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Published Version

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Morselli, F., Faconti, L., Mills, C. E. ORCID: <https://orcid.org/0000-0002-8313-3700>, Morant, S., Chowienczyk, P. J., Yeung, J. A., Cavarape, A., Cruickshank, J. K. and Webb, A. J. (2021) Dietary nitrate prevents progression of carotid subclinical atherosclerosis through blood pressure-independent mechanisms in patients with or at risk of type 2 diabetes mellitus. *British Journal of Clinical Pharmacology*, 87 (12). ISSN 0306-5251 doi: 10.1111/bcp.14897 Available at <https://centaur.reading.ac.uk/101982/>

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To link to this article DOI: <http://dx.doi.org/10.1111/bcp.14897>

Publisher: Wiley-Blackwell

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ORIGINAL ARTICLE

Dietary nitrate prevents progression of carotid subclinical atherosclerosis through blood pressure-independent mechanisms in patients with or at risk of type 2 diabetes mellitus

Franca Morselli^{1,2,6}  | Luca Faconti^{1,6}  | Charlotte E. Mills^{3,4,6}  |
 Steven Morant⁵ | Philip J. Chowieniczky^{1,3,6}  | Joshua Au Yeung^{1,6} |
 Alessandro Cavarape² | J. Kennedy Cruickshank^{3,6}  | Andrew J. Webb^{1,6} 

¹Department of Clinical Pharmacology, King's College London British Heart Foundation Centre, School of Cardiovascular Medicine and Sciences, UK

²Dipartimento di Area Medica, Clinica Medica, Università degli Studi di Udine, Udine, Italy

³King's College London, Department of Nutritional Sciences, School of Life Course Sciences, London, UK

⁴Department of Food and Nutritional Sciences, School of Chemistry, Food and Pharmacy, University of Reading, Reading, UK

⁵Medicines Monitoring Unit (MEMO), University of Dundee, UK

⁶Biomedical Research Centre, Clinical Research Facility, 4th Floor, North Wing, St Thomas' Hospital, Guy's and St Thomas' NHS Foundation Trust, London, UK

Correspondence

Dr Andrew J. Webb, Senior Lecturer in Cardiovascular Clinical Pharmacology, Department of Clinical Pharmacology, King's College London, St Thomas' Hospital, London, SE1 7EH, UK.
 Email: andrew.1.webb@kcl.ac.uk

Funding information

Fukuda Denshi, Tokyo, Japan

Aims: To test if 6 months' intervention with dietary nitrate and spironolactone could affect carotid subclinical atherosclerosis and stiffness, respectively, vs. placebo/doxazosin, to control for blood pressure (BP).

Methods: A subgroup of participants in our double-blind, randomized-controlled, factorial VaSera trial had carotid imaging. Patients with hypertension and with/at risk of type 2 diabetes were randomized to active nitrate-containing beetroot juice or placebo nitrate-depleted juice, and spironolactone or doxazosin. Vascular ultrasound for carotid diameter (CD, mm) and intima-media thickness (CIMT, mm) was performed at baseline, 3- and 6-months. Carotid local stiffness (CS, m/s) was estimated from aortic pulse pressure (Arteriograph) and carotid lumen area. Data were analysed by modified intention to treat and using mixed-model effect, adjusted for confounders.

Results: In total, 93 subjects had a baseline evaluation and 86% had follow-up data. No statistical interactions occurred between the juice and drug arms and BP was similar between the juices and between the drugs. Nitrate-containing vs. placebo juice significantly lowered CIMT (-0.06 [95% confidence interval -0.12 , -0.01], $P = .034$), an overall difference of $\sim 8\%$ relative to baseline; but had no effect on CD or CS. Doxazosin appeared to reduce CS from baseline (-0.34 [-0.62 , -0.06]) however, no difference was detected vs. spironolactone (-0.15 [-0.46 , 0.16]). No differences were detected between spironolactone or doxazosin on CIMT and CD.

Conclusions: Our results show that 6 months' intervention with dietary nitrate influences vascular remodelling, but not carotid stiffness or diameter. Neither spironolactone nor doxazosin had a BP-independent effect on carotid structure and function.

Principal investigator: The authors confirm that the Principal Investigator for this paper is Professor J. Kennedy Cruickshank and that he had direct clinical responsibility for patients. Clinical trial registration: ISRCTN registry: ISRCTN25003627/DOI 10.1186/ISRCTN25003627.

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KEYWORDS

arterial stiffness, beetroot juice, blood pressure, carotid, dietary nitrate, intima media thickness

1 | INTRODUCTION

Patients with type 2 diabetes (T2DM) are at increased risk of coronary heart disease, ischaemic stroke and vascular deaths. According to current guidelines, evaluation of cardiovascular risk in asymptomatic patients with T2DM should include the assessment of atherosclerotic burden. Although less powerful than coronary artery calcium screening for risk stratification, carotid intima-media thickness (CIMT) has been used as a widely available, simple and noninvasive marker of subclinical atherosclerosis. Also, some earlier studies suggested that CIMT may be a better tool than coronary artery calcium for predicting stroke,^{1,2} and it has been associated with coronary heart disease.^{3,4} As a measure of arterial wall thickness, an increased CIMT is considered to represent a quantitative expression of atherosclerotic burden and endothelial dysfunction.⁵⁻⁹

Key steps in the atherogenic process include a decline in nitric oxide (NO) synthesis, increased reactive oxygen species production, diminished NO bioavailability and endothelial dysfunction.¹⁰ Dietary nitrate, as found in green leafy vegetables and beetroot, is a source of NO via the nitrate (NO_3^-)-nitrite (NO_2^-)-NO pathway and confers several beneficial actions on the cardiovascular system.^{11,12} Studies in humans show that provision of dietary nitrate may enhance endothelial function,¹³ and suppresses microvascular inflammation.¹⁴

With respect to atherogenesis, studies in animals show that nitrate/nitrite may limit the development of intimal hyperplasia and smooth muscle cell proliferation, vascular ageing and atherosclerosis.¹⁵ Alef et al., found that sodium nitrite (24-h oral supplementation, a single intraperitoneal injection or via inhalation), limited intimal hyperplasia and smooth muscle cell proliferation following balloon injury of the rat carotid artery, whereas a low nitrate/nitrite diet increased intimal hyperplasia; notably, this could be reversed by the late introduction of nitrite.¹⁶ Studies performed in mice found mixed effects. Fourteen weeks' supplementation with sodium nitrate had no effect on atherogenesis in low density lipoprotein receptor knockout mice ($\text{LDLR}^{-/-}$).¹⁷ However, studies in apolipoprotein E knockout ($\text{ApoE}^{-/-}$) mice have shown benefit. Ten weeks' supplementation with low and moderate dose nitrate significantly improved endothelial function and atherosclerotic plaque composition in $\text{ApoE}^{-/-}$ mice fed a high-fat diet.¹⁸ Twelve weeks' supplementation with dietary nitrate, caused reductions in macrophage accumulation and elevations in smooth muscle accumulation within atherosclerotic plaques of $\text{ApoE}^{-/-}$ mice, suggesting plaque stabilization, albeit without effect on total plaque area; effects were seen with normal chow and high fat diets.¹⁹ Eight weeks' nitrate supplementation decreased atherosclerotic plaque area and inhibited vascular NADPH oxidase activity and oxidative stress in $\text{ApoE}^{-/-}$ mice fed a high fat diet.²⁰

Epidemiological data suggest that higher vegetable nitrate intake is associated with a lower CIMT and a lower risk of ischaemic

cerebrovascular disease events and atherosclerotic vascular disease mortality in 1226 older women,^{21,22} and of new cases of cardiovascular disease-related complications over 15-years of follow-up in 5324 middle-aged women.²³

However, direct evidence in humans is scarce, with no randomized-controlled trial data reported to date on whether chronic supplementation with dietary nitrate affects carotid structure and stiffness. To our knowledge, there is only 1 study testing the effects of organic nitrates on CIMT: Sekiya et al.,²⁴ studied 42 patients with ischaemic heart disease randomly allocated to receive 3 months' nicorandil (15 mg daily) or isosorbide dinitrate (ISDN; 40 mg daily). However, they found that ISDN increased/worsened CIMT, whilst nicorandil had no effect.²⁴ In contrast to inorganic nitrate/nitrite, organic nitrates are associated with impairment of endothelial function and tolerance/loss of effect. Indeed, in that study, ISDN was found to worsen brachial artery flow mediated dilatation.

This contrasts with antihypertensives, some of which have been extensively studied for their effects on CIMT. Mineralocorticoid receptor antagonists such as spironolactone have been shown to reduce the progression of CIMT over 2 years in nondiabetic patients on haemodialysis treated vs. placebo.²⁵ In an uncontrolled study, eplerenone was found to improve CIMT in patients with primary aldosteronism over 6 and 12 months, though this was also associated with decreased blood pressure (BP).²⁶ Over a prolonged period of 6 years, treatment of patients with primary aldosteronism with spironolactone had a similar significant effect on regression of CIMT as surgical treatment in patients with unilateral forms of primary aldosteronism, with no significant difference in BP between treatments.²⁷ However, whether spironolactone confers specific effects on CIMT vs. other antihypertensives/independently of BP has not been tested.

Thus, in a subgroup of participants who took part in our VaSera trial, we performed serial carotid ultrasound scans to a priori evaluate the effect of dietary nitrate on carotid structure and function. We have already reported on the primary outcome of the main study, which was to assess whether 6 months' intervention with dietary nitrate (vs. placebo), spironolactone (vs. doxazosin) or both (in a factorial design) could reduce arterial stiffness independently of BP,^{28,29} and the echocardiography subgroup findings.³⁰

2 | METHODS

2.1 | Study design and interventions

Among the patients with or at risk of T2DM who took part in the double-blind, randomized-controlled, factorial VaSera trial, a sub-population had carotid ultrasound performed at baseline and

throughout the study as part of the cardiovascular measurements. Recruitment occurred between 2013 and 2015 at Guy's and St. Thomas' Hospitals, London, UK and surrounding areas. Inclusion criteria were age between 18 and 80 years; clinical diagnosis or at risk of T2DM (i.e. body mass index ≥ 27 kg/m², positive family history or glucose intolerance after 75 g challenge); capability of understanding and following the protocol. Exclusion criteria included interfering chronic illness, allergy/intolerance to either drug and/or beetroot, eGFR <45 mL/min, HbA1c > 11% (97 mmol/mol), pregnancy, breast feeding or atrial fibrillation. The study protocol was approved by South London Research Ethics Committee. After being consented at visit 1 (V1), participants were randomized at visit 2 (V2) to spironolactone (12.5 mg titrated to 25 mg, twice daily) or doxazosin (4 to 8 mg, twice daily) and dietary nitrate as Beet It beetroot juice (HeartBeet Ltd., Suffolk, UK, 4.5 mmol nitrate increased to 11.2 mmol once daily) or nitrate-free Beet It juice, a specific placebo, recommended to be taken daily with their existing treatment. Cardiovascular measures were performed at baseline (V2) and repeated at 3 months (visit 5, V5) and 6 months (visit 6, V6). The results for primary outcome, aortic arterial stiffness (measured using aortic pulse wave velocity [PWV]), central systolic pressure and augmentation are published elsewhere.²⁸ Peripheral BP was measured according to guidelines,³¹ using a validated BP monitor (Intellisense 705IT, Omron, UK). Carotid ultrasound was performed by 2 expert operators, using a GE Vivid 7 Ultrasound System, and all images analysed by a single operator blinded to the intervention. Acquisitions respected the current recommendations for ultrasound evaluation of carotid arteries and common CIMT measurement^{32,33} Carotid ultrasound image sequences were analysed using a highly automated method (Carotid

Analyzer, Medical Imaging Application, LLC) for detection of near and far wall border, detection of near and far intima border, vessel diameter measurement, and CIMT measurement. Additional carotid parameters were calculated as follows: diameter distension (ΔD , mm), the difference between systolic and diastolic diameter; diameter distensibility (%), the diameter distension with respect to the diastolic diameter; area distension (mm²), the difference between systolic and diastolic carotid lumen area; diameter compliance (mm/mmHg), the diameter distension with respect to pulse pressure (which is the difference between central systolic [SBP] and diastolic [DBP] BP); cross sectional distensibility (CSD, %), the area distension with respect to the diastolic area; cross sectional compliance (mm²/mmHg), the area distension with respect to the pulse pressure; CSD coefficient (kPa⁻¹ $\times 10^{-3}$), cross sectional compliance with respect to the pulse pressure; carotid stiffness, (CS, m/s), CSD coefficient corrected by blood density according to the Moens-Korteweg Equation.³⁴ A visual summary of the timing of interventions and measurements is shown in Figure 1.

2.2 | Statistical analysis

This was a prespecified/prospectively conducted, exploratory mechanistic part of the main VaSera trial, performed in a subgroup who had carotid imaging as part of their baseline and follow-up visits. The main trial was powered to detect a 20% (SD 8%) reduction in the primary outcome of PWVart with spironolactone, with minimum 80% power, at $P < .05$, requiring a sample size of 24 participants each arm, aiming for 30 to allow a 20% drop-out.³⁵ The sample size was achieved in

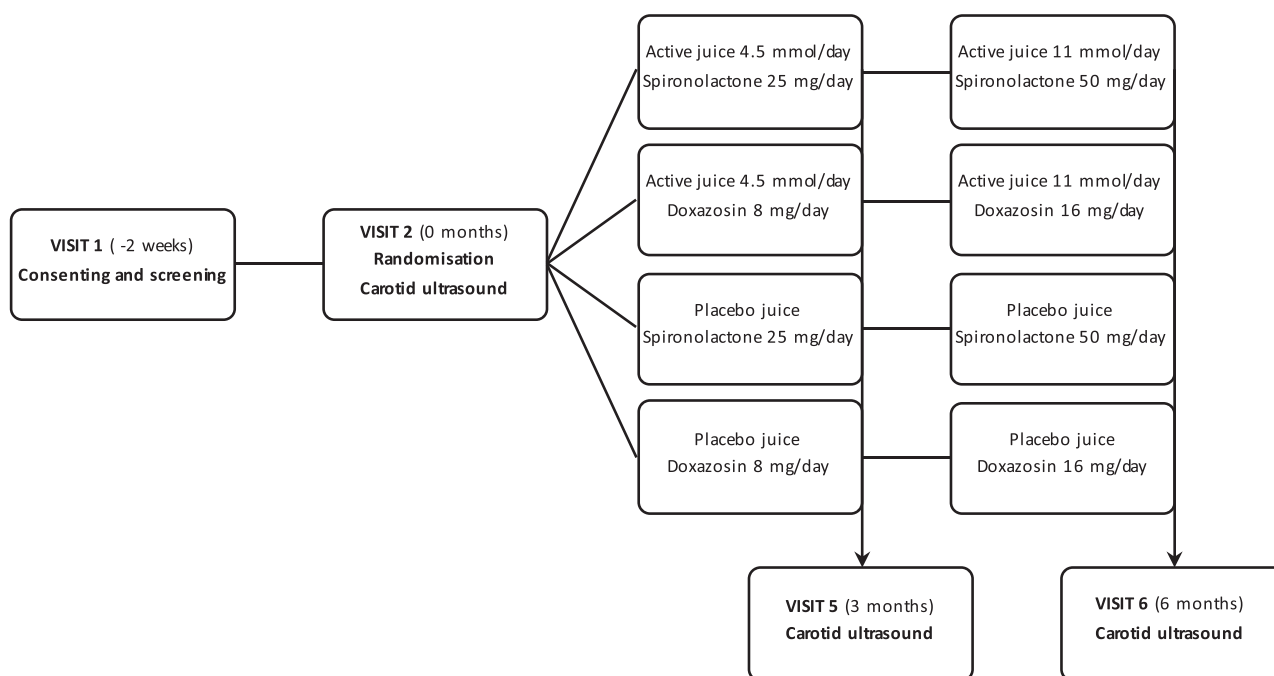


FIGURE 1 Study flow diagram. At Visit 2, participants were randomised to 1 of the 4 intervention arms and underwent baseline carotid ultrasound. Three months after (Visit 5), participants had follow-up imaging and doubled their dosage regimen as planned. A further 3 months after (Visit 6), participants had their last follow-up imaging

the main trial, with 126 patients randomised, and 110 and 106 patients completing mid-point and end of trial, respectively. An effect on CIMT was a tertiary outcome. Our independent biostatistician conducted a modified intention-to-treat analyses using SAS (Version 9.3) and included in the analysis all randomised participants with at least 1 outcome data at any follow-up visit but planned to exclude any randomised patient who failed to receive study medication or provided no follow-up for the primary outcome variable. The effect of the interventions was estimated using mixed-effects models, adjusted for sex, age, ethnicity (European, African-Caribbean, West African and others) and diagnosis of T2DM baseline value of the outcome as covariates. As per the statistical analysis plan, the models included an interaction term for drug \times nitrate juice, which allowed us to test for nonadditive effects. If no statistical interactions are detected between the juice and drug arms, the data are presented separately. The models also included baseline covariates and the baseline value of the covariate being analysed. Our data are exploratory and hypothesis-generating, rather than confirmatory and hypothesis-testing, thus, results are presented as least-square mean changes from baseline and difference between drugs and juices, averaged over both follow up visits for each outcome, with 95% CIs; in accordance with the recent editorial, *Statistical reporting of clinical pharmacology research*.³⁶ P-values are included when useful to demonstrate a statistically significant difference between interventions.

3 | RESULTS

Of the study participants, 93 had a baseline carotid ultrasound (V2) and 81 (86%) had follow-up imaging at V5 and V6 (Figure 2). No statistical interactions were detected between active beetroot or placebo juice arms and the spironolactone or doxazosin arms for any

of the haemodynamic or carotid outcomes, so data are presented separately. Baseline characteristics of the study population are listed in Table 1. Haemodynamic and carotid parameters compared to baseline are summarised in Table 2.

SBP and DBP did not differ between nitrate-containing and nitrate-depleted juice or between spironolactone and doxazosin (Table 2).

Data confirming adherence to the beetroot juice arm in the main study are published by Mills et al.²⁸ Baseline CIMT was similar across each intervention group, e.g. 0.72 mm in the placebo and 0.75 mm in the active juice group (Table 1). Compared to placebo juice, active nitrate-containing beetroot juice lowered CIMT (-0.06 mm [95% confidence interval -0.12 , -0.01], $P = .034$; Table 2, Figure 3); thus, representing a difference of $\sim 8\%$ relative to baseline. Compared to baseline, CIMT increased with the nitrate-depleted (0.05 mm [0.02, 0.09], $P = .005$; Table 2, Figure 3A).

Compared to placebo juice, active nitrate-containing beetroot juice resulted in no differences in maximum or minimum carotid diameter (CD; Table 2; Figure 4), or the difference between systolic and diastolic carotid diameter ΔD , diameter compliance, distensibility, cross-sectional compliance or CSD, or CS (Table 2; Figure 5).

Compared to doxazosin, spironolactone had no effect on CIMT, CD or CS. CS was reduced from baseline with doxazosin (-0.34 mm [-0.62 , -0.06], $P = .018$), but not with spironolactone (-0.15 mm [-0.46 , 0.16]; Table 2).

4 | DISCUSSION

Our study shows that 6 months' dietary nitrate as beetroot juice reduces CIMT, a marker of subclinical atherosclerosis, compared to nitrate-depleted placebo juice. This effect was independent of BP,

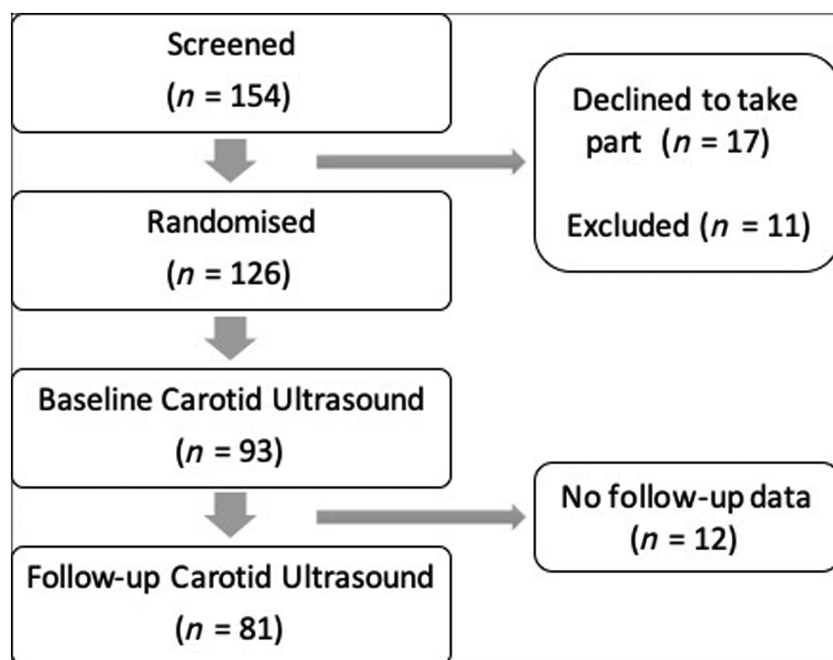


FIGURE 2 Consolidated standards of reporting trials (CONSORT) flow diagram for the VaSera trial, and subsample of participants who had carotid imaging at baseline and follow-up

TABLE 1 Baseline characteristics of the study population.

		Doxazosin		Spironolactone	
		Placebo juice	Active juice	Placebo juice	Active juice
Patients	<i>n</i>	22	17	21	21
Sex, female	<i>n (%)</i>	7 (32)	5 (29)	8 (38)	8 (38)
Type 2 diabetes	<i>n (%)</i>	16 (73)	11 (65)	15 (71)	11 (52)
Cardiovascular events	<i>n (%)</i>	1 (5)	2 (12)	2 (10)	2 (10)
Smoker	<i>n (%)</i>	2 (9)	2 (12)	2 (12)	1 (5)
Age (y)	<i>Mean (SD)</i>	59.1 (11.3)	57.8 (14.6)	57.4 (12.1)	56.6 (15)
BMI (kg/m²)	<i>Mean (SD)</i>	32.3 (5.8)	32.6 (7.8)	33.2 (5.7)	31.6 (4.3)
SBP (mmHg)	<i>Mean (SD)</i>	142.2 (20.74)	130.6 (19.05)	131.6 (14.08)	141 (25.98)
DBP (mmHg)	<i>Mean (SD)</i>	81.7 (13.3)	75.4 (12.2)	80.1 (11.2)	83.1 (13.5)
CIMT (mm)	<i>Mean (SD)</i>	0.72 (0.16)	0.75 (0.18)	0.71 (0.23)	0.75 (0.14)
Max CD (mm)	<i>Mean (SD)</i>	7.99 (1.07)	7.99 (0.85)	7.84 (1.22)	8.11 (0.84)
Min CD (mm)	<i>Mean (SD)</i>	7.43 (1.02)	7.53 (0.80)	7.31 (1.13)	7.51 (0.84)
ΔD (mm)	<i>Mean (SD)</i>	−0.28 (0.16)	−0.36 (0.13)	−0.31 (0.19)	−0.24 (0.09)
DD (%)	<i>Mean (SD)</i>	0.85 (0.16)	0.77 (0.09)	0.83 (0.14)	0.89 (0.14)
ΔA (mm²)	<i>Mean (SD)</i>	0.8 (0.16)	0.73 (0.13)	0.76 (0.19)	0.85 (0.14)
DC (mm/mmHg)	<i>Mean (SD)</i>	−1.95 (0.20)	−1.99 (0.16)	−2.01 (0.22)	−1.97 (0.13)
CSD (%)	<i>Mean (SD)</i>	1.17 (0.16)	1.08 (0.09)	1.15 (0.19)	1.21 (0.14)
CSC1 (mm²/mmHg)	<i>Mean (SD)</i>	−0.88 (0.20)	−0.91 (0.16)	−0.94 (0.22)	−0.89 (0.13)
CSDc (kPa^{−1} × 10^{−3})	<i>Mean (SD)</i>	−2.49 (0.20)	−2.55 (0.16)	−2.56 (0.22)	−2.52 (0.18)
CS (m/s)	<i>Mean (SD)</i>	5.53 (1.35)	5.91 (1.20)	6.02 (1.43)	5.74 (1.21)

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CIMT, carotid intima-media thickness; Max CD, maximum (systolic) carotid diameter; min CD, minimum (diastolic) carotid diameter; ΔD, difference between systolic and diastolic carotid diameter; DD, diameter distensibility; ΔA, difference between systolic and diastolic carotid lumen; DC, diameter compliance; CSD, cross sectional distensibility; CSC1, cross sectional compliance; CSDc, cross sectional distensibility coefficient; CS, carotid stiffness. SD, standard deviation

which did not differ between nitrate-rich and placebo juice, in line with the results from the overall study.^{28,30} The absence of changes in other morphological measures, such as carotid diameters in systole and diastole, supports a mechanism whereby nitrate has chronic, sustained actions predominantly on the most internal layer, the intima, rather than transient, reversible effects on smooth muscle of the media. No other comparative differences were found with either nitrate vs. placebo juice or spironolactone vs. doxazosin: notably, in contrast to nitrate, spironolactone was without effect on CIMT compared to doxazosin.

The effects of dietary nitrate on BP are well-described,^{37–39} and there is growing interest around its role for the reduction of cardiovascular risk.⁴⁰ Our findings highlight the potential of dietary nitrate to decelerate the atherosclerotic process in a population at increased risk, and this is the first randomized, placebo-controlled clinical trial demonstrating such an effect in humans. As we described in the Introduction, thickening of the carotid intima-media on ultrasound is an early marker of atherosclerosis, and the early involvement of the intima in the atherogenic process is well-established.^{7–9} Also, diminished production and availability of NO are important factors in the earliest phases of atherogenesis.¹⁰ Thus, dietary nitrate might be useful to supply the endothelium with NO and prevent oxidative stress,

alongside other measures to control risk factors for atherosclerosis.^{11,12,15} Indeed, as we described above, evidence of beneficial effects of long-term dietary nitrate on atherosclerosis has been previously reported from some, but not all studies performed in rats and Apo E^{−/−} mice,^{18–20} and an association has been observed in epidemiological studies.^{21,22} The cohort study by Bondonno et al.²¹ which included older Australian women aged 70–85 years, and without prevalent atherosclerotic vascular disease and/or diabetes, evaluated the nitrate intake from vegetables. Higher vegetable nitrate intake was associated with lower CIMT: for each 1 standard deviation or 29 mg/d (i.e. ~0.5 mmol/d) increase in nitrate intake, CIMT was decreased (−0.012 mm, *P* = .006); this was associated with a 17% lower risk of 14.5-year ischaemic cerebrovascular events,²¹ and diminished atherosclerosis related mortality.⁴¹ In our study, the nitrate intake was ~7.5 mmol/d for 3 months, which was increased to ~11.2 mmol/d from 3–6 months and resulted in a decrease in CIMT of 0.06 mm (vs. nitrate-free Beet It juice) over 6 months. To our knowledge, our study provides the first randomized, placebo-controlled trial evidence for the potential favourable direct effect of dietary nitrate on CIMT, and in a population comprising high risk males as well as females. Very recently, a single-arm, open-label phase 2 proof of concept study by Hughan et al., showed that 12 weeks' of

TABLE 2 Haemodynamic and carotid parameters (Model 2: fully-adjusted model for sex and age, plus ethnicity and diagnosis of diabetes).

	Active juice		Placebo juice		Active vs. placebo		Spironolactone		Doxazosin		Spironolactone vs. doxazosin	
	(n = 38)		(n = 43)				(n = 42)		(n = 39)			
Δ SBP (mmHg)	-7.59 (-10.7, -4.51)		-6.52 (-9.70, -3.34)		-1.08 (-5.56, 3.40)		-7.42 (-10.6, -4.28)		-6.69 (-9.79, -3.59)		-0.74 (-5.20, 3.73)	
Δ DBP (mmHg)	-5.05 (-6.80, -3.31)		-4.85 (-6.65, -3.05)		-0.21 (-2.74, 2.33)		-5.15 (-6.93, -3.38)		-4.75 (-6.50, -3.00)		-0.40 (-2.92, 2.11)	
Δ CIMT (mm)	-0.01 (-0.05, 0.03)		0.05 (0.02, 0.09)		-0.06 (-0.12, -0.00)		0.05 (0.01, 0.09)		0.00 (-0.04, 0.04)		0.05 (-0.01, 0.11)	
Δ max CD (mm)	-0.09 (-0.26, 0.07)		-0.13 (-0.29, 0.02)		0.04 (-0.19, 0.27)		-0.17 (-0.33, -0.01)		-0.06 (-0.22, 0.10)		-0.11 (-0.35, 0.12)	
Δ min CD (mm)	-0.10 (-0.26, 0.05)		-0.19 (-0.34, -0.05)		0.09 (-0.13, 0.31)		-0.17 (-0.32, -0.02)		-0.13 (-0.28, 0.03)		-0.04 (-0.27, 0.18)	
$\Delta(\Delta D)$ (mm) *	0.99 (0.91, 1.08)		1.08 (0.99, 1.17)		0.92 (0.82, 1.04)		0.99 (0.91, 1.08)		1.08 (0.99, 1.17)		0.92 (0.82, 1.04)	
Δ DD (%) *	1.01 (0.92, 1.10)		1.10 (1.01, 1.19)		0.92 (0.81, 1.04)		1.01 (0.93, 1.11)		1.09 (1.00, 1.19)		0.93 (0.82, 1.05)	
$\Delta(\Delta A)$ (mm ²) *	0.98 (0.90, 1.07)		1.06 (0.97, 1.15)		0.93 (0.82, 1.05)		0.97 (0.89, 1.07)		1.07 (0.98, 1.16)		0.91 (0.80, 1.04)	
Δ DC (mm/mmHg) *	1.03 (0.93, 1.14)		1.10 (1.01, 1.22)		0.93 (0.81, 1.08)		1.03 (0.92, 1.14)		1.11 (1.01, 1.22)		0.93 (0.80, 1.07)	
Δ CSD (%) *	1.01 (0.92, 1.11)		1.10 (1.01, 1.20)		0.91 (0.81, 1.04)		1.01 (0.93, 1.11)		1.10 (1.00, 1.20)		0.93 (0.81, 1.06)	
Δ CSC1 (mm ² /mmHg) *	1.02 (0.92, 1.10)		1.08 (0.98, 1.20)		0.94 (0.81, 1.10)		1.01 (0.90, 1.13)		1.09 (0.98, 1.20)		0.92 (0.78, 1.07)	
Δ CSDc (kPa ⁻¹ × 10 ⁻³) *	1.04 (0.94, 1.16)		1.14 (1.03, 1.26)		0.92 (0.79, 1.06)		1.05 (0.94, 1.17)		1.13 (1.02, 1.25)		0.93 (0.80, 1.08)	
Δ CS (m/s)	-0.11 (-0.40, 0.18)		-0.38 (-0.66, -0.10)		0.27 (-0.14, 0.68)		-0.15 (-0.46, 0.16)		-0.34 (-0.62, -0.06)		3.19-0.24, 0.62	

Change from baseline, active nitrate-containing beetroot juice vs. placebo nitrate-depleted juice, and spironolactone vs. doxazosin. SBP, systolic blood pressure; DBP, diastolic blood pressure; CIMT, carotid intima-media thickness; Max CD, maximum (systolic) carotid diameter; min CD, minimum (diastolic) carotid diameter; Δ D, difference between systolic and diastolic carotid diameter; DD, diameter distensibility; Δ A, difference between systolic and diastolic carotid lumen; DC, diameter compliance; CSD, cross sectional distensibility; CSC1, cross sectional compliance; CSDc, cross sectional distensibility coefficient; CS, carotid stiffness. The data presented are least squares mean (LSM) changes from baseline (or relative changes, indicated by *) and their 95% confidence intervals (CIs), estimated from a mixed-effects model and adjusted for sex and age, plus ethnicity and diagnosis of diabetes. Estimates that were significant at $P < .05$ in these models are highlighted in bold

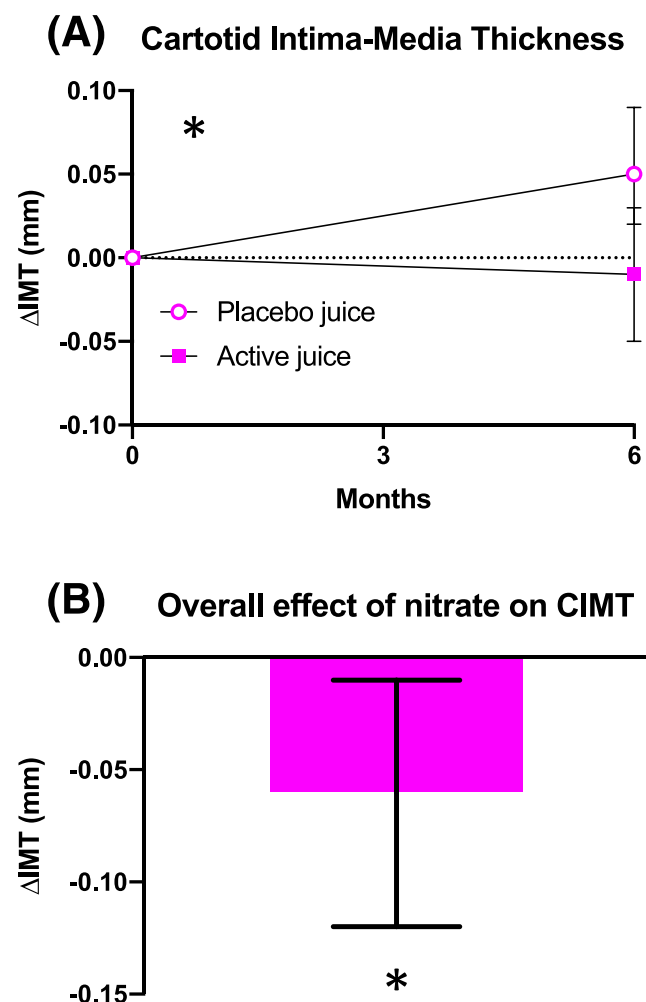


FIGURE 3 Effect of dietary nitrate as beetroot juice on carotid intima-media thickness (CIMT) measured by B-mode ultrasound. (A) Change from baseline in CIMT for active nitrate-rich juice and placebo nitrate-depleted juice. (B) Overall effect of active juice vs. placebo juice on CIMT. Data shown as least-square means with 95% s. * $P < .05$

oral sodium nitrite (40 mg 3 times a day) reduced CIMT by 0.04 mm from 0.773 ± 0.02 mm at baseline to 0.730 ± 0.02 at 12 weeks ($P = .0054$) in 20 patients with hypertension and metabolic syndrome.⁴² This study provides valuable mechanistic support for an effect of nitrite on CIMT, albeit accompanied by a significant decrease in semi-recumbent SBP and DBP and a significant $\sim 11\%$ increase in resting conduit artery diameter (brachial) from 0.36 ± 0.02 mm to 0.40 ± 0.02 mm after 12 weeks of nitrite therapy ($P = .0009$); this is similar to the acute vasodilatory effect of systemically-infused nitrite we had previously observed.⁴³ As commented above, our observed effect of dietary nitrate on CIMT was in the absence of changes in BP or carotid diameter.

Whilst not part of the main comparative analysis, inspection of the remaining data reveals that there was evidence of a decrease in CS from baseline with placebo juice and with doxazosin, (also, similar nonsignificant trends for nitrate-rich juice and for spironolactone).

However, no difference was found between the juices, or the between drugs, for CS reduction. We recently reported, as part of the main findings of this VaSera study, that doxazosin decreased arterial stiffness as aortic PWV.²⁸ Similar to its effect on muscular arteries, doxazosin is likely to reduce vascular tone and arterial stiffness in larger elastic arteries, such as the aorta and its branches. Differences in measurement techniques between aortic stiffness (measured using PWV) and carotid stiffness (measured using echo-tracking) and sample size may account, at least in part, for the lack of a significant effect of doxazosin (vs. spironolactone) on CS. In addition, previous studies have shown that risk factors such as high BP and type 2 diabetes stiffen the aorta more than the carotid.⁴⁴ Thus, the aortic response to doxazosin (PWV) might have been more evident than the carotid response (CS), in a population such as ours with these risk factors. Also, we have recently found that the relationship between circulating plasma nitrite concentration and pulse pressure, which is related to large artery stiffness, is more complex than we anticipated, albeit in an acute crossover study. Whilst active beetroot juice significantly decreased pulse pressure vs. placebo juice, addition of grapefruit juice, further decreased pulse pressure despite decreasing plasma nitrite concentration.⁴⁵

Overall, our results show that dietary nitrate influences vascular remodelling, probably acting on those layers of the vascular wall that are mainly involved in the atherogenic process. This might be due to the provision of an alternative source of NO, in the face of diminished NO production seen in the early stages of atheroma formation.

We acknowledge that current guidelines in Europe⁴⁶ and the USA⁴⁷ do not recommend routine CIMT evaluation as part of the cardiovascular risk assessment, and this is mainly due to conflicting results and lack of standardisation of measurements methods. CIMT remains an established marker of subclinical atherosclerosis, and further research is needed to clarify its role in the cardiovascular risk evaluation of asymptomatic individuals.^{9,48}

Whilst this was a well-controlled study (double blind placebo for nitrate) and the first trial of its kind for dietary nitrate—with 6 months' intervention, there were several limitations. For example, CIMT, together with other carotid vascular parameters, were not the primary outcome of the VaSera trial. Also, a relatively small subsample of 81 patients had follow-up data. Thus, our analysis is exploratory, and our results require further confirmation from larger studies. We did not assess physical activity and we had decided in the original design of the study, that habitual diet, including dietary nitrate intake, would not be controlled for, as we wished to reflect a *real-world* situation, and thus this could also be viewed as a strength; this was intentionally a pragmatic trial. Instead, we measured plasma nitrate and nitrite concentrations in the main study and found that they were elevated in patients on active (nitrate-containing) vs. placebo (nitrate-depleted) beetroot juice,²⁸ providing evidence of reasonable adherence and with an elevation above a variable background nitrate intake. Whilst food frequency questionnaires have some limitations, the development of a reference database for assessing dietary nitrate in vegetables,⁴⁹ which can be used in conjunction with a food frequency questionnaire, provides a useful tool for future interventional studies,

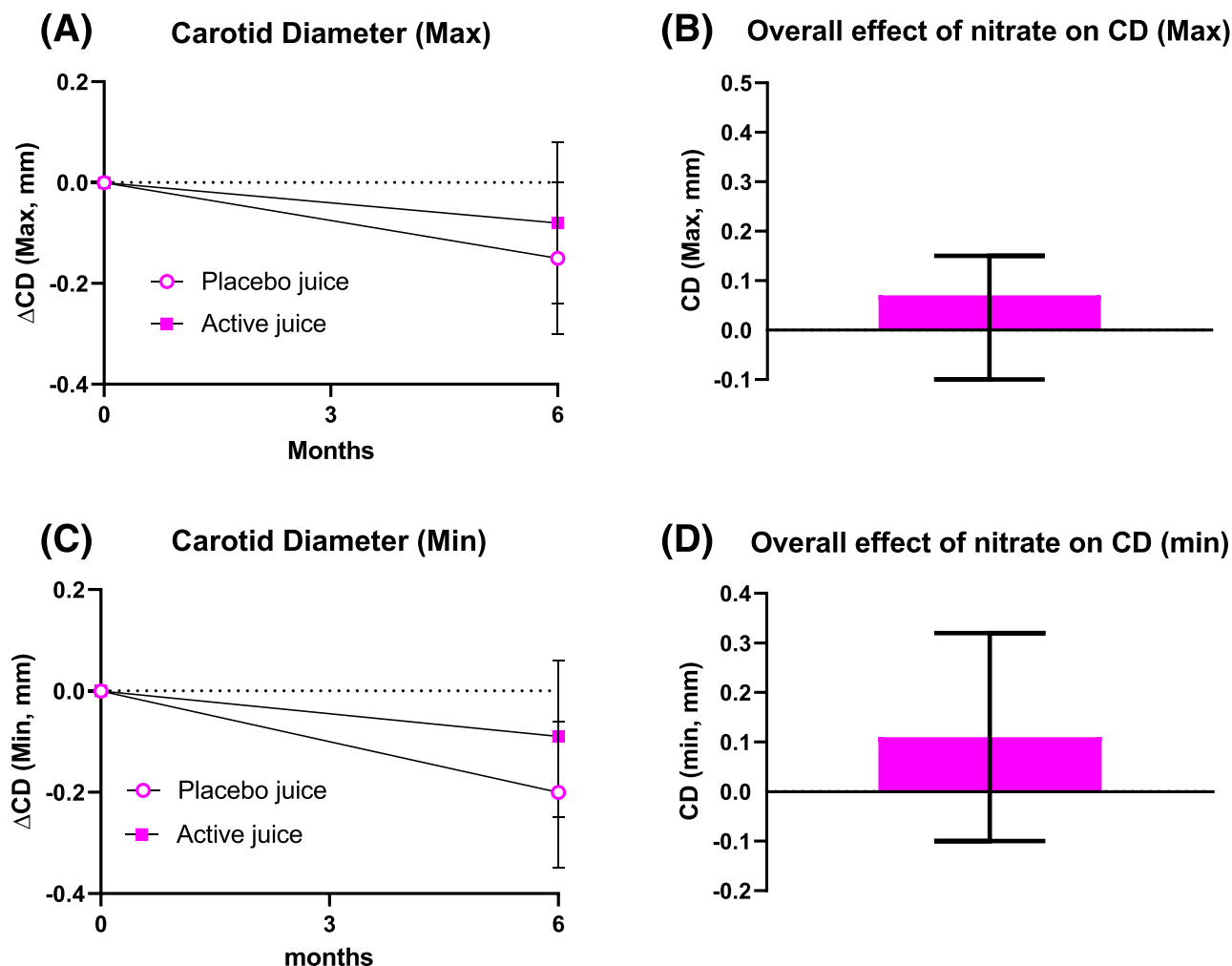


FIGURE 4 Effect of dietary nitrate as beetroot juice on carotid diameter (CD) measured by B-mode ultrasound. Change from baseline in maximum (A) and minimum (C) CD for active nitrate-rich juice and placebo nitrate-depleted juice. Overall effect of active juice vs. placebo juice on maximum (B) and minimum (D) CD. Data shown as least-square means with 95% confidence intervals

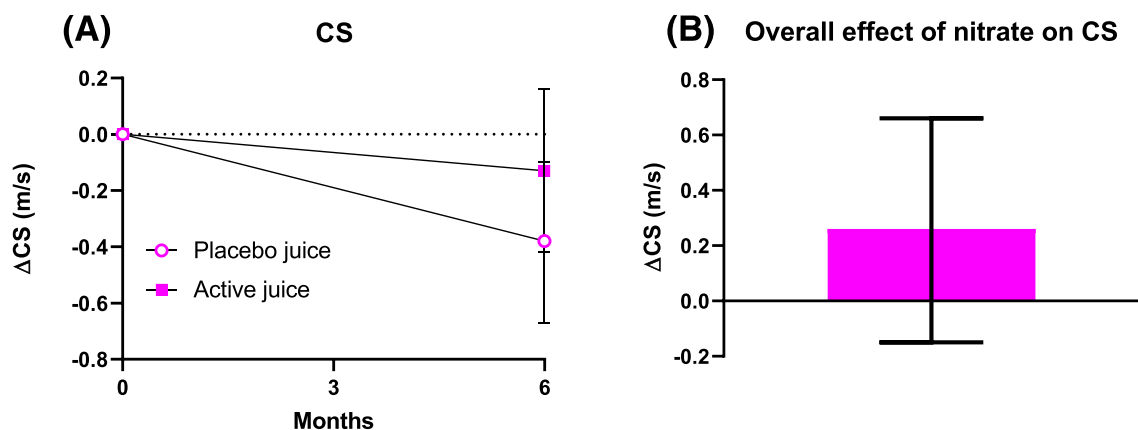


FIGURE 5 Effect of dietary nitrate as beetroot juice on carotid stiffness (CS) measured by B-mode ultrasound. (A) Change from baseline in CS for active nitrate-rich juice and placebo nitrate-depleted juice. (B) Overall effect of active juice vs. placebo juice on CS. Data shown as least-square means with 95% confidence intervals

besides the observational/epidemiological studies. Also, we have recently found that environmental nitrogen dioxide, including from gas appliances such as gas cookers, increases plasma nitrite concentration and lowers SBP and DBP.⁵⁰ Thus there is potential for confounding of the effect of dietary nitrate-derived circulating nitrite by nitrogen dioxide-derived circulating nitrite from atmospheric pollution and domestic gas appliances.

5 | CONCLUSION

CIMT was lower after 6 months of active beetroot juice intake compared to placebo juice. Other vascular parameters, such as carotid diameter and stiffness, did not differ between interventions. Thus, our results suggest that dietary nitrate might have a beneficial effect on intima-media remodelling, which is involved in atherogenesis, and thus represent a potential intervention to prevent or treat atherosclerosis.

ACKNOWLEDGEMENTS

The authors would like to thank the research nurses at Clinical Research Facility at St Thomas' Hospital for their assistance in running the study, as well as the study participants. We also thank Karen McNeill for managing the blinding and randomization of the interventions, and Suzanne Barrett, who worked as research administrator. The work was funded by Fukuda Denshi, Tokyo, Japan. We acknowledge internal infrastructure financial support from King's College London British Heart Foundation Centre; National Institute for Health Research (NIHR), Clinical Research Facility at Guy's & St Thomas' NHS Foundation Trust, NIHR Biomedical Research Centre, based at Guy's and St Thomas' NHS Foundation Trust, and King's College London. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

COMPETING INTERESTS

A.J.W. holds shares in HeartBeet Ltd, which receive a royalty from James White Drinks Ltd who manufacture the active nitrate-containing beetroot juice and placebo nitrate-depleted juice used in this study. The other authors have stated explicitly that there are no conflicts of interest in connection with this article. This work was funded by Fukuda Denshi, Tokyo, Japan.

CONTRIBUTORS

A.J.W. and J.K.C. both led the design of the VaSera trial, oversaw the data acquisition and analysis, and were involved in the interpretation of the results and revising the manuscript drafts. F.M. contributed to data acquisition, interpretation of the results and led drafting the manuscript. L.F. and C. M. were involved in research design, data acquisition and interpretation, and revised the manuscript drafts. S.V.M. led the data analysis and contributed to the manuscript drafts. P.C., J.A.Y. and A.C. were involved in the interpretation of the results and contributed to manuscript drafts. All authors approved the final version of the manuscript and have agreed accountability for the research.

DATA AVAILABILITY STATEMENT

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

ORCID

Franca Morselli  <https://orcid.org/0000-0002-6186-4780>

Luca Faconti  <https://orcid.org/0000-0002-1066-6899>

Charlotte E. Mills  <https://orcid.org/0000-0002-8313-3700>

Philip J. Chowienzyk  <https://orcid.org/0000-0003-4507-038X>

J. Kennedy Cruickshank  <https://orcid.org/0000-0001-8126-1760>

Andrew J. Webb  <https://orcid.org/0000-0002-8109-1877>

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How to cite this article: Morselli F, Faconti L, Mills CE, et al. Dietary nitrate prevents progression of carotid subclinical atherosclerosis through blood pressure-independent mechanisms in patients with or at risk of type 2 diabetes mellitus. *Br J Clin Pharmacol*. 2021;87(12):4729-4739. <https://doi.org/10.1111/bcp.14897>