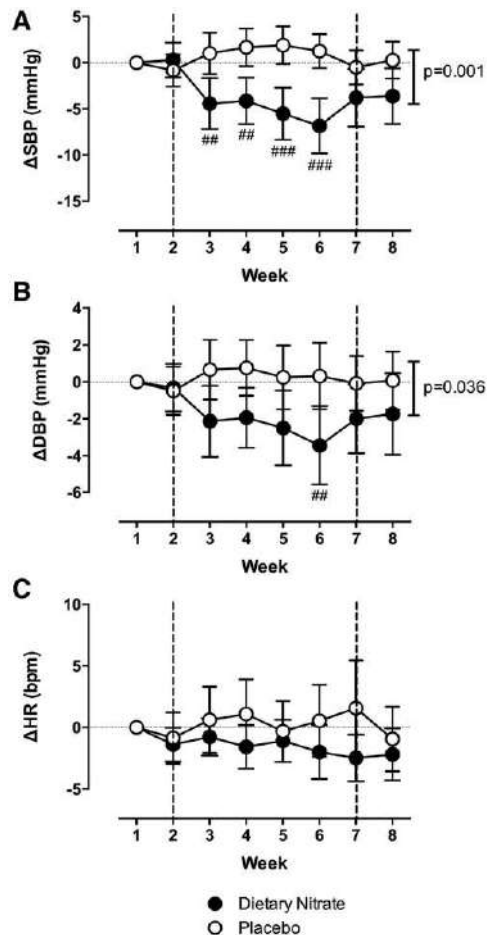
change in SBP measured in clinic and at home, but not with ABP (Figure S6A–S6C).

Dietary nitrate consumption was also associated with improvements in vascular function (Figure 4). Pulse wave velocity (PWV) was reduced after dietary nitrate consumption by 0.59 m/s (95% CI, 0.24–0.93; *P* < 0.01) compared with baseline and 0.58 m/s (95% CI, 0.05–1.10; *P* < 0.05) compared with placebo. Augmentation index was also reduced after dietary nitrate consumption by 5.2% (95% CI, 2.9–7.5; *P* < 0.001) compared with baseline and 6.1% (95% CI, 3.0–9.1; *P* < 0.01) compared with placebo. Baseline

brachial artery diameter and time to peak dilatation after reactive hyperemia were similar across all treatment groups and visits (Table S3). However, dietary nitrate consumption was associated with an increase in peak flow-mediated dilatation (FMD) of 1.0% (95% CI, 0.3–1.5; *P* < 0.001) not evident in the placebo limb.

A priori subgroup analyses were conducted by stratification for baseline hypertension treatment status (drug-naïve or treated). We recruited *n* = 34 into each subgroup. Four patients dropped out before receiving intervention: all 4 were in the treated hypertension arm, split equally between intervention





**Figure 3.** Dietary nitrate consumption reduces home blood pressure (BP) over entire 4-week intervention period in patients with hypertension. The effects of 4 weeks dietary nitrate consumption (beetroot juice 250 mL daily) or placebo (nitrate-depleted beetroot juice 250 mL daily) on change in weekly (A) systolic blood pressure (SBP) and (B) diastolic blood pressure (DBP) and (C) heart rate (HR) from baseline (week 1) measured at home. Data are expressed as mean $\pm$ SD. Significance shown for comparisons between treatment allocations for 2-way ANOVA; followed by ## $P<0.01$  and ### $P<0.001$  for Bonferroni post hoc test. The vertical dotted lines at 2 and 7 weeks signify the end of the 2-week run-in and the beginning of the washout periods.

and placebo arms. There were no differences in baseline demographics, characteristics and BPs of the subgroups (Table S4), and between the placebo and dietary nitrate subgroups.

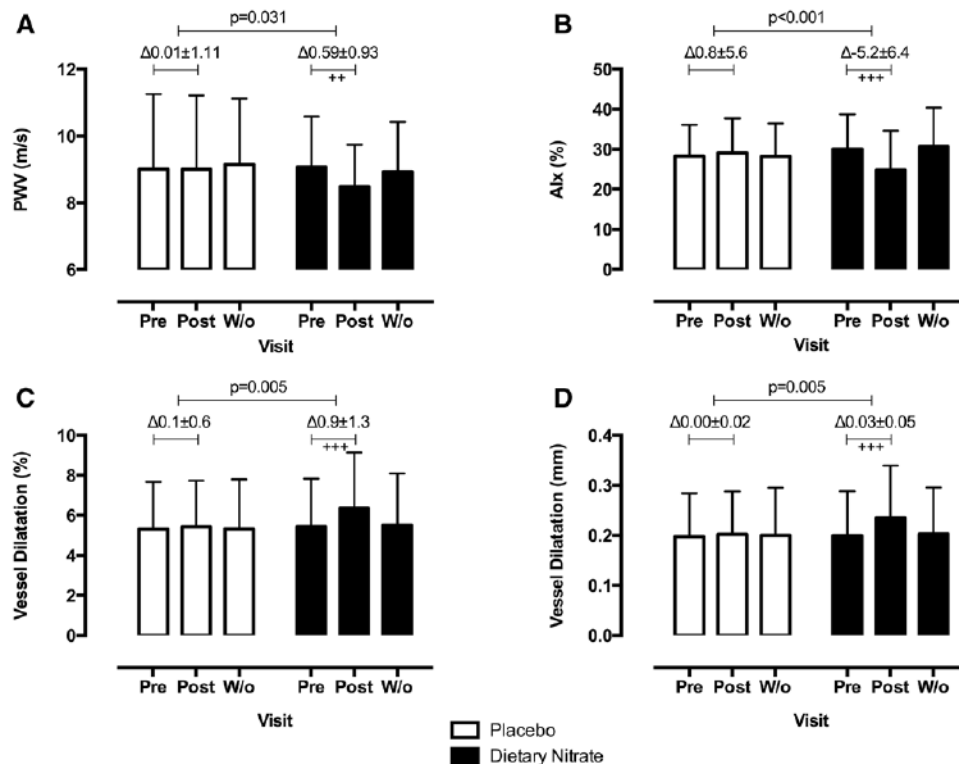
Dietary nitrate consumption produced similar rises in plasma, urine, saliva nitrite and nitrate, and plasma cGMP in both stratified limbs (Table S5). Importantly, BP was reduced compared with placebo by all methods used in both subgroups (Figures S7 and S8). PWV velocity was not altered in drug-naïve patients after dietary nitrate consumption, although endothelial function trended to improvement ( $P=0.06$ ) though this failed to reach conventional levels of statistical significance (Figure S7C and S7D). In the treated hypertensives, PWV velocity was reduced after dietary nitrate ( $0.82\pm0.87$  m/s;  $P<0.01$ ), and endothelial function was improved (change in FMD compared with placebo: 1.1%; 95% CI, 0.1–2.0;  $P=0.04$ ; Figure S8C and S8D).

## Discussion

Previously the oxidation of endogenously generated NO to nitrite, and nitrate was viewed as a 1-way, linear termination of NO activity. However, the discovery of the  $\text{NO}_3^-$ - $\text{NO}_2^-$ -NO reductive pathway, dependent on the enterosalivary circuit, has led to a radical revision of our understanding of the pathways that govern endogenous NO generation and NO metabolism.<sup>26</sup> This novel paradigm reveals the nitrogen oxides to be in an NO cycle that can be augmented through the provision of inorganic nitrate, given either by dietary or inorganic supplementary route. By capitalizing on this cycle, herein we show that a once daily dietary nitrate intervention augments NO generation through this pathway in patients with hypertension to lower BP. We have demonstrated that the intervention is well tolerated, safe, and is associated with robust BP reductions measured in and out of clinic.

Dietary nitrate supplementation providing  $\approx 6$  mmol nitrate daily for 4 weeks, caused substantial increases in plasma nitrate concentrations ( $\approx 5.5$ -fold), and is similar to the peak fold increases in previous studies with similar doses.<sup>23</sup> Plasma nitrite concentration was elevated by  $\approx 2.7$ -fold from baseline; the reduced fold increase compared with plasma nitrate concentrations reflecting use of the enterosalivary circulation in the bioconversion of ingested nitrate to nitrite that is critically dependent on the symbiotic relationship between oral bacteria and host.<sup>27</sup> In this study, dietary nitrate consumption elevated both saliva nitrate and nitrite concentrations with a ratio of saliva nitrate:nitrite of  $\approx 3$ . This is similar to the ratios identified in healthy volunteers after single dose nitrate supplementation,<sup>28,29</sup> suggesting that the enterosalivary circulation is intact and functioning normally in patients with hypertension. In these processes, dietary (or inorganic) nitrate can be thought of as a prodrug for the generation of plasma nitrite. The half-life of nitrite in the circulation after a single oral dosing has been estimated to be  $\approx 30$  minutes.<sup>30</sup> In contrast, nitrate has a half-life of  $\approx 6$  hours after oral dosing of inorganic nitrate.<sup>31</sup> The recirculation of nitrate, however, also extends the apparent half-life of nitrate-derived nitrite, with plasma nitrite peaking at  $\approx 3$  hours after inorganic nitrate ingestion and remaining elevated for at least a further 3 hours in both healthy and hypertensive subjects.<sup>17,19,23</sup>

In healthy volunteers, it has been shown that at least some of the nitrite in the swallowed saliva enters the circulation where it is chemically reduced by the action of  $\geq 1$  mammalian nitrite reductases to generate vasodilator NO.<sup>32</sup> That this occurs in patients with hypertension also is demonstrated biochemically in this study by increases in the downstream secondary messenger cGMP, that is elevated between 3–24 hours after dietary (or inorganic) nitrate ingestion,<sup>19,23</sup> confirming production of bioactive NO. Peak BP reductions coincide with these peak plasma nitrite elevation and plasma cGMP elevation, at  $\approx 3$  hours following dietary (or inorganic) nitrate ingestion,<sup>17,19,23</sup> and in this study, the increases in plasma nitrite concentration after dietary nitrate were associated with BP reductions measured both in- and out-of-clinic, providing further evidence that nitrite reduction to NO underlies the BP-lowering effects seen with dietary nitrate consumption.



**Figure 4.** Dietary nitrate consumption improves arterial function in patients with hypertension. The effects of 4 weeks dietary nitrate consumption (beetroot juice 250 mL daily) or placebo (nitrate-depleted beetroot juice 250 mL daily) on clinic measures of (A) pulse wave velocity (PWV), (B) augmentation index (Aix), and (C) % and (D) absolute increase in vessel diameter with flow-mediated dilatation (FMD). Data are expressed as mean $\pm$ SD. Significance shown for comparisons between treatment allocations of the change between pre and post for unpaired Student *t* test; and as ++*P*<0.01 and +++*P*<0.001 for Dunnett post hoc test comparison to baseline (pre) after 2-way ANOVA for changes within each treatment allocation cohort. Pre indicates first visit preintervention; post, second visit postintervention; and W/o, third visit washout.

Out-of-clinic BP measurements (ie, home and ABP) are recognized to be more predictive of target organ damage and mortality in population cohort studies<sup>33,34</sup> and underlies the use of all 3 methods in this study. Interestingly, the magnitude of BP reduction after dietary nitrate consumption was similar across all 3 methods of measurement with clinic BP reduced by 7.7/2.4 mmHg, 24-hour ABP by 7.7/5.2 mmHg, and home BP by 8.1/3.8 mmHg. Irrespective of the method of measurement, the magnitude of BP reduction is of clinical significance because it resembles the average BP reduction achieved with a single antihypertensive medication at standard dose (9.1/5.5 mmHg).<sup>35</sup> Most pharmacological treatments for raised BP give larger maximal BP reductions dependent on higher baseline BP values,<sup>35</sup> including following acute, single inorganic, and dietary nitrate dosing in healthy and drug-naïve stage 1 patients with hypertension.<sup>17,19,23</sup> Thus, one may postulate that the BP-lowering effects seen in these patients with mild hypertensive phenotypes may be greater in patients with more severe hypertension. However, this has not been tested in our study and is not known at this time and there is the possibility that in patients on multiple antihypertensive agents with established vascular damage from long-standing uncontrolled hypertension, the effects could be attenuated rather than amplified.

It is noteworthy that the home BP measurements over the 4 weeks demonstrate, if anything, an increasing magnitude of the effect with time with a reversal occurring only on washout.

These findings are in agreement with primate studies demonstrating no tachyphylaxis to repeated and continuous systemic nitrite administration over 2 weeks,<sup>36</sup> confirming an absence of the development of tolerance: a characteristic that has profoundly limited the clinical utility of the organic nitrates in CVD.<sup>37</sup>

Dietary nitrate consumption also improved indices of vascular function including aortic PWV, augmentation index, and FMD. PWV is the gold standard measure of arterial stiffness and is recognized as a powerful predictor of cardiovascular events.<sup>38</sup> The exact underlying mechanism for these changes in arterial stiffness is uncertain, but preclinical studies of age-induced arterial stiffening in mice suggest that systemic nitrite therapy reduces oxidative stress and advanced glycation end products that are associated with arterial stiffening.<sup>39</sup> However, it is also possible that the improvement in endothelial function measured by FMD plays a role in the improved PWV. A recent report in healthy, elderly subjects supplemented with 0.1 mmol/kg inorganic nitrate ( $\approx 6\text{--}8$  mmol/d) for 4 weeks demonstrated a modest increase in FMD of 0.5%, compared with no change in the placebo group.<sup>40</sup> We have previously demonstrated in young healthy individuals that while a single acute dose of 8 mmol of nitrate does not alter FMD (in healthy individuals the FMD response ranges from between 7% and 14%),<sup>28</sup> that single acute doses ranging between 6 and 24 mmol do protect against transient endothelial dysfunction induced by forearm ischemia–reperfusion injury.<sup>17,19</sup>

Finally, in all of the analyses, there were no significant changes in any functional parameter measured after consumption of the nitrate-free placebo intervention. This substantiates the proposal that inorganic nitrate is responsible for the beneficial effects seen with beetroot juice. The average intake of nitrate from regular food sources (predominantly vegetables) is 1.5 to 2 mmol daily,<sup>41</sup> and the acceptable daily intake is set by the World Health Organization at 3.7 mg/kg per day<sup>42</sup> ( $\approx 4.2$  mmol daily for a 70 kg person) because of concerns over methemoglobinemia and carcinogenesis. In this study, no suggestion of significant methemoglobinemia was evident, and previous studies that were associated with micromolar plasma nitrite concentration have not demonstrated clinically significant methemoglobinemia.<sup>20,43</sup> Although there are well established links between preformed nitrosoamines and carcinogenesis,<sup>44</sup> recent US National Toxicology Program reports on 2-year rodent feeding studies with nitrite<sup>45</sup> and a comprehensive World Health Organization report summarizing epidemiological cohort studies in humans evaluating the risk of cancer with nitrate intake have largely assuaged concerns on carcinogenesis.<sup>42</sup> Importantly, vegetable intake is associated with small reductions in cancer incidence, rather than increases.<sup>46</sup> Patients predisposed to oxalate renal stones may need to avoid certain high-nitrate vegetables that also contain oxalate, such as spinach and beetroot. Although study participants reported no adverse effects apart from expected discoloration of urine (beeturia) and feces from the purple betacyanin pigments in beetroot, we cannot be certain that prolonged intake of beetroot juice is reliably acceptable as a therapeutic source of dietary nitrate. There is batch-to-batch variation in nitrate content of vegetables and their juices and clinical studies using beetroot juice with varying concentrations delivering between  $\approx 4$  and 24 mmol. This natural variation could be controlled by use of anion exchange resin to provide fixed nitrate concentrations.<sup>25</sup> Our previous studies have suggested that 4 mmol is a threshold dose for BP lowering healthy volunteers, although it is not clear from our data whether this is also true for hypertensives or what dose of dietary nitrate might provide maximal BP lowering is not known.

Overall, our results presented in this study demonstrate that dietary nitrate provision to patients with hypertension provides robust BP lowering that is dependent on the conversion of nitrate to nitrite and thence NO, with no suggestion of tachyphylaxis over a 4-week period.

## Perspectives

The potential importance of our findings is substantial when one considers that each 2 mmHg increase in SBP increases mortality because of ischemic heart disease and stroke by 7% and 10%, respectively.<sup>47</sup> Although the time frame of the study is too short to make any supported claims to long-term BP control or to be able to extrapolate with any confidence to target organ damage reduction or CVD events, these appropriately powered data are the first to demonstrate robust, sustained BP-lowering with dietary nitrate in patients with hypertension that require BP control (rather than healthy subjects) and as such are encouraging and should spur large-scale, long-term outcome studies to explore the utility of a dietary nitrate-based therapeutics approach to hypertension and CVD risk

mitigation. Moreover, dietary nitrate provides a viable option to finally exploit the NO pathway, which has been implicated at multiple steps in the genetic architecture of BP and is a therapeutic modality targeted at gene products directly implicated in raised BP.<sup>48</sup> With large populations of inadequately treated patients with hypertension at higher risk of CVD,<sup>5,6</sup> an additional strategy, based on intake of nitrate-rich vegetables, may prove to be both cost-effective, affordable, and favorable for a public health approach to hypertension.

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## Disclosures

A. Ahluwalia is a director of Heartbeat Ltd. The other authors report no conflicts.

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## Novelty and Significance

### What Is New?

- Once a day dietary nitrate intervention for 4 weeks provides sustained blood pressure lowering in patients with hypertension.
- This dietary intervention also results in improved function of the blood vessels.
- Prolonged dietary nitrate use is not associated with any tachyphylaxis of the beneficial effects on the cardiovascular system.
- A once a day dietary nitrate intervention results in further blood pressure lowering in patients taking 1 to 4 other blood pressure medications.

### What Is Relevant?

- Dietary nitrate exerts potent and long-lasting blood pressure decrease in hypertension that is sustained with once a day dosing for 4 weeks.

- Daily dietary nitrate ingestion provides additional blood pressure lowering beyond conventional pharmacotherapy.

### Summary

A once a day dietary nitrate regimen offers a strategy to lower blood pressure in hypertension either as monotherapy or in conjunction with conventional pharmacotherapy.



## **On-line Supplement**

**Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: a randomised, double-blind, placebo-controlled clinical study.**

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Short title: Dietary Nitrate in Hypertension

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## Materials and Methods

### Study Patients

This study protocol was granted full ethical approval by the East London Research Ethics Committee. Patients were recruited through both newspaper adverts and posters, as well as directly through the Barts Blood Pressure Clinic. Treatment-naïve patients were on no medications of any type at all. Treated hypertensive patients were on at least one anti-hypertensive and could additionally be on a *statin* and anti-platelet agent. Patients provided informed consent after satisfying inclusion/exclusion criteria:

#### Inclusion criteria:

1. Adult male and females between 18 and 85 years of age, inclusive.
2. Uncontrolled BP on ambulatory BP (ABP) monitoring (daytime BP > 130/85 mmHg) as per British Hypertension Society guidelines.<sup>1</sup>
3. To be eligible, female subjects will be required to state that they are not pregnant, and will not become pregnant during the course of the study.
4. Body mass index (BMI) between 18 and 40 kg/m<sup>2</sup>.
5. The subject is able to understand and comply with protocol requirements, instructions and protocol-stated restrictions.

#### Exclusion criteria:

1. History of symptomatic coronary artery disease, stroke, or other known atherosclerotic disease.
2. History of chronic viral hepatitis (including presence of hepatitis B surface antigen or hepatitis C antibody), or other chronic hepatic disorders.
3. History of increased liver function tests (ALT, AST) due to acute or chronic liver conditions, 3x above the upper limit of normal or bilirubin 1.5x above the upper limit of normal at screening.
4. Renal impairment with creatinine clearance (eGFR) of <50 ml/min at screening.
5. Current poorly controlled diabetes mellitus, defined as HbA1c >10% at Screen.
6. Subjects with LDLc, >7.5 mmol/l. Fasting TG level >6mmol/l.
7. History of heart failure defined as NYHA class II - IV or those with known severe LV dysfunction (EF<30%) regardless of symptomatic status
8. History of malignancy within the past 5 years, other than non-melanoma skin cancer.
9. Current life-threatening condition other than vascular disease (e.g., very severe chronic airways disease, HIV positive, life-threatening arrhythmias) that may prevent a subject from completing the study.

10. Alcohol or drug abuse within the past 6 months.
11. Use of an investigational device or investigational drug within 30 days or 5 half-lives (whichever is the longer) preceding the first dose of study medication.
12. Subjects who will commence or who are likely to commence treatment with non-steroidal anti-inflammatory drugs (NSAIDs) (other than aspirin), from screening until study completion.
13. Any non-stable dosing of ongoing medication regimens throughout the study trial.
14. The subject has a three-month prior history of regular alcohol consumption exceeding an average weekly intake of > 28 units (or an average daily intake of greater than 3 units) for males, or an average weekly intake of > 21 units (or an average daily intake of greater than 2 units) for females. 1 unit is equivalent to a half-pint (284mL) of beer/lager; 25mL measure of spirits or 125mL of wine; or a positive alcohol breath test at the screening visit
15. If used, a positive urine test for drugs of abuse or alcohol at screening or prior to study medication administration.
16. Any other subject whom the Investigator deems unsuitable for the study (e.g., due to either medical reasons, laboratory abnormalities, expected study medication noncompliance, or subject's unwillingness to comply with all study-related study procedures).
17. Subjects with rheumatoid arthritis, connective tissue disorders and other conditions known to be associated with chronic inflammation (e.g. Inflammatory Bowel Disease).
18. Subjects with any acute infection, or significant trauma (burns, fractures).
19. Subjects who have donated more than 500 mL of blood within 56 days prior to the study.

#### Randomization and Blinding

Patients were randomized 1:1 to receive dietary nitrate or placebo, with stratification for baseline treatment status (drug-naïve or treated), using a binary random number sequence ([www.random.org](http://www.random.org)). No block randomization was applied and for each patient the next available code was used. Treatment assignment for patients in both the dietary nitrate and placebo groups remained blinded until data lock and statistical analysis at the end of the trial.

#### Blood pressure measurement:

Home BP: Patients were provided a validated BP monitor (705IT, Omron Corp., Tokyo, Japan) and appropriate sized upper-arm cuff and were given appropriate training and instruction on use. Patients were asked to record home BP and heart rate (HR) at 8am in triplicate in a seated position after 5 minutes rest and transcribe into provided diaries.

Clinic BP: BP and HR measurements were taken in triplicate in the seated position using a validated BP monitor (705IT, Omron Corp., Tokyo, Japan) and appropriate sized upper-arm cuff after 5 minutes rest. The readings were blinded to both the researcher and subject and an average of the second and third readings at each time-point were taken for analysis. BP was measured every 15 minutes for 1-hour to establish a baseline clinic BP.

Ambulatory BP: 24-hour ABP monitoring was performed using a validated machine (90207, Spacelabs Healthcare Ltd., Issaquah, USA) and appropriately sized cuff. The following protocol for measurements was used: 0700-2300 – one reading every 20 min; 2300-0700 – one reading every hour. Proprietary software was used to download readings and produce 24-hour, day-time (0700-2300) and night-time (2300-0700) mean BP and HR readings (90256 ABP Report Management System, Spacelabs Healthcare, Issaquah, USA).

#### Vascular function:

Pulse wave analysis (PWA): After 5 min supine rest, a 100 mm inflatable cuff was attached to the non-dominant arm and statically inflated to 65 mmHg. Brachial artery waveforms were digitally computed by Vicorder (Skidmore Medical Limited, Bristol, UK) using a volume-displacement method for at least 10 cardiac cycles to calculate augmentation index (AIx).<sup>2</sup>

Pulse wave velocity (PWV): After 5 min supine rest, Vicorder was used to simultaneously record the pulse wave from the carotid and femoral arteries using an oscillometric method. A small, inflatable neck pad is placed directly over a single carotid artery and secured around the neck by a Velcro tab and a cuff is placed around the subject's ipsilateral upper thigh. Both carotid and femoral cuffs are inflated automatically to 65 mmHg and the corresponding oscillometric signal from each cuff is digitally analysed to extract the pulse-time delay. The distance between the sternal notch and the thigh cuff is measured and used as a standard estimate for the aortic length. From these measurements aortic pulse wave velocity (PWV) can be derived as  $PWV = \text{aortic distance} / \text{pulse time delay}$ .<sup>3</sup>

Endothelial function: In a subset of patients (placebo n=28; dietary nitrate n=25), endothelial function was assessed by measuring brachial artery diameter in response to reactive hyperaemia (flow-mediated dilatation, FMD) as previously described in *gold standard* guidelines.<sup>4</sup> Briefly, a B-mode scan of the brachial artery was obtained in longitudinal section above the antecubital fossa using a 7.0-MHz linear array transducer and a standard Acuson Aspen system (Acuson, Mountain View, UK). Arterial diameter over a 1-2-cm section was determined for each image with the use of automatic edge-detection software (Brachial Tools, LCC Medical Imaging Applications, Iowa City, USA) and synchronised by 3-lead electrocardiography to



the R-wave of the QRS complex. Blood flow was altered in the brachial artery by a 7-cm-wide BP cuff placed immediately below the antecubital fossa around the forearm. After 1 min of baseline flow, the cuff was inflated to 300 mm Hg for 5 min and released, resulting in a brief episode of reactive hyperaemia and changes in brachial artery diameter in response to blood flow were assessed for an additional 5 min. Brachial artery diameter and brachial artery dilation expressed as percentage and absolute increase from baseline diameter and time to peak dilatation were determined using the software Vascular Analysis Tools (LCC Medical Imaging Applications, Iowa City, USA).

#### Biological sample collection:

Urine samples: Mid-stream urine samples were collected into sterile gallipots and aliquots were stored at -80°C until analysis at a later date.

Saliva samples: Unstimulated saliva was collected into sterile eppendorfs and centrifuged (14000g, 4°C, 10 minutes) and the supernatant was collected and stored at -80°C until analysis at a later date.

Blood samples: Blood was collected by standard venepuncture into duplicate 4mL pre-chilled lithium heparin vacutainer tubes (Becton, Dickinson & Co, Franklin Lakes, USA) and was immediately centrifuged (1300g, 4°C, 10 min) and plasma separated and deproteinated by filtration (centrifugation 14000g, 4°C, 60min) using 3kDa filters (Vivaspin 500, Sartorius Biotech, Aubagne, France) and the filtrate stored at -80°C, until analysis at a later date. Further 4mL sodium ethylenediaminetetraacetate (EDTA), gel separator and sodium fluoride vacutainer tubes were collected and sent to the Clinical Haematology and Biochemistry laboratory at Barts Health NHS Trust for standard haematological and clinical biochemical analysis.

Nitrate/nitrite concentration: Nitrate and nitrite (collectively termed  $NO_x$ ) concentrations in biological samples were measured as previously described using ozone-based chemiluminescence.<sup>5</sup> To determine total  $NO_x$  concentration, samples were added to 0.1 mol/L vanadium (III) chloride in 1 mol/L hydrochloric acid refluxing at 95°C under nitrogen. Nitrite concentration was determined by addition of samples to 0.09 mol/L potassium iodide in glacial acetic acid under nitrogen at room temperature. Nitrate concentration was calculated by subtraction of the nitrite concentration from the total  $NO_x$ . Samples measured were compared to a standard curve generated that day from known standards.

cGMP concentration: cGMP levels in samples were determined using an enzyme immunoassay (cGMP Enzymeimmunoassay Biotrak System RPN226) according to the manufacturer's instructions (GE Healthcare, GE Healthcare, Little Chalfont, UK), using a 96-well plate spectrophotometer. Blood samples were incubated immediately after venepuncture with a competitive, non-selective PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 0.1 mmol/L, Sigma-Aldrich Corp., St. Louis, USA) to prevent cleavage of cGMP and

then separated by centrifugation as above to produce plasma samples. The assay is based on competition between unlabelled cGMP and a fixed quantity of peroxidase-labelled cGMP, for binding sites on a cGMP-specific antibody. Samples measured were compared to a standard curve generated that day from known standards.

### Statistical analyses

The data in the manuscript and in the figures are presented as mean $\pm$ standard deviation or 95% confidence intervals (CIs) for comparisons between treatment allocations, unless otherwise specified. All statistical analyses were performed using Graphpad Prism™ software v6. For full statistical methods, including power analyses, pre-specified end-points and sub-groups, see online supplement. Power analyses (G\*Power v3.0) indicated that using an expected difference in clinic BP of 7.0 $\pm$ 5.1 mmHg<sup>6</sup> between groups that with an  $\alpha=0.05$ , 1- $\beta=0.95$  a sample size of  $n=15$  was required in each treatment group. We increased the  $n$  to 17 to account for dropouts and as we planned pre-specified sub-group analysis in both drug-naïve and treated hypertensive patients, we recruited 68 patients to the study.

The pre-specified research objective was to determine whether once-daily administration of dietary nitrate lowered BP and improved additional measures of arterial health in hypertensive patients. The pre-specified primary outcome for the study was change in BP (clinic, home and ABP) after consumption of dietary nitrate compared to change after placebo. Pre-specified secondary outcomes included change in plasma nitrite concentrations and endothelial function measured by % increase from baseline during FMD and arterial stiffness measured by aortic PWV. Sub-group analysis was conducted on a pre-specified basis on drug-naïve and treated hypertensive patients.

For the primary and secondary outcomes the change in each parameter was compared between placebo and dietary nitrate limbs by unpaired Student t-test. Changes in parameters over time were compared by repeated measures ANOVA with Dunnett's *post hoc* tests (to account for multiple comparisons) for comparison to baseline (pre-intervention measures) and Bonferroni *post hoc* tests (to account for multiple comparisons) for comparisons between groups. Determination of correlations between plasma [nitrite] with BP were determined using Pearson's correlation coefficient analysis. The level for statistical significance taken was  $P<0.05$  for all analyses.

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### Supplementary Tables and Table legends

Biochemical/Haematological Index	Pre	<u>Placebo</u>	W/o	Pre	<u>Dietary Nitrate</u>	W/o
		Post			Post	
Serum creatinine ( $\mu\text{mol/L}$ )	80.3 $\pm$ 17.3	80.0 $\pm$ 19.0	79.9 $\pm$ 17.6	74.7 $\pm$ 13.5	73.7 $\pm$ 15.5	73.2 $\pm$ 14.2
Serum sodium (mmol/L)	142 $\pm$ 2	142 $\pm$ 3	142 $\pm$ 3	142 $\pm$ 2	143 $\pm$ 2	143 $\pm$ 2
Serum potassium (mmol/L)	4.4 $\pm$ 0.3	4.6 $\pm$ 0.3	4.5 $\pm$ 0.4	4.5 $\pm$ 0.3	4.4 $\pm$ 0.3	4.4 $\pm$ 0.3
Serum total cholesterol (mmol/L)	5.6 $\pm$ 1.2	5.6 $\pm$ 1.4	5.6 $\pm$ 1.3	5.4 $\pm$ 0.9	5.2 $\pm$ 0.9	5.4 $\pm$ 1.0
Serum LDL-cholesterol (mmol/L)	3.2 $\pm$ 1.0	3.2 $\pm$ 1.0	3.2 $\pm$ 1.0	3.1 $\pm$ 0.9	2.9 $\pm$ 0.8	3.1 $\pm$ 0.9
Serum HDL-cholesterol (mmol/L)	1.9 $\pm$ 0.9	1.9 $\pm$ 0.9	1.9 $\pm$ 1.0	1.8 $\pm$ 0.4	1.8 $\pm$ 0.5	1.8 $\pm$ 0.4
Serum triglycerides (mmol/L)	1.2 $\pm$ 0.7	1.2 $\pm$ 0.6	1.2 $\pm$ 0.7	1.1 $\pm$ 0.5	1.1 $\pm$ 0.7	1.2 $\pm$ 0.7
Plasma glucose (fasting) (mmol/L)	5.1 $\pm$ 0.5	5.0 $\pm$ 0.5	4.9 $\pm$ 0.5	5.3 $\pm$ 0.7	5.2 $\pm$ 0.8	5.2 $\pm$ 1.3
Glycated haemoglobin (mmol/mol)	36.0 $\pm$ 4.8	37.2 $\pm$ 3.8	36.0 $\pm$ 4.0	38.7 $\pm$ 6.2	39.9 $\pm$ 5.1	38.7 $\pm$ 4.4
Haemoglobin (g/L)	145 $\pm$ 12	142 $\pm$ 11	143 $\pm$ 11	139 $\pm$ 10	137 $\pm$ 13	139 $\pm$ 13
Total leucocyte count ( $\times 10^9/\text{L}$ )	5.4 $\pm$ 1.2	5.8 $\pm$ 1.5	5.9 $\pm$ 1.6	6.0 $\pm$ 1.6	6.1 $\pm$ 1.8	5.9 $\pm$ 1.2
Platelet count ( $\times 10^9/\text{L}$ )	232 $\pm$ 51	231 $\pm$ 52	237 $\pm$ 59	256 $\pm$ 55	255 $\pm$ 52	253 $\pm$ 50

**Table S1** Biochemical and haematological indices by treatment allocation. The effects of 4 weeks dietary nitrate consumption (beetroot juice 250 mL daily) or placebo (nitrate-depleted beetroot juice 250 mL daily) on biochemical and haematological indices. Data are expressed as mean $\pm$ SD. No significance found after 2 way ANOVA. (Pre=1<sup>st</sup> visit pre-intervention; Post=2<sup>nd</sup> visit post-intervention; W/o=3<sup>rd</sup> visit washout).



Treatment Allocation	Placebo (n=32)	Dietary Nitrate (n=32)	Significance
<b>Clinic BP (mmHg)</b>			
SBP	137.8±12.1	138.4±17.1	0.87
DBP	84.1±9.0	82.8±11.2	0.62
HR (bpm)	70.6±8.8	68.0±10.0	0.32
<b>ABP (mmHg)</b>			
SBP	136.9±9.8	139.3±11.0	0.36
DBP	81.8±7.2	84.4±10.2	0.26
HR (bpm)	70.5±8.5	72.3±10.2	0.45
<b>Home BP (mmHg)</b>			
SBP	139.3±9.7	139.5±10.5	0.94
DBP	81.6±8.7	81.9±8.7	0.86
HR (bpm)	67.5±9.0	71.0±8.7	0.14

**Table S2** Baseline BP measured by 3 methods stratified by treatment allocation. Data are presented as mean±SD. Significance shown in the last column for unpaired Student t test. (ABP=ambulatory blood pressure; BP=blood pressure; DBP=diastolic blood pressure; HR=heart rate; SBP=systolic blood pressure).

Treatment Allocation	Placebo (n=32)	Dietary Nitrate (n=26)	Significance
<b>Vessel Diameter (mm)</b>			
Pre	3.82±0.69	3.70±0.58	ns
Post	3.83±0.71	3.69±0.61	ns
W/o	3.87±0.70	3.71±0.60	ns
<b>Time to peak dilatation (s)</b>			
Pre	58.0±25.2	54.4±23.4	ns
Post	56.5±22.5	52.3±18.3	ns
W/o	59.2±23.0	48.3±19.3	ns

**Table S3** Baseline vessel diameter and time to peak dilatation for FMD measures of endothelial function, stratified by treatment allocation. Data are presented as mean±SD. Significance shown in the last column for Bonferroni *post hoc* test following 2 way ANOVA. (Pre=1<sup>st</sup> visit pre-intervention; Post=2<sup>nd</sup> visit post-intervention; W/o=3<sup>rd</sup> visit washout).

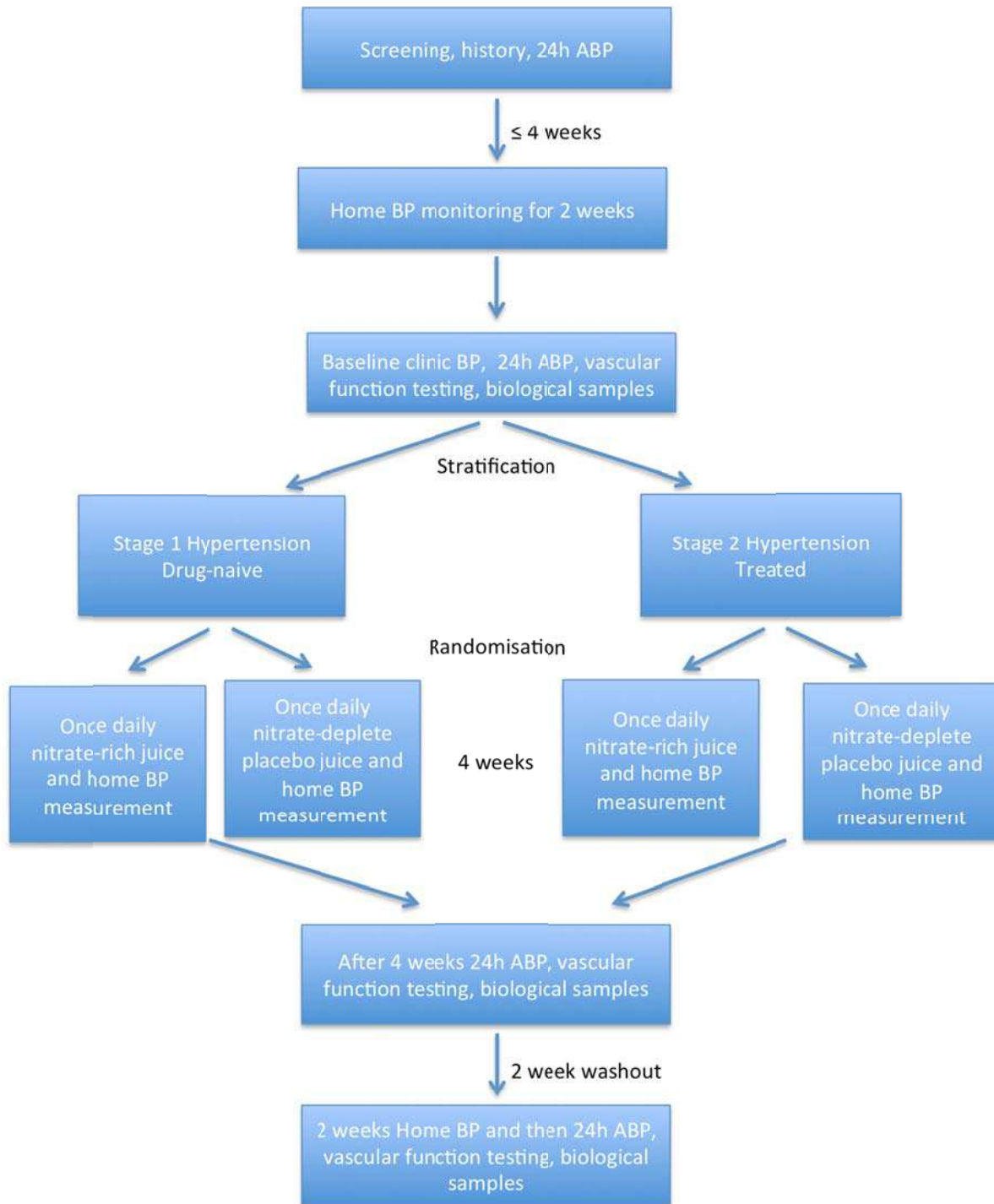
Treatment Allocation	Placebo	Dietary Nitrate	Significance
<b>Drug-naïve</b>			
<b>Demographics</b>			
n (female)	17 (7)	17 (11)	0.30
age (y)	56.5±14.2 (49.2-63.8)	58.8±13.1 (52.1-65.5)	0.61
BMI (kg/m <sup>2</sup> )	25.9±4.5 (23.6-28.2)	27.2±6.1 (24.1-30.4)	0.47
<b>Medications</b>			
hypertension drugs	0	0	
statins	0	0	
antiplatelet drugs	0	0	
<b>Screening ABP (mmHg)</b>			
SBP	141.0±5.1 (138.3-143.6)	141.0±5.0 (138.5-143.6)	0.95
DBP	83.6±4.0 (81.5-85.6)	84.8±6.7 (81.4-88.3)	0.51
HR	68.9±9.5 (64.1-73.8)	69.1±8.3 (64.9-73.4)	0.94
<b>Biochemistry</b>			
eGFR (mL/min)	80.4±16.3 (71.0-89.8)	86.7±17.0 (77.3-96.1)	0.32
Total Cholesterol:HDL-C	3.4±1.3 (2.6-4.1)	3.1±0.7 (2.8-3.5)	0.57
<b>Treated hypertensives</b>			
<b>Demographics</b>			
n (female)	15 (9)	15 (10)	1.00
age (y)	56.1±18.8 (46.5-65.8)	58.8±13.1 (52.1-65.5)	0.97
BMI (kg/m <sup>2</sup> )	27.0±3.6 (25.2-28.9)	27.2±6.1 (24.1-30.4)	0.62
<b>Medications</b>			
hypertension drugs	2.1±1.0 (1.6-2.6)	1.9±1.0 (1.4-2.4)	0.73
patients on (n):			
ACE-i/ARB	10	11	
β-blocker	3	3	
CCB	14	10	
diuretic	5	4	
α-blocker	2	4	
aldosterone antagonist	1	1	
statins	3	4	
antiplatelet drugs	0	0	
<b>Screening ABP (mmHg)</b>			
SBP	155.4±8.3 (151.1-159.6)	157.0±9.5 (152.1-161.9)	0.60
DBP	92.9±8.4 (88.6-97.2)	93.0±10.8 (87.5-98.6)	0.96
HR	72.2±6.8 (68.7-75.7)	76.7±11.8 (70.7-82.7)	0.18
<b>Biochemistry</b>			
eGFR (mL/min)	77.9±16.8 (68.6-87.1)	83.4±16.7 (74.2-92.6)	0.37
Total Cholesterol:HDL-C	3.4±1.3 (2.7-4.1)	3.1±0.8 (2.7-3.6)	0.51

**Table S4** Baseline characteristics stratified by baseline hypertension treatment status and treatment allocation. Data are presented as mean±SD (95%CI). Significance shown in the last column for unpaired Student t-test, except for analysis of sex for which Fisher's exact test was performed. (ABP=ambulatory blood pressure; ACE-i=angiotensin converting enzyme inhibitor; ARB=angiotensin receptor blocker; CCB=calcium channel blocker; DBP=diastolic blood pressure; eGFR=estimated glomerular filtration rate; HDL-C=high-density lipoprotein cholesterol; HR=heart rate; SBP=systolic blood pressure).

Biological sample	<u>Drug-naïve</u>		<u>Treated</u>	
	$\Delta$ to baseline	$\Delta$ to placebo	$\Delta$ to baseline	$\Delta$ to placebo
Plasma nitrate (μmol/L)	158±163 <sup>+++</sup>	161 (80-242) <sup>***</sup>	129±68 <sup>+++</sup>	128 (90-166) <sup>***</sup>
Plasma nitrite (μmol/L)	0.57±0.23 <sup>+++</sup>	0.61 (0.47-0.74) <sup>***</sup>	0.46±0.48 <sup>+++</sup>	0.45 (0.17-0.73) <sup>***</sup>
Urine nitrate (μmol/L)	5018±2253 <sup>+++</sup>	4701 (3502-5901) <sup>***</sup>	1293±416 <sup>+++</sup>	4236 (3124-5348) <sup>***</sup>
Urine nitrite (μmol/L)	0.53±0.20 <sup>+++</sup>	0.50 (0.40-0.61) <sup>***</sup>	0.44±0.20 <sup>+++</sup>	0.43 (0.30-0.54) <sup>***</sup>
Saliva nitrate (μmol/L)	3197±1991 <sup>+++</sup>	3224 (2212-4236) <sup>***</sup>	2891±1737 <sup>+++</sup>	2793 (1826-3759) <sup>***</sup>
Saliva nitrite (μmol/L)	1082±692 <sup>+++</sup>	1071 (722-1419) <sup>***</sup>	968±468 <sup>+++</sup>	944 (683-1205) <sup>***</sup>
Plasma cGMP (nmol/L)	2.44±3.16 <sup>+</sup>	2.88 (0.62-5.13) <sup>*</sup>	2.60±3.56 <sup>+</sup>	4.21 (1.84-6.58) <sup>**</sup>

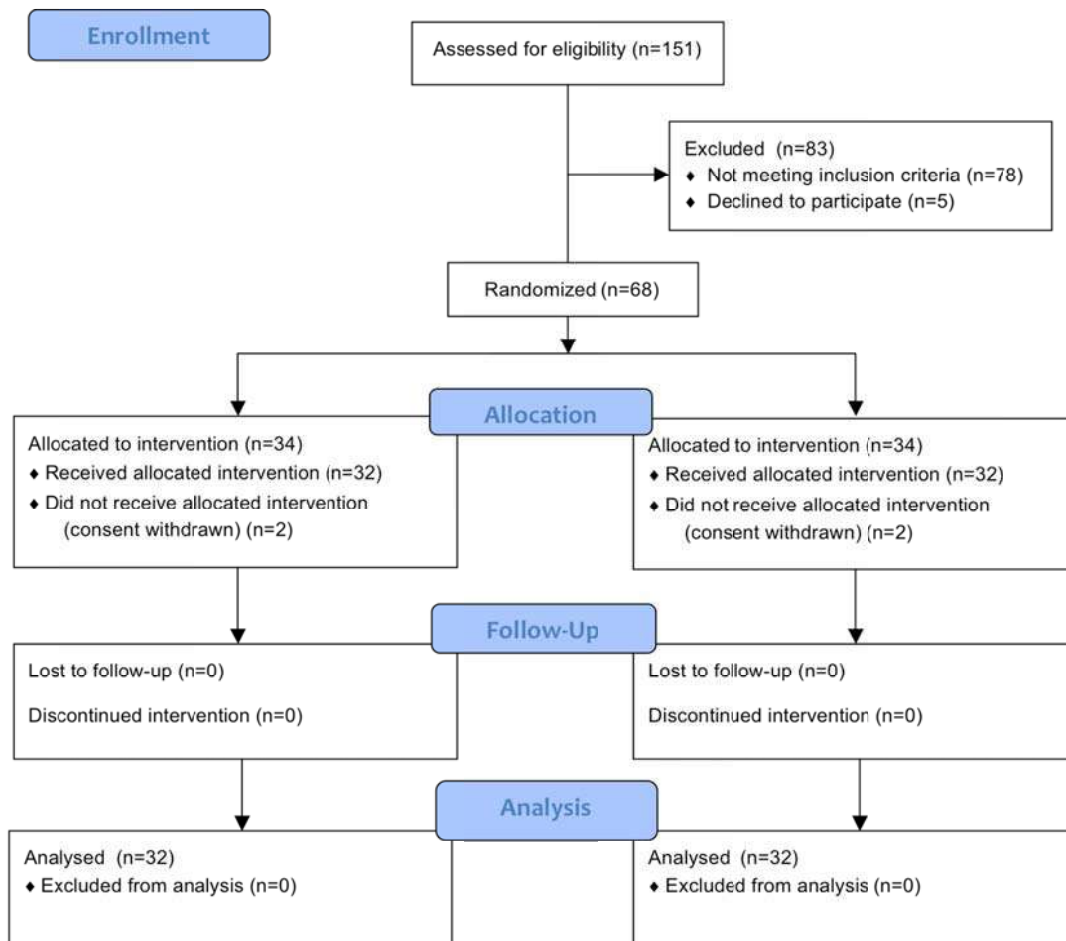
**Table S5** NO<sub>x</sub> and cGMP in biological compartments stratified by anti-hypertensive treatment allocation. The effects of 4 weeks dietary nitrate consumption (beetroot juice 250 mL daily) on nitrate, nitrite and cGMP concentrations in biological matrices. Data are expressed as mean±SD. Significance shown as +p<0.05 and +++p<0.001 for Dunnett's *post hoc* test comparison to baseline (Pre) following 2 way ANOVA; and for comparisons to placebo of the change between Pre and Post as \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 for unpaired Student t test. (cGMP=cyclic guanosine monophosphate; Pre=1<sup>st</sup> visit pre-intervention; Post=2<sup>nd</sup> visit post-intervention).



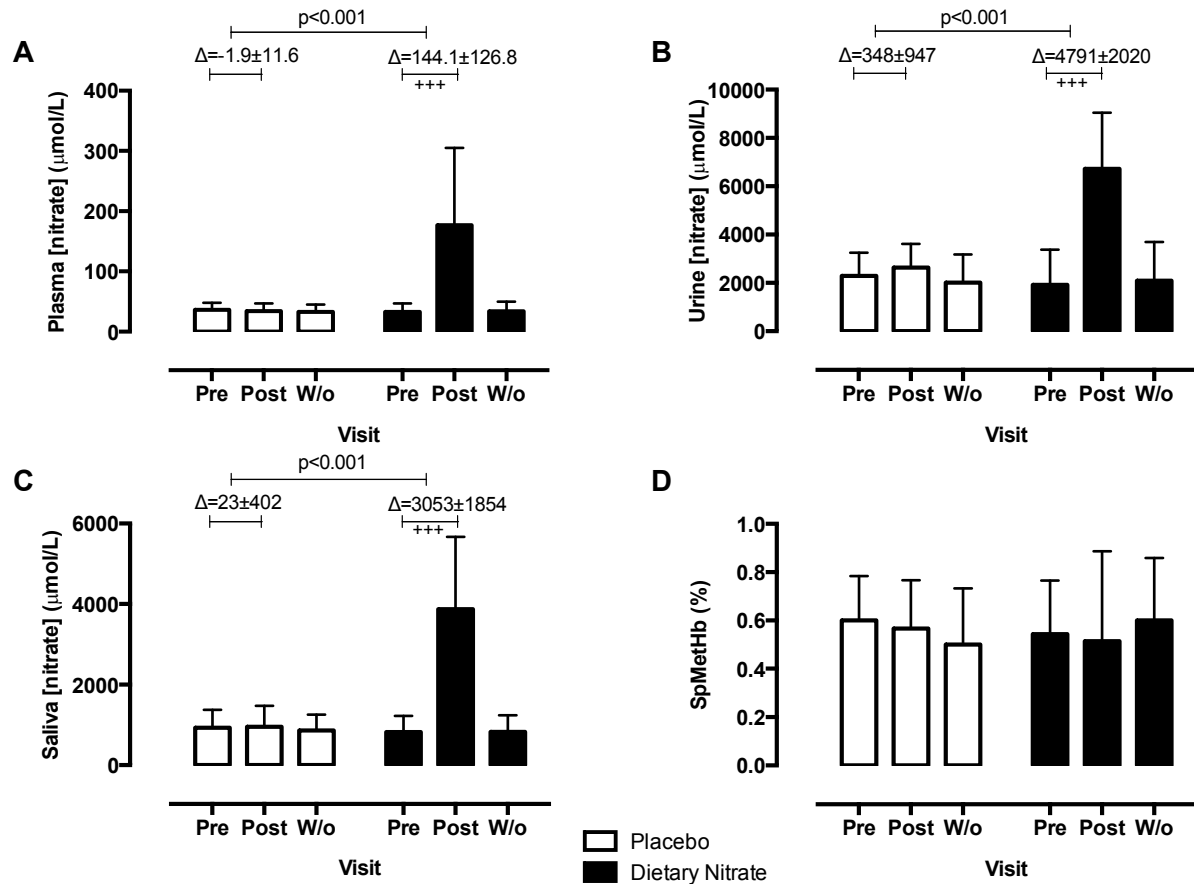


**Figure S1** Diagram of patient protocol

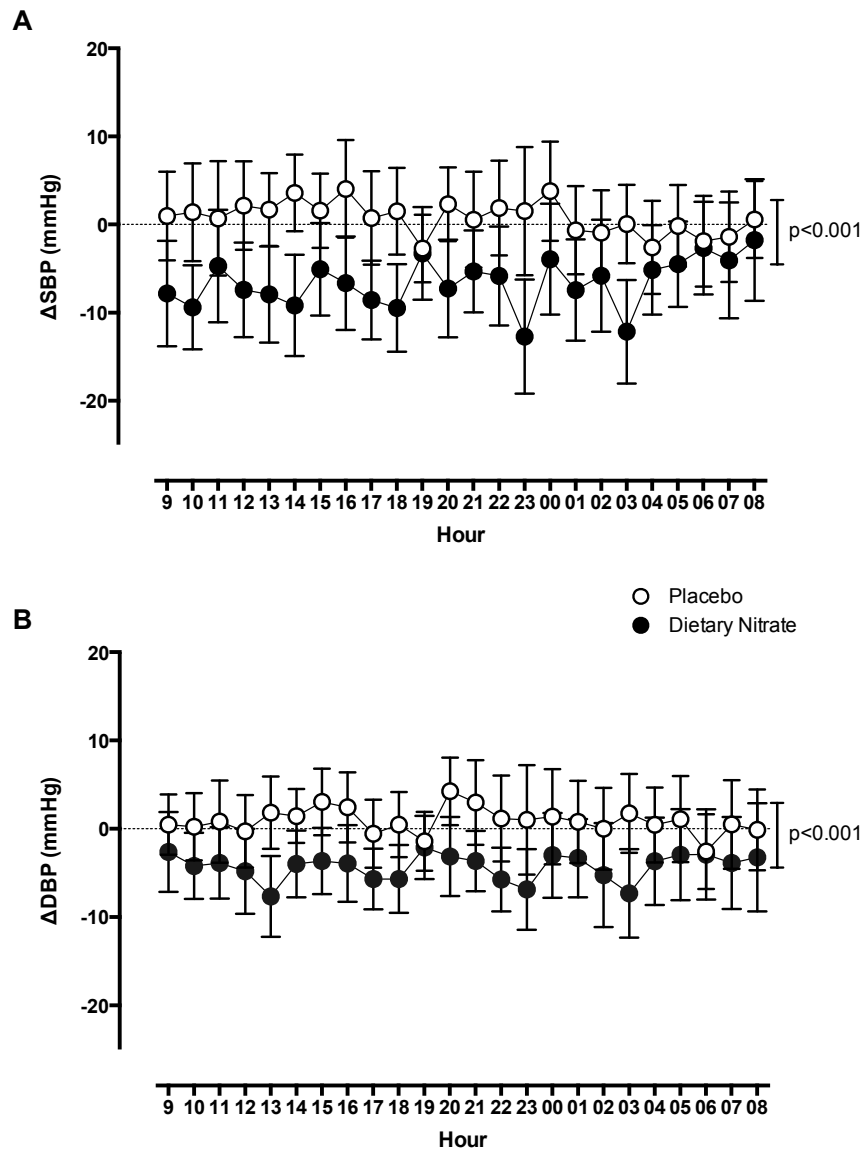
## CONSORT Flow Diagram



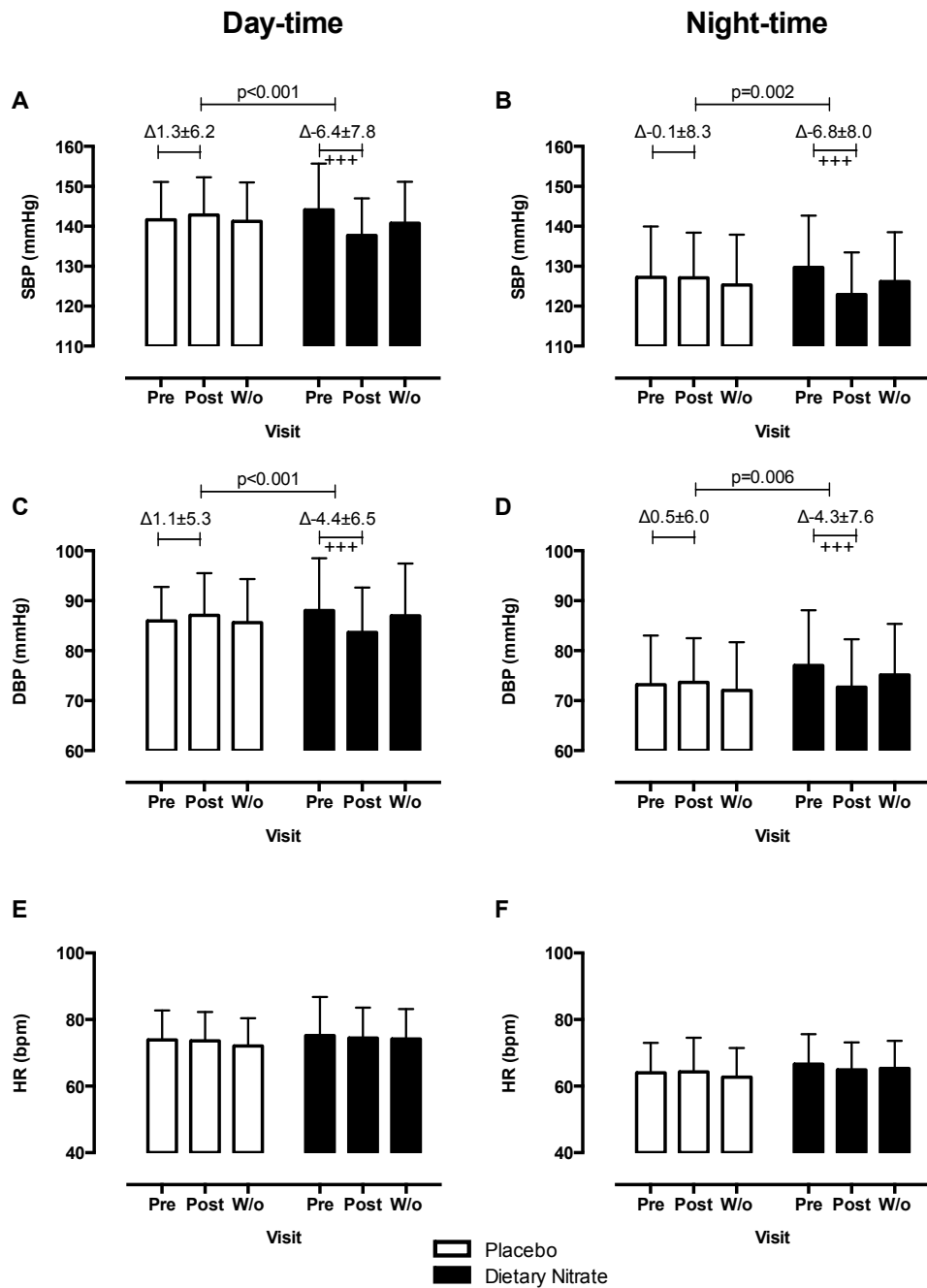
**Figure S2** CONSORT flow diagram



**Figure S3** Dietary nitrate consumption elevates nitrate concentration in hypertensive patients. The effects of 4 weeks dietary nitrate consumption (beetroot juice 250 mL daily) or placebo (nitrate-depleted beetroot juice 250 mL daily) on nitrate concentrations in (A) plasma, (B) urine (C) saliva and (D) transcutaneous arterial methaemoglobin concentrations. Data are expressed as mean $\pm$ SD. Significance shown for comparisons between treatment allocations of the change between Pre and Post as \*\*\* $p < 0.001$  for unpaired Student t test; and as +++ $p < 0.001$  for Dunnett's *post hoc* test comparison to baseline (Pre) following 2 way ANOVA for changes within each treatment allocation cohort. (Pre=1<sup>st</sup> visit pre-intervention; Post=2<sup>nd</sup> visit post-intervention; W/o=3<sup>rd</sup> visit washout).

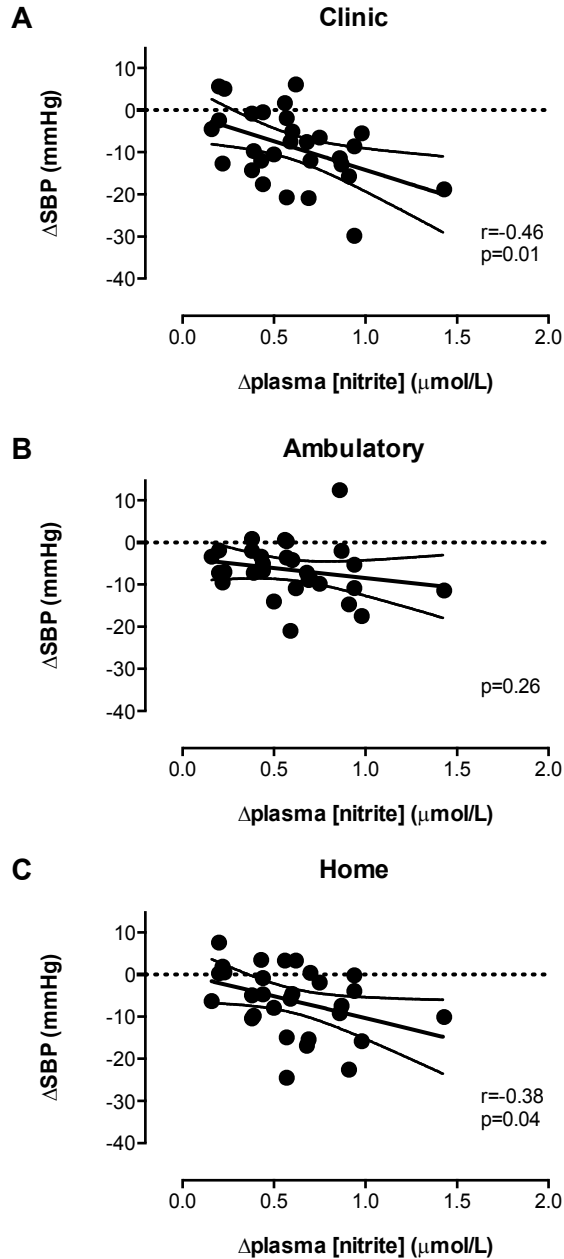


**Figure S4** Dietary nitrate consumption reduces ABP over entire 24h profile in hypertensive patients. The effects of 4 weeks dietary nitrate consumption (beetroot juice 250 mL daily) or placebo (nitrate-depleted beetroot juice 250 mL daily) on change in hourly (A) SBP and (B) DBP measured by ABP from Post to Pre. Data are expressed as mean±SD. Significance shown for comparisons between groups for 2-way ANOVA. (ABP=ambulatory blood pressure; BP=blood pressure; DBP=diastolic blood pressure; SBP=systolic blood pressure; Pre=1<sup>st</sup> visit pre-intervention; Post=2<sup>nd</sup> visit post-intervention).

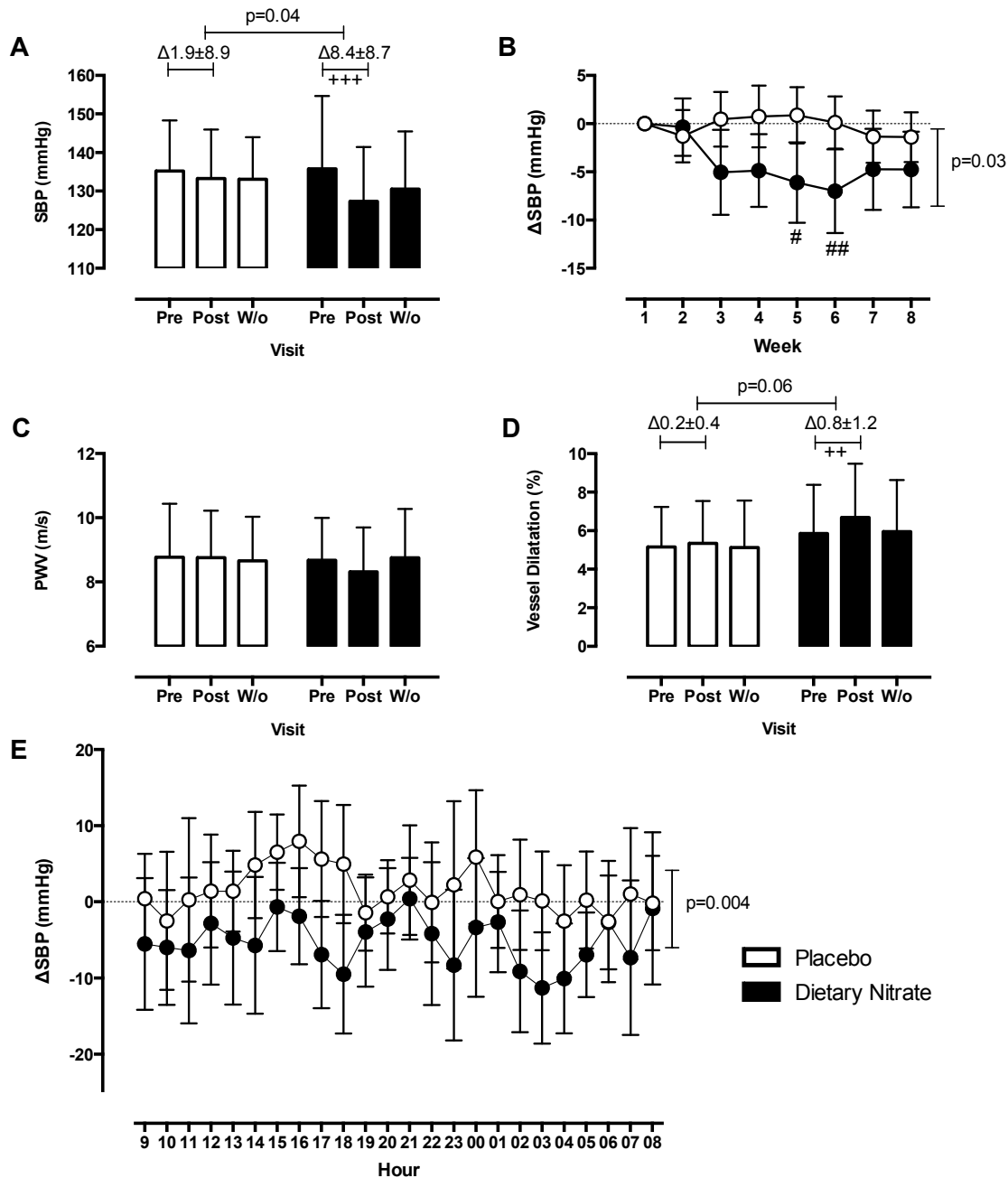


**Figure S5** Dietary nitrate consumption reduces daytime and nighttime ABP in hypertensive patients. The effects of 4 weeks dietary nitrate consumption (beetroot juice 250 mL daily) or placebo (nitrate-depleted beetroot juice 250 mL daily) on clinic measures of (A) SBP and (C) DBP and (E) HR; and on 24h ABP measures of (B) SBP and (D) DBP and (F) HR. Data are expressed as mean $\pm$ SD. Significance shown for comparisons between treatment allocations of the change between Pre and Post for unpaired Student t test; and as +++ $p < 0.001$  for Dunnett's *post hoc* test comparison to baseline (Pre) following 2 way ANOVA for changes within each treatment allocation

cohort. (ABP=ambulatory blood pressure; BP=blood pressure; DBP=diastolic blood pressure; HR=heart rate; SBP=systolic blood pressure; Pre=1<sup>st</sup> visit pre-intervention; Post=2<sup>nd</sup> visit post-intervention; W/o=3<sup>rd</sup> visit washout).



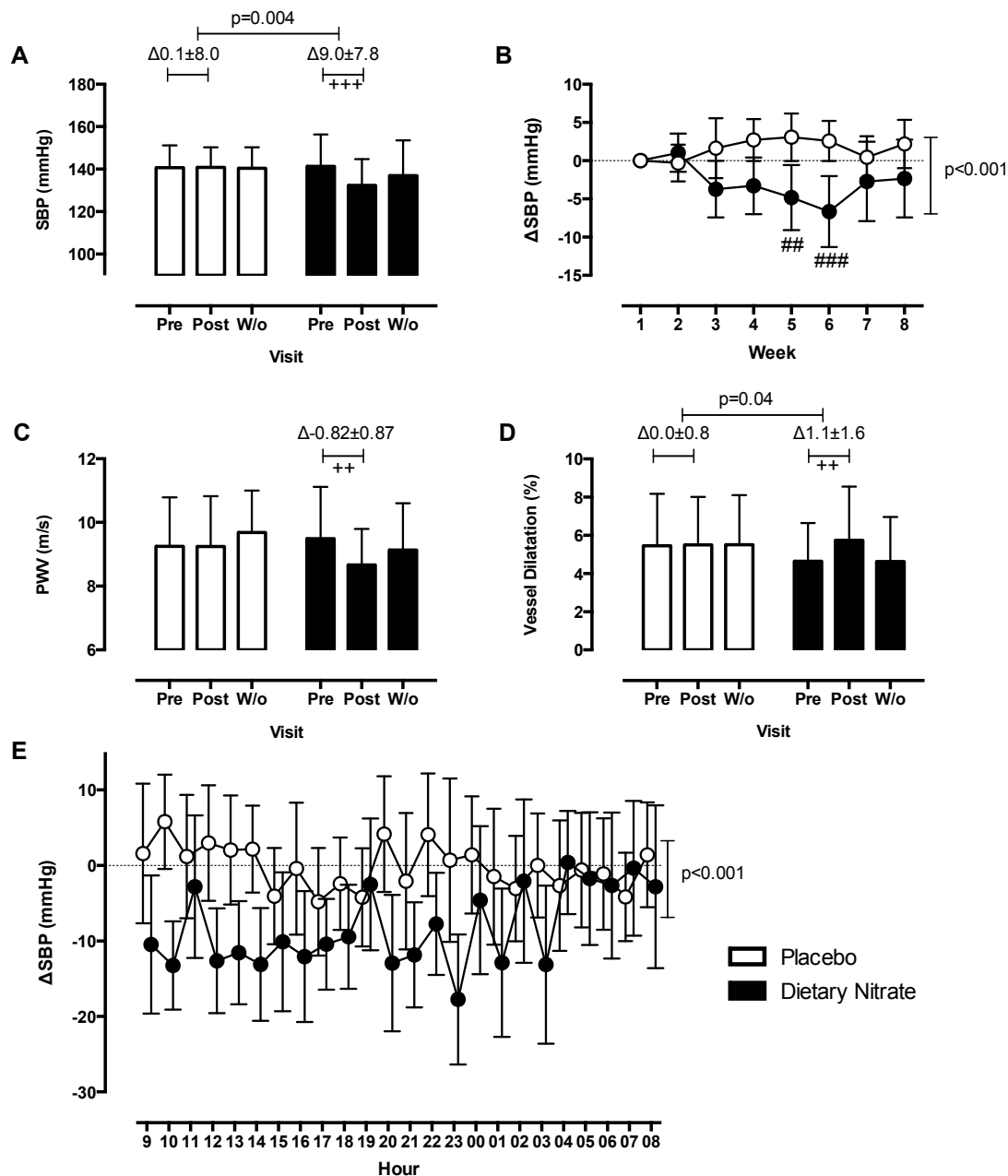
**Figure S6** The relationship between plasma nitrite concentration and BP. The change in plasma nitrite concentration and (A) clinic, (B) 24h ABP and (C) home SBP from Pre to Post in hypertensive patients allocated to dietary nitrate consumption for 4 weeks. Correlations determined using Pearson's correlation coefficient determination showing line of best fit and 95%CI's. (ABP=ambulatory blood pressure; SBP=systolic blood pressure; Pre=1<sup>st</sup> visit pre-intervention; Post=2<sup>nd</sup> visit post-intervention).



**Figure S7** Dietary nitrate consumption lowers BP in drug-naïve hypertensive patients. The effects of 4 weeks dietary nitrate consumption (beetroot juice 250 mL daily) or placebo (nitrate-depleted beetroot juice 250 mL daily) on (A) clinic SBP, (B) change in weekly home SBP compared to baseline (Week 1), (C) PWV, (D) % increase in FMD and (E) change in hourly SBP measured by ABP from Post to Pre. Data are expressed as mean $\pm$ SD. Significance shown for comparisons between treatment allocations of the change between Pre and Post for unpaired Student t test; as + $p<0.05$  and +++ $p<0.001$  for Dunnett's *post hoc* test comparison to baseline (Pre); by # $p<0.05$  and ## $p<0.01$  for Bonferroni *post hoc* test following 2 way ANOVA for changes within each treatment



allocation cohort. (ABP=ambulatory blood pressure; BP=blood pressure; cGMP=cyclic guanosine monophosphate; DBP=diastolic blood pressure; FMD=flow mediated dilatation; PWV=pulse wave velocity; SBP=systolic blood pressure; Pre=1<sup>st</sup> visit pre-intervention; Post=2<sup>nd</sup> visit post-intervention; W/o=3<sup>rd</sup> visit washout).



**Figure S8** Dietary nitrate consumption lowers BP in treated hypertensive patients. The effects of 4 weeks dietary nitrate consumption (beetroot juice 250 mL daily) or placebo (nitrate-depleted beetroot juice 250 mL daily) on (A) clinic SBP, (B) change in weekly home SBP compared to baseline (Week 1), (C) PWV, (D) % increase in FMD and (E) change in hourly SBP measured by ABP from Post to Pre. Data are expressed as mean $\pm$ SD. Significance shown for comparisons between treatment allocations of the change between Pre and Post for unpaired Student t test; as ++ $p<0.01$  and +++ $p<0.001$  for Dunnett's *post hoc* test comparison to baseline (Pre) following 2 way

ANOVA for changes within each treatment allocation cohort; and  $p < 0.001$  for 2-way ANOVA followed by  $p < 0.01$  and  $p < 0.001$  for Bonferroni *post hoc* test. (ABP=ambulatory blood pressure; BP=blood pressure; cGMP=cyclic guanosine monophosphate; DBP=diastolic blood pressure; FMD=flow mediated dilatation; PWV=pulse wave velocity; SBP=systolic blood pressure; Pre=1<sup>st</sup> visit pre-intervention; Post=2<sup>nd</sup> visit post-intervention; W/o=3<sup>rd</sup> visit washout

## Dietary Nitrate Provides Sustained Blood Pressure Lowering in Hypertensive Patients: A Randomized, Phase 2, Double-Blind, Placebo-Controlled Study

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