

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

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

4

5 **Short running title:** Spironolactone, nitrate and artery stiffness

6

7 Charlotte E. MILLS ^{a,b,c}, Virginia GOVONI ^{a,b}, Luca FACONTI ^{b,d}, Maria-Linda
8 CASAGRANDE ^{a,b}, Steven V. MORANT ^e, Hannah CRICKMORE ^a, Fahad IQBAL ^a, Perry
9 MASKELL ^e, Alisha MASANI ^e, Elisa NANINO ^{a,b}, Andrew J. WEBB ^{b,d}, J. Kennedy
10 CRUICKSHANK ^{a,b*}

11

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

14

15 ^a Cardiovascular Medicine group, Department of Nutritional Sciences, School of Life
16 Course Sciences, King's College London, UK

17 ^b Biomedical Research Centre, Clinical Research Facility, 4th Floor, North Wing, St
18 Thomas' Hospital, Guy's and St Thomas' NHS Foundation Trust, London, UK

19 ^c Hugh Sinclair Human Research Unit, Food and Nutritional Sciences, University of
20 Reading, UK

21 ^d King's College London British Heart Foundation Centre, Cardiovascular Division,
22 Department of Clinical Pharmacology, UK

23 ^e Medicines Monitoring Unit (MEMO), University of Dundee, UK

24

25

26

27 *Corresponding author: kennedy.cruickshank@kcl.ac.uk

28

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30 royalty from James White Drinks Ltd who manufacture the active nitrate-containing
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34

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

37

38 **Abstract**

39 *Aim*

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

43 *Methods*

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

52 *Results*

53 Spironolactone did not reduce stiffness, with evidence for reduced CAVI on doxazosin
54 rather than spironolactone (mean difference [95% confidence intervals]; 0.25[-0.3, 0.5]
55 units, $p=0.080$), firmer for PWVart (0.37[0.01, 0.7] ms^{-1} , $p=0.045$). There was no
56 difference in systolic pressure reduction between spironolactone and doxazosin (0.7[-
57 4.8, 3.3]mmHg, $p=0.7$). Circulating nitrate and nitrite increased on active versus
58 placebo juice, with central systolic pressure lowered -2.6[-4.5, - 0.8]mmHg, $p=0.007$
59 more on the active juice, but did not reduce CAVI, PWVart, nor peripheral pressure.
60 Change in nitrate and nitrite concentrations were 1.5-fold [1.1-2.2] and 2.2-fold [1.3,
61 3.6] higher on spironolactone than on doxazosin respectively; both $p<0.05$.

62 *Conclusion*

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

67

68 **Clinical trial registration:** ISRCTN registry: ISRCTN25003627/ DOI

69 10.1186/ISRCTN25003627.

70 **Statement 1: What is already known about this subject**

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

76 **Statement 2: What this study adds**

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

83

84

85

86 **Introduction**

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

98
99 Reductions in PWV by lifestyle measures are reported particularly for exercise, weight
100 loss and specific dietary components, and by various pharmacological agents, including
101 anti-hypertensives, statins, some anti-diabetic medications and advanced glycation end-
102 product breakers [12]. However, PWV reduction formally independent of BP is seldom
103 examined. Doing so is important as PWV is intrinsically linked to BP hence it can be
104 hard to distinguish the two.

105
106 Spironolactone, a mineralocorticoid receptor antagonist was recently found highly
107 effective in reducing BP in proven resistant hypertension [13]. Initial trials for
108 specifically reducing arterial stiffness, in early kidney disease [14], untreated
109 hypertensives [15] and dilated cardiomyopathy [16] appeared promising. These trials
110 were generally not designed to test impact on PWV formally independent of BP change.

111

112 Inorganic (dietary) nitrate, abundant in green leafy vegetables and beetroot [17]
113 reduces BP in healthy [18] and hypertensive volunteers [19] via the nitrate-nitrite-NO
114 pathway [20], but not in patients with T2DM, [21-22] or with their inclusion in a meta-
115 analysis of 24h ambulatory BP monitoring [23]. PWV reductions with inorganic
116 (dietary) nitrate have also been noted in healthy and hypertensive volunteers, but over
117 too short a period for vessel remodelling; these were likely BP-dependent reductions
118 [24]. We found that inorganic *nitrite* selectively lowers aortic, relative to peripheral, BP,
119 with reductions also in PWV that seem to be via selective normoxia-dependent conduit
120 (radial) artery dilatation in healthy volunteers [25-26], and selectively dilated epicardial
121 coronary arteries in patients undergoing coronary angiography [27]. While tolerance
122 develops to organic nitrates [28], it has not been described for inorganic (dietary)
123 nitrate [19], perhaps this due to the mechanisms of bioactivation of inorganic nitrite to
124 nitric oxide, and suppression of reactive oxygen species (ROS)[29]. Longer-term effects
125 of inorganic (dietary) nitrate beyond 6 weeks have not yet been tested.

126

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

132

133

134 **Methods**

135 *Study design and interventions*

136 A single centre, double-blind, parallel, randomised controlled intervention trial in a 2 x
137 2 factorial design was carried out in accordance with the Declaration of Helsinki and
138 U.S. Code of Federal Regulations.
139 Participants were assigned to one of 4 arms using computer randomization in blocks of
140 6, by an independent party. Interventions were spironolactone (12.5mg daily for 1
141 week, 12.5mg twice daily for 11 weeks, increased to 25mg twice daily to 24 weeks) with
142 doxazosin as its control (4mg similarly titrated to 8mg twice daily) and dietary nitrate
143 as beetroot juice (7.5mmol nitrate increased at 12 weeks to 11.2mmol nitrate, as
144 measured in our lab) or nitrate-free beetroot juice as placebo (0mmol nitrate), (see
145 Supplementary text). Spironolactone and doxazosin were prepared in indistinguishable
146 brown bottles by St Thomas' Hospital pharmacy, London, UK. Commercially available
147 beetroot juice, 'Beet It' and 'Beet It SPORT' were supplied as 15 x 70 mL bottles,
148 indistinguishable between active and control juice, prepared and supplied by James
149 White Drinks, Ltd, Suffolk UK.

150

151 Participants with or at risk of T2DM were recruited from Guy's and St Thomas'
152 Hospitals, London, UK and surrounding areas between 2013-2015. Inclusion criteria
153 were age 18-80years, clinically diagnosed T2DM *or* at risk of T2DM (as body mass index
154 (BMI) ≥ 27 kg/m², positive family history or glucose intolerance after 75g challenge),
155 ability to understand and comply with the protocol. Exclusion criteria: interfering
156 chronic illness, adverse reaction to either drug, known allergy to beetroot, eGFR < 45
157 mL min⁻¹, HbA1c >11% (97mM/M), pregnant, breast feeding or atrial fibrillation.

158 Written informed consent was obtained from all participants. The protocol was
159 approved by South London Research Ethics Committee.

160

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

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

169

170 After anthropometry, CAVI was measured using the VS-1500N, VaSera machine
171 (Fukuda Denshi Ltd, Japan) as described [30]. Microphone-detected heart sounds were
172 monitored, with BP cuffs on each arm and above each ankle, with pulse waves detected
173 by the cuffs at 30-50mmHg. CAVI was calculated from PWV, as pulse wave transit times
174 from aortic valve (2nd sound) to ankle: $CAVI = \frac{[2\rho/\Delta P] \cdot PWV^2}{\ln SBP/\ln DBP}$, with path
175 length estimated from height [31]. CAVI was measured in duplicate and averaged. CAVI₀
176 data were calculated as described previously [32]. PWVart, peripheral systolic, diastolic
177 and central BP, aortic and brachial augmentation index and heart rate from 6-8 cardiac
178 cycles were measured using appropriately sized cuffs by Arteriograph 24TM device
179 (TensioMed Kft. Hungary), analysing mean of duplicate good quality readings. Quality
180 was pre-specified with Arteriograph and VaSera waveforms checked by the
181 manufacturers, blinded to other data. PWV with standard deviations (SDs) >1 were
182 excluded.

183

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

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

188

189 *Statistical analysis*

190 Sufficient data from CAVI interventions were not available for sample size calculations.

191 We used previous studies on BP with beetroot juice [33-34] and the 1-year study of

192 PWVart on spironolactone [14] aiming to detect a 20% reduction over 6 months in PWV

193 (standard deviation (SD) 8%) with minimum 80% power, at $p < 0.05$. We estimated we

194 needed 24 participants per each of 4 arms, aiming for 30 per group allowing for 20%

195 drop out, for 24 patients in each to finish the trial.

196

197 A modified intention-to-treat analysis was performed using SAS (version 9.3); data are

198 presented as least-square means estimated from mixed effects models (log-transformed

199 where not normally distributed), adjusted as pre-specified for baseline, and any

200 difference in final SBP *change* between the two arms being analysed. To estimate

201 independence from BP change, changes in PWVart were adjusted for change in SBP.

202 Least square mean data were averaged over the 2 follow-up visits (3 and 6 months).

203 Regression analyses assumed linear relationships, with some predictor variables (renin,

204 nitrate and nitrite) log-transformed.

205

206 **Results**

207 *Baseline*

208 Of 154 patients eligible and agreeing to attend, 11 were not eligible (4 for high HbA1c, 2

209 for previous adverse reactions, 2 with atrial fibrillation, 3 for ill health); 17 then

210 declined to participate. The remaining 126 participants were randomised

211 (Supplementary Figure 1). Baseline characteristics were generally well-matched
212 between arms (Table 1 and Supplementary Table 1) both between drugs and by
213 nitrate/nitrate-free juices (Tables 2-3). Of randomized participants, 62% had T2DM
214 with mean HbA1c 50mM/M (6.7%). The remaining 38% were 'at risk' (mean HbA1c
215 <40 mM/M, 5.8%, BMI 32.5kg/m²).

216

217 *Follow up*

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

224

225 *Treatment effects*

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

230

231 SPIRONOLACTONE VS. DOXAZOSIN: In adjusted models, spironolactone and doxazosin
232 reduced BP similarly (SBP, least-square mean [95% CI]: -7.0 [-9.9, -4.2] vs. -6.3 [-9.1, -
233 3.5] mmHg respectively, p= 0.7, Figure 1C and diastolic (DBP), -5.6 [-7.4, -3.7] vs. -4.7 [-
234 6.5, -2.9] mmHg respectively, p= 0.5, Supplementary Figure 2A). The direction in
235 difference for the primary endpoint, change in CAVI between drugs, was *contrary* to our

236 hypothesis, borderline significant towards doxazosin (0.14 [-0.06, 0.34] vs. -0.11 [-0.30,
237 0.08] units $p=0.08$, for spironolactone and doxazosin respectively, Figure 1A). When
238 transposed to CAVI₀, our data was not significant -0.04(-0.44, 0.35) vs. 0.24 (-0.19,
239 0.67), $p= 0.34$ (doxazosin vs. spironolactone)[19]. However, the difference in PWVart
240 change between spironolactone and doxazosin was significant (-0.07 [-0.33, 0.18] vs. -
241 0.44 [-0.69, -0.19] ms^{-2} , $p=0.045$, Figure 1B) towards doxazosin, again contrary to our
242 hypothesis. There were also no other differences in other hemodynamic parameters
243 estimated by the Arteriograph for the drug arm, in central BP (-7.6 [-9.0, -6.3] vs. -7.2 [-
244 8.5, -5.9] mmHg, $p=0.6$; Figure 1 D), augmentation index (Supplementary Figure 2 B-C),
245 or heart rate.

246 Although no drug/ juice interactions in terms of hemodynamic variables were noted,
247 nitrate and nitrite concentrations were higher on spironolactone than on doxazosin by
248 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.
249 Unadjusted data are in Supplementary Table 1.

250

251 BEETROOT VS. PLACEBO JUICE: There were no adjusted differences in change in
252 arterial stiffness change as CAVI (0.02 [-0.18, 0.21] vs. 0.01 [-0.18, 0.21], $p=0.98$, Figure
253 2A) CAVI₀ 0.12(-0.29, 0.53) vs. 0.08(-0.34, 0.50), $p= 0.898$ (active vs. control) [19] nor
254 PWVart (-0.23 [-0.48, 0.01] vs. -0.28 [-0.54, -0.03], $p=0.8$, Figure 2B), nor in
255 brachial BP between active and placebo juice (SBP, -6.4 [-9.2, -3.6] vs. -6.9 [-9.8, -4.0]
256 mmHg, $p= 0.8$, Figure 2C, nor DBP, $p= 0.9$ (Supplementary Figure 3A). However,
257 difference in change in central (aortic) SBP between active and control juices was highly
258 significant (-8.7[-10, -7.4] vs. -6.1[-7.4, -4.8] mmHg, $p=0.007$, Figure 2D). Decreases in
259 aortic (-3 [-5.1, -0.9] vs. -0.3 [-2.4, 1.9] %, $p=0.08$) and brachial augmentation index (-5.9

260 [-10.0, -1.76] vs. -0.49 [-4.72, 3.74] %, $p=0.08$) were also borderline (Supplementary
261 Figure 3B-C).

262 Plasma nitrate levels rose as expected in those on active compared with placebo juice (a
263 4.3[3.4, 5.5]-fold increase vs. 1.3[1.02, 1.71], $p<0.001$, Figure 2 E); nitrite levels
264 increased 1.6[1.1, 2.2]-fold vs. 0.9[0.6, 1.2], $p=0.02$, Figure 2 F). These data confirm
265 adherence to the beetroot juice arm. Unadjusted data are in Supplementary Table 1.

266

267 *Adverse effects*

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

278

279 *Further regression analyses*

280 Relationships between baseline plasma renin, nitrate and nitrite and change in CAVI,
281 PWVart and central BP (from baseline to follow-up) were examined. Change in central
282 BP was significantly related to baseline plasma renin ($r= 0.36$, $p<0.001$), so that for a
283 10-fold reduction in plasma renin, there was an 8.6 mmHg greater fall in central BP
284 (Figure 3C). This result was not specific to either the drug or juice arm. There were no

285 relationships between change in CAVI or PWVart and renin ($r=0.02$, $p=0.8$, and $r=0.05$,
286 $p=0.6$, respectively - Figure 3A-B). There were also no relationships between change in
287 CAVI, PWVart or central BP and nitrate (Supplementary Figure 4 A-C; $r=0.07$, $p=0.6$; $r=-$
288 0.04 , $p=0.7$; $r=0.01$, $p=0.9$, respectively) or nitrite (Supplementary Figure 5A-C; $r=0.13$,
289 $p=0.3$; $r=0.02$, $p=0.87$; $r=-0.06$, $p=0.7$, respectively).

290

291

292 **Discussion**

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

297 *Spironolactone versus doxazosin*

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

310 formulated to produce CAVI₀ [32,36]; when we transposed our data based on CAVI₀
311 suggested by Spronk et al they were not significant [37].

312

313 Here, adequate daily doses of ≤16 mg doxazosin, as alpha receptor blockade, were
314 compared with ≤50 mg spironolactone, as mineralocorticoid receptor antagonist. Our
315 results contrast with previous work, which suggested spironolactone at just 25 mg
316 reduced PWV by 0.8 m/s versus placebo, apparently with little change in BP ¹⁴, in
317 patients with mild kidney impairment; in that study, spironolactone had been added to
318 angiotensin converting enzyme inhibitors and angiotensin receptor blockers. While the
319 change in PWV and aortic distensibility was significant, so also was the change in either
320 24-hour ambulatory, or in office systolic BP; i.e.: one was not independent of the other.
321 Left ventricular (LV) mass also changed, likely in response to the decrease BP. In our
322 study, we found that LV mass index between the 2 active BP drugs was not significant
323 [38]. Here, 71% patients were on prior anti-hypertensive medication of many types, and
324 the 62% with T2DM generally on metformin and other gluco-centric agents. The
325 difference in change of (office) BP was not significant, despite adjusting for the small
326 change in favour of spironolactone. Although there are suggestions that doxazosin may
327 reduce arterial stiffness [39-40], neither of those studies was a formal trial nor adjusted
328 for any BP change, and its use in arterial function has not to our knowledge been
329 examined in T2DM. From a physiological point of view the action of doxazosin can be
330 easily explained. Vascular tone does influence arterial stiffness in muscular arteries [41-
331 42] and is likely to have a similar action in larger arteries (although this influence is
332 difficult to assess due to concomitant effect in BP).

333

334 The absolute reduction in BP for those who finished 6 months' treatment was a similar
335 7 mmHg SBP reduction in both drug groups, but the least square mean fall in BP was a
336 non-significantly greater 2.3 mmHg on spironolactone than doxazosin, using a higher
337 dose than in our recent blinded, rotational Pathway Trial where the difference was 4.5
338 mmHg [13]. The different patient population and the lower dose of doxazosin likely
339 contributed to different treatment responses there to here.

340

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

352 *Effects of inorganic nitrate*

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

359 in peripheral BP with nitrate may underlie the lack of effect on PWV, suggesting dietary
360 nitrate has no direct effect on arterial stiffness. Further the lack of reduction on PWV
361 with dietary nitrate is in line with acute effects seen previously with glyceryl trinitrate
362 [46]. Plasma nitrate and nitrite did increase, some 4-fold and only 2-fold respectively.
363 The two other diabetes studies [21-22] also found significant increases in plasma nitrite
364 similar to that in healthy participants [33] and hypertensives [19].

365

366 Central SBP decreased on nitrate-containing juice, with similar if borderline changes in
367 augmentation index, simultaneous to the significant rise in plasma nitrite, without
368 peripheral BP changes. Although this could be as a result of venodilation with reduced
369 preload, indeed decreased central SBP was observed with decreased preload (induced
370 by lower limb venous occlusion) [47], in an echocardiogram sub-study (data not
371 presented here), we saw only very small differences in stroke volume between
372 treatments and so, although it might be contributory this is unlikely to be an alternative
373 mechanism [38]. This selective central SBP change is entirely consistent with our
374 previous findings of normoxia-dependent conduit artery dilatation after inorganic
375 nitrite, selectively reducing central SBP [28]. A measurable increase in plasma nitrite in
376 healthy volunteers also led to decreased *brachial*-femoral PWV, independently of
377 peripheral BP [28]. A different more muscular brachial conduit artery arterial path was
378 studied there. However, whether currently measured central BP has clinical impact
379 beyond peripheral BP in the general population, as some claim [48], remains uncertain,
380 as recently reported from Framingham [49], in part related to calibration issues [50-
381 51]. However, central aortic pressure may be especially relevant in specific populations,
382 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 ≤ 45 mL/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

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

419

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

425

426

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440

441 **Data sharing statement:**

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

444

445 **Authors contributions**

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

455

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617

TABLES

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

Juice:	Spironolactone		Doxazosin	
	Active (n= 35)	Placebo (n= 30)	Active (n= 29)	Placebo (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)

^a‘Active’ is nitrate containing beetroot juice; ^b‘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
<u>Vascular</u>						
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)
<u>Plasma</u>						
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)
<u>Urine</u>						
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)

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

	Baseline		Midpoint		End	
	Active	Placebo	Active	Placebo	Active	Placebo
<u>Vascular</u>						
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)
<u>Plasma</u>						
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)

^aAnalyzed in log units and geometric means presented

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

634 FIGURE LEGENDS

635 **Figure 1.** Change in vascular parameters in response to spironolactone and
636 doxazosin

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

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

644

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

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

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

655

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

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

660 PWV (pulse wave velocity by Arteriograph), BP (blood pressure), CAVI (cardio-ankle vascular
661 index), n=64.
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667 supplementary tables: 1
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