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DIETARY NITRATE DOES NOT MODIFY BLOOD PRESSURE AND CARDIAC OUTPUT AT REST AND DURING EXERCISE IN OLDER ADULTS: A RANDOMISED CROSS OVER STUDY

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Running title: Ageing, dietary nitrate and cardiovascular outcomes

Keywords: nitric oxide, exercise, vascular health, cardiac function, ageing

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#Equal Contribution

The material presented in this manuscript is original and it has not been submitted for publication elsewhere while under consideration for International Journal of Food Sciences and Nutrition

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27   **Conflict of interest statement:** The authors have no conflict of interest to declare.

28

29   **Abstract: 149**

30   **Manuscript: 5680**

31   **Tables: 1**

32   **Figures: 4**

33   **References: 37**

34   **OSM: 1**

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43   **ABSTRACT**

44   Dietary nitrate (NO_3^-) supplementation has been associated with improved vascular and

45   metabolic health. We conducted a double-blind, cross-over, placebo-controlled RCT to

46   investigate the effects of 7-day consumption of beetroot juice compared with placebo on 1)

47   blood pressure (BP) measured in resting conditions and during exercise, 2) cardiac and

48   peripheral vascular function and 3) biomarkers of inflammation, oxidative stress and

49   endothelial integrity.

50   Twenty non-smoking healthy participants aged 60-75y and BMI 20.0-29.9kg/m^2 were

51   recruited. Measurement were conducted before and after each 7-day intervention period.

52   Consumption of NO_3^- had no effect on resting systolic and diastolic BP. NO_3^- consumption

53   did not improve indexes of central and peripheral cardiac function responses during

54   cardiopulmonary exercise testing. Dietary NO_3^- supplementation did not modify biomarkers

55   of inflammation, oxidative stress and endothelial integrity.

56   This study do not support the short-term benefits of dietary NO_3^- supplementation on

57   physiological and biochemical markers of vascular health in older healthy adults.

58   **Trial Registration:** ISRCTN19064955

INTRODUCTION

Ageing is a primary risk factor for atherosclerosis and cardiovascular diseases (CVD)(North and Sinclair 2012). Cardiac ageing is characterised by prominent changes in cardiovascular tissues including hypertrophy, altered left ventricular (LV) diastolic function and cardiac output (CO), and increased arterial stiffness. In older adults, resting CO is preserved by an increase in LV end-diastolic volume with a consequent augmentation of stroke volume (SV). With the increase in energy demands during exercise, older adults achieve people a higher SV and mean arterial blood pressure but lower heart rate and peak oxygen consumption compared to younger subjects. Therefore, SV during exercise in older adults is preserved by an increase in end-diastolic volume whereas in younger subjects is maintained by a progressive decrease in end-systolic volume(Cheitlin 2003, Houghton et al. 2016). Augmentation index was significantly higher in older than younger participants and was inversely related CO in older participants [3].

Nitric oxide (NO) appears to have pleiotropic effects on cardiac physiology(North and Sinclair 2012), being produced by all myocardial cells and is involved in the regulation of coronary vasodilation and cardiomyocyte contractility(Massion et al. 2003). NO is synthesised by vascular and endocardial endothelial nitric oxide synthases (NOS), as well as neuronal and inducible NOS(Rastaldo et al. 2007). The effects of NO on myocardial contractility appear to be mediated by the opening of sarcolemmal voltage-operated and sarcoplasmic ryanodin receptor Ca(2+) channels(Rastaldo, et al. 2007). NO is also involved in the modulating post-ischemic cardiac remodeling infarction which may be mediated by a decreased mitochondrial permeability(Di Lisa et al. 2001).

NO is involved in several other physiological functions such as maintenance of vascular tone, platelet adhesion, angiogenesis, mitochondrial oxygen consumption, muscular performance and control of immunity and inflammation signalling pathways (Kelm 1999). Inorganic NO_3^-

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84   represents the final, stable end product of nitric oxide (NO) metabolism and it is mainly

85   excreted in urine (~70%). Approximately 25-30% of circulating blood NO_3^- enters a non-

86   enzymatic NO synthetic pathway involving salivary glands, oral microbiota and gastric acidic

87   environment (Lundberg et al. 2009). Inorganic NO_3^- from food can also enter the non-

88   enzymatic NO pathway, increase NO production and induce positive effects on

89   cardiovascular function and muscle performance(Lundberg, et al. 2009). The role of ageing

90   as a modifier of the effects of inorganic NO_3^- on cardiovascular outcomes remains unknown.

91   Convincing evidence on health benefits of dietary NO_3^- on cardiovascular outcomes

92   currently exists for young and middle-aged individuals (Gee and Ahluwalia 2016, Lara,

93   Ashor, et al. 2015), whereas contrasting findings have been reported in older populations(Gee

94   and Ahluwalia 2016, Lara, et al. 2015, Omar et al. 2016, Siervo et al. 2013). In addition,

95   limited information is available on the effects of inorganic NO_3^- consumption on cardiac

96   function at rest and during exercise in healthy adults (DeVan et al. 2015, Lee et al. 2015) and

97   in patients with heart disease (Eggebeen et al. 2016, Zamani et al. 2015).

98   We hypothesise that dietary NO_3^- consumption can increase systemic NO bio-availability and

99   have a positive effect on central and peripheral hemodynamic responses of healthy older

100   adults measured at rest, during different exercise intensities (low, moderate and high) and

101   post-exercise recovery. We evaluated the effects of beetroot juice, chosen as a rich source of

102   dietary NO_3^-, on blood pressure (BP), augmentation index (AIx) and hemodynamic

103   parameters of cardiac function including CO, stroke volume (SV), cardiac index (CI) and

104   heart rate (HR) measured at rest and during graded exercise on a stationary bike. We also

105   evaluated whether dietary NO_3^- consumption induced changes in circulating biomarkers of

106   inflammation, oxidative stress and endothelial integrity to provide mechanistic insights into

107   the effects of dietary NO_3^- on circulatory biomarkers closely involved in the regulation of

108   vascular function.

METHODS

The trial was approved by the North of Scotland Research Ethics committee (14/NS/0061) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. The study was a double-blind, cross-over, placebo-controlled RCT which took place between May and August 2014 across two sites (Newcastle upon Tyne and Sheffield). The duration of the each intervention was one week with a wash-out period between treatments of at least one week. This trial was registered in the International Standard Randomised Controlled Trial Number Register (ISRCTN19064955).

Participants: Twenty older healthy people (10 male, 10 female) were recruited (10 participants per site). Participants were included in the study if they did not have medical conditions or were not taking medications that might influence the study outcomes. A full list of the inclusion and exclusion criteria is provided in the **Online Supplementary Material**. Participants were asked to maintain their habitual diet and to avoid using chewing gum or mouth wash for at least 48 prior to the baseline visits (first and third visit) and during each of the one-week supplementation periods.

Study Overview: A telephone screening was performed to check eligibility to the trial's inclusion and exclusion criteria. Eligible participants were asked to arrive at the research facilities after a 12-hour overnight fast and having avoided strenuous physical activity for three days preceding the visit. Eligibility to the study was confirmed by measuring BMI, resting blood pressure and conducting a resting 12 lead electrocardiogram. Participants were randomised to a cross-over intervention and the assessment continued with the measurement of body composition and collection of blood and urine samples and the assessment of physical capability (reported elsewhere). Participants then rested for one hour and consumed a meal providing approximately 300kcal (CHO=85%, PRO=3%, FAT=12%). After the 1 hour rest period the exercise test was explained to the participants and they and they became

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134 accustomised to the bicycle ergometer. The exercise protocol is described in **Figure S1 of the**

135 **Online Supplementary Material**. After the vascular measurements and the exercise test,

136 instructions were provided for self-administration of the nutritional intervention (14 bottles of

137 either NO_3^--rich or NO_3^--depleted beetroot juice; 70ml x 2/day; Beet It, James White Ltd,

138 UK) and asked to consume one bottle of beetroot juice each morning and evening for the

139 subsequent 7 days. The daily dose of NO_3^--rich (intervention) or NO_3^--depleted (placebo)

140 beetroot juice contained ~12mmol and ~0.003mmol of NO_3^-, respectively. This concluded

141 Visit 1 of the trial. Participants returned to the research facilities in the morning of day eight

142 after they had completed a seven-day supplementation period. Measurements were conducted

143 approximately after 12 hours from drinking the beetroot juice as participants were asked to

144 fast overnight before arriving to the research centre. The resting 12 lead ECG was performed

145 and if normal the visit continued with a repeat of the assessments performed at visit 1. At the

146 end of the second visit, participants were asked to resume their habitual diet and physical

147 activity. After a wash out period of at least seven days the second phase (including Visits 3

148 and 4) was conducted similar to the first phase with the exception that participants crossed-

149 over experimental arms i.e. consumed the other intervention agent.

150 Resting and Daily Blood Pressure: Resting BP was measured in triplicate using an automated

151 BP monitor (Omron M3, Omron Healthcare, UK) at each clinic visit with the participant

152 seated comfortably for 15 min prior to the measurement and the arm supported at the level of

153 the heart. The same BP monitor (Omron M3, Omron Healthcare, UK) was provided to each

154 participant for the measurements of daily resting BP at home. Participants were asked to

155 conduct duplicate measurements in a seated position in the morning before drinking the juice

156 and in the evening before going to bed. Agreement of the daily BP monitoring was verified

157 against the BP recordings obtained from the 24-hr ABPM (systolic BP, r=0.71, p<0.001,

158 n=84; diastolic BP, r=0.80, p<0.001, n=84)(Jajja et al. 2014).

159   Resting and exercise central hemodynamics: All subjects performed a maximal graded

160   cardiopulmonary exercise test using an electro-magnetically controlled bicycle ergometer

161   (Corival, Lode, Groningen, Netherlands) with online gas exchange measurements (Metalyzer

162   3B, Cortex, Leipzig, Germany). The maximal progressive exercise test included cycling with

163   10-watt increments every minute until volitional exhaustion. The 12-lead ECG (Custo,

164   CustoMed GmbH, Ottobrunn, Germany) was continuously monitored and blood pressure

165   (Tango, SunTech Medical, Morrisville, NS, USA) recorded at rest, during exercise and

166   recovery (Newcastle Centre only, N=10). The test was terminated when the subject was

167   unable to pedal at a cadence of 50 revolutions per minute or they reached maximal oxygen

168   consumption. Peak oxygen consumption was defined as the average oxygen uptake during

169   the last minute of exercise. Non-invasive central hemodynamics parameters (SV, CO and CI)

170   were measured by bioreactance method (NICOM, Cheetah Medical, Deleware,

171   USA).(Jakovljevic 2014) CO was estimated under resting and exercise stress testing

172   conditions using the bio-reactance method which analysis the frequency of relative phase

173   shifts of electrical current applied across the thorax using four dual-surface electrodes.

174   Signals were applied to and recorded from the left and right sides of the thorax; these signals

175   are processed separately and averaged after digital processing. The signal processing unit of

176   the system determines the relative phase shift between the input signal relative to the output

177   signal. The phase shift occurs due to instantaneous changes in blood flow in the aorta.

178   Cardiac output is subsequently estimated as the product of stroke volume and heart rate.

179   Cardiac Index (CI) is calculated by adjusting the CO for body surface area. A graphical

180   description of the protocol is described in **Figure S2 of the Online Supplementary**

181   **Material**.

182   Augmentation Index: A high fidelity micro-manometer was used to apply a gentle pressure

183   and therefore flatten the radial artery in the non-dominant hand at the wrist under resting

184 condition using the SphygmoCor (AtCor Medical, NSW, Australia). Central aortic pressure

185 and augmentation index was then calculated automatically using the SphygmoCor software.

186 AIx was calculated as the difference between the first systolic peak and the second systolic

187 peak of the central arterial waveform, which was expressed as a percentage of pulse pressure.

188 Anthropometry, Dietary and Lifestyle Questionnaires: Body weight and height were

189 measured to the nearest 0.1 kg and 0.5 cm, respectively. The 9-item short form of the

190 International Physical Activity Questionnaire (IPAQ) was used to record levels of physical

191 activity: 1) vigorous-intensity activity 2) moderate-intensity activity, 3) walking and 4)

192 sitting. A combined total physical activity score was calculated and expressed in MET-

193 minutes/week(CRAIG et al. 2003). The EPIC Food Frequency Questionnaire (FFQ) was

194 administered at baseline and the FETA software used to extract dietary (energy and nutrient)

195 information(Mulligan et al. 2014).

196 Blood and Urine Collection: Fasting blood samples were collected at the beginning of each

197 visit and centrifuged at 3,000rpm for 10 min at 4 °C within 30min of collection. Aliquots of

198 plasma and serum were frozen and stored at −80 °C for subsequent analyses. Mid-stream

199 urine samples were collected, in fasting conditions, into sterile containers and stored at −20

200 °C for subsequent analyses.

201 Biomarker Analysis: A modified version of the gas chromatography mass spectrometry (GC-

202 MS) method proposed by Tsikas et al(Tsikas 2000) was used to determine NO_3^- and NO_2^-

203 concentrations in urine and plasma samples and sum of NO_3^- and NO_2^- (NOx) was calculated.

204 However, blood samples were not immediately processed (~30-45 minutes) to preserve NO_2^-

205 and therefore NO_3^- is the main contributor to the total concentration of NOx. The protocol

206 and validation of the modified GC-MS method have been described elsewhere(Qadir et al.

207 2013). Methods for the measurement of glucose, insulin, IL-6, 3-NT, cGMP, ET-1, P-

208 selectin, E-Selectin, intercellular adhesion molecule-3 (ICAM-3) and thrombomodulin are

209 reported in the **Online Supplementary Material**.

210 Statistical Analysis: Repeated-Measures General Linear Models (GLM) were used to test the

211 effect of NO_3^- consumption on measures of vascular function and blood biomarkers.

212 Treatment (nitrate vs placebo) was entered as a group factor (Tr) and the time points of the

213 incremental exercise test as the repeated factor (Ti). Post-hoc comparison between treatment

214 groups at each time point was performed using the Fisher LSD test. Analyses were conducted

215 using Statistica 10 for Windows (StatSoft.Inc, Tulsa, OK, USA). Statistical significance was

216 set at <0.05.

217 **RESULTS**

218 *Participants' characteristics* and safety: Twenty participants were randomised to the

219 interventions. One person developed an ischemic event during the physical exercise testing

220 performed at the second visit and he was excluded from the study (**Figure 1**). The remaining

221 19 participants (mean age 64.7±3.0 years) reported no side effects apart for the expected

222 urine discoloration related to the excretion of beetroot juice pigment (beeturia). Baseline VO_2

223 max of participants was 23.6±5.8 ml/kg/min for men and 20.5±2.8 ml/kg/min for women.

224 Body weight, dietary Intake and self-reported physical activity: Mean baseline BMI was

225 25.6±3.4 kg/m^2. Body weight did not change during the study in either groups (p=0.51)

226 (**Table S1 of the Online Supplementary Material**). Changes in self-reported physical

227 activity were again not different between the placebo and the NO_3^- arms (p=0.99) (**Table S1**

228 **of the Online Supplementary Material**).

229 Resting Clinic and Daily Blood Pressure: Baseline resting systolic and diastolic BP were

230 127.4 ± 16.1 mmHg (range: 100.0 – 168.0 mmHg) and 76.2 ± 9.6 mmHg (range: 61.6 – 95.7

231 mmHg), respectively. Clinic systolic BP were not significant after NO_3^- consumption

232 compared to placebo (-5.05±9.45 vs -2.64±9.04 mmHg respectively, p=0.42) (**Figure 2a**).

233 Similarly, daily BP was not significant for both systolic (p=0.75) and diastolic (p=0.63)

234 readings measured over the 7-day period (**Figure 2b**).

235 Augmentation Index: NO_3^- consumption did not have a significant effect on AIx (p=0.87,

236 **Figure S3**).

237 Blood Pressure and Cardiac Function during Standardised Exercise: NO_3^- consumption did

238 not influence systolic BP response (p=0.92, **Figure 3a**) during exercise whereas a non-

239 significant trend for lower diastolic BP after NO_3^- supplementation (p=0.08, **Figure 3b**).

240 Specifically, lower diastolic BP readings were recorded during moderate sub-maximal

241 exercise intensities (work rate: 40W, 60W and 80W). Dietary NO_3^- consumption did not

242 modify parameters of cardiac function (CO, HR, SV and CI) measured at rest, during

243 exercise and post-exercise recovery (**Figure 4a-d**). In addition, one week dietary NO_3^-

244 consumption did not modify the association between CO and oxygen consumption (VO_2)

245 (nitrate, B±SE= 6.04±0.34, R^2=0.60, p<0.001; placebo, B±SE= 6.68±0.42, R^2=0.55, p<0.001)

246 measured at different levels of exercise intensities (**Figure S4 of the Online Supplementary**

247 **Material**).

248 Laboratory Biomarkers: Concentrations of nitrite plus nitrate (NO_2^-+NO_3^-, NOx) in plasma

249 and urine increased after NO_3^- consumption by 150±77% and 979±488% compared to

250 placebo (-9±33% and -13±34%, respectively). NO_3^- consumption did not modify

251 concentrations of fasting glucose (p=0.41), insulin (p=0.95) and HOMA-IR (p=0.88). NO_3^-

252 consumption also did not induce any changes in biomarkers of endothelial function (cGMP,

253 endothelin-1, E-Selectin, P-Selectin, thrombomodulin and ICAM-3), inflammation (IL-6) and

254 oxidative stress (3-NT) (**Table 1**).

255 **DISCUSSION**

256 This study does not support a beneficial effect in the short-term of dietary NO_3^- ingestion on

257 cardiac and peripheral vascular health in older healthy adults. In particular, a lack of effect

258 was observed for BP and central hemodynamic responses measured both at rest and during

259 increased metabolic demands. These physiological measurements were complemented by a

260 panel of circulating biomarkers of metabolic control, oxidative stress and endothelial

261 integrity. None of these measurements were altered by one-week dietary NO_3^- ingestion,

262 which stimulate further discussion on uncovering the factors that may explain the lack of

263 efficacy in older populations and the contrast with the more consistent beneficial effects

264 observed in younger populations.

265 Extensive work has been conducted in the last decade to test the effects of dietary NO_3^- on

266 BP but, despite numerous trials, the evidence is still limited due to the small sample size and

267 short duration of completed trials(Gee and Ahluwalia 2016, Khatri et al. 2016, Mills et al.

268 2016, Siervo, et al. 2013). Further research is especially needed in older populations,

269 although patients with comorbidities such as peripheral arterial disease or heart failure (HF)

270 appear to receive greater health benefits from dietary NO_3^- consumption(Eggebeen, et al.

271 2016, Kenjale et al. 2011, Zamani, et al. 2015). However, DeVan et al(DeVan, et al. 2015)

272 have recently reported that sodium nitrite supplementation was well-tolerated and improved

273 endothelial function and lessens carotid artery stiffening in middle-aged and older adults.

274 Conversely, our group has recently reported non-significant effects of dietary NO_3^-

275 consumption on endothelial function and on 24-hr ambulatory blood pressure (BP) in older

276 adults with and without type 2 diabetes (>60years)(Lara, Ogbonmwan, et al. 2015, Siervo et

277 al. 2015). These results have also recently been corroborated by Bondonno et al who found

278 no effect of seven-day dietary NO_3^- consumption on home and 24-hr ambulatory BP in

279 patients with raised BP (age range: 30-70y)(Bondonno et al. 2015, Bondonno et al. 2014).

280 However, Kapil et al showed that a four-week intervention in drug-naïve hypertensive

281 subjects (age range: 18-85y) significantly reduced clinic, home and 24-hr ambulatory BP and

282 improved endothelial function(Kapil et al. 2015). The divergence of results is again part of

283 the discussion around the effects of dietary NO_3^- on health outcomes and priority is now

284 being assigned to the identification of factors accounting for the mixed findings. These

285 factors may include phenotypic characteristics (i.e., age, BMI, health status) of the

286 populations, dose of dietary nitrate and duration of supplementation, study design,

287 measurement protocols of BP an vascular health, type of cardiopulmonary fitness test

288 protocols. The dynamic BP responses during exercise have been investigated in young,

289 healthy populations(Bond et al. 2014, Lee, et al. 2015) and in patients with COPD(Berry et

290 al. 2015) and HF(Coggan et al. 2015, Coggan and Peterson 2016, Eggebeen, et al. 2016). In

291 healthy young populations, two studies reported a decline of sub-maximal systolic BP after

292 acute (single dose) and short-term (15 days) dietary NO_3^- consumption (Bond, et al. 2014,

293 Lee, et al. 2015). In older COPD patients, dietary NO_3^- decreased systolic BP at rest whereas

294 only diastolic BP showed a significant decline during 5W pedaling and 75% of maximal

295 work rate(Berry, et al. 2015). In patients with HF, acute dietary NO_3^- did not improve BP

296 responses during knee extension or cycle ergometry tests at sub-maximal and maximal

297 efforts(Coggan and Peterson 2016). However, one week of daily dosing with dietary NO_3^-

298 significantly improved submaximal BP in elderly patients with HF with preserved ejection

299 fraction(Eggebeen, et al. 2016). Our study is the first trial to investigate resting and dynamic

300 BP responses in older healthy adults and our results do not support a beneficial effect of

301 dietaryNO_3^-on oxygen consumption (data not shown) as well as vascular responses during

302 exercise. Precisely why there is a lack of response to dietary NO_3^- in our study is not known

303 since we have supplemented subjects for one week and administered a NO_3^- dose

304 considerably higher compared to other studies (>700mg/day). The factors explaining these

305 divergent age-dependent responses are still largely undetermined, which could potentially be

306 related to a decline in the reducing capacity to convert NO_3^- into NO_2^- or sensitivity of

307 cellular targets to NO.

308   In mice, NO_3^- consumption have been showed to increase the expression of calcium handling

309   proteins in the heart, resulting in increased cardiomyocyte calcium signaling and improved

310   left ventricular contractile function(Pironti et al. 2016). These findings have provided

311   preliminary support to the role of dietary NO_3^- as a cardiac modulator, which have then been

312   translated into clinical interventions in populations without and with impaired cardiac

313   function. In healthy populations, acute dietary NO_3^- consumption did not modify resting or

314   sub-maximal CO(Bond, et al. 2014) whereas an improvement of CO and SV was observed

315   after a one-week dietary NO_3^- consumption(Lee, et al. 2015). The only study testing the acute

316   effects of dietary NO_3^- on cardiac function in HF patients with preserved ejection fraction

317   found greater reductions in systemic vascular resistance, aortic augmentation index and

318   increased CO during exercise(Zamani, et al. 2015). We tested whether dietary NO_3^- could

319   minimise the age-related decline in myocardial contractility and ejection fraction, which

320   could prompt compensatory myocardial cardiac hypertrophy(North and Sinclair 2012). This

321   enhances in the short-term CO but the long-term effect of LV hypertrophy are known as

322   represents an important step in the development of HF and coronary syndromes(Gosse 2005).

323   Our scope was to evaluate whether dietary NO_3^- could represent an effective and simple

324   nutritional intervention that may minimise age-related changes in cardiac function and

325   impact, from a primary prevention perspective, on the risk for HF. However, these

326   preliminary results may not support the beneficial effects of dietary NO_3^- on cardiac function

327   in older healthy populations but, if confirmed in more robust trials, dietary NO_3^- may still

328   represent a promising nutritional strategy in patients with impaired cardiac function.

329   The small sample size and the short duration are important limitations of this trial and a

330   cautious interpretation of the results is needed; nevertheless, our study is to date one of the

331   longest trials testing the effects of dietary NO_3^- on resting and exercise vascular responses in

332   older participants. We did not assess daily dietary intake during the trial. However,

333 participants were asked to maintain their habitual dietary intake during the study and the

334 differences in nitrate intake between intervention and placebo groups were clearly

335 demonstrated by the large differences in plasma and urinary nitrate concentrations. Plasma

336 NO_2^- concentrations were not measured since it was not possible due to logistic constraints to

337 process the samples immediately after collection to minimise the immediate NO_2^- degradation

338 (half-life: ~5 minutes). However, in previous studies testing the effects of dietary

339 NO_3^- consumption in older participants where plasma NO_2^- concentration was measured, an

340 increase in plasma NO_3^- and NOx concentrations similar to the amount observed in this study

341 occurred alongside a significant rise in plasma NO_2^- concentrations(Gilchrist et al. 2013). In

342 addition, the measurement of NOx was critical to assess the compliance to the interventions

343 as well as the attainment of a significant rise in plasma NO_3^- to enable an increased NO

344 generation via the NO non-enzymatic pathway. Finally, the limitations of bio-reactance for

345 the assessment of cardiac hemodynamic profiles have to be taken into account for the

346 interpretation of the results.

347 Testing the efficacy of dietary NO_3^- consumption on cardiovascular health is an attractive

348 research area due to the potential use of natural products to increase NO_3^- intake and

349 applicability in long-term dosing. However, this short term intervention showed that dietary

350 NO_3^- consumption did not modify physiological and biochemical markers of vascular health

351 in healthy older adults. However, these findings are preliminary and require corroboration in

352 studies with longer duration and larger samples of healthy older individuals as well as in

353 older patients with increased cardiovascular risk.

354

355

356 **Author contributions**

357 M.S. is the guarantor of this work and, as such, had full access to all the data in the study and

358 takes responsibility for the integrity of the data and the accuracy of the data analysis. M.S.

359 and E.W. designed the study. M.S. wrote the manuscript and researched data; C.O., D.J.,

360 C.C., A.W.A., A.R., M.R., M.K., E.W. collected the data. All authors contributed to

361 discussion and reviewed/edited manuscript.

376 **Conflicts of interest**

377 None to report

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380   **References**

381   Berry MJ, Justus NW, Hauser JI, Case AH, Helms CC, Basu S, Rogers Z, Lewis MT, Miller
382   GD. 2015. Dietary nitrate supplementation improves exercise performance and decreases
383   blood pressure in COPD patients. Nitric Oxide.48:22-30.
384   Bond V, Jr., Curry BH, Adams RG, Millis RM, Haddad GE. 2014. Cardiorespiratory function
385   associated with dietary nitrate supplementation. Appl Physiol Nutr Metab.39:168-172.
386   Bondonno CP, Liu AH, Croft KD, Ward NC, Shinde S, Moodley Y, Lundberg JO, Puddey
387   IB, Woodman RJ, Hodgson JM. 2015. Absence of an effect of high nitrate intake from
388   beetroot juice on blood pressure in treated hypertensive individuals: a randomized controlled
389   trial. Am J Clin Nutr.102:368-375.
390   Bondonno CP, Liu AH, Croft KD, Ward NC, Yang X, Considine MJ, Puddey IB, Woodman
391   RJ, Hodgson JM. 2014. Short-term effects of nitrate-rich green leafy vegetables on blood
392   pressure and arterial stiffness in individuals with high-normal blood pressure. Free Radic Biol
393   Med.77:353-362.
394   Cheitlin MD. 2003. Cardiovascular physiology-changes with aging. Am J Geriatr
395   Cardiol.12:9-13. Epub 2002/12/28.
396   Coggan AR, Leibowitz JL, Spearie CA, Kadkhodayan A, Thomas DP, Ramamurthy S,
397   Mahmood K, Park S, Waller S, Farmer M, Peterson LR. 2015. Acute Dietary Nitrate Intake
398   Improves Muscle Contractile Function in Patients With Heart Failure: A Double-Blind,
399   Placebo-Controlled, Randomized Trial. Circ Heart Fail.8:914-920.
400   Coggan AR, Peterson LR. 2016. Dietary Nitrate and Skeletal Muscle Contractile Function in
401   Heart Failure. Curr Heart Fail Rep.
402   CRAIG CL, MARSHALL AL, SJÖSTRÖM M, BAUMAN AE, BOOTH ML,
403   AINSWORTH BE, PRATT M, EKELUND U, YNGVE A, SALLIS JF, OJA P. 2003.
404   International Physical Activity Questionnaire: 12-Country Reliability and Validity. Medicine
405   & Science in Sports & Exercise.35:1381-1395.
406   DeVan AE, Johnson LC, Brooks FA, Evans TD, Justice JN, Cruickshank-Quinn C,
407   Reisdorph N, Bryan NS, McQueen MB, Santos-Parker JR, Chonchol MB, Bassett CJ, Sindler
408   AL, Giordano T, Seals DR. 2015. Effects of sodium nitrite supplementation on vascular
409   function and related small metabolite signatures in middle-aged and older adults. Journal of
410   Applied Physiology.
411   Di Lisa F, Menabò R, Canton M, Barile M, Bernardi P. 2001. Opening of the Mitochondrial
412   Permeability Transition Pore Causes Depletion of Mitochondrial and Cytosolic NAD+ and Is
413   a Causative Event in the Death of Myocytes in Postischemic Reperfusion of the Heart.
414   Journal of Biological Chemistry.276:2571-2575.
415   Eggebeen J, Kim-Shapiro DB, Haykowsky M, Morgan TM, Basu S, Brubaker P, Rejeski J,
416   Kitzman DW. 2016. One Week of Daily Dosing With Beetroot Juice Improves Submaximal
417   Endurance and Blood Pressure in Older Patients With Heart Failure and Preserved Ejection
418   Fraction. JACC Heart Fail. Epub 2016/02/15.
419   Gee LC, Ahluwalia A. 2016. Dietary Nitrate Lowers Blood Pressure: Epidemiological, Pre-
420   clinical Experimental and Clinical Trial Evidence. Curr Hypertens Rep.18:17. Epub
421   2016/01/28.
422   Gilchrist M, Winyard PG, Aizawa K, Anning C, Shore A, Benjamin N. 2013. Effect of
423   dietary nitrate on blood pressure, endothelial function, and insulin sensitivity in type 2
424   diabetes. Free Radic Biol Med. Epub 2013/02/12.
425   Gosse P. 2005. Left ventricular hypertrophy as a predictor of cardiovascular risk. J Hypertens
426   Suppl.23:S27-33.

427   Houghton D, Jones TW, Cassidy S, Siervo M, MacGowan GA, Trenell MI, Jakovljevic DG.
428   2016. The effect of age on the relationship between cardiac and vascular function. Mech
429   Ageing Dev.153:1-6. Epub 2015/11/22.
430   Jajja A, Sutyarjoko A, Lara J, Rennie K, Brandt K, Qadir O, Siervo M. 2014. Beetroot
431   supplementation lowers daily systolic blood pressure in older, overweight subjects. Nutrition
432   Research.
433   Jakovljevic DG, Trenell, M.I., MacGowan, G.A. 2014. Bioimpedance and bioreactance
434   methods for monitoring cardiac output. Best Practice & Research Clinical
435   Anaesthesiology.28:381-394.
436   Kapil V, Khambata RS, Robertson A, Caulfield MJ, Ahluwalia A. 2015. Dietary nitrate
437   provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2,
438   double-blind, placebo-controlled study. Hypertension.65:320-327.
439   Kelm M. 1999. Nitric oxide metabolism and breakdown. Biochimica et Biophysica Acta -
440   Bioenergetics.1411:273-289.
441   Kenjale AA, Ham KL, Stabler T, Robbins JL, Johnson JL, Vanbruggen M, Privette G, Yim
442   E, Kraus WE, Allen JD. 2011. Dietary nitrate supplementation enhances exercise
443   performance in peripheral arterial disease. J Appl Physiol (1985).110:1582-1591.
444   Khatri J, Mills CE, Maskell P, Odongerel C, Webb AJ. 2016. It is Rocket Science - Why
445   dietary nitrate is hard to beet! Part I: Twists and turns in the realisation of the nitrate-nitrite-
446   NO pathway. Br J Clin Pharmacol.
447   Lara J, Ashor AW, Oggioni C, Ahluwalia A, Mathers JC, Siervo M. 2015. Effects of
448   inorganic nitrate and beetroot supplementation on endothelial function: a systematic review
449   and meta-analysis. Eur J Nutr.
450   Lara J, Ogbonmwan I, Oggioni C, Zheng D, Qadir O, Ashor A, Brandt K, Mathers JC, Siervo
451   M. 2015. Effects of handgrip exercise or inorganic nitrate supplementation on 24-h
452   ambulatory blood pressure and peripheral arterial function in overweight and obese middle
453   age and older adults: A pilot RCT. Maturitas.82:228-235.
454   Lee JS, Stebbins CL, Jung E, Nho H, Kim JK, Chang MJ, Choi HM. 2015. Effects of chronic
455   dietary nitrate supplementation on the hemodynamic response to dynamic exercise. Am J
456   Physiol Regul Integr Comp Physiol.309:R459-466.
457   Lundberg JO, Gladwin MT, Ahluwalia A, Benjamin N, Bryan NS, Butler A, Cabrales P,
458   Fago A, Feelisch M, Ford PC, Freeman BA, Frenneaux M, Friedman J, Kelm M, Kevil CG,
459   Kim-Shapiro DB, Kozlov AV, Lancaster Jr JR, Lefer DJ, et al. 2009. Nitrate and nitrite in
460   biology, nutrition and therapeutics. Nat Chem Biol.5:865-869.
461   Massion PB, Feron O, Dessy C, Balligand JL. 2003. Nitric oxide and cardiac function: ten
462   years after, and continuing. Circ Res.93:388-398.
463   Mills CE, Khatri J, Maskell P, Odongerel C, Webb AJ. 2016. It is rocket science - why
464   dietary nitrate is hard to Beet! part II: further mechanisms and therapeutic potential of the
465   nitrate-nitrite-NO pathway. Br J Clin Pharmacol.
466   Mulligan AA, Luben RN, Bhaniani A, Parry-Smith DJ, O'Connor L, Khawaja AP, Forouhi
467   NG, Khaw KT. 2014. A new tool for converting food frequency questionnaire data into
468   nutrient and food group values: FETA research methods and availability. BMJ
469   Open.4:e004503. Epub 2014/03/29.
470   North BJ, Sinclair DA. 2012. The intersection between aging and cardiovascular disease. Circ
471   Res.110:1097-1108.
472   Omar SA, Webb AJ, Lundberg JO, Weitzberg E. 2016. Therapeutic effects of inorganic
473   nitrate and nitrite in cardiovascular and metabolic diseases. J Intern Med.279:315-336.
474   Pironti G, Ivarsson N, Yang J, Farinotti AB, Jonsson W, Zhang SJ, Bas D, Svensson CI,
475   Westerblad H, Weitzberg E, Lundberg JO, Pernow J, Lanner J, Andersson DC. 2016. Dietary

476 nitrate improves cardiac contractility via enhanced cellular Ca(2+) signaling. Basic Res
477 Cardiol.111:34.
478 Qadir OK, Teh J, Siervo M, Seal CJ, Brandt K. 2013. Method using gas chromatography
479 mass spectrometry (GC-MS) for analysis of nitrate and nitrite in vegetables. NUTRIHORT :
480 Nutrient management, innovative techniques and nutrient legislation in intensive horticulture
481 for an improved water quality.
482 Rastaldo R, Pagliaro P, Cappello S, Penna C, Mancardi D, Westerhof N, Losano G. 2007.
483 Nitric oxide and cardiac function. Life Sci.81:779-793. Epub 2007/08/21.
484 Siervo M, Lara J, Jajja A, Sutyarjoko A, Ashor AW, Brandt K, Qadir O, Mathers JC,
485 Benjamin N, Winyard PG, Anning C, Shore A, Gilchrist M. 2015. Ageing modifies the
486 effects of beetroot juice supplementation on 24-hour blood pressure variability: An individual
487 participant meta-analysis. Nitric Oxide.47:97-105.
488 Siervo M, Lara J, Ogbonmwan I, Mathers JC. 2013. Inorganic nitrate and beetroot juice
489 supplementation reduces blood pressure in adults: a systematic review and meta-analysis. J
490 Nutr.143:818-826. Epub 2013/04/19.
491 Tsikas D. 2000. Simultaneous derivatization and quantification of the nitric oxide metabolites
492 nitrite and nitrate in biological fluids by gas chromatography/mass spectrometry. Anal
493 Chem.72:4064-4072. Epub 2000/09/20.
494 Zamani P, Rawat D, Shiva-Kumar P, Geraci S, Bhuva R, Konda P, Doulias PT, Ischiropoulos
495 H, Townsend RR, Margulies KB, Cappola TP, Poole DC, Chirinos JA. 2015. Effect of
496 inorganic nitrate on exercise capacity in heart failure with preserved ejection fraction.
497 Circulation.131:371-380; discussion 380.
498

FIGURE LEGENDS

Figure 1: Plasma and urinary nitrate after either 7-day consumption of nitrate-rich or nitrate-depleted beetroot juice. Data presented as mean±1SE.

Figure 2: Resting (Panel A) and daily (Panel B) systolic and diastolic blood pressure (BP) measured during a one week oral consumption (End) with either nitrate-rich or nitrate-depleted beetroot juice. Data presented as mean±SEM. SBP= systolic blood pressure; DBP= diastolic blood pressure.

Figure 3: Systolic (Panel A) and diastolic (Panel B) blood pressure (BP) at rest, during incremental exercise and post-exercise recovery after one week oral consumption with either nitrate-rich or nitrate-depleted beetroot juice. Data presented as mean±SEM.

Figure 4: Heart rate (Panel A), stroke volume (Panel B), cardiac output (Panel C) and cardiac index (Panel D) at rest, during incremental exercise and post-exercise recovery after one week oral consumption with either nitrate-rich or nitrate-depleted beetroot juice. Data presented as mean±SEM.