

**Dietary nitrate does not have an effect on physical activity outcomes in healthy older adults : a randomized, cross-over trial**

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1 **DIETARY NITRATE DOES NOT HAVE AN EFFECT ON PHYSICAL**  
2 **ACTIVITY OUTCOMES IN HEALTHY OLDER ADULTS: A**  
3 **RANDOMIZED, CROSS-OVER TRIAL**

4  
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## 42 **Abbreviations**

43 BMI= body mass index; HGS= hand-grip strength; TUG= time-up-and-go; RCRT=repeated-

44 chair-rising-test; WLS=10m walking speed; NO= nitric oxide; ATP= Adenosine

45 triphosphate; PAD= peripheral arterial disease; COPD= chronic obstructive pulmonary

46 disease; BP= blood pressure; eNOS= endothelial Nitric Oxide Synthase; ROS= reactive

47 oxygen species; ECG = electrocardiography; CHO= carbohydrate; PRO= protein; FAT= fat;

48 BIA= Bioelectrical impedance analyses; FM= fat mass; FFM= fat free mass; WC= waist

49 circumference; IPAQ= International Physical Activity Questionnaire; EPIC= European

50 Prospective Investigation into Cancer and Nutrition; FFQ= Food Frequency Questionnaire;

51 RER= respiratory exchange ratio; GC-MS= gas chromatography mass spectrometry; GLM=

52 General Linear Models; HOMA-IR= Homeostatic Model of Insulin Resistance;

53 MET=Metabolic Equivalent of Task.

54

55 **ABSTRACT**

56 Dietary nitrate ( $NO_3^-$ ) ingestion appears to enhance exercise capacity and performance in  
57 young individuals whereas inconclusive findings have been reported in older people. We  
58 conducted a double-blind, cross-over randomized clinical trial in older normal weight and  
59 overweight healthy participants testing whether beetroot juice (a rich source of  $NO_3^-$ ) for one  
60 week may increase nitric oxide bioavailability via the non-enzymatic pathway and enhance 1)  
61 exercise capacity during an incremental exercise test, 2) physical capability and 3) free-living  
62 physical activity.

63 Twenty non-smoking healthy participants aged 60-75y and BMI 20.0-29.9kg/m<sup>2</sup> were  
64 included. Pre and post supplementation resting, sub-maximal, maximal and recovery gas  
65 exchanges were measured. Physical capability was measured by hand-grip strength (HGS),  
66 time-up-and-go (TUG), repeated-chair-rising-test (RCRT), and 10m walking speed (WLS).  
67 Free-living physical activity was assessed by triaxial accelerometry. Changes in urinary and  
68 plasma  $NO_3^-$  concentrations were measured by gas chromatography mass spectrometry.  
69 Nineteen participants (M/F=9/10) completed the study. Beetroot juice increased significantly  
70 both plasma and urinary  $NO_3^-$  concentrations ( $p<0.001$ ) compared to placebo. Beetroot juice  
71 did not influence resting, sub-maximal and maximal oxygen consumption during the  
72 incremental exercise test. In addition, measures of physical capability and physical activity  
73 levels measured in free-living conditions were not modified by beetroot juice ingestion.

74 The positive effects of beetroot juice ingestion on exercise performance seen in young  
75 individuals were not replicated in healthy, older adults. Whether aging represents a modifier  
76 of the effects of dietary  $NO_3^-$  on muscular performance is not known and mechanistic studies  
77 and larger trials are needed to test this hypothesis.

78  
79 **Keywords:** inorganic nitrate, nitric oxide, exercise, oxygen consumption, aging

## 80 1. INTRODUCTION

81 Aging is characterized by a progressive decline in muscle mass and strength which are risk  
82 factors for physical disability[1]. Aging is also associated with modifications of  
83 mitochondrial bioenergetics with consequent effects on muscular performance[2]. Dietary  
84 nitrate ( $NO_3^-$ ) supplementation enhances muscular efficiency in humans[3, 4], a finding  
85 which can be explained by increased nitric oxide (NO) bioavailability and the role of NO in  
86 modulating mitochondrial coupling and bioenergetics of muscular activity[5, 6]. However,  
87 the majority of  $NO_3^-$  supplementation studies have been conducted in healthy, physically  
88 active young adults[7, 8] and few studies have evaluated the effects of dietary  $NO_3^-$  on  
89 physical or muscular function in older people[9-11]. Larsen et al in 2007[12] was the first to  
90 report reduced sub-maximal  $O_2$  uptake in young healthy adults after three-day oral  
91 supplementation with potassium  $NO_3^-$ . Kenjale et al[10] observed delayed onset of  
92 claudication after three days of oral  $NO_3^-$  supplementation in older patients with peripheral  
93 arterial disease (PAD). However, subsequent studies reported contrasting results for the  
94 effects of dietary  $NO_3^-$  on exercise performance in healthy older people[9, 13] as well as in at  
95 risk populations (i.e., those with diabetes[11], heart failure[14], and chronic obstructive  
96 pulmonary disease (COPD)[15, 16]). All studies employed a double-blind randomized cross-  
97 over study design and administered beetroot juice to increase  $NO_3^-$  intake. However,  
98 differences in study duration,  $NO_3^-$  dose or assessment of exercise capability likely  
99 contributed to the observed heterogeneous responses. For example, outcomes have included  
100 sub-maximal [12, 15, 17] or maximal oxygen ( $O_2$ ) uptake [18-20] assessed with incremental  
101 standardised tests [12, 15, 21] as well as time trials [22-24] or physical capability tests [9,  
102 25], all of which were performed in controlled settings. No study has investigated the effects  
103 of dietary  $NO_3^-$  supplementation on free living physical activity.

104 We hypothesized that dietary  $NO_3^-$  supplementation would increase NO bioavailability,  
105 muscular energetics and exercise performance – with significant changes expected in sub-  
106 maximal, maximal and recovery  $O_2$  uptake – which may translate into beneficial effects on  
107 physical capability and free living physical activity. To test these hypotheses, we conducted a  
108 double-blind, cross-over, placebo controlled RCT in older healthy adults to investigate the  
109 effects of beetroot juice, chosen as a rich source of dietary  $NO_3^-$ , on physical activity  
110 outcomes measured in research ( $O_2$  uptake during incremental cycle ergometer exercise,  
111 walking speed, time-up-and-go, repeated chair rising test and hand grip strength) and free  
112 living (accelerometry) settings.

## 113 2. METHODS and MATERIALS

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

121 *2.1 Participants:* Twenty male and female, older (60-75 y) non-obese adults (BMI range:  
122 18.5 - 29.9  $kg/m^2$ ) were enrolled in the study. Participants were non-smokers and weight  
123 stable. Participants were included in the study if they did not have medical conditions or were  
124 not taking medications that might influence the study outcomes. A full list of the inclusion  
125 and exclusion criteria is provided in the **Online Supplementary Material**. Participants were  
126 asked to maintain their habitual diet and to avoid using chewing gum or mouth wash for at  
127 least 48 prior to the baseline visits (first and third visit) and during each of the one-week  
128 supplementation periods.

129 2.2 *Randomization*: A randomization list for each site was generated by a member of staff not  
130 involved in the study using [www.sealedenvelopes.com](http://www.sealedenvelopes.com). Each participant was randomized to  
131 the cross-over interventions (i.e., placebo →  $NO_3^-$  or  $NO_3^-$  → placebo). Intervention agents  
132 were dispensed at each baseline visit by two members of staff not involved in the study who  
133 had access to the stored beetroot juice and ensured the correct treatment allocation.

134 2.3 *Study Overview*: A telephone screening was performed to check eligibility according to  
135 the trial inclusion and exclusion criteria. Eligible participants were invited for a further  
136 screening visit at the research facilities including measurement of BMI, resting BP and  
137 resting 12-lead electrocardiography (ECG). Participants were asked to arrive after a 12-hour  
138 overnight fast and having avoided strenuous physical activity for three days preceding the  
139 visit. If eligible, participants were randomized to a cross-over intervention and the baseline  
140 assessment continued with the measurement of body composition, collection of blood and  
141 urine samples and assessment of physical capability. Participants then rested for one hour and  
142 consumed a meal providing approximately 300kcal (CHO=85%, PRO=3%, FAT=12%). In  
143 addition, during this one-hour rest period, participants completed a series of questionnaires to  
144 assess dietary intake and physical activity. After the one-hour rest, participants were  
145 explained the exercise test while they accustomed to the ergometer. The exercise protocol is  
146 described in **Figure S1 of the Online Supplementary Material**. After the exercise test,  
147 instructions were provided for self-administration of the nutritional intervention (14 bottles of  
148 either  $NO_3^-$  -rich or  $NO_3^-$  -depleted beetroot juice; 70ml x 2/day; Beet It, James White Ltd,  
149 UK) and asked to consume one bottle of beetroot juice each morning and evening for the  
150 subsequent 7 days. The daily dose of  $NO_3^-$  -rich (intervention) or  $NO_3^-$  -depleted (placebo)  
151 beetroot juice contained ~12mmol and ~0.003mmol of  $NO_3^-$ , respectively. Participants were  
152 provided with instructions and forms for recording wearing time of the accelerometer. This  
153 concluded Visit 1 of the trial. Participants returned to the research facilities in the morning of

154 day eight after they had completed a seven-day supplementation period. A detailed medical  
155 interview was conducted to ascertain any side effects experienced during the supplementation  
156 period. A resting 12-lead ECG was performed and, if normal, the study visit was completed  
157 by repeating the same assessments as performed during Visit 1. At the end of the second visit,  
158 participants were asked to resume their habitual diet and physical activity. After a wash out  
159 period of at least seven days the second phase (including Visits 3 and 4) was conducted  
160 similar to the first phase with the exception that participants crossed-over experimental arms  
161 i.e. consumed the other intervention agent.

162 *2.4 Body Composition:* Bioelectrical impedance analyses (BIA) (Newcastle: TANITA  
163 418MA, Tanita Ltd, Japan; Sheffield: InBody 720 Analyser, InBody Bldg, Korea) was used  
164 to assess fat mass (FM) and fat free mass (FFM). Body weight, height and waist  
165 circumference (WC) were measured using standardized protocols.

166 *2.5 Resting Blood Pressure:* Resting BP was measured in triplicate using an automated BP  
167 monitor (Omron M3, Omron Healthcare, UK) with the participant seated comfortably for 15  
168 min prior to measurement and the arm supported at the level of the heart. The recorded value  
169 was calculated as the mean of the three measurements.

170 *2.6 Physical Capability:* A battery of tests (hand grip strength (HGS), timed up and go  
171 (TUG), repeated chair rise test (RCRT) and 10m walking speed (WLS)), performed in the  
172 same order at each visit, was completed at baseline and at the end visit of each phase.  
173 Triplicate measurements of HGS were performed in both arms at baseline and after  
174 intervention using a digital dynamometer (Takei 5401, Takei, Japan). The average of six  
175 measurements was calculated. To complete the TUG, participants were asked to stand up  
176 from a chair, walk three meters at a self-selected comfortable speed, cross a line on the floor,  
177 turn around, walk back, and sit down again. The RCRT was completed using a standard chair  
178 without armrests. Participants had both arms crossed against the chest, starting from the



179 seated position and standing up (legs straight) and sitting down (full weight on the chair) and  
180 the test calculates the time required (in seconds) to complete five repeated chair stands. For  
181 the WLS, a 10-m path with a flying start was used to avoid acceleration/deceleration effects  
182 associated with starting and stopping during this assessment. The middle 6-m of this path  
183 were used for the measurement. Patients were instructed to “walk as fast as they can” and the  
184 time (in seconds) to complete the 6-m path was recorded.

185 *2.7 Objective Measurement of Free Living Physical Activity:* Participants were asked to wear  
186 a triaxial accelerometer (GT3X ActiGraph accelerometer (Pensacola, FL, USA)) above the  
187 right hip for eight consecutive days during waking hours and to remove it only for water  
188 activities (for example, swimming or bathing). Accelerometry data were collected in one-  
189 minute epochs. Non-wear time was defined as 60 min or more of consecutive zero counts.  
190 One participant experienced a device malfunction and data were excluded from subsequent  
191 analysis. Counts per minute were converted into minutes of sedentary time (less than or equal  
192 to 100 counts per min), light (100-759 counts per min), moderate (1952–5724 counts per  
193 min) and vigorous-intensity (5725+ counts per min) physical activity[26]. Physical activity  
194 energy expenditure was calculated using the Freedson approach[26].

195 *2.8 Dietary and Lifestyle Questionnaires:* The 9-item short form of the International Physical  
196 Activity Questionnaire (IPAQ) was used to record duration of four intensity levels of  
197 physical activity: 1) vigorous-intensity activity, 2) moderate-intensity activity, 3) walking,  
198 and 4) sitting. A combined total physical activity score was calculated and expressed in  
199 MET-minutes/week[27]. The EPIC Food Frequency Questionnaire (FFQ) was administered  
200 at baseline and the FETA software used to extract dietary (energy and nutrient)  
201 information[28].

202 *2.9 Exercise Test:* An incremental exercise test was performed at baseline and at the end of  
203 each intervention period to assess pulmonary gas exchange variables at rest, during sub-

204 maximal and maximal intensities and in the post-exercise recovery phase. Briefly, each  
205 participant underwent cardiopulmonary exercise testing on an electronically-braked cycle  
206 ergometer. The protocol included a five-minute resting phase followed by a 20 watts stepwise  
207 increase in workload every three minutes while they were invited to maintain a stable  
208 pedalling rate (60-70 rpm). After reaching 80 watts, participants were asked to exercise until  
209 exhaustion (ramp protocol: 10 watts/minute), which was followed by a five-minute passive  
210 recovery period. A graphical description of the protocol is described in **Figure S1 of the**  
211 **Online Supplementary Material**. Pulmonary gas exchange and ventilation were measured  
212 (Newcastle: MetaMax 3B, Cortex Biophysik, Leipzig, Germany; Ultima Cardio2,  
213 Medgraphics, St Paul, MN, USA). Heart rate (HR) was measured during all tests using  
214 cardio-thoracic impedance. Oxygen uptake ( $\dot{V}O_2$ ), minute ventilation ( $\dot{V}E$ ), carbon dioxide  
215 excretion rate ( $\dot{V}CO_2$ ), and respiratory exchange ratio (RER) were assessed.  $\dot{V}O_2$  assessed  
216 during the last minute of the incremental exercise test was recorded as  $\dot{V}O_{2peak}$ . Ventilatory  
217 threshold was calculated using the V-slope method[29].

218 *2.10 Blood and Urine Collection:* Fasting blood samples were collected at the beginning of  
219 each visit and centrifuged at 3,000rpm for 10 min at 4 °C within 30min of collection.  
220 Aliquots of plasma and serum were frozen and stored at -80 °C for subsequent analyses.  
221 Mid-stream urine samples were collected, in fasting conditions, into sterile containers and  
222 stored at -20 °C for subsequent analyses.

223 *2.11 Biomarker Analysis:* A modified version of the gas chromatography mass spectrometry  
224 (GC-MS) method proposed by Tsikas et al[30] was used to determine  $NO_3^-$  concentrations in  
225 urine and plasma samples. The protocol and validation of the modified GC-MS method have  
226 been described elsewhere[31]. This method showed good repeatability, with coefficients of  
227 variation for replicate analyses of 7.8%, 8.6% and 12.0% for saliva, urine and plasma  
228 samples, respectively.

229 2.12 *Sample size*: The primary outcome of the study was the effect of  $NO_3^-$  supplementation  
230 on  $\dot{V}O_2$  consumption during sub-maximal exercise. Data on the expected effect size were  
231 obtained from a previous cross-over design study testing the effects of incremental exercise  
232 on sub-maximal and maximal  $O_2$  consumption in young adults after a six-day nitrate  
233 supplementation[32] which showed that  $\dot{V}O_2$  during moderate exercise was  $1.53 \pm 0.12$  L·min<sup>-1</sup>  
234 and  $1.45 \pm 0.12$  L·min<sup>-1</sup> in the placebo and nitrate groups respectively. On this basis, 20  
235 participants were needed in a cross-over randomized trial to detect a difference of  $0.08 \pm 0.12$   
236 L·min<sup>-1</sup> with a power of 0.80 and alpha of 0.05.

237 2.13 *Statistical Analyses*: Repeated-Measures General Linear Models (GLM) were used to  
238 test the effect at the end of each intervention of  $NO_3^-$  supplementation on measures of  
239 exercise performance and physical capability. Treatment ( $NO_3^-$  vs placebo) was entered as a  
240 group factor (Tr) and the time points of the incremental exercise test as the repeated factor  
241 (Ti). Post-hoc comparison between treatment groups at each time point was performed using  
242 the Fisher LSD test. The area under the curve (AUC) for  $\dot{V}O_2$  consumption during the  
243 incremental exercise test was calculated at baseline and end of study using the trapezoidal  
244 method. A paired t test was used to compare differences between the two interventions for the  
245 AUCs and free living physical activity outcomes. Data were presented as means  $\pm$  SD or  
246 means  $\pm$  95% confidence intervals (95% CI). Analyses were conducted using Statistica 10 for  
247 Windows (StatSoft.Inc, Tulsa, OK, USA). Statistical significance was set at  $<0.05$ .

### 248 3. RESULTS

249 3.1 *Participants' characteristics, safety and Compliance with Interventions*: Twenty  
250 participants were randomized to the intervention. One person developed an ischemic event  
251 during the physical exercise testing performed at the second visit and he was excluded from  
252 the study (**Figure 1**). The remaining 19 participants (mean age  $64.7 \pm 3.0$  years (range: 60 - 75  
253 years)) reported no side effects apart for the expected urine discoloration related to the

254 excretion of beetroot juice pigment (beeturia). All participants reported that they consumed  
255 all the intervention drinks provided and all of them completed all the measurements included  
256 in the study protocol. This included high compliance with wearing of the accelerometer (total  
257 wear time: ~7.5-8.0 days out of maximum 8 days).

258 *3.2 Dietary Intake and Self-Reported Physical Activity:* Energy intake was  $2728 \pm 1430$   
259 kcal/day with  $47 \pm 8\%$ ,  $35 \pm 7\%$  and  $18 \pm 4\%$  of energy provided by carbohydrates, fats and  
260 protein respectively. Self-reported physical activity was again not different between the  
261 placebo and the  $NO_3^-$  arms as participants in both groups reported an average increase in total  
262 physical activity of approximately 300 METs/week ( $p=0.99$ ) (**Table 1 and Table S2 of the**  
263 **Online Supplementary Material**).

264 *3.3 Body Composition:* Mean baseline BMI was  $25.6 \pm 3.4$  kg/m<sup>2</sup> with 12 participants being in  
265 the overweight category ( $25 \leq \text{BMI} < 30$  kg/m<sup>2</sup>). Body weight was stable across the study with  
266 changes of  $0.01 \pm 0.85$  kg in the placebo and  $-0.16 \pm 0.57$  kg in the intervention group ( $p=0.51$ ).  
267 Similarly, no statistically significant between-treatment differences were found for FFM  
268 ( $0.02 \pm 1.00$  kg vs  $0.11 \pm 0.77$  kg,  $p=0.65$ ) and FM ( $-0.03 \pm 0.79$  kg vs  $0.27 \pm 0.75$  kg,  $p=0.86$ )  
269 (**Table 1 and Table S2 of the Online Supplementary Material**).

270 *3.4 Resting Blood Pressure:* Baseline resting systolic and diastolic BP ranged from 100.0 to  
271 168.0 mmHg and 62.0 to 97.0 mmHg, respectively. The decrease in systolic BP ( $-5.05 \pm 9.45$   
272 mmHg) with  $NO_3^-$  supplementation was approximately double that observed with the placebo  
273 ( $-2.64 \pm 9.04$  mmHg) but this difference was not significant ( $p=0.48$ ). Both interventions  
274 produced similar falls in diastolic BP ( $-3.70 \pm 5.59$  vs  $-3.49 \pm 6.42$  mmHg,  $p=0.90$ ) (**Table 1**  
275 **and Table S2 of the Online Supplementary Material**).

276 *3.5 Laboratory biomarkers:* Concentrations of nitrite plus nitrate ( $NO_2^- + NO_3^-$ , NOx) in  
277 plasma and urine increased substantially after  $NO_3^-$  supplementation by  $150 \pm 77\%$  and  
278  $979 \pm 488\%$  but not after the placebo intervention ( $-9 \pm 33\%$  and  $-13 \pm 34\%$ , respectively).

279 3.6 Gas-Exchange during Standardized Exercise: Nitrate supplementation had no significant  
280 effect on pulmonary gas exchange ( $O_2$  and  $CO_2$ ) measured during resting, sub-maximal,  
281 maximal and recovery phases of the incremental exercise test.  $O_2$  consumption increased  
282 linearly with the intensity of the workload and  $O_2$  consumption at exhaustion was  $1.67\pm 0.51$   
283 and  $1.64\pm 0.55$   $L\cdot min^{-1}$  following  $NO_3^-$  and placebo interventions ( $p=0.86$ ), respectively. There  
284 was a steady and comparable decline in  $O_2$  consumption during the 5-minute recovery phase  
285 with return to baseline resting values for both interventions (**Figure 3A**). The AUCs for  $O_2$   
286 consumption for both treatments were similar ( $p=0.89$ , data not showed). Similarly, weight-  
287 adjusted  $O_2$  consumption did not significantly different between the  $NO_3^-$  and placebo groups  
288 ( $p=0.99$ , **Figure S2 of the Online Supplementary Material**).  $O_2$  consumption at ventilatory  
289 threshold was similar for the  $NO_3^-$  ( $0.90\pm 0.39$   $L\cdot min^{-1}$ ) and placebo ( $0.91\pm 0.39$   $L\cdot min^{-1}$ )  
290 treatments ( $p=0.35$ ) and no differences between the two interventions were observed for  $CO_2$   
291 production, RER,  $\dot{V}E$  and HR (**Figure 3B to 3E**). Time to exhaustion was shorter following  
292 the  $NO_3^-$  intervention but the difference was not significant ( $p=0.10$ , **Figure 3F**). The  
293 adjustment of the analyses for baseline values of gas exchanges did not modify the results  
294 (data not showed). A summary of the data for each time point is provided in **Table S3 of the**  
295 **Online Supplementary Material**.

296 3.7 Physical Capability and Objective Assessment of Free Living Physical Activity: Physical  
297 performance was assessed using a battery of tests measuring strength, performance and  
298 balance.  $NO_3^-$  supplementation produced small improvements in performance for all tests but  
299 the effects were not statistically significant (**Table 2**). Similarly,  $NO_3^-$  supplementation had  
300 no significant effect on total energy physical activity or on each type of physical activity (i.e.,  
301 sedentary, light, moderate, vigorous) (**Table 3**).

## 302 4. DISCUSSION

303 *4.1 Summary of Research Findings:* This is the first study to evaluate the effects of dietary  
304  $NO_3^-$  supplementation on physical performance assessed in research settings and free-living  
305 conditions in healthy older participants. Contrary to the large body of evidence supporting a  
306 positive effect of dietary  $NO_3^-$  supplementation on exercise performance, our study showed  
307 no effects of  $NO_3^-$  supplementation on  $O_2$  consumption during sub-maximal and maximal  
308 exercise performance in older **healthy** participants. In addition, there were no significant  
309 effects of dietary  $NO_3^-$  supplementation on measures of physical capability and free-living  
310 physical activity.

311 *4.2 Comparison with Body of Evidence:* Research into the effects of dietary  $NO_3^-$  on exercise  
312 performance has been influenced by two significant events: 1) first paper published by Larsen  
313 et al in 2007[12] reporting a reduced sub-maximal  $O_2$  consumption after three-day oral  $NO_3^-$   
314 supplementation and 2) development of a  $NO_3^-$ -depleted and  $NO_3^-$ -enriched concentrated  
315 beetroot juice which has allowed the design of robust double-blind, randomized nutritional  
316 interventions[11]. **Since 2007, several RCTs have tested the effects of dietary  $NO_3^-$  on**  
317 **exercise performance in humans. A small number of these trials supplemented participants**  
318 **with pharmacological preparation (sodium or potassium  $NO_3^-$ )[3, 12, 19, 21, 33-35] whereas**  
319 **the majority of the trials used beetroot juice as a way to increase dietary  $NO_3^-$  intake[9-11, 16,**  
320 **23, 25, 36].** Most of the studies recruited mainly young, physically fit participants and only a  
321 few trials [9-11, 13, 15-17, 37, 38] have tested the effects of dietary  $NO_3^-$  in older participants  
322 (mean age range: 63 – 70 years). The first study in older participants was conducted in eight  
323 patients with PAD who received 3.5 hours before the exercise testing either 500ml of  
324 beetroot juice or orange juice[10]. The study found an increased exercise time before onset of  
325 claudication pain and time to exhaustion. The remaining studies in older participants have  
326 reported contrasting results, which may be explained by differences in the duration of  
327 supplementation (range: 2.5 hours[15] to 14 days[11]), type of population (healthy[9, 13],

328 PAD[11], COPD[15, 16], type 2 diabetes[11], heart failure[14, 17]), dose of  $NO_3^-$  (range: ~  
329 300 – ~ 700mg) or exercise test (walking test[9, 10, 16, 25], incremental exercise[10, 14],  
330 forearm exercise[13]). Overall, the results have showed a reduced responsiveness of older  
331 participants to dietary  $NO_3^-$  supplementation. Negative results were seen in healthy older  
332 participants[9] and patients with diabetes[25] and COPD[16], whereas improved exercise  
333 performance was observed in patients with heart failure[14] and PAD[10]. Our study  
334 confirmed that dietary  $NO_3^-$  supplementation for one week in older adults produced no  
335 beneficial effects on physical capability or exercise performance measured in standardized  
336 clinical settings. In addition, we reported for the first time a lack of effect of  $NO_3^-$   
337 supplementation on free living physical activity, which may entail a re-examination of the  
338 usefulness of dietary  $NO_3^-$  supplementation as a viable nutritional population strategy to  
339 enhance physical performance.

340 *4.3 Biological Mechanisms:* Dietary  $NO_3^-$  is converted to NO in a two-step reduction process  
341 proceeding via the intermediate formation of  $NO_2^-$ . The first step is performed by saprophytic  
342 bacteria with reductase activity colonizing the dorsal area of the tongue.  $NO_2^-$  is then either  
343 converted to NO in the acidic gastric environment or transported in blood and reduced  
344 enzymatically in areas of tissues with lower oxygen tension and pH where metabolic  
345 demands are higher[39]. The latter conditions are frequently encountered in areas of  
346 contracting muscles, which favour the  $NO_2^-$  conversion into NO to enhance coupling between  
347 muscle perfusion and metabolic activities[5]. The improved metabolic activity reported in  
348 previous studies appears to be related to an increased mitochondrial efficiency and/or  
349 reduction of the energetic cost of muscle contractions[6]. This raises important questions  
350 about why  $NO_3^-$  supplementation does not improve physical capability or function in older  
351 people and stimulate future studies to investigate mechanisms that may explain the reduce  
352 effects of  $NO_3^-$  supplementation on muscular performance with aging. Putative mechanisms

353 may involve altered reductase capacity to convert  $NO_3^-$  into NO or reduced effects of NO on  
354 skeletal muscle mediated by age-related changes in mitochondrial function and contractile  
355 efficiency. Whether higher doses or longer supplementation periods may overcome the  
356 alleged age-related decline in muscular response to dietary  $NO_3^-$  supplementation is currently  
357 not known.

358 *4.4 Limitations:* The small sample size and the relatively short duration of the intervention  
359 are important limitations of this study and therefore the results may require a careful  
360 interpretation. While we measured plasma  $NO_2^-$  concentrations using GCMS, due to logistic  
361 constraints it was not possible to process the samples immediately after collection to  
362 minimise  $NO_2^-$  degradation. These results are therefore unavailable. However, previous  
363 studies involving dietary  $NO_3^-$  supplementation in older participants where plasma  
364  $NO_2^-$  concentration was measured, an increase in plasma  $NO_3^-$  concentrations similar to the  
365 amount observed in this study occurred alongside a significant rise in  
366 plasma  $NO_2^-$  concentrations [40].

## 367 5. CONCLUSIONS

368 We tested for the first time the ergogenic effects of dietary  $NO_3^-$  supplementation in older  
369 participants on exercise performance and free-living physical activity and found that, overall,  
370 dietary  $NO_3^-$  supplementation had no effects. The results seem to indicate that aging may  
371 modify the muscular response to dietary  $NO_3^-$  supplementation. However, these results await  
372 confirmation in future studies with larger samples size and in targeted populations with  
373 impaired muscular performance.



374 **Author contributions**

375 M.S. is the guarantor of this work and, as such, had full access to all the data in the study and  
376 takes responsibility for the integrity of the data and the accuracy of the data analysis. M.S.  
377 and E.W. designed the study. M.S. wrote the manuscript and researched data; C.O., D.J.,  
378 D.H., C.C., A.W.A., A.R., M.R., M.K., E.W. researched data. All authors contributed to  
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525 **FIGURE LEGENDS**

526 **Figure 1:** Description of recruitment phases

527 **Figure 2:** Changes in plasma and urinary nitrate after either one-week supplementation of  
528 nitrate-rich or nitrate-depleted beetroot juice in 19 older healthy adults. Data presented as  
529 means $\pm$ 95%CI. A paired t test was applied to test differences between the two interventions  
530 at baseline and end of the study.

531 **Figure 3:** Differences in gas exchanges and heart rate after one-week supplementation with  
532 either nitrate-rich or nitrate-depleted (placebo) beetroot juice in 19 older healthy adults. Data  
533 presented as means $\pm$ 95%CI. A repeated-measure ANOVA model was applied to test  
534 differences between the two interventions at the end of each intervention.  $\dot{V}O_2$  = oxygen  
535 volume;  $\dot{V}CO_2$  = carbon dioxide volume; RER= respiratory exchange ratio;  $\dot{V}E$ = pulmonary  
536 ventilation; HR= heart rate.

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<b>Table 1: Baseline characteristics (N=19)</b>		
	<i>Means</i>	<i>SD</i>
<b>M/F</b>	9/10	
<b>Age (years)</b>	64.7	3.0
<b>BMI (kg/m<sup>2</sup>)</b>	25.6	3.4
<b>WC (cm)</b>	88.5	13.9
<b>FM (kg)</b>	22.0	6.3
<b>FFM( kg)</b>	50.2	11.5
<b>Resting Systolic BP (mmHg)</b>	127.4	16.1
<b>Resting Diastolic BP (mmHg)</b>	76.2	9.6
<b>Energy Intake (Kcal/day)</b>	2728	1431
<b>CHO (g/day)</b>	308	152
<b>FAT (g/day)</b>	107	73
<b>PRO (g/day)</b>	103	57
<b>Saturated Fat (g/day)</b>	35.6	26.5
<b>Unsaturated Fat (g/day)</b>	14.1	10.4
<b>Fibre (g/day)</b>	23.9	13.0

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N= number of participants; M= Male; F= Female; Body mass index= body mass index; WC= waist circumference; FM= fat mass; FFM= fat free mass; BP= blood pressure; CHO= carbohydrate; FAT= fat; PRO= protein;

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**Table 2:** Measures of physical capability before and after supplementation with either nitrate-rich or nitrate-depleted (placebo) beetroot juice for **one** week.

	Placebo		Nitrate		
	Baseline	End	Baseline	End	Main Effect
<b>Hand-Grip Strength (kg)</b>	28.92±9.09	29.49±9.26	29.24±9.34	29.51±9.92	0.53
<b>Time Up and Go (seconds)</b>	5.44±0.76	5.62±0.76	5.67±1.07	5.58±1.00	0.53
<b>Repeated Chair Standing (seconds)</b>	8.03±2.24	7.65±1.73	7.73±1.77	7.60±1.73	0.41
<b>10m Walking Test (seconds)</b>	2.83±0.60	2.80±0.44	2.94±0.53	2.84±0.54	0.79

Data presented as means±SD. A repeated-measure ANOVA model was applied to test differences between the two interventions at the end of each intervention in 19 older healthy adults.

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**Table 3:** Measures of free living physical activity after supplementation with either nitrate-rich or nitrate-depleted (placebo) beetroot juice measured over each one-week intervention with either placebo or nitrate.

	Placebo	Nitrate	Δ	P
<b>Total Physical Activity (kcal)</b>	3378.66±1615.62	3066.11±1274.17	-312.55 ± 904.17	0.14
<b>Average Length of Sedentary Bouts (minutes)</b>	170.15±41.57	175.73±68.76	5.57±68.73	0.72
<b>Daily Average of Sedentary Bouts (minutes)</b>	184.10 ± 194.84	136.10 ± 155.60	-48.01 ± 85.25	0.40
<b>Average Length of Sedentary Breaks (minutes)</b>	110.31±42.81	129.10±86.42	18.78±100.36	0.42
<b>Daily Average of Sedentary Breaks (minutes)</b>	331.05 ± 102.62	322.68 ± 94.62	-8.38 ± 56.28	0.79
<b>Time in Sedentary Activity (minutes)</b>	8993.68±984.47	8473.15±2139.85	-520.52±1782.42	0.21
<b>Time in Light Activity (minutes)</b>	2690.31±1194.68	2520.63±1171.47	-169.68±806.39	0.37
<b>Time in Moderate Activity (minutes)</b>	249.42±149.13	222.42±144.92	-26.94±97.83	0.24
<b>Time in Vigorous Activity (minutes)</b>	32.94±94.86	20.52±56.40	-12.42±54.82	0.19

Data presented as means±SD. Δ= difference between placebo and beetroot juice groups. A paired t test was used to compare differences between the two interventions for free living physical activity outcomes in 19 older healthy adults.

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