

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
5 **Mario SIERVO^{1*}, Clio OGGIONI¹, Djordje G JAKOVLJEVIC^{2,3}, Michael TRENELL^{2,3},**
6 **John C MATHERS^{1,3}, David HOUGHTON², Carlos CELIS-MORALES¹, Ammar W**
7 **ASHOR¹, Alan RUDDOCK⁴, Mayur RANCHORDAS⁴, Markos KLONIZAKIS⁴, Elizabeth**
8 **A WILLIAMS⁵**

9
10 ¹*Human Nutrition Research Centre, Institute of Cellular Medicine, Newcastle University,*
11 *Campus for Ageing and Vitality, Newcastle on Tyne, NE4 5PL, UK*

12 ²*Institute of Cellular Medicine, MoveLab, Newcastle University, Newcastle upon Tyne NE2*
13 *4HH, UK,*

14 ³*RCUK Centre for Ageing and Vitality, Newcastle University, Newcastle upon Tyne NE2*
15 *4HH, UK*

16 ⁴*Centre for Sport and Exercise Science, Sheffield Hallam University, Sheffield, UK*

17 ⁵*Human Nutrition Unit, Department of Oncology, Faculty of Medicine, Dentistry and*
18 *Health, University of Sheffield, SheffieldS10 2RX,UK*

19

20

21 *Corresponding author: Dr Mario Siervo (mario.siervo@ncl.ac.uk)

22

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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/m² 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. 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 ± 0.12 L·min⁻¹
234 and 1.45 ± 0.12 L·min⁻¹ 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 ± 0.12
236 L·min⁻¹ 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 ± 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 ± 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 ± 3.4 kg/m² with 12 participants being in
265 the overweight category ($25 \leq \text{BMI} < 30$ kg/m²). Body weight was stable across the study with
266 changes of 0.01 ± 0.85 kg in the placebo and -0.16 ± 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 ± 1.00 kg vs 0.11 ± 0.77 kg, $p=0.65$) and FM (-0.03 ± 0.79 kg vs 0.27 ± 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 ± 9.45
272 mmHg) with NO_3^- supplementation was approximately double that observed with the placebo
273 (-2.64 ± 9.04 mmHg) but this difference was not significant ($p=0.48$). Both interventions
274 produced similar falls in diastolic BP (-3.70 ± 5.59 vs -3.49 ± 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 ± 0.51
283 and 1.64 ± 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 ± 0.39 $L\cdot min^{-1}$) and placebo (0.91 ± 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
379 discussion and reviewed/edited manuscript.

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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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Table 1: Baseline characteristics (N=19)		
	<i>Means</i>	<i>SD</i>
M/F	9/10	
Age (years)	64.7	3.0
BMI (kg/m²)	25.6	3.4
WC (cm)	88.5	13.9
FM (kg)	22.0	6.3
FFM(kg)	50.2	11.5
Resting Systolic BP (mmHg)	127.4	16.1
Resting Diastolic BP (mmHg)	76.2	9.6
Energy Intake (Kcal/day)	2728	1431
CHO (g/day)	308	152
FAT (g/day)	107	73
PRO (g/day)	103	57
Saturated Fat (g/day)	35.6	26.5
Unsaturated Fat (g/day)	14.1	10.4
Fibre (g/day)	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		Main Effect
	Baseline	End	Baseline	End	
Hand-Grip Strength (kg)	28.92±9.09	29.49±9.26	29.24±9.34	29.51±9.92	0.53
Time Up and Go (seconds)	5.44±0.76	5.62±0.76	5.67±1.07	5.58±1.00	0.53
Repeated Chair Standing (seconds)	8.03±2.24	7.65±1.73	7.73±1.77	7.60±1.73	0.41
10m Walking Test (seconds)	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
Total Physical Activity (kcal)	3378.66±1615.62	3066.11±1274.17	-312.55 ± 904.17	0.14
Average Length of Sedentary Bouts (minutes)	170.15±41.57	175.73±68.76	5.57±68.73	0.72
Daily Average of Sedentary Bouts (minutes)	184.10 ± 194.84	136.10 ± 155.60	-48.01 ± 85.25	0.40
Average Length of Sedentary Breaks (minutes)	110.31±42.81	129.10±86.42	18.78±100.36	0.42
Daily Average of Sedentary Breaks (minutes)	331.05 ± 102.62	322.68 ± 94.62	-8.38 ± 56.28	0.79
Time in Sedentary Activity (minutes)	8993.68±984.47	8473.15±2139.85	-520.52±1782.42	0.21
Time in Light Activity (minutes)	2690.31±1194.68	2520.63±1171.47	-169.68±806.39	0.37
Time in Moderate Activity (minutes)	249.42±149.13	222.42±144.92	-26.94±97.83	0.24
Time in Vigorous Activity (minutes)	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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