

## Effects of acute dietary nitrate supplementation on aortic blood pressures and pulse wave characteristics in post-menopausal women

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### ABSTRACT

**Purpose:** Consumption of nitrate-rich beetroot juice can lower blood pressure in peripheral as well as central arteries and may exert additional hemodynamic benefits (e.g. reduced aortic wave reflections). The specific influence of nitrate supplementation on arterial pressures and aortic wave properties in postmenopausal women, a group that experiences accelerated increases in these variables with age, is unknown. Accordingly, the primary aim of this study was to determine the effect of consuming nitrate-rich beetroot juice on resting brachial and aortic blood pressures (BP) and pulse wave characteristics in a group of healthy postmenopausal women, in comparison to a true (nitrate-free beetroot juice) placebo.

**Methods:** Brachial (oscillometric cuff) and radial (SphygmoCor) pressures and derived-aortic waveforms were measured during supine rest in thirteen healthy postmenopausal women ( $63 \pm 1$  yr) before and 100 min after consumption of 140 ml of either nitrate-rich (9.7 mmol, 0.6 gm  $\text{NO}_3^-$ ) or nitrate-depleted beetroot juice on randomized visits approximately 10 days apart (cross-over design). Ten young premenopausal women ( $22 \pm 1$  yr) served as a reference (non-supplemented) cohort.

**Results:** Brachial and derived-aortic variables showed the expected age-associated differences in these women (all  $p < 0.05$ ). In post-menopausal women, nitrate supplementation reduced ( $p < 0.05$  vs. placebo visit) brachial systolic BP ( $\text{BR}_{\text{nitrate}} - 4.9 \pm 2.1$  mmHg vs  $\text{BR}_{\text{placebo}} + 1.1 \pm 1.8$  mmHg), brachial mean BP ( $\text{BR}_{\text{nitrate}} - 4.1 \pm 1.7$  mmHg vs  $\text{BR}_{\text{placebo}} + 0.9 \pm 1.3$  mmHg), aortic systolic BP ( $\text{BR}_{\text{nitrate}} - 6.3 \pm 2.0$  mmHg vs  $\text{BR}_{\text{placebo}} + 0.5 \pm 1.7$  mmHg) and aortic mean BP ( $\text{BR}_{\text{nitrate}} - 4.1 \pm 1.7$  mmHg vs  $\text{BR}_{\text{placebo}} + 0.9 \pm 1.3$  mmHg), and increased pulse pressure amplification ( $\text{BR}_{\text{nitrate}} + 4.6 \pm 2.0\%$  vs  $\text{BR}_{\text{placebo}} + 0.7 \pm 2.5\%$ ,  $p = 0.04$ ), but did not alter aortic pulse wave velocity or any other derived-aortic variables (e.g., augmentation pressure or index).

**Conclusions:** Dietary nitrate supplementation favorably modifies aortic systolic and mean blood pressure under resting conditions in healthy postmenopausal women. Acute supplementation of nitrate does not, however, appear to restore indices of aortic stiffness in this group. Future work should evaluate chronic, long-term effects of this non-pharmacological supplement.

### 1. Introduction

Cardiovascular disease risk rises exponentially after menopause, which is due in part to increases in systemic blood pressure [1]. Increases in aortic blood pressure, driven by progressive stiffening of large elastic arteries and an earlier return of the reflected pulse wave to

the heart, appear to contribute to the heightened cardiovascular disease risk of older women more so than men [2–4]. Examining the extent to which various vascular risk reduction therapies can reduce aortic blood pressures and favorably modify other prognostic pulse wave characteristics (e.g., augmentation index [7–9]) in older women is therefore clinically important.

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A major mechanism thought to underlie age-associated increases in blood pressures and central arterial stiffness/augmented wave reflections in women is the menopausal loss of estrogen [3,5,10] and its nitric oxide (NO)-mediated vasodilator and vasoprotective effects. Acute dietary nitrate supplementation has been shown to increase circulating nitrite (precursor of NO) via the NO synthase-independent NO production pathway (nitrate – nitrite – NO pathway) and lower resting peripheral blood pressure in older populations [11,12]. Hughes et al. recently reported that acute (single dose) consumption of nitrate-rich beetroot juice can also lower aortic blood pressure (but not aortic wave reflections) in healthy older adults [11]. That investigation did not compare the effects of beetroot juice to a nitrate-depleted placebo (a true control) and was not powered to investigate possible sex differences. To the best of our knowledge, all studies that have examined the effects of beetroot juice/nitrate supplementation have included either both sexes or only men. Thus, the potential aortic blood pressure lowering effects of nitrate supplementation per se have not been established in postmenopausal women, a cohort with considerable interest in non-pharmacological (non-estrogenic) blood pressure lowering therapies [13].

In the present study, using a crossover study design, we examined the acute effects of nitrate-rich beetroot juice on resting blood pressures and pulse wave characteristics in a group of healthy older women in comparison to a true (nitrate-free) placebo. We hypothesized that acute nitrate supplementation would lower aortic blood pressures in this specific cohort. A sample of young, premenopausal women served as a reference group to confirm baseline (non-supplemented) differences in these hemodynamic variables.

## 2. Methods

### 2.1. Subjects

Healthy postmenopausal and premenopausal women were recruited from the local university community. Interested volunteers provided written informed consent prior to enrollment. On the same day, medical screening was performed to determine eligibility. Participants were selected if they were low-to moderately active (i.e., < 3 days/week of exercise), free of overt chronic disease as assessed by a clinician-reviewed medical history questionnaire and venous blood chemistry (hematologic, liver, and kidney function), and met the following criteria: (i) resting seated blood pressure below 140/90 mmHg, (ii) body mass index between 18.5 and 35 kg/m<sup>2</sup>, (iii) fasting plasma glucose < 100 mg/dL or HbA1c < 6.0%, (iv) fasting plasma low-density lipoprotein < 130 and/or high-density lipoprotein > 40 mg/dL, (v) non-smoker, (vi) not currently taking any cardiovascular medications or hormone therapy, and (vii) had not donated blood or blood products in the past 3 months. The intervention component of this study (nitrate supplementation in postmenopausal subjects) was powered based on aortic mean arterial pressure (aMAP) responses observed in the first 5 postmenopausal women we tested (effect size = 0.47, power = 0.43). Characteristics of the 13 postmenopausal and 10 premenopausal women who completed this study are shown in Table 1.

All procedures were approved by the Office of Research Protections at Pennsylvania State University in agreement with the guidelines set forth by the Declaration of Helsinki. This trial was registered with [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT03380000) (NCT03380000).

### 2.2. Study timeline

Subjects were instructed to avoid using mouthwash on the morning of each study visit. All studies were performed in the morning hours, between 8AM and noon.

**Visit 1:** Upon arrival to the clinical research center (CRC), a venous blood sample was collected for determination of baseline plasma nitrate (NO<sub>3</sub><sup>-</sup>) and nitrite (NO<sub>2</sub><sup>-</sup>) concentrations. This was followed, after

**Table 1**

**Subject Characteristics.** \* indicates significant (p < 0.05) difference between premenopausal and postmenopausal subjects. BMI, body mass index; HDL, high-density lipoproteins; LDL, low-density lipoproteins; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial blood pressure; Pulse wave velocity (\*based on n = 9 postmenopausal). Data are means ± S.E.M.

	Premenopausal women	Postmenopausal women
Subjects (n)	10	13
Age (years)	22 ± 1	63 ± 1 *
Height (cm)	167.6 ± 1.7	162.5 ± 1.7 *
Weight (kg)	60.1 ± 3.0	66.7 ± 3.6
BMI (kg/m <sup>2</sup> )	21.4 ± 0.9	25.4 ± 1.4 *
Hematocrit (%)	40.0 ± 0.6	41.1 ± 0.7
Total cholesterol (mg/dL)	159.6 ± 7.3	216.4 ± 8.6 *
HDL (mg/dL)	63.6 ± 3.7	70.6 ± 6.9
LDL (mg/dL)	82.9 ± 5.5	125.2 ± 8.6 *
Fasting Glucose (mg/dL)	81.9 ± 2.0	93.3 ± 2.6 *
SBP (mmHg)	106 ± 2	117 ± 4 *
DBP (mmHg)	65 ± 2	70 ± 2
MAP (mmHg)	78 ± 2	88 ± 3 *
<sup>a</sup> Pulse wave velocity (m/s)	5.0 ± 0.1	7.5 ± 0.4*

10 min of quite supine rest, by the measurement of brachial blood pressure (HEM-705CP, Omron) and SphygmoCor-based estimates of aortic hemodynamics, reflected wave indices, and carotid-to-femoral PWV (AtCor Medical, CvM). All age comparisons (premenopausal vs. postmenopausal women) were based on visit 1 measurements.

**Visits 2 and 3** (beetroot juice study visits, postmenopausal women only): Subjects were instructed to avoid eating high nitrate containing foods such as spinach and lettuce on the day prior, and to consume only water for at least 8 h prior to these beetroot study visits. Upon arrival to the CRC, a venous blood sample was collected for determination plasma nitrate and nitrite concentrations. Following 10 min of quiet supine rest, brachial blood pressure, aortic hemodynamics, radial pulse wave characteristics, and PWV were measured using the same methods as during visit 1. Subjects then consumed either nitrate-rich beetroot juice (~9.7 mmol NO<sub>3</sub><sup>-</sup>, BR<sub>nitrate</sub>; 140 mL Beet-It Organic, James White Juice Company) or nitrate-depleted beetroot juice (BR<sub>placebo</sub>; 140 mL nitrate-depleted Beet-It Organic, James White Juice Company). A second venous blood sample was drawn 90 min after juice consumption to assess absorption (rise in plasma NO<sub>3</sub><sup>-</sup>) and conversion (plasma NO<sub>2</sub><sup>-</sup>) of the ingested nitrate (or lack thereof during the placebo juice visit). After 10 min of quiet supine rest (i.e., 100 min post-juice consumption), repeat measurements of brachial blood pressure, aortic hemodynamics, radial pulse wave characteristics, and PWV were performed. A third venous blood sample was drawn at the end of the entire study visit, which also included a subsequent handgrip exercise protocol (part of our larger registered clinical trial) that varied in duration/time to fatigue; thus the timing of this third and final blood sample varied, but was always at least 180 min after juice consumption.

The beetroot juice was concealed in a locked cabinet located in the CRC at a temperature maintained between 22 and 25 degrees Celsius. Research nurses with the CRC dispensed the supplements to subjects on these two study visits and the order of intervention was randomized and blinded for both subjects and researchers.

### 2.3. Pulse wave analysis and pulse wave velocity

Aortic blood pressure, central aortic indices, and carotid-to-femoral PWV (central PWV) were determined by a tonometry-based vascular profiling device (SphygmoCor CvM, AtCor Medical). Aortic blood pressures and other central aortic indices such as augmentation index (AI, which indicates the size of the increase or decrease in the pulse height as a result of the reflected wave), augmentation index @ HR75 (the augmentation index adjusted for a heart rate of 75 bpm), tension time index (indicating myocardial oxygen demand), and the diastolic

time index (indicating subendocardial flow) were derived and calculated from the radial pulse wave using the transfer function utilized in the vascular profiling device. Left ventricular wasted energy was calculated separately as  $E_w$  ( $\text{sec} \cdot \text{dyne} \cdot \text{cm}^2$ ) = Augmentation Pressure (mmHg) \* reflected wave duration (msec) \*  $\pi/2$  multiplied by the conversion factor 1.33 [14]. Rate pressure product (RPP) was calculated by multiplying SBP and HR. Carotid-to-femoral PWV was measured in a subset of postmenopausal women ( $n = 9$ ). Carotid-to-femoral PWV was calculated by dividing the estimated distance between the carotid and femoral arteries (i.e. carotid artery to sternal notch + sternal notch to femoral artery) by the pulse transit time between the two sites. Reported aortic BP and PWV values are either the mean of the two similar consecutive measurements or the median of the three consecutive measurements.

#### 2.4. Plasma nitrate and nitrite analysis

Venous blood samples were drawn into heparin tubes (4 mL lithium heparin tubes, BD Vacutainer, Franklin Lakes, N.J., USA) and immediately centrifuged at 3200 rpm (1590 g) and 4 °C for 10 min. After centrifugation, plasma was extracted and stored in  $-80$  °C freezer for later analysis of nitrate and nitrite concentration. The ENO-20 analyzer (EICOM, San Diego, Calif., USA) with a sensitivity of 0.1 pmol for nitrate and nitrite was used to measure nitrate and nitrite concentration in the plasma samples. Briefly, plasma was mixed with an equal volume of 100% methanol and centrifuged at 12,000 g for 10 min followed by sample loading into a 96-well plate. Nitrate and nitrite were then separated via column chromatography and individually reacted with a Griess reagent, synthesizing a diazo compound. The absorbance of this red diazo compound was read at a wavelength of 540 nm using a visible light detector.

#### 2.5. Statistical analysis

A Student *t*-test determined differences between pre- and post-menopausal women groups (visit 1). To assess differences between BR<sub>placebo</sub> and BR<sub>nitrate</sub>, two-way repeated measure analysis of variance (ANOVA) was used. Bonferroni's multiple comparisons test followed if a significant group  $\times$  time interaction effect was observed. The 95% confidence intervals, which provides insight into the potential clinical relevance of these changes, were also calculated. A paired *t*-test was used for the comparisons of changes from pre- to post-beetroot juice consumption. All statistical analyses were performed using Prism 7 (GraphPad Software, Inc., La Jolla, CA). Statistical significance was set at  $p < 0.05$ . All data are expressed as the mean  $\pm$  standard error.

### 3. Results

#### 3.1. Subject characteristics (Table 1)

Postmenopausal women were older, shorter, and had higher BMI than premenopausal women. Postmenopausal women also had significantly higher supine resting (arm cuff) SBP, MAP, total cholesterol, LDL, and fasting glucose compared to premenopausal women (all  $p < 0.05$ ).

#### 3.2. Plasma nitrate and nitrite (Fig. 1)

Baseline plasma nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ) concentrations (upon arrival to the CRC before beetroot juice consumption) were not different between study visits ( $\text{NO}_3^-$ ,  $p = 0.34$ ;  $\text{NO}_2^-$ ,  $p = 0.58$ ). Two-way repeated measures ANOVA revealed significant interaction effects for both plasma  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentration (all  $p < 0.05$ ) and follow-up pairwise comparisons showed that during the BR<sub>nitrate</sub> study visit, plasma  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentrations were significantly elevated at 100 min ( $\text{NO}_3^- = 15$  fold,  $\text{NO}_2^- = 4$  fold) and at the end of

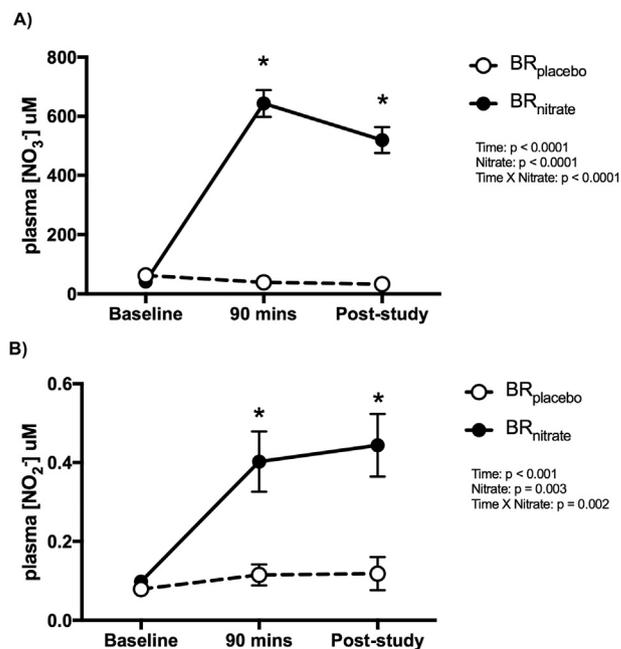


Fig. 1. Plasma nitrate and nitrite concentrations. Comparison of average A) plasma nitrate ( $\text{NO}_3^-$ ) concentration and B) plasma nitrite ( $\text{NO}_2^-$ ) concentration before, 90 min after consumption of BR<sub>placebo</sub> and BR<sub>nitrate</sub>, and at the end of the study visit (at least 180 min following the juice consumption) in postmenopausal women ( $n = 9$ ). Abbreviations: BR<sub>placebo</sub>, nitrate-depleted beetroot juice; BR<sub>nitrate</sub>, nitrate-rich beetroot juice. \* indicates significant ( $p < 0.05$ ) difference between BR<sub>placebo</sub> and BR<sub>nitrate</sub>. Data are means  $\pm$  S.E.M.

the study (post-study  $\text{NO}_3^- = 13$  fold,  $\text{NO}_2^- = 5$  fold) compared to the BR<sub>placebo</sub> study visit (all  $p < 0.001$ ).

#### 3.3. Age comparisons

Group data for aortic blood pressures and central hemodynamics in pre- and postmenopausal women are presented in Table 2. Resting aortic systolic, diastolic, and mean pressures, as well as pulse pressure, were all higher in postmenopausal women compared to premenopausal women (all  $p < 0.01$ ). In addition to aortic pressures, SphygmoCor-derived aortic reflected wave indices including augmentation pressure, augmentation index (AI), diastolic time index, and end systolic pressure were higher in postmenopausal women (all  $p < 0.05$ ). Reflected wave arrival time and pulse pressure amplification were lower, while rate pressure product,  $E_w$  and PWV were higher in postmenopausal women (all  $p < 0.05$  vs. premenopausal women).

#### 3.4. Effects of nitrate supplementation on brachial and aortic blood pressures

In postmenopausal women, nitrate supplementation reduced ( $p < 0.05$  vs. placebo visit) both brachial systolic BP (BR<sub>nitrate</sub>  $-4.9 \pm 2.1$  mmHg vs. BR<sub>placebo</sub>  $+1.1 \pm 1.8$  mmHg;  $p = 0.048$ ) and aortic systolic BP (BR<sub>nitrate</sub>  $-6.3 \pm 2.0$  mmHg vs. BR<sub>placebo</sub>  $+0.5 \pm 1.7$  mmHg;  $p = 0.008$ ; Fig. 2). Significant reductions with nitrate supplementation were also observed for brachial mean BP (BR<sub>nitrate</sub>  $-4.1 \pm 1.7$  mmHg vs. BR<sub>placebo</sub>  $+0.9 \pm 1.3$  mmHg;  $p = 0.007$ ) and aortic mean BP (BR<sub>nitrate</sub>  $-4.1 \pm 1.7$  mmHg vs. BR<sub>placebo</sub>  $+0.9 \pm 1.3$  mmHg;  $p = 0.007$ ). Nitrate supplementation did not elicit statistically significant reductions in diastolic BP (brachial or aortic).

**Table 2**

Age group comparison of aortic hemodynamics. Abbreviations: aSBP, aortic systolic blood pressure; aDBP, aortic diastolic blood pressure; aMAP, aortic mean arterial pressure; aPP, aortic pulse pressure; PPamp, pulse pressure amplification ratio; AP@HR75, augmentation pressure normalized to heart rate 75; AI@HR75, augmentation index normalized to heart rate 75; TTI, central tension time index; DTI, central diastolic time index; ED, ejection duration; T1R, time of the start of the reflected wave; ESP, end systolic pressure; SEVR, subendocardial viability ratio; Ew, left ventricular wasted energy; RPP, rate pressure product. \* indicates significant ( $p < 0.05$ ) difference between young and older subjects. Data are means  $\pm$  S.E.M.

	Premenopausal women n = 10	Postmenopausal women n = 13	P-value
aSBP (mmHg)	91 $\pm$ 2	113 $\pm$ 4	< 0.001*
aDBP (mmHg)	66 $\pm$ 2	72 $\pm$ 2	0.062
aMAP (mmHg)	78 $\pm$ 2	89 $\pm$ 3	0.005*
aPP (mmHg)	25 $\pm$ 1	41 $\pm$ 2	< 0.001*
PPamp (%)	158 $\pm$ 2	117 $\pm$ 2	< 0.001*
AP@HR75 (mmHg)	1 $\pm$ 1	11 $\pm$ 1	< 0.001*
AI@HR75 (%)	3 $\pm$ 3	29 $\pm$ 1	< 0.001*
TTI (mmHg*s <sup>-1</sup> *min <sup>-1</sup> )	1916 $\pm$ 74	2149 $\pm$ 98	0.071
DTI (mmHg*s <sup>-1</sup> *min <sup>-1</sup> )	2759 $\pm$ 84	3215 $\pm$ 122	0.006*
ED (ms)	37 $\pm$ 1	35 $\pm$ 1	0.213
T1R (ms)	154 $\pm$ 6	134 $\pm$ 3	0.012*
ESP (mmHg)	81 $\pm$ 3	102 $\pm$ 4	< 0.001*
SEVR (%)	145 $\pm$ 6	153 $\pm$ 9	0.455
Ew (s <sup>2</sup> *dyne*cm <sup>2</sup> )	393 $\pm$ 120	3247 $\pm$ 324	< 0.001*
RPP (mmHg*bpm)	6018 $\pm$ 278	6971 $\pm$ 313	0.033*

### 3.5. Effects of nitrate supplementation on pulse pressure amplification and indices of myocardial demand (Table 3)

In postmenopausal women, nitrate supplementation increased ( $p < 0.05$  vs. placebo visit) pulse pressure amplification ( $\Delta$ PPamp:  $BR_{\text{nitrate}} = 4.6 \pm 2.0\%$  vs.  $BR_{\text{placebo}} = 0.7 \pm 2.5\%$ ; Table 3, Fig. 3). Nitrate supplementation significantly reduced ( $p < 0.05$  vs. placebo visit) end-systolic pressure ( $\Delta$ ESP:  $BR_{\text{nitrate}} = -5.3 \pm 2.0$  mmHg vs.

$BR_{\text{placebo}} = 0.9 \pm 1.8$  mmHg), but did not significantly alter augmentation index (Fig. 3) or indices of myocardial demand/load such as tension time index, diastolic time index, reflected wave arrival time, sub-endocardial viability ratio, left ventricular wasted energy, or rate pressure product (Table 3).

### 3.6. Effects of nitrate supplementation on aortic pulse wave velocity

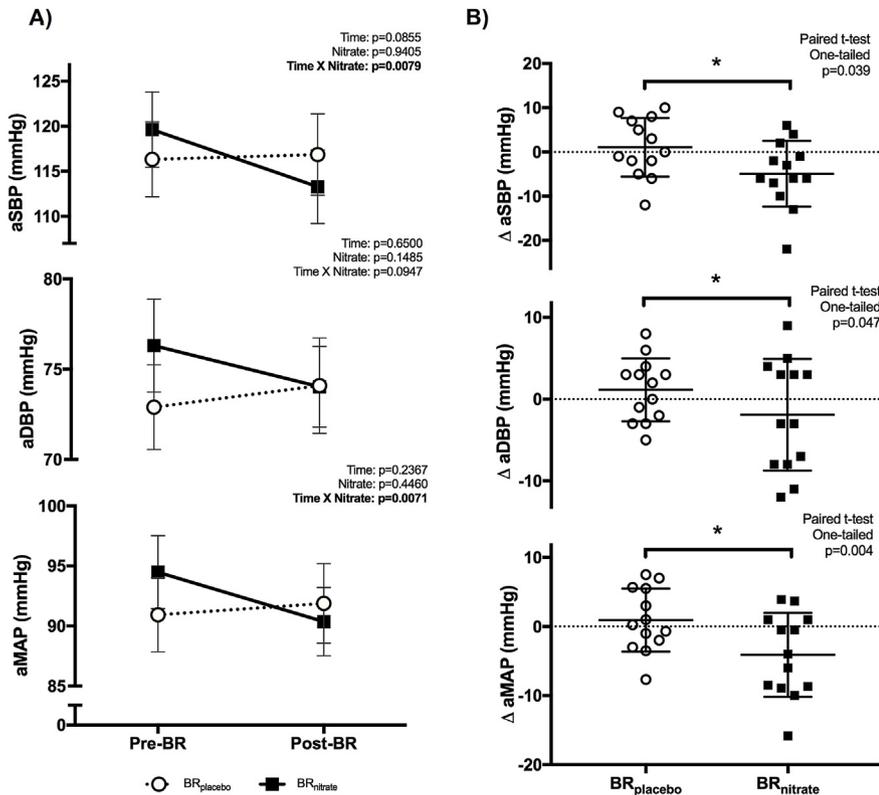
In postmenopausal women, nitrate supplementation did not significantly alter ( $p < 0.05$  vs. placebo visit) carotid-to-femoral PWV (Fig. 4) or  $\Delta$ PWV normalized to MAP (data not shown).

## 4. Discussion

The primary purpose of this study was to determine, in postmenopausal women, the effect of acute nitrate supplementation on resting brachial and aortic hemodynamics. Consistent with our hypothesis, we observed decreases in brachial and aortic (systolic and mean) blood pressures following nitrate-rich beetroot juice consumption. Brachial and aortic BP were not altered following consumption of a true placebo (nitrate-depleted beetroot juice), suggesting a hemodynamically significant influence of nitrate in these women. Additionally, we found significant between-visit differences in pulse pressure amplification (larger increase with nitrate). While this acute intervention did not appear to reverse any age-associated aortic stiffness or wave reflection differences in these subjects, the present study is the first to show both a peripheral and central BP lowering effect of dietary nitrate supplementation in postmenopausal women.

### 4.1. Nitrate supplementation and aortic blood pressures in post-menopausal women

Hughes et al. [11] were the first to report that acute consumption of nitrate-rich beetroot juice can lower brachial and aortic blood pressures in healthy older (as well as younger) adults. The present findings



**Fig. 2.** Effects of dietary nitrate supplementation on aortic blood pressures. Comparison of mean (panel A) and individual (panel B) aortic blood pressures before and 100 min after beetroot juice ( $BR_{\text{placebo}}$  or  $BR_{\text{nitrate}}$ ) consumption in postmenopausal women ( $n = 13$ ). Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure;  $BR_{\text{placebo}}$ , nitrate-depleted beetroot juice;  $BR_{\text{nitrate}}$ , nitrate-rich beetroot juice. \* indicates significant ( $p < 0.05$ ) difference between  $BR_{\text{placebo}}$  and  $BR_{\text{nitrate}}$ . Data are means  $\pm$  S.E.M.

**Table 3**

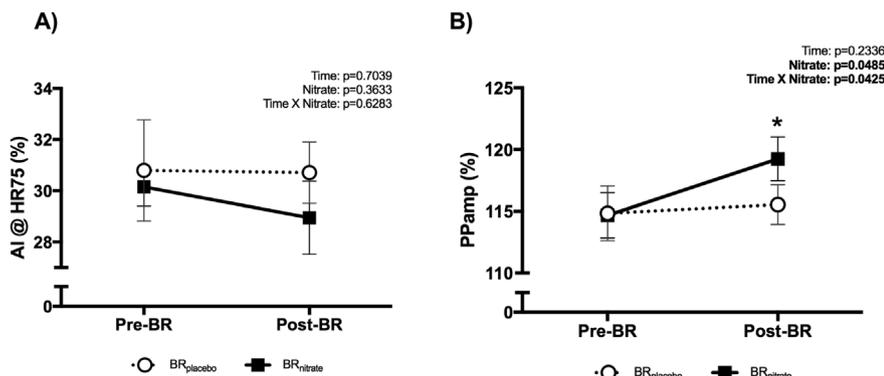
Effects of dietary nitrate supplementation on aortic hemodynamics (n = 13). Abbreviations: aSBP, aortic systolic blood pressure; aDBP, aortic diastolic blood pressure; aMAP, aortic mean arterial pressure; aPP, aortic pulse pressure; PPamp, pulse pressure amplification ratio; AP@HR75, augmentation pressure normalized to heart rate 75; AI@HR75, augmentation index normalized to heart rate 75; TTI, central tension time index; DTI, central diastolic time index; ED, ejection duration; T1R, time of the start of the reflected wave; ESP, end systolic pressure; SEVR, subendocardial viability ratio; Ew, left ventricular wasted energy; RPP, rate pressure product; CI, 95% confidence intervals. \* indicates significant (p < 0.05). Data are means ± S.E.M.

		Pre-juice consumption	Post-juice consumption	Changes (Δ)	Time	Nitrate	Time × Nitrate Interaction	Low CI	High CI
aSBP (mmHg)	BR <sub>placebo</sub>	116.3 ± 4.2	116.9 ± 6.3	0.5	p = 0.086	p = 0.941	p = 0.008*	-3.376	4.427
	BR <sub>nitrate</sub>	119.6 ± 4.2	113.3 ± 4.1	-6.3				-10.235	-2.432
aDBP(mmHg)	BR <sub>placebo</sub>	72.9 ± 2.4	74.1 ± 3.4	1.2	p = 0.650	p = 0.149	p = 0.095	-2.268	4.665
	BR <sub>nitrate</sub>	76.3 ± 2.6	74.0 ± 2.6	-2.3				-5.742	1.191
aMAP (mmHg)	BR <sub>placebo</sub>	90.9 ± 3.1	91.9 ± 4.5	0.9	p = 0.237	p = 0.446	p = 0.007*	-1.873	3.771
	BR <sub>nitrate</sub>	94.5 ± 3.0	90.4 ± 2.8	-4.1				-6.925	-1.280
aPP (mmHg)	BR <sub>placebo</sub>	43.4 ± 2.2	42.8 ± 3.3	-0.7	p = 0.010*	p = 0.208	p = 0.138	-4.523	3.177
	BR <sub>nitrate</sub>	43.3 ± 2.6	39.3 ± 2.6	-4.1				-7.908	-0.208
PPamp (%)	BR <sub>placebo</sub>	114.8 ± 2.2	115.5 ± 2.2	0.7	p = 0.234	p = 0.049*	p = 0.043*	-2.407	3.792
	BR <sub>nitrate</sub>	114.7 ± 1.8	119.2 ± 1.8	4.6				1.478	7.676
AP@HR75 (mmHg)	BR <sub>placebo</sub>	12.3 ± 1.2	12.2 ± 1.1	-0.2	p = 0.287	p = 0.153	p = 0.226	-1.926	1.567
	BR <sub>nitrate</sub>	12.0 ± 0.9	10.6 ± 0.8	-1.4				-3.156	0.336
AI@HR75 (%)	BR <sub>placebo</sub>	30.8 ± 2.0	30.7 ± 1.2	-0.1	p = 0.704	p = 0.363	p = 0.628	-4.154	3.975
	BR <sub>nitrate</sub>	30.1 ± 0.8	29.0 ± 1.4	-1.2				-5.270	2.860
TTI (mmHg*s <sup>-1</sup> *min <sup>-1</sup> )	BR <sub>placebo</sub>	2189 ± 118	2115 ± 121	-75	p = 0.017*	p = 0.538	p = 0.191	-176.611	26.983
	BR <sub>nitrate</sub>	2269 ± 100	2170 ± 95	-153				-254.573	-50.978
DTI (mmHg*s <sup>-1</sup> *min <sup>-1</sup> )	BR <sub>placebo</sub>	3269 ± 118	3402 ± 123	133	p = 0.749	p = 0.720	p = 0.055	-56.895	323.369
	BR <sub>nitrate</sub>	3400 ± 121	3309 ± 103	-91				-280.696	99.568
ED (ms)	BR <sub>placebo</sub>	35.3 ± 1.3	33.7 ± 1.2	-1.6	p = 0.074	p = 0.557	p = 0.341	-3.046	-0.146
	BR <sub>nitrate</sub>	35.4 ± 1.1	34.6 ± 1.0	-0.8				-2.252	0.649
T1R (ms)	BR <sub>placebo</sub>	135.3 ± 4.8	130.8 ± 1.9	-4.5	p = 0.248	p = 0.646	p = 0.851	-15.566	6.617
	BR <sub>nitrate</sub>	135.5 ± 2.9	132.2 ± 3.7	-3.3				-14.387	7.797
ESP (mmHg)	BR <sub>placebo</sub>	104.7 ± 3.9	105.6 ± 3.8	0.9	p = 0.179	p = 0.984	p = 0.012*	-2.920	4.651
	BR <sub>nitrate</sub>	107.8 ± 3.8	102.5 ± 3.5	-5.3				-9.093	-1.522
SEVR (%)	BR <sub>placebo</sub>	153.3 ± 8.0	165.3 ± 8.2	12.0	p = 0.045*	p = 0.580	p = 0.464	-1.141	25.231
	BR <sub>nitrate</sub>	153.0 ± 7.8	159.6 ± 7.1	6.5				-6.654	19.718
Ew (s*dyne*cm <sup>2</sup> )	BR <sub>placebo</sub>	3754 ± 407	3367 ± 330	-386	p = 0.067	p = 0.083	p = 0.297	-1015.239	242.863
	BR <sub>nitrate</sub>	3604 ± 321	2838 ± 274	-765				-1394.518	-136.416
RPP (mmHg*bpm)	BR <sub>placebo</sub>	7478 ± 415	7599 ± 443	120	p = 0.922	p = 0.668	p = 0.388	-289.200	530.123
	BR <sub>nitrate</sub>	7668 ± 371	7586 ± 333	-82				-491.829	327.495

support the brachial and aortic blood pressure lowering potential of this non-pharmacologic intervention, and extend the findings of Hughes et al. by confirming that these effects are due to nitrate-to-nitrite reduction (and presumably reduction to nitric oxide), and not to other vasoactive components found in beetroot juice (anti-oxidants, polyphenols, etc) or the acute effects of fluid ingestion per se [15,16]. The current findings also add to the evidence that aortic (mainly systolic) BP is responsive to dietary nitrate supplementation. In this regard, the reduction in aortic systolic BP in our small sample of healthy postmenopausal women is consistent with that observed following a 6 month nitrate-rich (vs. nitrate-depleted) beetroot juice consumption study involving 126 pre- and diabetic middle-aged adults, a substantial number of whom were women of post-menopausal age [17].

The significant reductions in aortic systolic, but not diastolic, BP after consumption of nitrate rich beetroot juice in the present sample of

post-menopausal women could reflect a selective dilation of muscular conduit arteries (relative to smaller arterioles) associated with the conversion of nitrite. This interpretation is based on several findings in the literature including 1) the fact that intravenous infusion of inorganic (sodium) nitrite reduces aortic systolic BP in humans through a selective dilatory effect in medium-size conduit arteries, but not small arterioles [16], 2) the effectiveness of organic nitrates (glyceryl trinitrate) in selectively vasodilating large muscular arteries [18], and 3) the observation of Cecelja et al. [2] of an increased aortic systolic pressure over 10 years in middle-aged women that was acutely reversed by sublingual nitroglycerin administration. Despite the different routes of administration, bioconversion pathways, and study populations, these collective findings from the literature and the present study suggest that organic and inorganic nitrate-nitrite conversion may operate by similar mechanisms leading to a reduction in aortic systolic BP in humans. The



**Fig. 3.** Effects of dietary nitrate supplementation on augmentation index and pulse pressure amplification. Comparison of A) augmentation index at heart rate 75 bpm (AI@HR75) and B) pulse pressure amplification, before and 100 min after beetroot juice (BR<sub>placebo</sub> or BR<sub>nitrate</sub>) consumption in postmenopausal women (n = 13). Abbreviations: AI, augmentation index; HR, heart rate; PPamp, pulse pressure amplification; BR<sub>placebo</sub>, nitrate-depleted beetroot juice; BR<sub>nitrate</sub>, nitrate-rich beetroot juice. \* indicates significant (p < 0.05) difference between BR<sub>placebo</sub> and BR<sub>nitrate</sub>. Data are means ± S.E.M.

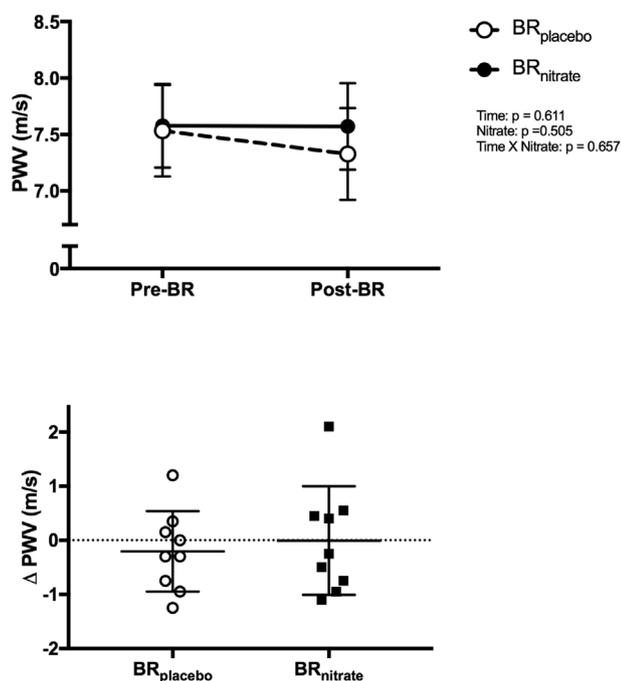


Fig. 4. Effects of dietary nitrate supplementation on aortic pulse wave velocity. Comparison of mean (top) and individual (bottom) pulse wave velocity (PWV) values measured before and 100 min after consumption of BR<sub>placebo</sub> or BR<sub>nitrate</sub> in postmenopausal women (n = 9). Abbreviations: PWV, pulse wave velocity; BR<sub>placebo</sub>, nitrate-depleted beetroot juice; BR<sub>nitrate</sub>, nitrate-rich beetroot juice. \* indicates significant (p < 0.05) difference between BR<sub>placebo</sub> and BR<sub>nitrate</sub>. Data are means ± S.E.M.

accelerated increase in brachial [1] and aortic [2] systolic BP with menopause in women, and the attendant rise in cardiovascular disease risk [2–4], suggests the need to further investigate the hemodynamic mechanisms and potential cardiovascular protective properties of dietary nitrate in this population.

#### 4.2. Nitrate supplementation and aortic pulse wave characteristics in older adults

We observed, as has been shown in multiple prior cross-sectional studies, an age-associated increase in aortic PWV and differences in several reflected pulse wave characteristics indicative of a stiffened cardiovascular system (Table 2). Such changes are likely due, in women, to the combined effects of chronological and ovarian aging [19–21]. Such effects are of particular clinical interest because of their association with cardiovascular [22] and cerebrovascular [23] risk in older adults and are thought to result from both structural changes in the arterial (aortic) wall [24–27] and alterations in endothelial function (less NO-mediated dilation, more endothelin-mediated constriction [28,29]). The lack of change in aortic PWV and wave reflection characteristics following nitrate supplementation in the present group of women, mostly in their 60s, likely reflects the inability of a single (albeit large) dose of nitrate to reverse age- and menopause-associated aortic remodeling. Another possible explanation is that 100 min post-juice consumption was not long enough, or the dose of nitrate not high enough, to induce these changes via the nitrate-nitrite-NO pathway. However, this is highly unlikely as we observed 15-fold and 4-fold increases in plasma nitrate and nitrite, respectively, in this timeframe in addition to the blood pressure lowering effects described above. It is noteworthy that Hughes et al. [11] also observed in their older cohort a peak degree of aortic blood pressure lowering over this time (i.e., 90 min and 120 min post consumption) without corresponding changes in measures of aortic wave reflection. Liu et al. [30] similarly observed

no alteration in augmentation index (AI) among healthy middle-aged and older adults following a single, nitrate-rich meal. Collectively, these studies suggest that a longer duration (i.e., weeks) of dietary nitrate-supplementation is likely needed to alter arterial wave reflection indices in older adults. Two studies of 4 weeks duration have indeed reported significant reductions in augmentation index in normotensive and hypertensive older adults (both sexes) following nitrate supplementation, although neither used a cross-over (within-subjects treatment) design [31,32].

The significant age group differences (Table 2) we observed in reflected wave transit time (faster in old), diastolic time index (higher in old), augmentation magnitude and index (both higher in old), and pulse pressure amplification (lower in old), are each indicative of greater systolic loading in the aged heart and likely reflect a higher myocardial work demand in these older women. The only afterload variable to show a significant alteration with acute nitrate supplementation in the present study was the pulse pressure amplification ratio. The significant increase in this variable after nitrate supplementation (p = 0.043) could indicate a favorable effect on ventricular afterload as is seen with acute organic nitrate administration (e.g. sublingual nitroglycerin; [28,33]) and/or a selective dilation of large muscular (vs. large central elastic) conduit arteries in these older women. However, there was also a borderline significant reduction in the diastolic time index (DTI; p = 0.055), an indication that myocardial perfusion during diastole may have been reduced with nitrate supplementation. A reduced DTI after nitrate-rich beetroot juice consumption is also evident in the older subjects studied by Hughes et al. [11]. The significance of these myocardial work-related effects of nitrate-rich beetroot juice in older adults of both sexes require further, longer duration, study.

#### 4.3. Experimental considerations

In the present study, we used a double blind, randomized crossover study design with rigorously screened subjects who followed standardized pre-visit test instructions on what to avoid (teeth brushing, mouthwash, etc) and who were each tested at a similar time of day. We also used a research quality nitrate supplement and placebo (Beet-It organic, James White Company) which produced large increases in plasma nitrate and nitrite with the active supplement in all participants (8–33-fold increase for nitrate; 2–7-fold increase for nitrite), with minimal increases seen after placebo consumption (Fig. 1). While we did not strictly control or monitor diet via daily logs, participants were instructed to consume only water for at least 8 h prior to the beetroot consumption visits, and to refrain from high nitrate containing food such as spinach and lettuce the day prior. Baseline plasma nitrate and nitrite concentrations did not differ on the two visits (p = 0.33 and 0.27, respectively; nitrate vs. placebo visit), suggesting adequate subject compliance with these instructions. We cannot extrapolate our results to smaller or larger doses of nitrate supplementation or to other populations, but we used a volume of beetroot juice (140 ml) and dose of nitrate (9.7 mmol) that produced hemodynamically significant effects without adverse side effects and was well tolerated in this cohort of healthy postmenopausal women. We did not examine these effects in younger or premenopausal women and thus cannot confirm whether age or estrogen deficiency contributed to the null findings with respect to aortic pulse wave (forward or reflected) changes. Lastly, while this study was sufficiently powered to detect the effects of dietary nitrate supplementation on our primary outcomes (aortic systolic and mean BP), the relatively small number of women studied (13 postmenopausal) may have been insufficient to detect significant changes in some of our other outcomes.

#### 4.4. Translational perspective

Traditional pharmacological management of blood pressure has not been highly effective in older women [34–36] and there are few low

risk, estrogen-based therapies for improving vascular health in postmenopausal women [37,38]. This is also a group with considerable interest in non-pharmacological therapies [13]. The current finding that dietary nitrate supplementation can acutely lower brachial and aortic systolic blood pressures in older postmenopausal women supports the conduct of longer duration nitrate supplementation studies in this population.

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## Author contributions

Task	DJK	CAR	YS	DJM	MAB	MF	DKS	SB	MDM	DNP
Conception & Design	X			X	X	X				X
Perform Experiments	X	X	X	X	X					
Analyze Data	X	X	X				X	X		X
Interpret Results	X	X	X				X	X	X	X
Prepare Figures	X									
Draft Manuscript	X									X
Edit/revise Manuscript	X						X	X	X	X
Approve Manuscript	X	X	X	X	X	X	X	X	X	X

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