**Effects of dietary nitrate supplementation, from beetroot juice, on blood pressure in hypertensive pregnant women: a randomised, double-blind, placebo-controlled feasibility trial**

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**Short title:** Dietary nitrate supplementation in pregnancy

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**Abstract:**

Chronic hypertension in pregnancy is associated with significant adverse pregnancy outcomes, increasing the risk of pre-eclampsia, fetal growth restriction and preterm birth. Dietary nitrate, abundant in green leafy vegetables and beetroot, is reduced *in vivo* to nitrite and subsequently nitric oxide, and has been demonstrated to lower blood pressure, improve vascular compliance and enhance blood flow in non-pregnant humans and animals. The primary aims of this study were to determine the acceptability and efficacy of dietary nitrate supplementation, in the form of beetroot juice, to lower blood pressure in hypertensive pregnant women. In this double-blind, placebo-controlled feasibility trial, 40 pregnant women received either daily nitrate supplementation (70mL beetroot juice, n=20) or placebo (70mL nitrate-depleted beetroot juice, n=20) for 8 days. Blood pressure, cardiovascular function and uteroplacental blood flow was assessed at baseline and following acute (3 hours) and prolonged (8 days) supplementation. Plasma and salivary samples were collected for analysis of nitrate and nitrite concentrations and acceptability of this dietary intervention was assessed based on questionnaire feedback. Dietary nitrate significantly increased plasma and salivary nitrate/nitrite concentrations compared with placebo juice (p<0.001), with marked variation between women. Compared with placebo, there was no overall reduction in blood pressure in the nitrate-treated group; however there was a highly significant correlation between changes in plasma nitrite concentrations and changes in diastolic blood pressure in the nitrate-treated arm only (r=-0.6481; p=0.0042). Beetroot juice supplementation was an acceptable dietary intervention to 97% of women. This trial confirms acceptability and potential efficacy of dietary nitrate supplementation in pregnant women. Conversion of nitrate to nitrite critically involves oral bacterial nitrate reductase activities. We speculate that differences in efficacy of nitrate supplementation relate to differences in the oral microbiome, which will be investigated in future studies.

**Key words:**

Dietary nitrate, nitrite, nitric oxide, beetroot juice, blood pressure, pregnancy

## 1. Introduction:

Chronic hypertension in pregnancy affects up to 3% of all pregnancies [1] and is defined by blood pressure (BP) greater than 140mmHg systolic (SBP) and/or 90mmHg diastolic (DBP) on two separate occasions before 20 weeks' gestation/beyond 12 weeks postpartum [2; 3]. Pregnancies complicated by chronic hypertension are increasingly prevalent, likely secondary to increasing maternal age [4], obesity [5] and metabolic syndrome [6]. Women with chronic hypertension are less likely to undergo successful cardiovascular adaptations in mid-pregnancy, including the normal reduction in BP and peripheral vascular resistance, which play a vital role in fetoplacental development. Consequently, these women are at increased risk of complications including fetal growth restriction (FGR; [7], placental abruption [8], indicated preterm birth [9] and pre-eclampsia (PE; 8-fold increase; [10; 11]). Despite the prevalence of adverse outcomes in this group of women and their association with level of hypertension [12], treatment options for hypertension in pregnancy are limited, making the development of new antihypertensive agents suitable for use in pregnancy a key research priority [2].

The development and use of pharmacological interventions in pregnancy is often hindered by a paucity of safety data and anxiety from women and clinicians alike of causing harm to the fetus. In recent years, there have been concerns that antihypertensive treatments might reduce uteroplacental perfusion, thereby negatively impacting on fetal growth. This was not demonstrated in the CHIPS trial, however [13], which was an unblinded multi-centre randomised controlled trial investigating the consequences of less-tight (DBP≤100mmHg)

versus tight (DBP≤95mmHg) BP control, using standard antihypertensive medications, in pregnancy. It contrasted previous smaller studies [14] in demonstrating no significant difference in adverse perinatal outcomes between the two groups. Instead, there was a significantly increased risk of severe hypertension in women whose blood pressure was under less-tight control [13]. There is ongoing debate regarding blood pressure targets in women with non-severe hypertension (SBP 130-144mmHg, DBP 80-94mmHg), and this group of women remain under-researched in intervention trials. As the CHIPS trial data highlights, it may be important to control blood pressure in borderline hypertensive pregnant women in order to prevent severe hypertension and subsequent poor pregnancy outcomes.

Nitric oxide (NO) is a potent vasodilator, crucial in maintaining a low resistance cardiovascular system and key in mediating maternal cardiovascular adaptations of pregnancy. In both humans and animals, NO levels are reported to increase across pregnancy [15; 16; 17; 18; 19] and, in some reports, reductions in NO production have been associated with pregnancy pathologies, including FGR and PE [15; 16; 17]. Several therapies aiming to enhance NO bioavailability are currently in obstetric trials [20]. Dietary nitrate (abundant in green leafy vegetables and beetroot) provides an alternative source of nitric oxide (NO), and in recent years the therapeutic potential for nitrate supplementation has been demonstrated in a number of clinical [21; 22; 23; 24] and preclinical [23; 25; 26] studies. Dietary nitrate is absorbed and subsequently reduced to nitrite by commensal bacteria via the entero-salivary pathway. Nitrite then enters the systemic circulation, and can be reduced to NO in the blood or in tissues [27]. The current trial draws on recent evidence demonstrating that dietary nitrate supplementation significantly lowers BP in both normotensive [24] and hypertensive [23] subjects out-with pregnancy. A single dose of beetroot juice, which is a rich source of nitrate, was sufficient to reduce BP, an effect that peaked at ~3 hours post-ingestion and

persisted for at least 24 hours [23]. Beetroot juice has also been shown to increase blood flow and reduce vascular resistance in non-pregnant animal models [25]. More recently, preliminary data from our laboratory in a mouse model of hypertension in pregnancy has shown that 6 days of supplementation with beetroot juice can reduce BP and improve maternal uterine artery endothelial function [Cottrell et al., unpublished]. In addition to these promising effects on blood pressure regulation and vascular function, it is likely that a dietary intervention such as beetroot juice might be more acceptable to pregnant women than anti-hypertensive medications. Thus, this feasibility study aimed to determine the acceptability and efficacy of dietary nitrate supplementation, from beetroot juice, as an intervention to reduce blood pressure in pregnant women with borderline hypertension.

## 2. Subjects and Methods

### 2.1 Trial design

This study was a single-site, double-blind, randomised controlled trial (RCT) and was prospectively registered at clinicaltrials.gov (study identifier: NCT02520687). Study protocol and documentation were granted full ethical approval by the local research ethics committee (REC reference 15/NW/0383; full protocol available on request). Participants were recruited after being identified and approached through the Maternal and Fetal Health Research Centre (MFHRC) high-risk antenatal clinics. All participants provided informed consent. Participants were recruited between October 2015 and February 2017; the final participant delivered in April 2017. All data were collected during study appointments at the MFHRC (St Mary's Hospital, Manchester, UK) by specialist research midwives and obstetricians. The intervention (a total of 8 days of beetroot juice supplementation) was completed between

22+0 and 35+6 weeks' gestation. Pregnancy outcome data was collected for all women following delivery.

## 2.2 Eligibility criteria

Women with a diagnosis of borderline chronic hypertension (SBP 130-144mmHg and/or DBP 80-94mmHg before 20 weeks' gestation), who were not taking anti-hypertensive medication, and who were between 22 and 35+6 weeks of gestation were included in the study. Exclusion criteria were: pre-existing diabetes (type I or II), multiple pregnancy, concomitant antihypertensive medication, age under 16 and incapacity to provide informed consent.

## 2.3 Intervention

Participants were given 70mL treatment/placebo juice daily (James White Drinks Ltd), for a total of 8 days. The treatment juice contained ~400mg nitrate in 70 mL of beetroot juice (92.4 ± 11.9 mmol/L); placebo treatment was 70mL of a nitrate-depleted beetroot juice (containing <0.01 mmol/L nitrate; James White Drinks Ltd), identical otherwise in taste and appearance [28]. Women were advised to continue their normal diet during the study, and were asked to take the allocated treatment at approximately the same time each day. In all women, blood samples and blood pressure/cardiovascular parameters were determined at baseline (0 hours), 3 hours and 24 hours after the first dose of treatment, and then on day 8, prior to the final treatment dose (see detailed description below and **Supplementary Figure 1** for overview of study design).

## 2.4 Randomisation method and blinding

Block randomisation was performed in advance (using an online programme; block size of 4,

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1:1 allocation) and sequentially numbered prefilled treatment envelopes (containing the week of supplementation) were created by a researcher independent of the study team consenting/administering and performing measurements (MW). Thus, both the participant and the member of the research team assessing the clinical outcomes were blinded to the treatment arm.

**2.5 BP measurements**

BP and heart rate (HR) were measured in each arm in the sitting position (once in each arm, data averaged to obtain mean sitting BP) and 4 repeated BP measurements were taken in the lying position in the left arm during the ultrasound scan (using Alere Microlife BP monitors; Cheshire, UK). The Arteriograph® (Tensiomed, Budapest, Hungary) was used to measure HR, SBP, DBP and to calculate pulse wave velocity (PWV) and augmentation index (both measures of arterial stiffness) in both left lateral (LL) and supine positions; this was recorded via the manufacturer's software. Non-invasive cardiac output monitoring (NICOM) system (Cheetah Medical (UK) Ltd, Maidenhead, UK) was used to measure HR, SBP, DBP and peripheral vascular resistance. Uteroplacental Doppler measurements (uterine artery, umbilical artery/vein) were performed in triplicate (Voluson E6, GE Healthcare; measurements made using a 4-8mHz curvilinear transabdominal probe). Ambulatory blood pressure measurement (24-hour ABPM) was performed using validated machines (Model 90207, Spacelabs Healthcare Ltd, Hertfordshire, UK) on day one and day 8 of the treatment period. Between 0700-2300h, one reading every 30 minutes was taken; from 2300-0700, one reading every hour was taken. At each visit, measurements were taken in the same order for each participant; salivary and blood sampling was completed after cardiovascular/Doppler measurements and prior to treatment ingestion.

## 2.6 Outcomes

The primary process outcome for this study was recruitment rate (calculated as number recruited/total number of eligible women contacted). The secondary process outcome was the acceptability of the intervention, based on feedback from a questionnaire (see Supplementary Data). The primary clinical outcome was to identify a reduction in clinic BP (using sitting position BP values) in the treatment group, compared with placebo. Secondary clinical outcomes were to determine (1) average day and night time ABPM measurements (measured on day 1 and day 8); (2) pulse-wave velocity (PWV); (3) total peripheral vascular resistance (TPRI); (4) uteroplacental blood flow parameters (uterine and umbilical artery resistance (RI) and pulsatility indices (PI)); and (5) changes in salivary and plasma nitrate/nitrite concentrations in response to treatment. We also aimed *a priori* to determine whether BP or other cardiovascular variables correlated with changes in plasma nitrite concentrations, as seen in previous studies [22].

## 2.7 Biochemical measurements

Venous blood samples were collected at 0, 3, 24 hour and day 8 timepoints into collection tubes (BD 364938) and EDTA added to a final concentration of 2mM. Blood was centrifuged immediately and plasma removed and stored at -80°C until analysis. Saliva was collected in parallel at these same timepoints, directly into 1.5mL Eppendorf tubes, frozen immediately and stored at -80°C until analysis. Plasma and salivary content of nitrate and nitrite was measured using a high-performance liquid chromatography (HPLC) system (ENO-20; Eicom) as previously described [26].

**2.8 Sample size calculation**

Whilst primarily a feasibility study, this study was powered to detect a reduction in BP of 5mmHg with 80% power and a 95% level of confidence. This is a conservative yet clinically relevant change, which is in line with previous studies investigating beetroot juice administration in non-pregnant subjects [22; 24]. Assuming a standard deviation of 6.2 mmHg in BP measurements in pregnancy (based on studies measuring BP by the same means as this trial [29]), and using a one-sided t-test, the target sample size was 40 completing participants (1:1 allocation). The block randomisation list included 44 participants to allow for non-completion.

**2.9 Statistical analysis**

Variables were checked for normality using the Jarque-Bera skewness-kurtosis test and outliers identified using the ROUT method (with Q=0.1% for removal of definitive outliers). Reproducibility of repeated measurements and agreement between BP/HR measurements using different modalities were assessed using intraclass correlation coefficients (ICC) and Bland Altman analyses, respectively. Comparisons between devices were made using lyin (supine) position measurements only, to account for postural effects on BP. The primary outcome (blood pressure change) was compared using t-test, between time 0 and 3hr, 24hr and day 8 (168 hour) time points. The remaining outcomes were compared using Mann-Whitney and ANOVA as appropriate; $p < 0.01$ was considered significant (to account for multiple comparisons). Associations between biochemical and BP measurements were assessed using Pearson's linear correlation, with $p < 0.01$ considered significant. All statistical analyses were performed using Stata/IC (version 14.1, StataCorp, Texas, USA) or GraphPad Prism (version 7.00, GraphPad Software, La Jolla California USA).

## 3. Results

Study population characteristics and pregnancy outcome data are summarised in Table 1. Out of the 58 eligible women approached, 44 women agreed to participate, and a total of 40 women (40/58; 69%) completed the study (Figure 1). For all 14 women who declined to participate, time commitment was cited as the primary reason. Of the 44 recruited women, 2 withdrew (1 on the first visit and the other prior to the final visit) and 2 were excluded due to the development of pregnancy complications during the trial period. The participant who withdrew before the final visit was included in baseline, 3 and 24 hour analyses (Figure 1).

| | Nitrate (n=20) | Placebo (n=21) | All |
|---|---|---|---|
| **Gestation (weeks + days)** Median (range) | 28+1 (22+5 – 35+5) | 27+1 (22+5 – 34+6) | |
| **Ethnicity;** N (%) | | | |
| White | 13 (65.00%) | 14 (66.67%) | 27 (65.85%) |
| Black | 5 (25.00%) | 7 (33.33%) | 12 (29.27%) |
| Asian | 1 (5.00%) | 0 | 1 (2.44%) |
| Other | 1 (5.00%) | 0 | 1 (2.44%) |
| **BMI (kg/cm$^2$)** Median (range), [IQR] | 34.01 (19.87 – 49.61) [11.64] | 26.99 (19.29 – 40.03) [9.46] | 29.97 (19.29 – 49.61) [11.20] |
| **Booking SBP (mmHg)** Median (range) | 135 (110 – 171) | 129 (110 – 166) | 130 (110 – 171) |
| **Booking DBP (mmHg)** Median (range) | 81 (64 – 100) | 80 (60 – 90) | 80 (60 – 100) |
| **Gestation at delivery (weeks + days);** Median (range) | 38+6 (36+0 – 40+5) | 38+6 (32+2 – 40+5) | 38+6 (32+2 – 40+5) |
| **Preterm;** N (%) | 2 (10.00%) | 2 (9.52%) | 4 (9.76%) |
| **Birthweight <10$^{th}$ centile (n)** | 5 (25.00%) | 4 (19.05%) | 9 (21.95%) |
| **Pre-eclampsia;** N (%) | 0 | 1 (4.76%) | 1 (2.44%) |

| Birth weight (BW) centile Median (range) | 35.06 (1.06 – 94.29) | 28.87 (4.09 – 96.66) | 29.97 (1.06 – 96.66) |
|---|---|---|---|
| Fetal demise in utero; N (%) | 0 | 0 | 0 |

**Table 1:** Baseline clinical characteristics and demographics of study group (n=41). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; BW, birth weight.
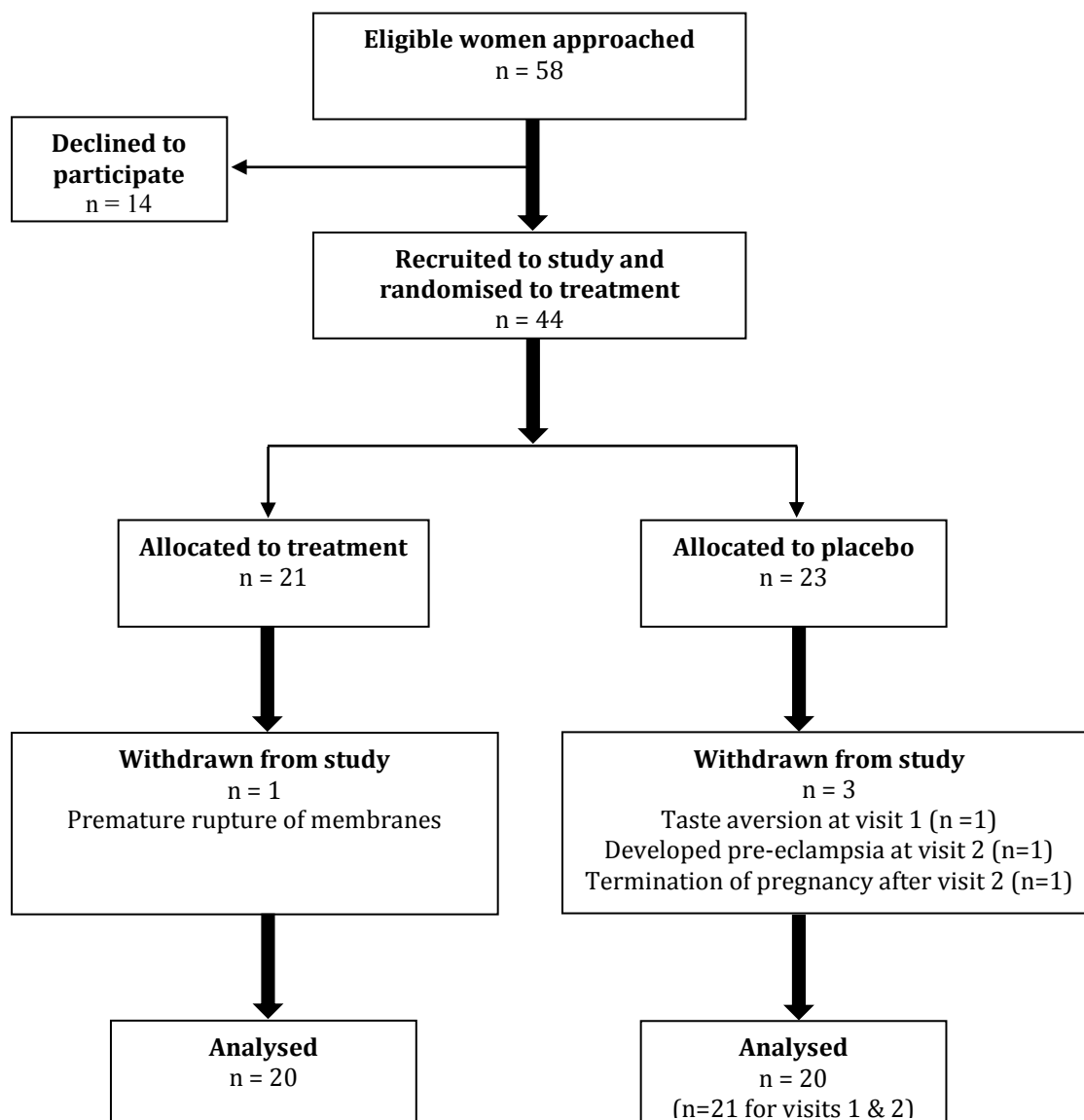


**Figure 1:** Flow diagram of trial participants.

Of the 40 women who completed the full trial, 37/40 (92.5%) completed the acceptability

questionnaire (see **Supplementary Data File**). 23/37 (62.2%) found it easy to take the

beetroot juice daily; 1/37 (2.7%) found it difficult and 13/37 (35.1%) found it neither easy

nor difficult. 20/37 (54.1%) women found it palatable. In terms of overall acceptability, 36/37

(97.3%) said that if it was found to be beneficial, they would take it in the future. Both

treatment interventions were well tolerated, and no adverse events were reported.

The agreement of BP measurements between different modalities (clinic BP measured using

the Alere Microlife monitors, arteriograph and NICOM measurements) is shown in Figure 2,

and highlights the significant variability of BP measurements between modalities in pregnant
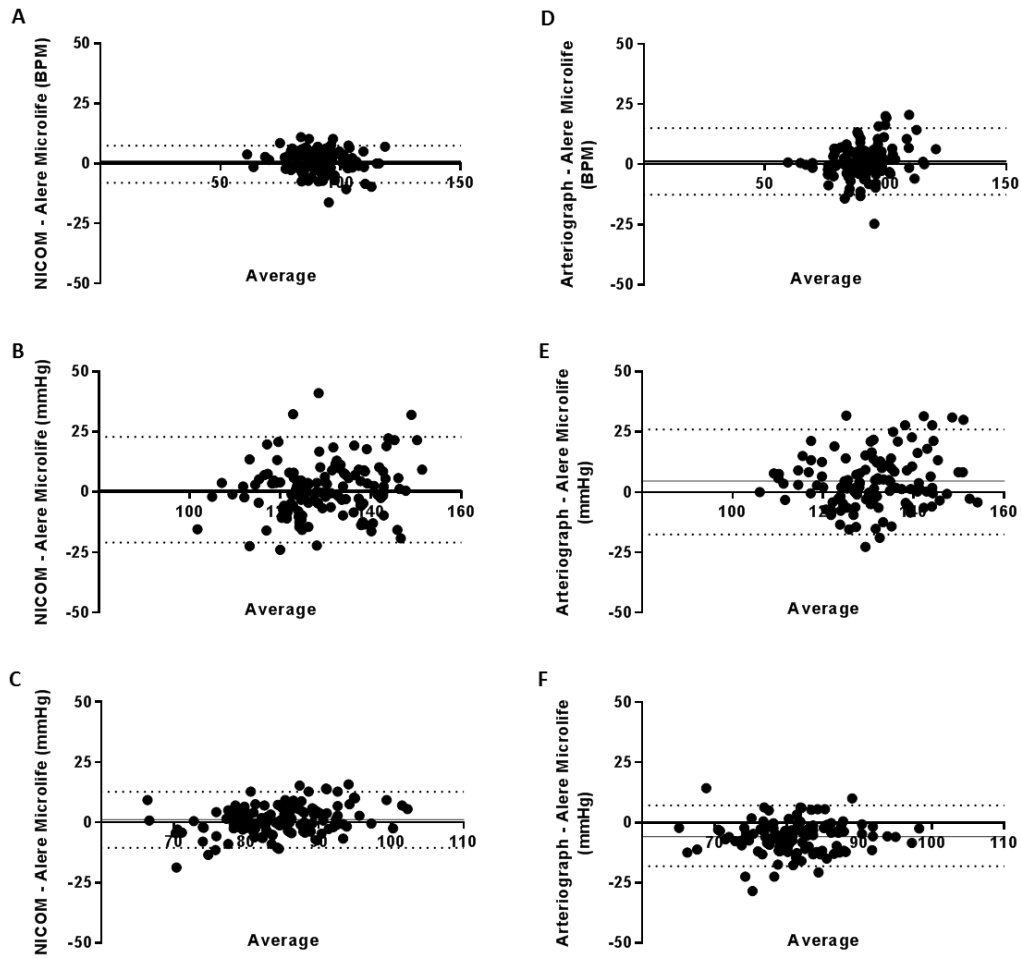
women, particularly apparent for SBP.

**Figure 2: Variation in heart rate and blood pressure across different measurement modalities**.  Bland-Altman plots showing differences in heart rate (A & D), systolic blood pressure (B & E) and diastolic blood pressure (C & F) between measurement devices (Alere Microlife, NICOM and arteriograph monitors).  All measurements were performed with participant in the supine position. The average (mean) of both readings are shown on the x-axis, with between-modality difference expressed on the y-axis. Dotted lines indicate 95% limits of agreement and line of bias as solid line (not visible where value approaches 0). BPM = beats per minute.

As shown in Table 2, there was no significant effect of nitrate supplementation compared with placebo on clinic BP at any time point across the study.

| | Time (hours) | Nitrate | | Placebo | | P value |
|---|---|---|---|---|---|---|
| | | Mean (SD) [C.I.] | Delta (from 0h) | Mean (SD) [C.I.] | Delta (from 0h) | |
| **Sitting SBP** | 0 | 133.90 (9.28) [129.56 – 138.24] | | 132.45 (11.44) [127.25 – 137.66] | | |
| | 3 | 129.89 (9.40) [125.37 – 134.42] | -3.74 (8.83) [-7.99 – 0.52] | 129.76 (9.99) [125.21 – 134.31] | -2.69 (7.83) [-6.26 – 0.88] | 0.693 |
| | 24 | 130.25 (10.70) [125.24 – 135.26] | -3.65 (7.36) [-7.09 – -0.21] | 130.50 (9.10) [126.36 – 134.64] | 1.95 (11.23) [-7.06– 3.16] | 0.572 |
| | 168 | 133.45 (9.83) [128.85 – 138.05] | -0.45 (7.85) [-4.12 – 3.22] | 129.38 (9.55) [124.90 – 133.85] | -3.65 (9.32) [-8.01– 0.71] | 0.247 |
| **Sitting DBP** | 0 | 85.43 (6.07) [82.58 – 88.27] | | 86.33 (7.48) [82.93 – 89.74] | | |
| | 3 | 83.03 (6.83) [79.73 – 86.32] | -2.50 (4.33) [-4.59 – -0.41] | 82.83 (7.48) [79.06 – 86.61] | -3.5 (8.48) [-7.36 – 0.36] | 0.647 |
| | 24 | 83.08 (8.26) [79.21 – 86.94] | -2.35 (7.02) [-5.63 – 0.93] | 85.07 (7.93) [81.46 – 88.68] | -1.26 (7.27) [-4.57– 2.05] | 0.629 |
| | 168 | 83.23 (7.17) [79.87 – 86.58] | -2.20 (5.99) [-5.00– 0.60] | 85.43 (7.26) [82.03 – 88.82] | -0.88 (6.71) [-4.01– 2.26] | 0.514 |

**Table 2:** Dietary nitrate supplementation does not significantly reduce sitting BP compared with placebo. Data are presented as mean (±SD), [95% CIs]. N=20-21 per group.

Similarly, there was no significant different between nitrate and placebo groups on BP measured using any other modalities (arteriography, NICOM measurements; **Supplementary Table 1**). Changes in vascular resistance/vessel stiffness and uteroplacental Doppler parameters were also comparable between dietary nitrate and placebo groups (summarised in

**Supplementary Tables 2 and 3**). Compliance amongst women for day 8 ABPM measurements was poor, with only 26/40 (65%) having daytime ABPM monitoring and 19/40 (47.5%) completing the full 24 hours, and with an unequal split of treatment/placebo participants. As a result, insufficient data for statistical analysis were obtained using this modality of BP measurement.

Following a single dose of dietary nitrate, both plasma nitrate and nitrite concentrations were significantly elevated at 3 hours post-ingestion compared with placebo levels ($p<0.001$ for both; Figure 3). At 24 hours after the initial dose, concentrations had returned to baseline values and after one week of daily supplementation, there were no significant differences in plasma nitrate or nitrite (day 8 measurements were taken prior to ingestion of the final treatment dose; Figure 3). Salivary nitrate/nitrite concentrations across the intervention period showed similar responses, with significant elevations in both nitrate and nitrite at the 3 hour time point ($p<0.001$ for both; Figure 3). However, in contrast to plasma concentrations, salivary nitrate concentrations were elevated on day 8 in the nitrate treatment arm, compared with placebo ($p<0.01$, Figure 3), reflecting a sustained effect of treatment in this biological compartment.

The biochemical data highlighted significant variability in the assimilation and/or conversion of ingested nitrate to nitrite amongst the women in this study. In order to determine whether changes in BP were related to differences in treatment responses, pre-defined correlations were performed between the change in BP and change in plasma nitrite concentrations across the acute (0-3 hour) phase following treatment ingestion, where nitrite concentrations were maximal. These analyses revealed a significant correlation between the change in DBP and plasma nitrite across this timeframe, specifically in the dietary nitrate group (Figure 4).

Similar associations were seen between plasma nitrite and BP responses as measured using different modalities (arteriography, NICOM; **Supplementary Figure 2**), suggesting that a key determinant of BP responses to beetroot juice supplementation involves the assimilation and conversion of nitrate to nitrite.
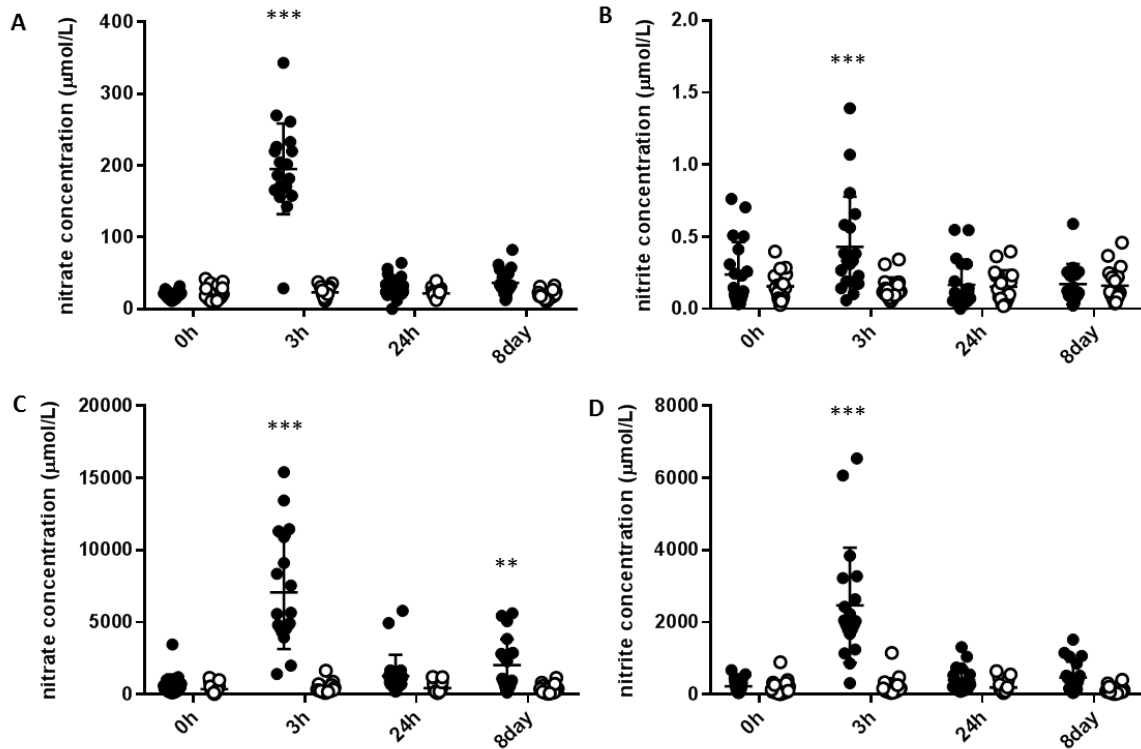


**Figure 3: Dietary nitrate elevates plasma and salivary nitrate and nitrite concentrations.** The effects of dietary nitrate (filled circles) or placebo (open circles) ingestion on (A) plasma nitrate, (B) plasma nitrite, (C) salivary nitrate and (D) salivary nitrite. Individual data points are shown, with mean ± SD indicated, and were analysed by 2-way ANOVA followed by Sidak's multiple comparisons test. Significance shown between nitrate and placebo groups as **$p<0.01$ and ***$p<0.001$. N=17-21 per group.

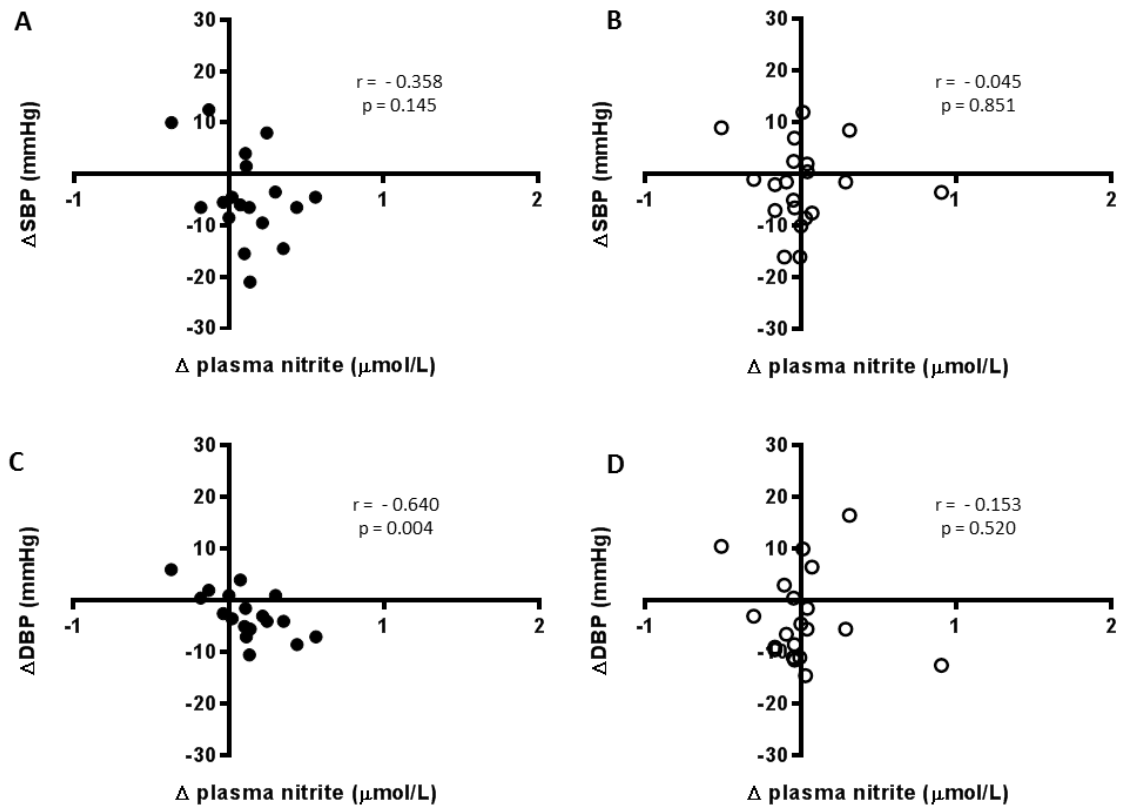**Figure 4: Changes in plasma nitrite determine BP responses following acute nitrate ingestion.** Correlation of changes ($\Delta$) in plasma nitrite vs. changes in SBP (A&B) and DBP (C&D) in nitrate (filled circles, A&C) and placebo (open circles B&D) between 0-3 hour timepoints (i.e. following acute nitrate ingestion). BP measured using Alere microlife BP monitors. Data analysed using Pearson linear regression. N=17-21 per group.

## 4. Discussion:

To our knowledge, this is the first trial to investigate the effects of dietary nitrate supplementation on blood pressure in pregnancy. In this study, we have confirmed acceptability and feasibility of beetroot juice supplementation in pregnancy, but we did not demonstrate a difference in blood pressure post treatment between the treatment and placebo groups (primary clinical outcome). However, despite considerable variability in BP measurements, in a pre-specified analysis we did identify significant correlations between changes in plasma nitrite concentrations and DBP responses following nitrate supplementation. Previous studies in non-pregnant individuals have demonstrated that the peak change in plasma nitrite (and blood pressure) occurs between 2-4 hours after nitrate ingestion [22; 24; 27], corresponding with the time taken for enterosalivary nitrate-nitrite reduction [30]. We therefore targeted this time point for investigation in the present study, and demonstrated a similar, significant relationship between BP and plasma nitrite in pregnant women to that shown previously in non-pregnant populations [22]. Our data from this small feasibility trial therefore suggest that dietary nitrate may represent an effective intervention for the lowering of blood pressure in hypertensive pregnant women, but that the efficacy likely depends on the ability of the individual to assimilate and convert dietary nitrate to nitrite.

There is a growing body of evidence illustrating the importance of oral bacteria in determining both normal, physiological blood pressure regulation [27], as well as in determining BP responses to dietary nitrate supplementation [31]. Elegant studies using antibacterial mouthwash to deplete the normal bacteria present in the oral cavity showed significantly reduced plasma and salivary nitrite levels; these changes were associated with a corresponding increase in BP, and ablation of oral nitrate reductase activity [27]. Smoking is

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also associated with a significant reduction in oral nitrate reductase activity [32]. Interestingly, the one subject in the treatment arm of our study who was a smoker failed to show an increase in plasma nitrite levels at the 3 hour timepoint following nitrate ingestion; nitrite concentrations in fact were reduced over this timeframe, and BP in this subject increased. Whilst in our study this is one observation, data from other studies continue to highlight the important associations between oral bacterial populations and the nitrate-nitrite-NO pathway *in vivo*. Although we have not directly investigated the role of oral bacterial nitrate reductase activity in this feasibility trial, our data suggest that this aspect will be critical to consider in future studies.

Of note, in our study we observed a decline in BP across all women (i.e. in both the treatment and placebo arms of the study) across the initial 3-hour period. This lowering of blood pressure by both nitrate-replete beetroot juice and a matched placebo control has been shown previously [31], although the authors did not address the finding directly. A recent meta-analysis of clinical trials in which beetroot juice was used as the dietary intervention to deliver nitrate for lowering of blood pressure showed an attenuated effect size in studies utilising the placebo (nitrate-depleted beetroot juice control), as opposed to water or other control [33], suggesting nitrate-independent effects of beetroot juice on blood pressure reduction. In the present study, it could be that environmental factors (e.g. women acclimatising to the clinic environment, or being reassured of their baby's wellbeing post-ultrasound scan), may explain in part this response, in addition to potential effects of beetroot juice *per se*; any or all of these factors could lead to a physiological lowering of BP. It is also possible that the raised BMI of women in the treatment arm of the study could have attenuated the ability of nitrate to lower peripheral blood pressure, as has been shown recently in non-pregnant individuals [34].

The present study has also highlighted the significant variability associated with measuring BP in pregnant women in the clinical setting. Indeed, our measured standard deviation in the present dataset for SBP was ~9-10 mmHg, compared with the estimated 6.2 mmHg used in power calculations. We attempted to gain a more holistic view of BP and cardiovascular changes across the intervention period by determining measurements of vessel stiffness/compliance (PWV) and vascular resistance (TPRI) in response to treatment. Comparing the different modalities, we found that the most reproducible measure in terms of BP was the standard, validated clinic BP monitor, and additionally found that SBP exhibited a higher degree of variability between modalities compared with DBP. Interestingly, a recent systematic review and meta-analysis carried out to assess the effects of dietary nitrate on BP found that the significant BP-lowering effect of nitrate was found only when resting clinic BP measures were used; non-significant differences were observed using 24-hour ABPM and home BP monitoring [35]. The information gained from this feasibility study will inform the design of future studies assessing BP responses to treatment interventions in pregnant women, a challenging group in which to run well-controlled clinical trials.

The strengths of this study are its randomised double-blind controlled design and a high level of compliance in taking the daily dietary intervention (all women reported successfully completing the 8-day treatment period). In terms of limitations, our BP data indicated a higher degree of variability than predicted, thus this feasibility trial has highlighted methodological issues with obtaining reproducible BP measurements in pregnant women. Future trials will need to ensure that multiple BP measurements are taken, ideally following ultrasound scan reassurance of fetal well-being and a defined acclimatisation period, in order to reduce variability. We were also unable to obtain ABPM measurements from a significant

proportion of participants, as women were unable to tolerate this intervention. The main reason cited was the disturbed sleep as a result of these measurements being taken every hour during the overnight period. In addition, we did not strictly control the diets of the participants during the supplementation period, which will likely have contributed to the variability seen in terms of plasma nitrate/nitrite concentrations, as well as influencing the BP and other cardiovascular variables measured but again dietary restrictions or enforced fasting periods can pose issues in pregnant women. These factors, combined with a relatively diverse population (in terms of ethnicity, body mass index and baseline BP), are likely to have contributed to the degree of variability seen in salivary and plasma nitrate/nitrite levels, as well as in the response to treatment. Despite these limitations, our data suggest that dietary nitrate may be a promising therapeutic intervention for use in pregnancy amongst women who are able to utilise it.

Given the paucity of treatment options for hypertensive pregnant women, the development of new interventions that improve BP regulation and lead to sustained BP lowering represent an ongoing unmet need. If efficacious, dietary approaches are more likely to deliver realistic interventions, given issues of compliance and risk that surround pharmacological treatment of pregnant women. Importantly, the current study has identified that dietary nitrate supplementation, via beetroot juice, is well tolerated and acceptable to pregnant women. A recent review has highlighted the need for further preclinical and clinical studies to address the safety of dietary nitrate supplementation [36]. Our ongoing preclinical studies and planned future clinical trials are addressing this knowledge gap; however, the results of this feasibility trial have shown that ingestion of 400mg of nitrate for 8 days after 22 weeks gestation is safe and acceptable for both mother and baby.

Our data suggest that the ability to convert nitrate to nitrite determines the BP-lowering efficacy of dietary nitrate supplementation. Further studies are required to confirm this finding, and to investigate the potential for manipulation of bacterial populations (e.g. concurrent probiotic supplementation) that would promote the utilisation of dietary nitrate, its conversion to nitrite, and ultimately enhance the BP lowering effects of this dietary intervention.

## 5. Acknowledgements:

## 6. Sources of support:

## 7. Conflict of Interest

The authors declare no conflict of interest.

## 8. Authors' contributions

JEM, MW, SLG, CPS, EDJ and ECC Designed research; LO, CC, SLG, TT, JOL, EW, CN, EDJ and ECC conducted research; LO, JEM and ECC analysed data; LO, JEM and ECC wrote the paper; EC had primary responsibility for final content. All authors read and approved the final manuscript.

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