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A randomised, factorial trial to reduce arterial stiffness independently of blood pressure: Proof of concept? The 'VaSera' trial testing dietary nitrate and spironolactone

Short running title: Spironolactone, nitrate and artery stiffness

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*PI statement: 'The authors confirm that the Principal Investigator for this paper is Professor J K Cruickshank and that he had direct clinical responsibility for patients.'

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Keywords: beetroot juice, dietary nitrate, blood pressure, arterial stiffness, nitrate-nitrite-NO pathway, type 2 diabetes

Abstract

Aim

To test if spironolactone or dietary nitrate from beetroot juice could reduce arterial stiffness as aortic pulse wave velocity (PWVart), a potential treatment target, independently of blood pressure.

Methods

Daily spironolactone (≤50mg) versus doxazosin (control ≤16mg) and 70mL beetroot juice ('Beet-It' ≤11mmol nitrate) versus nitrate-depleted juice (placebo; 0mmol nitrate) were tested in people at risk or with type-2 diabetes using a double-blind, 6-month factorial trial. Vascular indices (baseline, 12, 24 weeks) were cardiac-ankle vascular index ('CAVI'), a nominally pressure-independent stiffness measure (primary outcome), pulse wave velocity (PWVart) secondary, central systolic pressure and augmentation. Analysis was intention-to-treat, adjusted for systolic pressure differences between trial arms.

Results

Spironolactone did not reduce stiffness, with evidence for reduced CAVI on doxazosin rather than spironolactone (mean difference [95% confidence intervals]; 0.25[-0.3, 0.5] units, p=0.080), firmer for PWVart (0.37[0.01, 0.7] ms^{-1}, p=0.045). There was no difference in systolic pressure reduction between spironolactone and doxazosin (0.7[-4.8, 3.3]mmHg, p=0.7). Circulating nitrate and nitrite increased on active versus placebo juice, with central systolic pressure lowered -2.6[-4.5, - 0.8]mmHg, p=0.007 more on the active juice, but did not reduce CAVI, PWVart, nor peripheral pressure. Change in nitrate and nitrite concentrations were 1.5-fold [1.1-2.2] and 2.2-fold [1.3, 3.6] higher on spironolactone than on doxazosin respectively; both p<0.05.

Conclusion

Contrary to our hypothesis, in at-risk/type-2 diabetes patients, spironolactone did not reduce arterial stiffness, rather PWVart was lower on doxazosin. Dietary nitrate elevated plasma nitrite, selectively lowering central systolic pressure, observed previously for nitrite.

Clinical trial registration: ISRCTN registry: ISRCTN25003627/ DOI 10.1186/ISRCTN25003627.

Statement 1: What is already known about this subject

- Arterial stiffness is a predictor of mortality, independently of BP and diabetes
- Inorganic dietary nitrate has been shown to reduce blood pressure and arterial stiffness via the nitrate-nitrite, nitric oxide pathway
- Spironolactone is reported to reduce arterial stiffness, but if this is BP-independent is not clear

Statement 2: What this study adds

- The longest trial to test inorganic nitrate on vascular parameters to date
- Inorganic dietary nitrate selectively reduced central systolic BP which parallels previous data
- Despite lowering BP slightly more than did the α-blocker, doxazosin, spironolactone did not reduce arterial stiffness, which was marginally lowered on doxazosin

Introduction

Type 2 diabetes mellitus (T2DM) is characterized by excess cardiac and vascular disease even before 'formal' diagnosis [1,2]. Arterial stiffness measured as aortic pulse wave velocity (PWVart) is amongst the most powerful 21predictors of both cardiovascular and all-cause mortality, crucially independent of mean or systolic blood pressure (SBP) and other standard risk factors, including glycaemia [3]. Reducing arterial stiffness could be particularly valuable in overweight people at increased risk of or already with overt T2DM, because of its predictive impact in glucose intolerance/ T2DM [4], and its high prevalence in these people since early measures of arterial stiffness were used [5-7]. The pathology of arterial stiffness involves elastin degradation and collagen deposition with fibrosis from inflammatory stimuli including dysregulation of nitric oxide (NO) [8] and up-regulation of pro-fibrotic factors [9-11].

Reductions in PWV by lifestyle measures are reported particularly for exercise, weight loss and specific dietary components, and by various pharmacological agents, including anti-hypertensives, statins, some anti-diabetic medications and advanced glycation end-product breakers [12]. However, PWV reduction formally independent of BP is seldom examined. Doing so is important as PWV is intrinsically linked to BP hence it can be hard to distinguish the two.

Spironolactone, a mineralocorticoid receptor antagonist was recently found highly effective in reducing BP in proven resistant hypertension [13]. Initial trials for specifically reducing arterial stiffness, in early kidney disease [14], untreated hypertensives [15] and dilated cardiomyopathy [16] appeared promising. These trials were generally not designed to test impact on PWV formally independent of BP change.

Inorganic (dietary) nitrate, abundant in green leafy vegetables and beetroot [17] reduces BP in healthy [18] and hypertensive volunteers [19] via the nitrate-nitrite-NO pathway [20], but not in patients with T2DM, [21-22] or with their inclusion in a meta-analysis of 24h ambulatory BP monitoring [23]. PWV reductions with inorganic (dietary) nitrate have also been noted in healthy and hypertensive volunteers, but over too short a period for vessel remodelling; these were likely BP-dependent reductions [24]. We found that inorganic *nitrite* selectively lowers aortic, relative to peripheral, BP, with reductions also in PWV that seem to be via selective normoxia-dependent conduit (radial) artery dilatation in healthy volunteers [25-26], and selectively dilated epicardial coronary arteries in patients undergoing coronary angiography [27]. While tolerance develops to organic nitrates [28], it has not been described for inorganic (dietary) nitrate [19], perhaps this due to the mechanisms of bioactivation of inorganic nitrite to nitric oxide, and suppression of reactive oxygen species (ROS)[29]. Longer-term effects of inorganic (dietary) nitrate beyond 6 weeks have not yet been tested.

In the trial reported here, we hypothesised that spironolactone and dietary nitrate would reduce arterial stiffness independently of BP reduction in people with or at risk of T2DM. We tested this hypothesis in a double-blind, controlled, factorial design 24-week trial using cardio-ankle vascular index (CAVI) as the primary measure of stiffness and PWVart adjusted for BP change as the secondary outcome.

Methods

Study design and interventions

A single centre, double-blind, parallel, randomised controlled intervention trial in a 2 x 2 factorial design was carried out in accordance with the Declaration of Helsinki and U.S. Code of Federal Regulations.

Participants were assigned to one of 4 arms using computer randomization in blocks of 6, by an independent party. Interventions were spironolactone (12.5mg daily for 1 week, 12.5mg twice daily for 11 weeks, increased to 25mg twice daily to 24 weeks) with doxazosin as its control (4mg similarly titrated to 8mg twice daily) and dietary nitrate as beetroot juice (7.5mmol nitrate increased at 12 weeks to 11.2mmol nitrate, as measured in our lab) or nitrate-free beetroot juice as placebo (0mmol nitrate), (see Supplementary text). Spironolactone and doxazosin were prepared in indistinguishable brown bottles by St Thomas' Hospital pharmacy, London, UK. Commercially available beetroot juice, 'Beet It' and 'Beet It SPORT' were supplied as 15 x 70 mL bottles, indistinguishable between active and control juice, prepared and supplied by James White Drinks, Ltd, Suffolk UK.

Participants with or at risk of T2DM were recruited from Guy's and St Thomas' Hospitals, London, UK and surrounding areas between 2013-2015. Inclusion criteria were age 18-80years, clinically diagnosed T2DM *or* at risk of T2DM (as body mass index (BMI) ≥27 kg/m^2, positive family history or glucose intolerance after 75g challenge), ability to understand and comply with the protocol. Exclusion criteria: interfering chronic illness, adverse reaction to either drug, known allergy to beetroot, eGFR < 45 mL min^{-1}, HbA1c >11% (97mM/M), pregnant, breast feeding or atrial fibrillation. Written informed consent was obtained from all participants. The protocol was approved by South London Research Ethics Committee.

The primary outcome was change in arterial stiffness, nominally independent of BP, as measured by CAVI. Secondary outcomes were arterial stiffness, as measured by PWVart, with central BP and augmentation index. Both primary and secondary outcomes were to be adjusted for differences in peripheral baseline BP and BP change between trial arms, start-finish.

At St Thomas' Hospital Clinical Research Facility, London, participants rested supine in a temperature-controlled room for 20 minutes. Vascular measures were then performed supine in random order according to institutional guidelines.

After anthropometry, CAVI was measured using the VS-1500N, VaSera machine (Fukuda Denshi Ltd, Japan) as described [30]. Microphone-detected heart sounds were monitored, with BP cuffs on each arm and above each ankle, with pulse waves detected by the cuffs at 30-50mmHg. CAVI was calculated from PWV, as pulse wave transit times from aortic valve (2^{nd} sound) to ankle: CAVI= [ln SBP/ln DBP]. [2ρ/ΔP]. PWV^2, with path length estimated from height [31]. CAVI was measured in duplicate and averaged. $CAVI_0$ data were calculated as described previously [32]. PWVart, peripheral systolic, diastolic and central BP, aortic and brachial augmentation index and heart rate from 6-8 cardiac cycles were measured using appropriately sized cuffs by Arteriograph 24™ device (TensioMed Kft. Hungary), analysing mean of duplicate good quality readings. Quality was pre-specified with Arteriograph and VaSera waveforms checked by the manufacturers, blinded to other data. PWV with standard deviations (SDs) >1 were excluded.

Non-fasted blood (Hb, HbA1c, plasma glucose, sodium, potassium, creatinine, aldosterone and renin mass concentrations) and urinary sodium, potassium, creatinine

were measured by our accredited laboratory. Plasma nitrate and nitrite concentrations were measured by chemiluminescence as described [25,33].

Statistical analysis

Sufficient data from CAVI interventions were not available for sample size calculations. We used previous studies on BP with beetroot juice [33-34]and the 1-year study of PWVart on spironolactone [14] aiming to detect a 20% reduction over 6 months in PWV (standard deviation (SD) 8%) with minimum 80% power, at $p<0.05$. We estimated we needed 24 participants per each of 4 arms, aiming for 30 per group allowing for 20% drop out, for 24 patients in each to finish the trial.

A modified intention-to-treat analysis was performed using SAS (version 9.3); data are presented as least-square means estimated from mixed effects models (log-transformed where not normally distributed), adjusted as pre-specified for baseline, and any difference in final SBP *change* between the two arms being analysed. To estimate independence from BP change, changes in PWVart were adjusted for change in SBP. Least square mean data were averaged over the 2 follow-up visits (3 and 6 months). Regression analyses assumed linear relationships, with some predictor variables (renin, nitrate and nitrite) log-transformed.

Results

Baseline

Of 154 patients eligible and agreeing to attend, 11 were not eligible (4 for high HbA1c, 2 for previous adverse reactions, 2 with atrial fibrillation, 3 for ill health); 17 then declined to participate. The remaining 126 participants were randomised

(Supplementary Figure 1). Baseline characteristics were generally well-matched between arms (Table 1 and Supplementary Table 1) both between drugs and by nitrate/nitrate-free juices (Tables 2-3). Of randomized participants, 62% had T2DM with mean HbA1c 50mM/M (6.7%). The remaining 38% were 'at risk' (mean HbA1c <40 mM/M, 5.8%, BMI 32.5kg/m^2).

Follow up

Time from randomization to midpoint dose increase was 13±3 weeks and from midpoint-final visit 12±3weeks, totaling 24±5 weeks from randomization to end-of-study. Between baseline and 12 weeks' follow-up, 16 participants dropped out (6 no reason, 1 unrelated illness, 4 not re-contacted and 5 with side effects: 2 dizziness, 2 elevated glucose, 1 breathlessness). There were no follow-up measures for these participants.

Treatment effects

No statistical interactions occurred between beetroot or placebo juice arms and the spironolactone vs. doxazosin arm for any of the main/ haemodynamic outcomes, so data are presented separately (Tables 2-3). Supplementary Table 1 shows absolute, unadjusted changes of vascular and biological parameters for the 4 arms.

SPIRONOLACTONE VS. DOXAZOSIN: In adjusted models, spironolactone and doxazosin reduced BP similarly (SBP, least-square mean [95% CI]: -7.0 [-9.9, -4.2] vs. -6.3 [-9.1, -3.5] mmHg respectively, p= 0.7, Figure 1C and diastolic (DBP), -5.6 [-7.4, -3.7] vs. -4.7 [-6.5, -2.9] mmHg respectively, p= 0.5, Supplementary Figure 2A). The direction in difference for the primary endpoint, change in CAVI between drugs, was *contrary* to our

hypothesis, borderline significant towards doxazosin (0.14 [-0.06, 0.34] vs. -0.11 [-0.30, 0.08] units p=0.08, for spironolactone and doxazosin respectively, Figure 1A). When transposed to $CAVI_0$, our data was not significant -0.04(-0.44, 0.35) vs. 0.24 (-0.19, 0.67), p= 0.34 (doxazosin vs. spironolactone)[19]. However, the difference in PWVart change between spironolactone and doxazosin was significant (-0.07 [-0.33, 0.18] vs. -0.44 [-0.69, -0.19] ms^{-2}, p=0.045, Figure 1B) towards doxazosin, again contrary to our hypothesis. There were also no other differences in other hemodynamic parameters estimated by the Arteriograph for the drug arm, in central BP (-7.6 [-9.0, -6.3] vs. -7.2 [-8.5, -5.9] mmHg, p=0.6; Figure 1 D), augmentation index (Supplementary Figure 2 B-C), or heart rate.

Although no drug/ juice interactions in terms of hemodynamic variables were noted, nitrate and nitrite concentrations were higher on spironolactone than on doxazosin by 1.5-fold [1.1-2.2] and 2.2-fold [1.3, 3.6] respectively; both $p<0.05$; see Figure 1 E-F. Unadjusted data are in Supplementary Table 1.

BEETROOT VS. PLACEBO JUICE: There were no adjusted differences in change in arterial stiffness change as CAVI (0.02 [-0.18, 0.21] vs. 0.01 [-0.18, 0.21], p=0.98, Figure 2A) $CAVI_0$ 0.12(-0.29, 0.53) vs. 0.08(-0.34, 0.50), p= 0.898 (active vs. control) [19] nor PWVart (-0.23 [-0.48, 0.01] vs. -0.28 [-0.54, -0.03], p=0.8, Figure 2B), nor in brachial BP between active and placebo juice (SBP, -6.4 [-9.2, -3.6] vs. -6.9 [-9.8, -4.0] mmHg, p= 0.8, Figure 2C, nor DBP, p= 0.9 (Supplementary Figure 3A). However, difference in change in central (aortic) SBP between active and control juices was highly significant (-8.7[-10, -7.4] vs. -6.1[-7.4, -4.8] mmHg, p=0.007, Figure 2D). Decreases in aortic (-3 [-5.1, -0.9] vs. -0.3 [-2.4, 1.9] %, p=0.08) and brachial augmentation index (-5.9

[-10.0, -1.76] vs. -0.49 [-4.72, 3.74] %, p=0.08) were also borderline (Supplementary Figure 3B-C).

Plasma nitrate levels rose as expected in those on active compared with placebo juice (a 4.3[3.4, 5.5]-fold increase vs. 1.3[1.02, 1.71], p<0.001, Figure 2 E); nitrite levels increased 1.6[1.1, 2.2]-fold vs. 0.9[0.6, 1.2], p=0.02, Figure 2 F). These data confirm adherence to the beetroot juice arm. Unadjusted data are in Supplementary Table 1.

Adverse effects

From randomization, all adverse effects were documented and assessed after unblinding, which did not occur until after the trial finished. Of 126 participants randomized, 12 reported effects deemed to be related to the drug interventions (5 dizziness of whom 4 taking doxazosin, 2 rashes (1 taking spironolactone), 1 reported incontinence (doxazosin), nausea (spironolactone), heartburn (spironolactone), tachycardia (doxazosin), breathlessness (spironolactone)). In 8 of these patients, doses were adjusted or stopped; one participant willingly tolerated the effects. Throughout the study 5 patients withdrew consent due to adverse effects, 3 deemed related to the intervention (2 dizziness, 1 breathlessness); 1 patient reported dyspepsia, deemed related to juice. No participants were excluded.

Further regression analyses

Relationships between baseline plasma renin, nitrate and nitrite and change in CAVI, PWVart and central BP (from baseline to follow-up) were examined. Change in central BP was significantly related to baseline plasma renin (r= 0.36, p<0.001), so that for a 10-fold reduction in plasma renin, there was an 8.6 mmHg greater fall in central BP (Figure 3C). This result was not specific to either the drug or juice arm. There were no

relationships between change in CAVI or PWVart and renin (r=0.02, p=0.8, and r=0.05, p= 0.6, respectively - Figure 3A-B). There were also no relationships between change in CAVI, PWVart or central BP and nitrate (Supplementary Figure 4 A-C; r=0.07, p=0.6; r= -0.04, p= 0.7; r=0.01, p=0.9, respectively) or nitrite (Supplementary Figure 5A-C; r=0.13, p=0.3; r= 0.02, p= 0.87; r=-0.06, p=0.7, respectively).

Discussion

This randomized trial demonstrated a proof of concept that reduction of arterial stiffness, an independent predictor of mortality generally and in T2DM [4], could be measured and estimated independently of BP, as measured by CAVI and PWVart, adjusting for differences in achieved BP between trial groups.

Spironolactone versus doxazosin

The reduction in CAVI, which measures cardiac-ankle PWV, including a long more muscular arterial path, was borderline (p=0.07). However, contrary to our hypothesis, the result was in the opposite direction, towards the doxazosin, not the spironolactone arm. The consistency and direction of this effect on arterial stiffness was supported, again independent of BP change, by the significant impact on central PWVart, our other main outcome. Unlike aortic PWV measured as carotid-femoral [3] or down just the descending aorta [6], PWV in the extremities, down muscular arterial pathways such as the femoral- posterior tibial or cardiac-brachial routes, does not predict outcomes [34]. However, the ease of CAVI/ PWV measurement using the multi-cuff arm-ankle method, which includes the central aorta, and the microphone-detected 2nd sound timing for the precise initiation of the pressure/flow wave outweighs issues of including extremity pathways in its measurements. BP independence of CAVI has been discussed and re-

formulated to produce $CAVI_0$ [32,36]; when we transposed our data based on $CAVI_0$ suggested by Spronk et al they were not significant [37].

Here, adequate daily doses of ≤16 mg doxazosin, as alpha receptor blockade, were compared with ≤50 mg spironolactone, as mineralocorticoid receptor antagonist. Our results contrast with previous work, which suggested spironolactone at just 25 mg reduced PWV by 0.8 m/s versus placebo, apparently with little change in BP [14], in patients with mild kidney impairment; in that study, spironolactone had been added to angiotensin converting enzyme inhibitors and angiotensin receptor blockers. While the change in PWV and aortic distensibility was significant, so also was the change in either 24-hour ambulatory, or in office systolic BP; i.e.: one was not independent of the other. Left ventricular (LV) mass also changed, likely in response to the decrease BP. In our study, we found that LV mass index between the 2 active BP drugs was not significant [38]. Here, 71% patients were on prior anti-hypertensive medication of many types, and the 62% with T2DM generally on metformin and other glucocentric agents. The difference in change of (office) BP was not significant, despite adjusting for the small change in favour of spironolactone. Although there are suggestions that doxazosin may reduce arterial stiffness [39-40], neither of those studies was a formal trial nor adjusted for any BP change, and its use in arterial function has not to our knowledge been examined in T2DM. From a physiological point of view the action of doxazosin can be easily explained. Vascular tone does influence arterial stiffness in muscular arteries [41-42] and is likely to have a similar action in larger arteries (although this influence is difficult to assess due to concomitant effect in BP).

The absolute reduction in BP for those who finished 6 months' treatment was a similar 7 mmHg SBP reduction in both drug groups, but the least square mean fall in BP was a non-significantly greater 2.3 mmHg on spironolactone than doxazosin, using a higher dose than in our recent blinded, rotational Pathway Trial where the difference was 4.5 mmHg [13]. The different patient population and the lower dose of doxazosin likely contributed to different treatment responses there to here.

Results from the anti-hypertensive ALLHAT Trial are relevant; its doxazosin arm had to be stopped after ~2 years, due to excess heart failure and other cardiac events [43]. Having diabetes on doxazosin in the trial was a particular aggravating factor [44]. Whilst the change in PWVart and the borderline change in CAVI could be related to changes in cardiac function, our echocardiographic data [38] do not suggest that as the ejection fraction (EF), and global longitudinal strain (GLS) which is a well established markers of systolic function, were similar between the two drugs in our study; however, S' (a tissue-Doppler systolic function index) was increased by spironolactone versus doxazosin. Thus, while our data suggest we have shown 'proof of concept' that PWVart can be reduced independent of BP change, we have not shown it is independent of cardiac functional change.

Effects of inorganic nitrate

No effect of active (nitrate containing) beetroot juice, even at higher dose, was found on peripheral (brachial) BP, CAVI or PWV consistent with two previous dietary nitrate studies in patients with diabetes [21-22] and in line with our recent results that *acute* physiological elevations of plasma glucose and insulin, following an oral glucose tolerance test, result in a lack of BP-lowering with dietary nitrate in healthy adults [45]. Previous reductions in PWV were with peripheral BP reductions [22]; the lack of change

in peripheral BP with nitrate may underlie the lack of effect on PWV, suggesting dietary nitrate has no direct effect on arterial stiffness. Further the lack of reduction on PWV with dietary nitrate is in line with acute effects seen previously with glyceryl trinitrate [46]. Plasma nitrate and nitrite did increase, some 4-fold and only 2-fold respectively. The two other diabetes studies [21-22] also found significant increases in plasma nitrite similar to that in healthy participants [33] and hypertensives [19].

Central SBP decreased on nitrate-containing juice, with similar if borderline changes in augmentation index, simultaneous to the significant rise in plasma nitrite, without peripheral BP changes. Although this could be as a result of venodilation with reduced preload, indeed decreased central SBP was observed with decreased preload (induced by lower limb venous occlusion) [47], in an echocardiogram sub-study (data not presented here), we saw only very small differences in stroke volume between treatments and so, although it might be contributory this is unlikely to be an alternative mechanism [38]. This selective central SBP change is entirely consistent with our previous findings of normoxia-dependent conduit artery dilatation after inorganic nitrite, selectively reducing central SBP [28]. A measurable increase in plasma nitrite in healthy volunteers also led to decreased *brachial*-femoral PWV, independently of peripheral BP [28]. A different more muscular brachial conduit artery arterial path was studied there. However, whether currently measured central BP has clinical impact beyond peripheral BP in the general population, as some claim [48], remains uncertain, as recently reported from Framingham [49], in part related to calibration issues [50-51]. However, central aortic pressure may be especially relevant in specific populations, such as HFpEF [52].

383 The confirmation of a central BP effect here, as found previously, suggests that testing
384 for *central* aortic stiffening changes, affecting the aortic root, ascending aorta or arch
385 using other imaging methods including MR could be revealing. Other recent
386 Framingham work confirms that rather than flow-mediated dilation per se, poorer
387 forearm hyperemic mean blood flow velocity reflecting microvascular (smaller
388 resistance vessel) changes underlies some 8-13% of the overall stiffening effect
389 measured by PWV that predicts outcomes powerfully and independently of BP in that
390 cohort [53].

391

392 Despite observing no drug/ juice interactions in hemodynamic parameters, there was
393 an interesting finding of increased plasma nitrate and nitrite concentrations observed
394 on spironolactone versus doxazosin. This could be related to spironolactone's diuretic
395 effect, hemo-concentrating nitrate and nitrite, relative to the vasodilatory effect of
396 doxazosin, or via altering renal nitrate/nitrite excretion; unfortunately the latter was
397 not assessed in this study.

398

399

400 Adverse events attributable to the blinded interventions were small and minor, with
401 one person mentioning some increased reflux/acidity on the active, nitrate containing
402 beetroot juice. Potassium retention on spironolactone was not a problem at all,
403 probably because entry excluded people with eGFR values of $\leq$45mL.min.

404

405 This was intentionally a pragmatic trial testing general efficacy of the interventions. In
406 retrospect, the choice of doxazosin as the control antihypertensive agent for

spironolactone could be disputed, but few other drugs currently balance dosage and effect equivalently. Medication timing over the 6 months, and adherence to respective treatments could not be assured, although changes in nitrate concentrations on active juice suggested reasonable adherence to the juice overall. Participants were asked to take their treatment and juice on rising or around breakfast-time, since peak plasma nitrite concentrations after dietary nitrate ingestion occurs about 2.5 hours later [18]; however, the intervals between juice ingestion and visits to the Clinical Research Facility and hence blood collection may have been highly variable. Measurements were all made under standardized conditions in a Clinical Research Facility. We also recognize the limitation in our sample size calculation being based on PWV, and not CAVI, the primary outcome of the research; this was due to sufficient data not being available at the time of starting the trial.

Contrary to our hypothesis, arterial stiffness was not reduced on spironolactone, rather that occurred on the doxazosin arm independently of BP, as measured by PWVart, with a similar borderline effect on the longer muscular arterial pathway estimated by CAVI, in these patients with or at risk of T2DM. Whilst active nitrate-containing beetroot juice had no effect on arterial stiffness, central BP was significantly reduced by nitrate-nitrite.

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Data sharing statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors contributions

AJW and JKC both led the design of the research and oversaw the acquisition of the data and data analysis, they were involved in the interpretation of the results and revising the manuscript drafts. CEM contributed to research design, she led the data acquisition and was involved in the interpretation of the results and led drafting the manuscript. VG, LF and MLC were all involved in research design, data acquisition and interpretation and revising the manuscript drafts. SVM lead the data analysis and contributed to the manuscript drafts. HC, FI, PM, AM and EN were all involved in data acquisition and contributed to manuscript drafts. All authors approved the final version of the manuscript and have agreed accountability of the research.

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TABLES

Table 1. Mean (with standard deviation) baseline characteristics of patients randomized into the VaSera trial

	Spironolactone		Doxazosin	
Juice:	**Active**	**Placebo**	**Active**	**Placebo**
	(n= 35)	**(n= 30)**	**(n= 29)**	**(n= 33)**
Age, years	56.9 (13.9)	56.3 (11.0)	57.6 (12.3)	55.9 (14.0)
Female, n (%)	11 (31.4)	11 (36.7)	13 (44.8)	11 (33.3)
Weight, kg	96.4 (15.0)	94.8 (15.2)	98.1 (19.7)	88.4 (19.9)
Height, m	1.69 (0.10)	1.70 (0.10)	1.71 (0.10)	1.72 (0.12)
BMI, kg/m^2	33.9 (4.9)	33.0 (4.8)	33.3 (6.1)	30.2 (6.1)
Waist, cm	112 (29)	110 (10)	112 (13)	103 (14)
T2DM, n (%)	18 (51.4)	20 (66.7)	18 (62.1)	22 (66.7)
eGFR, mL/min/1.73m^2 [a]	81 (25)	80 (17)	84(23)	84 (18)
Patients treated with				
Metformin, n (%)	12 (36.4)	15 (50.0)	15 (51.7)	15 (46.9)
Insulin, n (%)	5 (14.7)	8 (26.7)	2 (6.9)	8 (24.2)
Other oral diabetic medication, n (%)	6 (17.6)	7 (23.3)	7 (24.1)	6 (18.2)
Anti hypertensive, n (%) [b]	20 (57.1)	20 (66.7)	25 (86.2)	24 (72.7)
Mean number of antihypertensives, n (%)[c]	1.6 (0.7)	1.9 (0.9)	1.7 (0.8)	1.7 (0.6)
Diuretics, n (%)	5 (14.7)	10 (33.3)	9 (31.0)	13 (40.6)
Statins, n (%)	15 (44.1)	15 (50.0)	17 (58.6)	15 (45.5)

'Active' is nitrate containing beetroot juice; 'placebo' is nitrate depleted beetroot juice.

Values are mean (standard deviation) unless stated otherwise. [a] Calculated using abbreviated MDRD equation, [b] represents those taking at least one anti hypertensive, [c] represents mean number of anti hypertensive drugs taken of those taking at least one

626 **Table 2.** Mean (with 95% confidence interval) vascular, plasma and urine parameters at baseline and follow-up visits for

627 spironolactone and doxazosin

	Baseline		Midpoint		End	
	Spironolactone	**Doxazosin**	**Spironolactone**	**Doxazosin**	**Spironolactone**	**Doxazosin**
Vascular						
CAVI, units	8.35(8.02,8.67)	8.07(7.68,8.47)	8.37(8.08,8.67)	8.05(7.57,8.52)	8.19(7.81,8.57)	8.04(7.56,8.52)
PWVart, ms^{-2}	9.4(8.9,9.9)	9.7(9.1,10.2)	9.3(8.8,9.7)	8.9(8.4,9.4)	9.3(8.9,9.7)	9.3(8.8,9.9)
SBP, mmHg	143.4(138.3,148.4)	140.1(136.2,144.0)	137.2(132.4,141.9)	136.7(132.1,141.3)	135.0(131.1,139.0)	137.7(132.7,142.7)
DBP, mmHg	88.0(84.7,91.2)	86.8(84.7,88.9)	84.5(81.8,87.1)	84.5(82.0,87.1)	82.6(79.5,85.7)	84.8(82.0,87.7)
aoSBP, mmHg	135.4(129.1,141.6)	130.0(123.7,136.3)	126.6(120.5,132.7)	125.0(119.5,130.5)	123.7(118.3,129.2)	123.9(119.2,128.6)
brAIX, %	-16.4(-24.8,-7.9)	-22.7(-31.6,-13.8)	-22.4(-30.2,-14.6)	-22.6(-32.7,-12.4)	-24.6(-32.9,-16.2)	-24.2(-32.7,-15.7)
aoAIX, %	29.4(25.1,33.6)	26.1(21.6,30.6)	26.3(22.3,30.2)	26.2(21.1,31.4)	25.2(21.0,29.4)	25.4(21.1,29.7)
HR, bpm	69.9(66.6,73.2)	71.7(68.2,75.2)	69.3(66.4,72.1)	69.6(66.5,72.6)	71.4(68.2,74.5)	69.6(66.3,72.9)
Plasma						
Glucose[a], mmol/L	6.4(5.8,7.0)	6.2(5.6,6.8)	7.1(6.3,8.0)	6.3(5.6,7.2)	6.7(5.8,7.8)	6.0(5.3,6.8)
HbA1c[a], %	6.7(6.3,7.0)	6.7(6.4,7.0)	6.9(6.6,7.3)	6.7(6.4,7.1)	6.8(6.4,7.2)	6.7(6.3,7.1)
Sodium, mmol/L	139.7(139.0,140.3)	139.7(139.0,140.4)	138.2(137.4,139.0)	140.0(139.3,140.7)	138.4(137.5,139.4)	139.7(139.0,140.4)

	Baseline		Midpoint		End	
	Spironolactone	**Doxazosin**	**Spironolactone**	**Doxazosin**	**Spironolactone**	**Doxazosin**
Potassium, mmol/L	4.27(4.16,4.39)	4.18(4.07,4.30)	4.53(4.42,4.64)	4.21(4.09,4.34)	4.60(4.49,4.72)	4.17(4.07,4.28)
Creatinine[a] µmol/L	81.6(76.6,87.0)	81.4(75.9,87.3)	83.1(77.4,89.3)	83.7(78.5,89.2)	84.7(78.5,91.4)	83.4(78.0,89.1)
Renin[a], mU/mL	31.8(18.7,54.0)	31.5(20.2,49.2)	63.2(38.5,103.7)	38.3(21.6,67.9)	66.1(39.3,111.4)	39.2(22.1,69.8)
Aldosterone[a], pmol/L	225(191,264)	229(199,264)	439(367,525)	281(241,327)	391(325,470)	300(251,358)
Nitrate[a], µM	37.4(29.6,47.4)	25.2(19.0,33.6)	78.1(55.8,109.4)	62.4(43.5,89.5)	97.8(66.5,144.0)	54.2(35.9,81.9)
Nitrite[a], nM	0.189(0.123,0.289)	0.147(0.098,0.222)	0.268(0.177,0.405)	0.133(0.076,0.233)	0.242(0.146,0.400)	0.115(0.065,0.203)
Urine						
Sodium[a], mmol/L	61.9(53.1,72.2)	64.0(52.7,77.8)	64.1(53.8,76.4)	64.6(53.1,78.5)	78.3(65.5,93.5)	70.0(58.8,83.5)
Potassium[a], mmol/L	50.4(43.4,58.5)	57.2(48.3,67.8)	66.1(56.5,77.5)	62.5(53.3,73.3)	61.6(52.0,73.0)	70.2(60.8,81.0)
Creatinine[a], mmol/L	7.99(6.60,9.68)	9.21(7.45,11.38)	8.86(7.34,10.70)	10.57(8.53,13.08)	8.09(6.71,9.75)	10.58(8.77,12.76)

[*]Analyzed in log units and geometric means presented.

PWVart, pulse wave velocity by Arteriograph; aoSBP, aortic blood pressure; aoAix aortic augmentation index; bpm, beats per minute; brAix, brachial augmentation index; CAVI, cardio-ankle vascular index; DBP, diastolic blood pressure; HR heart rate; SBP, systolic blood pressure. Values are mean (95% confidence interval)

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Table 3. Mean (with 95% confidence interval) vascular, plasma and urine parameters at baseline and two follow up visits for nitrate containing (active) and nitrate depleted (placebo) beetroot juice

	Baseline		Midpoint		End	
	Active	**Placebo**	**Active**	**Placebo**	**Active**	**Placebo**
Vascular						
CAVI, units	8.28(7.92,8.64)	8.14(7.77,8.51)	8.29(7.91,8.67)	8.13(7.72,8.54)	8.15(7.71,8.58)	8.08(7.65,8.52)
PWVart, ms^{-2}	9.7(9.1,10.3)	9.4(8.9,9.8)	9.3(8.8,9.8)	8.9(8.5,9.4)	9.5(9.0,9.9)	9.2(8.7,9.7)
SBP, mmHg	142.5(137.7,147.3)	140.9(136.7,145.1)	137.5(132.8,142.3)	136.4(131.7,141.1)	136.2(131.9,140.4)	136.7(131.9,141.5)
DBP, mmHg	88.5(85.6,91.5)	86.3(83.8,88.7)	84.4(81.5,87.2)	84.6(82.2,87.0)	83.4(80.7,86.1)	84.1(80.9,87.3)
aoSBP, mmHg	134.8(128.8,140.8)	130.6(124.0,137.1)	127.2(121.1,133.4)	124.4(118.9,129.9)	123.9(119.2,128.5)	123.8(118.3,129.2)
brAIX, %	-13.5(-21.5,-5.5)	-25.7(-34.8,-16.6)	-20.4(-28.7,-12.2)	-24.5(-34.1,-14.8)	-23.2(-31.4,-15.0)	-25.6(-34.3,-16.9)
aoAIX, %	30.8(26.7,34.9)	24.6(20.0,29.2)	27.3(23.1,31.5)	25.3(20.4,30.1)	25.9(21.8,30.0)	24.7(20.3,29.1)
HR, bpm	68.5(65.5,71.5)	73.0(69.3,76.7)	68.8(66.0,71.7)	69.9(66.9,73.0)	71.8(68.6,75.1)	69.1(66.0,72.2)
Plasma						
Glucose[a] mmol/L	5.91(5.36,6.52)	6.64(6.05,7.29)	7.04(6.15,8.05)	6.45(5.76,7.21)	6.49(5.66,7.45)	6.21(5.44,7.08)
HbA1c[a], %	6.63(6.30,6.97)	6.73(6.43,7.04)	6.81(6.42,7.23)	6.87(6.57,7.18)	6.65(6.24,7.09)	6.85(6.52,7.19)

	Baseline		Midpoint		End	
	Active	**Placebo**	**Active**	**Placebo**	**Active**	**Placebo**
Sodium, mmol/L	139.9(139.2,140.5)	139.5(138.8,140.2)	138.8(138.0,139.7)	139.3(138.6,140.1)	139.1(138.2,140.0)	139.0(138.1,139.8)
Potassium, mmol/L	4.26(4.15,4.37)	4.20(4.08,4.33)	4.46(4.32,4.59)	4.32(4.20,4.43)	4.41(4.28,4.55)	4.38(4.26,4.49)
Creatinine[a] µmol/L	81.3(75.4,87.6)	81.7(77.1,86.7)	83.4(77.1,90.3)	83.4(78.8,88.2)	85.3(78.6,92.5)	82.9(78.1,87.9)
Renin[†], mU/mL	23.5(14.7,37.6)	42.6(26.0,69.8)	41.6(24.7,70.0)	57.4(33.0,99.7)	49.8(29.6,83.7)	54.7(30.4,98.6)
Aldosterone[a], pmol/L	230(200,266)	224(190,263)	337(281,406)	363(305,431)	375(318,442)	319(260,391)
Nitrate[a], µM	28.8(22.5,36.9)	32.4(24.2,43.4)	125.4(94.0,167.3)	43.4(32.5,58.1)	118.0(82.4,169.0)	35.9(25.7,50.0)
Nitrite[a], nM	0.191(0.130,0.282)	0.144(0.092,0.226)	0.268(0.168,0.428)	0.139(0.083,0.233)	0.219(0.135,0.353)	0.107(0.056,0.203)
Urine						
Sodium[a], mmol/L	66.2(54.4,80.6)	60.0(51.7,69.6)	66.9(54.9,81.5)	61.8(52.0,73.3)	79.7(67.3,94.3)	68.9(57.3,82.9)
Potassium[a], mmol/L	51.6(44.2,60.1)	55.4(47.0,65.3)	63.0(53.2,74.7)	65.3(56.3,75.8)	65.8(56.9,76.0)	65.6(55.1,78.1)
Creatinine[a], mmol/L	8.96(7.35,10.93)	8.18(6.67,10.03)	9.58(7.82,11.73)	9.77(7.97,11.98)	9.28(7.68,11.22)	9.12(7.51,11.06)

*Analyzed in log units and geometric means presented

'Active' is nitrate containing beetroot juice; 'placebo' is nitrate depleted beetroot juice. PWVart, pulse wave velocity by Arteriograph; aoSBP, aortic blood pressure; aoAix aortic augmentation index; bpm, beats per minute; brAix, brachial augmentation index; CAVI, cardio-ankle vascular index; DBP, diastolic blood pressure; HR heart rate; SBP, systolic blood pressure. Values are mean (95% confidence interval)

FIGURE LEGENDS

Figure 1. Change in vascular parameters in response to spironolactone and doxasozin

Change in cardio-ankle vascular index, aortic pulse wave velocity, systolic, and central blood pressure and plasma nitrate and nitrite concentration on drug intervention

Data are least square means averaged over the two follow up visits with mean, 95% confidence intervals. * is $p<0.05$. A, CAVI (cardio-ankle vascular index), B, PWV (pulse wave velocity by Arteriograph), C, SBP (systolic blood pressure), D, aoSBP (aortic systolic blood pressure), E, [nitrate] (plasma nitrate concentration), F, [nitrite] (plasma nitrite concentration).

Figure 2. Change in vascular parameters in response to inorganic nitrate from beetroot juice and nitrate free, placebo beetroot juice

Change in cardio-ankle vascular index, aortic pulse wave velocity, systolic, and central blood pressure, aortic and brachial augmentation index and plasma nitrate and nitrite concentration on juice intervention.

Data are least square means averaged over the two follow up visits with mean, 95% confidence intervals. * is $p>0.05$, ** is $p< 0.01$, *** is $p<0.001$. A, CAVI (cardio-ankle vascular index), B, PWV (pulse wave velocity by Arteriograh), C, SBP (systolic blood pressure), D, aoSBP (aortic systolic blood pressure), E, [nitrate] (plasma nitrate concentration) F, [nitrite] (plasma nitrite concentration).

Figure 3. Correlation between change in vascular parameters and baseline plasma renin

Change in CAVI (A), PWV (B) and central BP (C) vs. baseline plasma nitrite concentration

660 PWV (pulse wave velocity by Arteriograph), BP (blood pressure), CAVI (cardio-ankle vascular
661 index), n=64.
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