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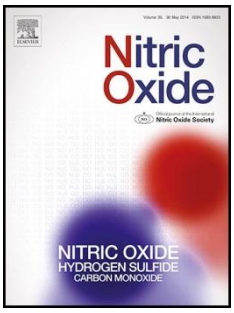

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Title: Ageing modifies the effects of beetroot juice supplementation on 24-hour blood pressure variability: an individual participant meta-analysis

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Running title: Beetroot juice and 24-hour blood pressure variability

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Highlights

- The effects of beetroot juice supplementation on BP variability have not been investigated.
- Beetroot juice decreased nocturnal systolic BP variability in subjects aged less than 65y.
- Greater changes in nitrite concentrations decreased nocturnal mean and variability of systolic BP.

Abstract

Objectives: Abnormal circadian oscillations of blood pressure (BP) and nocturnal-diurnal BP differences (i.e., dipping) increase cardiovascular risk. Whether inorganic nitrate supplementation influences 24-hr BP variability is currently unknown. We studied the effects of high-nitrate beetroot juice supplementation on BP variability measured by 24-hr ambulatory BP monitoring (24-hr ABPM) in older subjects.

Methods: Data from four independent randomised clinical trials were collated. Eighty-five older participants (age range: 55-76 years) were included in the final database. Two trials had an open-label, parallel design and two trials had a cross-over, double-blind design. Participants were randomised to either beetroot juice or placebo. Changes in 24-hr ABPM (daily, diurnal, nocturnal), variability (weighted-SDs), night-dipping, morning surge for systolic and diastolic BP were measured. Meta-analysis was conducted to obtain pooled estimates of the effect size for each BP outcome. Sub-group analyses were conducted to evaluate the influence of age, BMI, gender, BP status and changes in nitrite concentrations on the effect size.

Results The pooled effect of beetroot juice on all BP outcomes was not significant. Beetroot juice ingestion determined a significant decrease in nocturnal systolic BP variability in

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subjects aged less than 65y (2.8mmHg, -4.5 -1.0, p=0.002) compared to the older group (≥65y; 1.0mmHg, -2.2 4.2, p=0.54). A greater change in NO_2^- concentrations after beetroot supplementation was associated with significant differences for nocturnal mean (-3.4mmHg, -0.6 -2.4, p=0.02) and variability (-0.8mmHg, -1.5 -0.06, p=0.03) of systolic BP.

Conclusions: The vascular responsiveness to inorganic nitrate may be modified by mechanisms of vascular ageing influencing the reducing capacity to convert inorganic nitrate into nitrite and tissue-specific responses to dietary nitrate supplementation.

Keywords: ageing, ambulatory blood pressure, beetroot juice, inorganic nitrate, hypertension

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1. Introduction

Raised blood pressure (BP) is a leading cause of cardiovascular diseases and main contributor to the global burden of non-communicable diseases[1]. The haemodynamic effects of raised BP are responsible for the remodelling of cardiac ventricles[2] and intima-media thickening[3], which increase the risk of cardiovascular diseases such as heart failure or stroke[4]. There is a continuum of cardiovascular risk that increases as BP rises, and the theoretical minimum threshold of risk associated with systolic BP has been estimated to be approximately 115mmHg[5]. For each 2 mmHg rise in systolic BP there is a 7% increased risk of mortality from ischaemic heart disease and a 10% increased risk of mortality from stroke[6]. These statistics emphasise the importance of small reductions in BP for the effective management and prevention of hypertension-related comorbidities.

Effective nutritional and lifestyle interventions are key to prevent hypertension and related cardiovascular complications[7]. The reduction of salt intake is an example of a nutritional intervention with immediate benefits on BP regulation[8]. More recently, inorganic nitrate (NO_3^-) supplementation has been advanced as a potential, effective nutritional strategy to control BP[9; 10]. A recent meta-analysis showed a decrease in resting systolic BP of 4.4mmHg after either inorganic NO_3^- or beetroot juice supplementation[11]. In addition, dietary patterns rich in inorganic NO_3^-, such as the Dietary Approach to Stop Hypertension (DASH diet), have been associated with a reduction in resting systolic and diastolic BP of 5.2mmHg and 2.2mmHg, respectively[12].

Twenty-four hour ambulatory BP monitoring (24-hr ABPM) is a reference method for the diagnostic assessment of hypertension and monitoring of anti-hypertensive treatments[13; 14]. This method provides information on circadian BP rhythm such as mean diurnal and nocturnal BP, BP variability, night dipping and morning surge[15]. Abnormal values of any of these indexes are independently associated with a greater haemodynamic load and CVD risk[16]. The effects of inorganic NO_3^- supplementation on measures of 24-hr BP variability have not been investigated. We hypothesised that inorganic NO_3^- supplementation may increase nitric oxide (NO) bioavailability[9], via an NO-synthase independent NO generation, and influence both mean values and variability of systolic BP. We also predicted that these effects were more significant on nocturnal BP, which may be explained by the diminution of the putative, confounding effects of physical activity and mental stress on BP regulation.

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To address these hypotheses, we collated 24-hr ABPM data originally collected in four independent randomised clinical trials testing the effects of beetroot juice supplementation, as a rich source of inorganic NO_3^-, for a minimum of one week on 24-hr ABPM variability, diurnal and nocturnal BP, night dipping, morning surge and ambulatory arterial stiffness index (AASI) in older subjects (≥55 years). The individual data included in each trial were entered in a meta-analytical model to calculate the pooled effect size for each 24-ABPM systolic and diastolic outcome. Finally, we investigated whether the efficacy of inorganic NO_3^- on 24-hr ABPM outcomes was modified by ageing, gender, obesity, high BP and magnitude of post-supplementation rise in nitrite (NO_2^-) concentrations.

2. Methods

2.1 Study Design:

All trials were conducted in the UK and recruited older men and women aged 55 years and older. A description of the trial protocols has been previously reported for Trial-1[17] and Trial-3[18]. A description of the protocols of Trial-2 and Trial-4 is provided in the **Online Supplementary Material**. Briefly, two trials were conducted in Newcastle upon Tyne (Newcastle University, Trial-1, Trial-2) and two trials were conducted in Exeter (Exeter University, Trial-3, Trial-4). Both Newcastle-led trials had a two-arm, parallel study design. Trial-1 had a duration of three weeks and included blackcurrant juice as control (200 ml/day) and supplementation of 70 ml/day of concentrated beetroot juice (~4.5 mmol nitrate/day). Trial-2 had a duration of one week and included a negative control (diet only) and supplementation of 140 ml/day (~9.0 mmol nitrate/day) of concentrated beetroot juice. Both Exeter-led trials had a cross-over, double-blind, placebo controlled study design. Trials 3 and 4 had both a duration of two weeks and included alternate, random supplementation of 250 ml/day of NO_3^--rich beetroot juice (7.5mmol nitrate/day active) or 250 ml/day of NO_3^--depleted beetroot juice (0.002mmol nitrate/day, placebo). All beetroot juice supplements were provided by the same company (James White Drinks Ltd., UK).

2.2 Subjects:

A total of 85 non-smoking men and women (M/F: 50/35), aged 55–76 years old with body mass index (BMI) between 20.2 and 39.5 kg/m^2 were recruited at the Newcastle and Exeter research centres.Trial-1 was approved by the Newcastle University - Faculty of Medical Sciences Ethics Committee (Application No. 00628/2013). Trial-2 was approved by the North East - Northern & Yorkshire Research Ethics Committee (Study No. 12/NE/0134).

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Trials 3 and 4 were both approved by the Devon and Torbay Research Ethics Committee (Study No. 09/H0202/43).Written informed consent was obtained from all participants prior to participation in each trial.

2.3 Study Protocol

Newcastle: A telephone screening interview was conducted to ensure eligibility of participants. Participants attended the research facilities at Newcastle University in fasting conditions. Anthropometric measurements (weight, height and waist circumference) were performed and body mass index (BMI) calculated. Participants were then randomized to one of two interventions (Trial 1: beetroot or blackcurrant juice; Trial 2: beetroot or diet only) and baseline measurements including resting BP, collection of saliva (Trial-1) and plasma (Trial-2) samples, and completion of the International Physical Activity Questionnaire (IPAQ) for the assessment of physical activity. At the end of the visit participants were fitted with a 24-hour AMBP monitor to continuously assess BP over the next 24-hour period. Saliva samples were transferred to a −20 °C freezer within 2 hours of collection. Fasting blood samples were collected in lithium heparin tubes and centrifuged within 30min from collection. Plasma samples were then immediately transferred to a −80 °C freezer.

The intervention phase started immediately after the completion of the 24-hour BP monitoring period and lasted for 21 and 7 days for Trial-1 and Trial-2, respectively. During this phase, each participant was expected to comply with the assigned nutritional intervention and dietary plan to standardise NO_3^- intake. At the end of the intervention, participants returned to the research unit to repeat the same set of measurements performed at baseline.

Exeter: Subjects were recruited from the Exeter 10000 (EXTEND) bio-resource[18]. Eligible participants were randomized to begin, in either order, a 2-week period of supplementation with 250 ml of beetroot juice daily or 250 ml of NO_3^--depleted beetroot juice, followed by a 4-week washout period before entering the second arm of the study. Subjects were instructed to consume the juice along with their evening meal to minimize any potential glycaemic excursion, typically between 1800 and 2000 hours. Participants continued their usual antihypertensive medication and their usual hypoglycaemic medications including metformin. Hypoglycaemic agents were omitted on visits for which subjects were fasted. Twenty-four-hour blood pressure monitoring was performed from 0900 on day 13 of each supplementation arm. Fasting blood samples for NO_3^- and NO_2^- were collected into lithium heparin collection tubes. Samples were centrifuged immediately and plasma was immediately separated and flash-frozen in liquid nitrogen before transfer to a −80 °C freezer.

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2.4 Nutritional Supplementation:

Newcastle: Participants enrolled in Trial-1 and randomised to the intervention group were asked to every morning drink 70 ml of concentrated beetroot juice (Beet-It Sport Shot, James White company Ltd, Ipswich UK, 71 kcal). Each bottle (70 ml) provides approximately 300-400 mg of inorganic NO_3^-. Participants randomised to the control group were asked to every morning drink 200 ml of blackcurrant juice (Capri-Sun Blackcurrant Juice, 100kcal), 2.7±0.1 mg NO_3^- per bottle. Participants enrolled in Trial-2 and randomised to the intervention group were asked to drink 70 ml of concentrated beetroot juice in the morning and 70 ml in the evening. Participants randomised to the control group were asked to follow the diet only. Participants in both trials were required to follow a diet to standardize NO_3^- intake during the period of study. A description of the diet has been previously reported[17]. Participants were also asked not to change daily physical activities, to avoid the use of mouthwash during the study and to limit alcohol and caffeinated drink consumption during the study period.
Exeter: Participants were given either 250 ml beetroot juice (active) or 250 ml NO_3^--depleted beetroot juice (placebo) for two weeks. The untreated juice used in the active arm of the trial provided approximately 480 mg of NO_3^- per day and the placebo juice provided 0.15 mg of NO_3^- per day. Throughout the study patients were asked to maintain their normal diet apart from the juices given and not to change any other lifestyle factors. They were asked to continue their usual physical activity levels. Diet and activity levels were not monitored in the study.

2.5 Resting Blood Pressure

Newcastle: Resting BP was measured in triplicate using an automated BP monitor (Trial 1: Omron M2 Basic, Omron Healthcare, UK; Trial 2: CARESCAPE V100 monitor, GE Healthcare, UK) with the patient seated comfortably for 15 min prior to measurement and the arm supported at the level of the heart. The final value was calculated as the mean of the lowest two measurements. A large cuff was used for obese subjects.
Exeter: Resting BP was measured using an automated BP monitor (Omron M6, Kyoto, Japan) with the patient seated comfortably for 15 min prior to measurement and the arm supported at the level of the heart. Five measurements were taken in total and the mean of the last three was recorded. A large cuff was used for obese subjects.

2.6 24-hrABPM

Newcastle: A validated device approved by the British Hypertension Society was used to

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monitor 24-hr systolic and diastolic BP (Mobil-O-Graph NG, I.E.M. GmbH). All participants were instructed with respect to the use and the way the device operates. During monitoring BP was measured every 30min at daytime (between 0700 and 2200) and every 60min at night (between 2200 and 0700). Patients were advised to continue their normal activity during the monitoring period. All of the valid recordings were analysed to obtain average 24-hour systolic and diastolic BP.

Exeter: Each participant was fitted with a TM-2430 ambulatory blood pressure monitor (A&D Medical, Draycott, Gloucestershire, UK) (validated by the British Hypertension Society). The device was programmed to record BP every 15min between the hours of 0700 and 2200 and every 30min from 2200 to 0700. Participants were advised they could carry out their usual activities but to avoid strenuous exercise. All of the valid recordings were analysed to obtain average 24-hour systolic and diastolic BP.

2.7 24-hr Blood Pressure Outcomes

The same protocol was applied to the raw BP data to derive the ambulatory BP outcomes for both systolic and diastolic BP. 24-hr ABPM profiles were checked and systolic BP readings >250 or <70 mmHg, diastolic BP >150 or <40 mmHg were excluded. 24-hour mean BP is the average of the BP values recorded over 24-hours. Mean BP values were also calculated for diurnal (0715to 2145) and nocturnal (2200 to 0700). Weighted standard deviation (SD) for 24-hour, diurnal and nocturnal BP values was calculated as a measure of BP variability. Night BP dipping was calculated as the difference between nocturnal and diurnal BP. Morning surge was calculated as the difference between post-awakening BP (0715 to 0900) and nocturnal BP. The ambulatory arterial stiffness index (ASSI) was calculated as 1 minus the regression slope of DBP on SBP from ABPM[19].

2.8 Nitrate and Nitrite Concentrations

Newcastle: A modified version of the method proposed by Tsikas et al[20] was used to determine NO_3^- and NO_2^- concentrations in saliva (Trial-1) and plasma (Trial-2) samples using gas chromatography mass spectrometry (GC-MS). The validation and protocol of the modified GC-MS method has been described elsewhere[21]. This method showed a good repeatability, as coefficients of variation for replicate analyses of samples were 7.8%, 8.6% and 12.0% in saliva, urine and plasma samples, respectively.

Exeter: Before analysis, samples were deproteinized using a modification of the technique described by Higuchi and Motomizo[22]. Plasma NO_3^- and NO_2^- concentrations were

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determined using a Sievers nitric oxide analyzer (Sievers NOA 280; Analytix Ltd, Durham, UK) using the methods described by Bateman et al.[23]. The between-batch coefficient of variation for NO_3^- was 13% and for NO_2^- was 8%.

2.9 Statistical Analysis

All statistical analyses were completed using SPSS for Windows (SPSS, version 17.0; SPSS Inc, Chicago, Ill, USA). Summary data are presented as mean (SD or 95%CI). P values<0.05 (2-tailed) were considered as statistically significant.

Newcastle: A general linear model was used to test differences in BP outcomes between the two nutritional interventions (beetroot, control). Analyses were adjusted for baseline values of the selected outcome. Post-intervention means and 95%CI are reported for each trial.

Exeter: Paired t-test was used to compare differences in BP outcomes between the two nutritional interventions (beetroot, placebo). Post-intervention means and 95%CI are reported for each trial.

Meta-analysis: A two-step meta-analysis of individual data was performed to pool together the summary statistics of each trial[24]. Meta-analysis was performed by using Comprehensive Meta-Analysis software (version 2, Biostat, Englewood, New Jersey, USA). The post-intervention least-squares means and SD values of each 24-hr ABPM outcome for both intervention and control groups were extracted and used in the analysis of parallel trials. The end of intervention mean differences and SD values of the differences between intervention and control were used for the analysis of cross-over trials. Data synthesis, including calculation of effect sizes with 95%CI, was accomplished by employing fixed or random-effect models. Random effects models were employed when a substantial heterogeneity between trials was observed (I^2>50%)[25]. Heterogeneity was evaluated by the Cochran's Q test and I^2 calculated. Subgroup analyses were undertaken to investigate the variables which influenced the effects of beetroot juice supplementation on 24-hr ABPM outcomes. These factors included: age (<65y, ≥65y), gender (male, female), BMI (<30.0 kg/m^2, ≥30.0 kg/m^2), resting BP status (high, normal) and percent changes in saliva (Trial 1) and plasma (Trial 2, 3, 4) NO_2^- concentrations after supplementation. Percent changes relative to baseline were calculated for salivary and plasma changes in NO_2^- concentrations after beetroot supplementation. The median (50th centile) of the distribution was calculated to compare the effects between subjects with lower (<50th centile) *vs* higher (≥50th centile) changes in NO_2^- concentrations on 24-hr ambulatory BP outcomes. Meta-regression analysis was performed to evaluate whether changes in NO_2^- concentrations were associated with

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changes in 24-hr ambulatory BP outcomes. High BP was defined as having a systolic BP ≥140 mmHg or diastolic BP ≥90 mmHg. A mixed-effect model was used to evaluate within-factor (p_{within}) and between-factor ($p_{between}$) significant differences in effect size for each 24-hr ABPM outcome.

3. Results

Participants' Baseline Characteristics

A total of 50 males and 35 females older participants (63.8±5.2 years) were included in the final analysis. Seventeen healthy normal weight participants (BMI: 25.6±2.5 kg/m^2) were included in Trial-4; 21 and 20 overweight and obese participants were included in Trial-1 (BMI: 30.1±4.2 kg/m^2) and Trial-2 (BMI: 29.8±4.5 kg/m^2), respectively. Trial-3 included 27 obese type 2 diabetic participants (BMI: 30.7±3.1kg/m^2).The average resting systolic and diastolic BP were 138.7±16.4 mmHg and 79.5±9.6 mmHg, respectively and 36 subjects had a high resting systolic (154.1±11.2 mmHg) and diastolic (85.7±8.3 mmHg) BP. Baseline characteristics of the two parallel trials were not significantly different between interventions. **Table 1** shows the baseline characteristics of the subjects included in each trial. BP medications were prescribed only in 26 type 2 diabetic patients (Trial 3) whereas participants in the other three trials were free of anti-hypertensive medications. Additional baseline characteristics for each trial are reported in **Table S1 of the Online Supplementary Material.**

3.1 Individual Trials

Systolic BP: Overall, each trial showed no significant effect of beetroot juice supplementation on all 24-hr ambulatory BP outcomes. Only Trial-1 showed a significant increase in morning surge (11.0mmHg, 0.1, 22.9, p=0.04) which was not observed in the other trials (**Table 2**).

Diastolic BP: Overall, trials showed no significant effect of beetroot juice supplementation on all 24-hr ambulatory BP outcomes. Only Trial-1 and Trial-3 showed a significant increase in morning surge (9.0mmHg, -1.7 16.3, p=0.02) and night dipping (2.6 mmHg, -0.3 4.8, p=0.02), respectively. However, these results were not confirmed in the other trials (**Table 3**).

AASI: Beetroot juice supplementation was not associated with significant changes in AASI in each trial (**Table S2, Online Supplementary Material**).

3.2 Main Meta-Analysis

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Beetroot juice supplementation was not associated with significant changes in systolic and diastolic 24-hrABPM outcomes when results from the four trials were pooled together. Fixed models were applied to all BP outcomes because of the non-significant heterogeneity (I^2 range: 0 - 41%) with the exception of morning surge (systolic BP, I^2=61%; diastolic BP, I^2=74%) which employed a random model to derive the pooled estimates (**Table 4**). Beetroot juice supplementation was not associated with significant changes in AASI (**Table S3, Online Supplementary Material**).

3.3 Sub-group Meta-Analysis

Age: Beetroot juice supplementation determined a significant decrease in nocturnal systolic BP variability in subjects aged less than 65y (2.8 mmHg, -4.5 -1.0, p_{within}=0.002) compared to the older group (≥65y; 1.0mmHg, -2.2 4.2, p_{within}=0.54) and a significant difference between the two age groups was observed ($p_{between}$=0.04). In addition, beetroot juice had a significantly lower effect on night dipping in older subjects (3.3mmHg, 0.2 6.4, p_{within}=0.03) compared to younger subjects (-1.4mmHg, -4.2 1.4, p_{within}=0.33; $p_{between}$=0.02). No significant between-group differences were observed for diastolic BP (**Table 5**).

Gender: We did not observe significant differences for all 24-hr ambulatory systolic BP outcomes between male and female subjects. A marginal effect was observed for nocturnal diastolic BP variability with lower variability observed in female compared to male subjects ($p_{between}$=0.05) (**Table 5**).

BMI: Body size had a marginal effect on both systolic and diastolic BP outcomes. Normal weight and overweight subjects appeared to have a significant lower nocturnal systolic BP variability (-1.9 mmHg, -3.6 -0.2, p_{within}=0.03) compared to obese subjects (**Table 5**).

Resting BP: Overall, we did not observe significant differences for all 24-hr ambulatory systolic BP outcomes between subjects with normal or raised BP. A marginal effect was observed for nocturnal systolic BP variability in subject with normal BP (-2.1 mmHg, -4.2 0.08, p_{within}=0.06) (**Table 5**).

Δ%[NO_2^-]: The median (50th centile) of the percent change in NO_2^- concentrations after beetroot juice supplementation in the four trials was 30.9%. Subjects with greater changes in NO_2^- concentrations (≥50th centile) showed a significant difference for nocturnal mean systolic BP (-3.4 mmHg -0.6 -2.4, $p_{between}$=0.02), nocturnal systolic BP variability (-0.8 mmHg, -1.5 -0.06, p_{within}=0.03) and night dipping (-2.5 mmHg, -3.4 -1.9, p_{within}=0.01) (**Table 5**). Meta-regression showed a significant association between percent changes in NO_2^- concentrations with nocturnal mean systolic BP (β=-0.01±0.006 mmHg, p=0.04) (**Figure S1,**

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Online Supplementary Material). The association was not significant with other 24-hr BP outcomes (data not showed). Percent changes in NO_2^- concentrations were not associated with a significant effect on AASI (**Table S4, Online Supplementary Material**).

4. Discussion

This meta-analysis of individual participant data presents the most comprehensive evaluation to date on the effects of beetroot juice supplementation on 24-hr ABPM in older subjects. Our results showed that the main effect of inorganic NO_3^- on 24-hr ABPM outcomes was not significant. However, sub-group analyses revealed ageing and post-supplementation changes in NO_2^- concentrations as potential factors influencing the association between inorganic NO_3^- and vascular responses. The latter highlights the clustering of the population into two distinct phenotypes, named as"reducers" and "non-reducers", discriminated by their efficiency in reducing inorganic NO_3^- into NO_2^-, which is closely correlated with the vascular response. The mechanisms underpinning these individual differences are still largely under-investigated and they may involve the oral microbiota, gastric redox environment, oxygen tension and pH in the peripheral circulation or efficiency of enzymatic reductase activity (i.e., deoxy-haemoglobin, aldehyde dehydrogenase, xanthine oxido-reductase)[9; 26]. In addition, the ageing process may play a role in all these mechanisms. Ageing is associated with changes in oral microflora which may influence the efficiency of bacterial reductase activity in the conversion of NO_3^- into NO_2^-[27]. In addition, gastric acid production declines with age[28]and this process may affect the formation of NO in the acid stomach from the acid-mediated disproportionation of NO_2^-[29]. Hence, it is currently not known whether greater doses of inorganic NO_3^- are required in older people to account for the decline in redox potential and augment NO bioavailability. Ageing may also be associated with diminished sensitivity of vascular smooth muscular cells (VSMCs) to the dilatory effects of NO, thus higher doses may be required[30]. This reduced sensitivity causes an impaired NO-dependent vasodilation as demonstrated by a reduced *in vitro* response of VSMCs to NO with ageing[31], which may contribute to explain the reduced vascular responses in older participants.

The effects of beetroot juice supplementation mainly influence nocturnal systolic BP. A diminished nocturnal fall in BP is an independent risk factor for arterial stiffness and recent findings have identified mean nocturnal BP as a sensitive predictor of cardio- and cerebrovascular morbidity and mortality[32; 33]. Cardiovascular risk decreased by 17% for every 5 mmHg decrease in nocturnal systolic BP in both hypertensive and non-hypertensive populations even after adjusting for sex, age, diabetes, baseline BP and hypertension

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371 medications[34]. These results may suggest a putative role played by either physical activity
372 or emotional stress in confounding the effects of inorganic NO_3^- on diurnal BP measured by
373 24-hr ABPM. These two factors are known for modifying the reliability of the technique as
374 well as cardiovascular responses and, therefore, potentially introducing a bias in the
375 measurement of diurnal BP[35]. In addition, inorganic NO_3^- plays a role in skeletal muscle
376 energetics[36] and it may contribute to the non-significant effect of inorganic NO_3^- on diurnal
377 BP. A recent study has also suggested that skeletal muscle may serve as a nitrate reservoir,
378 for direct formation of nitrite and NO, and for determining levels of nitrate in other
379 organs[37]. We hypothesise that inorganic NO_3^- supplemented during the more active diurnal
380 hours may be utilised by the skeletal muscle and channelled towards metabolic functions,
381 which may reduce its availability for vascular regulatory mechanisms. Rassaf et al.[38] have
382 demonstrated that physical activity may unmask endothelial dysfunction and impaired
383 cardiovascular function by enhancing a greater conversion of NO_2^- into NO, by virtue of
384 lower PO_2 and pH in muscular tissue of subjects with major cardiovascular risk factors. The
385 same research group also demonstrated that healthy older subjects failed to adequately
386 increase circulating NO_2^- after exercise[39]. The effects of inorganic nitrate on muscular
387 metabolism and exercise energetics in older subjects and the partitioning of dietary nitrate
388 towards metabolic and vascular functions are a novel area of research and future studies are
389 needed to test these hypotheses.
390 This is the first analysis that has collected individual data from the only four randomised
391 clinical trials that, to date, have investigated the effects of inorganic NO_3^- supplementation on
392 24-hr ABPM. Three other trials have tested the effects of beetroot-based interventions on 24-
393 hr ABPM. Two trials have not been considered for inclusion as they have evaluated the acute
394 effects (i.e., one day) of a single dose of beetroot juice on 24-hr ABPM outcomes[40; 41].
395 The third trial was conducted in patients with hypertension aged 18 to 85 years. The trial
396 reported a significant effect of beetroot juice on 24-hr, daily and nocturnal systolic BP
397 whereas the effect on BP variability has not been reported[42]. An additional strength of our
398 analysis is represented by the sample size which is the largest available dataset testing the
399 effects of inorganic NO_3^- supplementation on 24-hr ABPM outcomes. *A posteriori* sample
400 size calculations showed a power of 81% to detect differences in BP of ±3.0mmHg (SD
401 7.5mmHg) between control and intervention groups. A limitation of the analysis is the
402 difference in study design between trials. However, the heterogeneity of the pooled estimates
403 ranged from moderate to low for all 24-hr ABPM outcomes, which denoted an overall
404 between-study agreement of the effects of beetroot juice on 24-hr ABPM outcomes. Morning

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surge was the only BP outcome with high heterogeneity and therefore a random model was applied to derive the pooled effect size. The trials showed differences in the collection of biological samples (saliva, blood) to test for changes in NO_3^- and NO_2^- during supplementation; however, the exclusion of the saliva samples from the analysis did not modify the association between nocturnal BP outcomes and changes in NO_2^- concentrations. Subjects with type 2 diabetes (Trial 3) were on anti-hypertensive medications during the beetroot juice supplementation, which may have also influenced the study outcomes. However, the exclusion of Trial 3 from the meta-analysis did not modify the pooled results as we still observed a significant influence of ageing and changes in NO_2^- on nocturnal mean, variability and dipping of systolic BP. An additional limitation of the analysis was the inability to test the biological mechanisms that may have explained the significant effects of beetroot juice supplementation on nocturnal systolic BP as well as the putative role of ageing in modifying the effects of dietary NO_3^- on vascular function. Biological factors may include NO_3^- partitioning between vascular and metabolic effects during diurnal activities and the role of ageing in the influencing the reducing steps converting NO_3^- into NO_2^- and NO and/or biological responsiveness of target cells to NO activity. This information was however not available in the four trials included in the meta-analyses. We therefore advocate for a careful interpretation of our results until these mechanisms will be tested in future studies.

5. Conclusions

Ageing and changes in NO_2^- concentrations modified the effects of beetroot juice, as a rich source of inorganic NO_3^-, on nocturnal systolic BP variability. The vascular responsiveness to inorganic NO_3^- may be modified by mechanisms of vascular ageing and efficiency of the reductase activity converting inorganic NO_3^- into NO_2^-. If confirmed in future studies, these findings may open novel opportunities to improve personalised nutrition for the management of hypertension.

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Contributions

The Corresponding Author (MS) is the guarantor for the manuscript and had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final version of the paper.

Conflicts of Interests:

Newcastle: All authors have no conflicts of interest to declare

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Table 1: Descriptive statistics of the four trials included in the analysis.										
	Trial 1^{PL}			Trial 2^{PL}			Trial 3^{CO}		Trial 4^{CO}	
	Beetroot	Control	p	Beetroot	Control	p	All	p	All	p
N	10	11		10	10		27	-	17	-
M/F	7/3	5/6	0.38	4/6	5/5	0.87	18/9	-		-
Age (years)	62.7±4.9	61.4±4.3	0.54	64.9±6.1	61.4±3.4	0.13	67.2±4.9	-	60.5±3.6	-
Height (m)	1.74±0.11	1.69±0.11	0.28	1.69±0.08	1.69±0.11	0.86	1.68±0.08	-	1.74±0.10	-
Weight (kg)	92.5±15.4	84.2±14.6	0.21	84.3±16.4	86.5±13.4	0.76	87.1±11.5	-	78.7±12.3	-
BMI (kg/m^2)	30.5±4.4	29.4±4.1	0.55	29.7±4.9	30.0±4.2	0.89	30.7±3.1	-	25.6±2.5	-
WC (cm)	103.8±12.8	100.7±10.1	0.55	103.0±11.9	97.8±24.5	0.77	106.1±8.0	-	93.3±10.5	-
Resting SBP	135.1±14.8	131.1±14.8	0.54	146.9±19.8	143.8±20.3	0.73	142.8±13.9	-	131.6±16.0	-
Resting DBP	77.4±9.5	76.1±10.9	0.78	79.3±8.5	77.8±12.7	0.66	81.1±9.1	-	80.0±9.57	-

580 Data are presented as mean±SD; N= number of subjects; M/F=male/female; BMI=body mass index; WC= waist circumference;
581 SBP= systolic blood pressure; DBP= diastolic blood pressure; PL= parallel study design; CO= cross-over study design.
582 T test for independent samples was used to compare the two groups in parallel trials.(Trial 1,2: Newcastle) and (Trial 3,4: Exeter)

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Table 2: Effects of beetroot juice supplementation on systolic blood pressure outcomes measured by 24-hr ambulatory blood pressure monitoring. Results are presented individually for each trial included in the meta-analysis.

	Trial 1[PL]				Trial 2[PL]				Trial 3[CO]				Trial 4[CO]			
	Beetroot	Control	Δ	p	Beetroot	Control	Δ	P	Beetroot	Control	Δ	p	Beetroot	Control	Δ	p
24-hour Mean	126.3 (119.9, 132.7)	127.9 (121.8, 134.0)	-1.5 (-10.4, 7.3)	0.72	127.6 (123.9, 131.4)	124.7 (120.9, 128.4)	2.9 (-2.4, 8.3)	0.26	135.1 (132.0, 135.8)	134.5 (131.2, 137.8)	0.6 (-2.7, 3.9)	0.72	131.0 (124.3, 137.8)	129.8 (124.5, 135.2)	1.1 (-2.1, 4.5)	0.46
24-hour SD	14.7 (11.4, 17.9)	15.4 (12.3, 18.5)	-0.7 (-5.2, 3.8)	0.75	16.5 (13.3, 19.7)	14.6 (11.4, 17.8)	1.9 (-3.8, 5.2)	0.38	20.9 (19.7, 22.1)	21.2 (19.6, 22.8)	-0.3 (-1.7, 1.2)	0.68	18.8 (16.6, 21.0)	19.3 (16.8, 21.8)	-0.5 (-2.6, 1.6)	0.64
Diurnal Mean	130.5 (123.6, 137.5)	131.2 (124.5, 137.9)	-0.7 (-10.4, 9.0)	0.88	132.2 (127.0, 137.3)	128.3 (123.2, 133.5)	3.8 (-3.5, 11.1)	0.29	138.8 (135.0, 142.6)	138.9 (135.0, 142.9)	-0.2 (-3.8, 3.5)	0.93	135.5 (129.2, 141.7)	134.3 (129.1, 139.4)	1.2 (-2.0, 4.3)	0.45
Diurnal SD	13.1 (9.4, 16.9)	14.8 (11.2, 18.4)	-1.6 (-6.9, 3.6)	0.52	14.6 (11.5, 17.6)	13.4 (10.4, 16.4)	1.2 (-3.2, 5.5)	0.58	19.7 (18.3, 21.1)	19.8 (18.2, 21.4)	-0.1 (-1.5, 1.4)	0.88	17.6 (15.2, 20.3)	17.9 (15.1, 20.6)	-0.3 (3.0, 2.4)	0.82
Nocturnal Mean	115.6 (109.0, 122.3)	121.2 (114.8, 127.6)	-5.5 (-14.8, 3.6)	0.22	111.1 (106.5, 115.7)	115.4 (110.8, 120.0)	-4.3 (-10.8, 2.1)	0.17	123.3 (120.2, 126.4)	121.2 (116.9, 125.5)	2.1 (-1.6, 5.8)	0.26	117.4 (107.9, 126.9)	116.1 (109.2, 123.0)	1.3 (-3.8, 6.4)	0.59
Nocturnal SD	11.1 (8.3, 13.9)	13.4 (10.7, 16.1)	-2.3 (-6.2, 1.5)	0.22	11.5 (8.6, 14.3)	11.8 (9.0, 14.7)	-0.3 (-4.3, 3.6)	0.85	16.9 (14.8, 19.1)	16.6 (14.4, 18.7)	0.3 (-2.0, 2.8)	0.75	13.0 (11.0, 15.1)	15.4 (12.2, 18.7)	-2.4 (-6.0, 1.2)	0.17
Dipping	-14.9 (-20.0, -9.8)	-9.9 (-14.7, -5.1)	-4.9 (-12.0, 2.1)	0.15	-18.7 (-24.8, -12.7)	-15.1 (-21.1, -9.0)	-3.6 (-12.2, 5.0)	0.39	-15.4 (-20.1, -10.8)	-17.7 (-22.9, -12.5)	2.3 (-1.8, 6.3)	0.26	-18.0 (-23.4, -12.6)	-18.1 (-22.2, -14.1)	0.1 (-3.9, 4.2)	0.94
Morning Surge	**15.5 (7.1, 23.8)**	**4.0 (-3.8, 12.0)**	**11.0 (0.1, 22.9)**	**0.04**	17.1 (8.4, 25.7)	12.0 (3.4, 20.0)	5.0 (-7.5, 17.6)	0.41	16.2 (11.6, 20.9)	20.8 (15.9, 25.8)	-4.5 (-10.7, 1.6)	0.58	14.2 (7.7, 20.8)	12.4 (7.6, 17.3)	1.8 (-5.1, 8.7)	0.14

Data are presented as mean (95%CI). SD= standard deviation. PL= parallel study design; CO= cross-over study design; Δ= difference between beetroot and control group. Significant results are highlighted in bold. (Trial 1,2: Newcastle) and (Trial 3,4: Exeter)

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Table 3: Effects of beetroot juice supplementation on diastolic blood pressure outcomes measured by 24-hr ambulatory blood pressure monitoring. Results are presented individually for each trial included in the meta-analysis.

	Trial 1[PL]				Trial 2[PL]				Trial 3[CO]				Trial 4[CO]			
	Beetroot	Blackcurrant	Δ	p	Beetroot	Control	Δ	p	Beetroot	Control	Δ	p	Beetroot	Control	Δ	p
24-hour Mean	79.4 (74.9, 83.9)	80.1 (75.8, 84.4)	-0.7 (-6.9, 5.5)	0.81	78.2 (76.1, 80.4)	76.6 (74.4, 78.8)	1.6 (-1.4, 4.7)	0.28	75.1 (73.0, 77.2)	76.4 (74.2, 78.6)	-1.2 (-3.1, 0.6)	0.17	79.9 (76.3.3, 83.5)	79.5 (76.1, 82.9)	0.4 (-2.1, 2.9)	0.74
24-hour SD	11.8 (9.9, 13.8)	11.0 (9.2, 12.9)	0.8 (-1.8, 3.5)	0.53	11.4 (9.7, 13.1)	10.9 (9.1, 12.6)	0.5 (-1.8, 2.9)	0.63	15.7 (14.1, 17.2)	16.5 (14.7, 18.2)	-0.8 (-2.8, 1.2)	0.40	15.1 (12.9, 17.3)	14.3 (12.3, 16.3)	0.8 (-1.4, 2.9)	0.46
Diurnal Mean	82.9 (77.9, 88.0)	82.8 (78.1, 87.6)	-0.1 (-6.0, 7.0)	0.97	81.8 (78.9, 84.7)	79.6 (76.7, 82.5)	2.1 (-2.0, 6.3)	0.29	77.2 (74.8, 79.6)	79.3 (76.7, 81.9)	-2.1 (-4.2, 0.1)	0.06	83.0 (79.7, 86.3)	82.6 (79.2, 85.9)	0.4 (-2.6, 3.5)	0.76
Diurnal SD	10.5 (8.7, 12.2)	9.4 (7.8, 11.0)	1.1 (-1.3, 3.4)	0.37	9.1 (7.6, 10.5)	9.1 (7.7, 10.6)	-0.08 (-2.1, 1.9)	0.93	15.7 (14.0, 17.4)	16.4 (14.5, 18.4)	-0.7 (-3.0, 1.5)	0.50	14.7 (11.9, 17.4)	13.8 (11.4, 16.0)	0.9 (-1.6, 3.5)	0.45
Nocturnal Mean	70.5 (65.6, 75.4)	74.1 (69.5, 78.8)	-3.6 (-10.3, 3.1)	0.27	66.0 (61.7, 70.2)	68.5 (64.3, 72.7)	-2.5 (-8.5, 3.4)	0.38	68.3 (66.0, 70.3)	67.8 (65.1, 70.5)	0.5 (-1.1, 2.1)	0.51	70.4 (65.0, 75.8)	69.9 (65.4, 74.5)	0.5 (-2.2, 3.1)	0.71
Nocturnal SD	9.5 (7.0, 12.0)	10.2 (7.8, 12.5)	-0.7 (-4.1, 2.7)	0.67	9.1 (6.4, 11.7)	10.5 (7.9, 13.1)	-1.4 (-5.1, 2.3)	0.43	11.9 (10.4, 13.5)	11.6 (9.7, 13.4)	0.3 (-1.9, 2.7)	0.74	10.0 (8.5, 11.6)	10.1 (8.1, 12.1)	-0.1 (-2.4, 2.2)	0.93
Dipping	-11.9 (-16.4, -7.4)	-9.1 (-13.4, -4.8)	-2.8 (-9.0, 3.5)	0.36	-14.4 (-18.3, -10.5)	-12.5 (-16.4, -8.6)	-1.9 (-7.5, 3.6)	0.48	**-8.8 (-11.6, -6.0)**	**-11.4 (-14.6, -8.2)**	**2.6 (-0.3, 4.8)**	**0.02**	-12.5 (-16.1, -8.9)	-12.6 (-15.8, -9.3)	0.1 (-3.1, 3.2)	0.97
Morning Surge	**14.6 (9.6, 19.7)**	**5.6 (0.9, 10.4)**	**9.0 (-1.7, 16.3)**	**0.02**	12.2 (7.3, 17.2)	8.5 (3.6, 13.5)	3.7 (-3.4, 10.8)	0.29	10.0 (6.3, 13.0)	14.9 (10.6, 19.1)	-4.8 (-9.9, 0.2)	0.06	12.5 (6.9, 18.0)	12.7 (9.3, 16.1)	-0.2 (-5.3, 4.8)	0.92

Data are presented as mean (95%CI). SD= standard deviation. PL= parallel study design; CO= cross-over study design; Δ= difference between beetroot and control group. Significant results are highlighted in bold. (Trial 1,2: Newcastle) and (Trial 3,4: Exeter)

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Table 4: Meta-analysis of pooled data (N=85) for each blood pressure (BP) outcome obtained from the analysis of 24-hr ambulatory BP monitoring.						
	Systolic BP (mmHg)			Diastolic BP (mmHg)		
	Effect Size	95%CI	P	Effect Size	95%CI	P
24-hour Mean	1.0	-0.9, 3.0	0.28	0.2	-1.4, 1.0	0.76
24-hour SD	0.2	-0.8, 1.3	0.65	0.2	-0.8, 1.3	0.65
Diurnal Mean	0.08	-0.1, 0.3	0.44	-0.6	-2.0, 0.8	0.41
Diurnal SD	-0.1	-1.2, 1.0	0.84	0.3	0.7, 1.3	0.58
Nocturnal Mean	0.1	-2.3, 2.5	0.91	0.2	-1.0, 1.4	0.80
Nocturnal SD	-0.8	-2.3, 0.7	0.30	-0.2	-1.4, 1.0	0.72
Dipping	0.1	-2.5, 2.2	0.90	1.0	-0.6, 2.5	0.23
Morning Surge^{R}	2.3	-4.0, 8.7	0.47	1.6	-4.0, 7.2	0.57

SD= standard deviation; 95%CI= 95% Confidence Intervals. Fixed-effect models were applied to derive pooled estimates for BP outcomes expect for Morning Surge (R) which was derived using a random-effect model (see methods section for more details).

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Table 5: Sub-group analysis to evaluate the effects of age, gender, body mass index (BMI), blood pressure (BP) and percent changes in nitrite concentrations ($\Delta[NO_2]$) on the pooled effect size for systolic and diastolic BP outcomes

			Systolic BP (mmHg)			Diastolic BP (mmHg)		
		N	Effect Size	95%CI	P	Effect Size	95%CI	P
24-hour Mean	Age <65y	46	0.7	-3.0, 4.4	0.99	1.3	-0.3, 3.0	0.28
	Age ≥65y	39	0.7	-3.6, 5.0		-0.2	-2.5, 2.0	
	Male	50	0.7	-1.8, 3.3	0.92	0.6	-1.9, 3.2	0.66
	Female	35	0.5	-2.4, 3.5		-0.01	-1.3, 1.3	
	BMI<30kg/m^2	53	0.2	-2.1, 2.5	0.95	0.1	-1.5, 1.7	0.80
	BMI≥30kg/m^2	32	0.09	-3.1, 3.3		-0.2	-2.2, 1.8	
	Raised Resting BP	36	1.3	-2.3, 5.0	0.88	0.1	-1.5, 1.8	0.66
	Normal Resting BP	49	1.0	-1.1, 3.1		0.7	-1.3, 2.8	
	$\Delta[NO_2]$<50th Centile	40	2.4	-0.7, 5.6	0.58	1.3	-0.7, 3.4	0.47
	$\Delta[NO_2]$≥50th Centile	45	1.1	-2.3, 4.6		0.1	-2.7, 2.8	
24-hour SD	Age <65y	46	-0.05	-1.5, 1.4	0.78	0.1	-1.2, 1.4	0.79
	Age ≥65y	39	0.2	-0.7, 1.1		0.4	-1.6, 2.4	
	Male	50	-0.5	-1.9, 0.9	0.86	0.4	-1.4, 2.1	0.78
	Female	35	-0.3	-1.8, 1.1		0.6	-0.5, 1.8	
	BMI<30kg/m^2	53	-0.5	-1.7, 0.7	0.43	-0.2	-1,2, 0.9	0.63
	BMI≥30kg/m^2	32	0.4	-1.4, 2.2		0.4	-1.7, 2.5	
	Raised Resting BP	36	-0.1	-2.6, 2.4	0.90	1.1	-1.1, 3.4	0.67
	Normal Resting BP	49	-0.3	-1.6, 1.0		0.6	-0.7, 1.9	
	$\Delta[NO_2]$<50th Centile	40	0.1	-1.6, 1.8	0.63	0.9	-0.7, 2.5	0.39
	$\Delta[NO_2]$≥50th Centile	45	-0.4	-1.6, 0.8		-0.2	-2.0, 1.6	
Diurnal Mean	Age <65y	46	1.9	-0.5, 4.4	0.16	0.8	-1.3, 3.0	0.92
	Age ≥65y	39	-1.2	-4.8, 2.4		0.6	-4.1, 5.4	
	Male	50	-0.2	-2.4, 2.0	0.93	-0.03	-0.3, 0.2	0.57
	Female	35	-0.3	-3.7, 3.0		0.1	-0.4, 0.7	
	BMI<30kg/m^2	53	1.0	-2.3, 4.5	0.52	0.5	-2.9, 3.9	0.64
	BMI≥30kg/m^2	32	-0.5	-4.3, 3.1		-0.5	-3.5, 2.3	
	Raised Resting BP	36	0.7	-4.7, 6.2	0.92	-0.06	-2.3, 2.2	0.96
	Normal Resting BP	49	0.5	-1.7, 2.6		0.1	-2.5, 2.3	
	$\Delta[NO_2]$<50th Centile	40	3.0	-0.1, 6.2	0.89	1.5	-1.0, 4.2	0.42
	$\Delta[NO_2]$≥50th Centile	45	3.7	-4.9, 12.3		-0.1	-3.2, 3.0	
Diurnal SD	Age <65y	46	0.08	-0.3, 0.4	0.72	0.6	-1.8, 2.9	0.60
	Age ≥65y	39	0.24	-0.5, 1.0		-0.2	-1.6, 1.3	
	Male	50	0.3	-1.7, 2.2	0.51	0.1	-0.7, 1.1	0.62
	Female	35	-0.5	-1.8, 0.8		0.6	-1.1, 2.4	
	BMI<30kg/m^2	53	0.1	-1.3, 1.1	0.83	0.2	-0.6, 0.9	0.85
	BMI≥30kg/m^2	32	0.1	-1.8, 2.1		0.4	-1.7, 2.5	
	Raised Resting BP	36	-0.7	-3.9, 2.4	0.60	0.5	-1.1, 2.3	0.73
	Normal Resting BP	49	0.2	-1.1, 1.5		0.2	-0.8, 1.2	
	$\Delta[NO_2]$<50th Centile	40	-0.1	-2.1, 1.9	0.81	0.9	-0.6, 2.4	0.28
	$\Delta[NO_2]$≥50th Centile	45	-0.4	-1.7, 0.8		-0.6	-2.8, 1.6	
Nocturnal Mean	Age <65y	46	**-1.9**	**-3.4, -0.4**	0.44	0.3	-1.6, 2.4	0.70
	Age ≥65y	39	0.4	-5.6, 3.5		1.0	-1.8, 3.8	
	Male	50	-3.1	-6.4, 0.06	0.42	-0.2	-2.3, 1.9	0.92
	Female	35	-1.3	-4.5, 1.9		-0.05	-2.1, 2.0	
	BMI<30kg/m^2	53	-0.6	-3.8, 2.5	0.63	0.4	-1.0, 1.9	0.39
	BMI≥30kg/m^2	32	-3.3	-13.7, 7.0		-4.0	-14.0, 6.0	
	Raised Resting BP	36	-2.1	-8.5, 4.1	0.36	-0.2	-2.7, 2.2	0.68
	Normal Resting BP	49	1.1	-1.7, 4.0		0.4	-1.2, 2.0	
	$\Delta[NO_2]$<50th Centile	40	1.4	-1.8, 4.7	**0.02**	0.4	-2.0, 2.8	0.44
	$\Delta[NO_2]$≥50th Centile	45	**-3.4**	**-0.6, -2.4**		-0.7	-1.9, 0.6	
Nocturnal SD	Age <65y	46	**-2.8**	**-4.5, -1.0**	**0.04**	-2.3	-6.8, 2.1	0.41
	Age ≥65y	39	1.0	-2.2, 4.2		0.6	-4.9, 6.1	
	Male	50	-0.8	-2.3, 0.7	**0.04**	1.0	-0.3, 2.2	0.05
	Female	35	2.1	-0.4, 4.7		-1.8	-4.2, 0.6	
	BMI<30kg/m^2	53	**-1.9**	**-3.6, -0.2**	0.09	-0.2	-1.4, 1.0	0.82
	BMI≥30kg/m^2	32	0.7	-1.9, 3.2		0.1	-2.4, 2.6	
	Raised Resting BP	36	0.7	-1.1, 2.4	**0.04**	0.6	-0.9, 2.1	0.23
	Normal Resting BP	49	**-2.2**	**-4.2, -0.1**		-0.8	-2.5, 0.9	
	$\Delta[NO_2]$<50th Centile	40	-0.6	-1.8, 0.5	0.86	-0.6	-2.5, 1.2	0.56

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	$\Delta[NO_2] \geq 50^{th}$ Centile	45	**-0.8**	**-1.5, -0.06**		0.1	-1.6, 1.9	
Dipping	Age <65y Age ≥65y	46 39	-1.4 **3.3**	-4.2, 1.4 **0.2, 6.4**	**0.02**	-0.3 **2.6**	-2.6, 1.9 **0.01, 5.1**	0.09
	Male Female	50 35	0.03 1.2	-2.9, 2.9 -3.0, 5.6	0.64	0.3 0.4	-2.6, 3,2 -2.4, 3.2	0.97
	BMI<30kg/m^2 BMI≥30kg/m^2	53 32	0.4 -4.4	-2.4, 3.3 -13.4, 4.5	0.31	0.9 -2.4	-1.8, 3.6 -11.9, 6.9	0.49
	Raised Resting BP Normal Resting BP	36 49	-3.3 0.4	-9.6, 3.9 -2.1, 2.9	0.28	-0.2 0.9	-3.9, 3.4 -1.0, 2.9	0.56
	$\Delta[NO_2] < 50^{th}$ Centile $\Delta[NO_2] \geq 50^{th}$ Centile	40 45	-0.5 **-2.5**	-2.6, 1.7 **-3.4, -1.9**	0.15	-0.5 -0.1	-3.6, 2.6 -3.4, 3.3	0.85
Morning Surge	Age <65y Age ≥65y	46 39	3.6 -0.7	-1.1, 8.5 -13.2, 11.8	0.52	2.0 -2.9	-1.3, 5.3 -9.6, 3.7	0.19
	Male Female	50 35	1.5 1.8	-4.2, 7.3 -6.4, 10.1	0.95	0.1 4.0	-5.2, 5.6 -2.6, 10.7	0.38
	BMI<30kg/m^2 BMI≥30kg/m^2	53 32	2.1 3.5	-2.5, 6.8 -13.6, 20.8	0.87	0.4 3.8	-3.4, 4.2 -7.3, 15.1	0.56
	Raised Resting BP Normal Resting BP	36 49	3.0 0.8	-3.9, 10.0 -8.0, 9.5	0.69	3.6 0.1	-4.0, 11.4 -5.4, 5.7	0.47
	$\Delta[NO_2] < 50^{th}$ Centile $\Delta[NO_2] \geq 50^{th}$ Centile	40 45	-1.4 7.0	-6.1, 3.2 -3.8, 18.0	0.15	-0.1 1.9	-4.1, 3.9 -3.8, 7.8	0.56

SD= standard deviation. 95%CI= 95% Confidence Intervals. Significant results are highlighted in bold. $\Delta[NO_2] < 50^{th}$ Centile corresponds to the median of the distribution for percent changes in nitrite concentrations in plasma (Trial 1, Trial 3 and Trial 4) and saliva (Trial 2).

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