

1 **Effects of dietary nitrate supplementation on the response to extremity cooling and**
2 **endothelial function in individuals with cold sensitivity. A double blind, placebo**
3 **controlled, crossover, randomised control trial.**

4
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21 **Key words:**

22 Nitrate
23 Nitric oxide
24 Microvascular
25 Beetroot
26 Non-freezing cold injury

27

28

29 **Abbreviations**

30 ACh acetylcholine

31 CVC cutaneous vascular conductance

32 MAP mean arterial pressure

33 NFCI non-freezing cold injury

34 NO• nitric oxide

35 RSNO S-Nitrosothiols

36 eNOS endothelial nitric oxide synthase

37

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42 **Abstract**

43 Individuals with cold sensitivity have low peripheral skin blood flow and skin temperature
44 possibly due to reduced nitric oxide (NO•) bioavailability. Beetroot has a high concentration
45 of inorganic nitrate and may increase NO-mediated vasodilation. Using a placebo-controlled,
46 double blind, randomised, crossover design, this study tested the hypotheses that acute
47 beetroot supplementation would increase the rate of cutaneous rewarming following a local
48 cold challenge and augment endothelium-dependent vasodilation in cold sensitive
49 individuals.

50 Thirteen cold sensitive participants completed foot and hand cooling (separately, in 15 °C
51 water for 2 minutes) with spontaneous rewarming in 30°C air whilst skin temperature and
52 cutaneous vascular conductance (CVC) were measured (Baseline). On two further separate
53 visits, participants consumed 140 ml of either concentrated beetroot juice (nitrate
54 supplementation) or nitrate-depleted beetroot juice (Placebo) 90 minutes before resting
55 seated blood pressure was measured. Endothelial function was assessed by measuring CVC

56 at the forearm, finger and foot during iontophoresis of 1% w/v acetylcholine followed by
57 foot and hand cooling as for Baseline.

58 Plasma nitrite concentrations significantly increased in nitrate supplementation compared
59 to Placebo and Baseline ($502 \pm 246 \text{ nmol.L}^{-1}$; $73 \pm 45 \text{ nmol.L}^{-1}$; $74 \pm 49 \text{ nmol.L}^{-1}$ respectively;
60 $n=11$; $P < 0.001$). Resting blood pressure and the response to foot and hand cooling did not
61 differ between conditions (all $P > 0.05$). Nitrate supplementation did not alter endothelial
62 function in the forearm, finger or foot (all $P > 0.05$) compared to Placebo.

63 Despite a physiologically meaningful rise in plasma nitrite concentrations, acute nitrate
64 supplementation does not alter extremity rewarming, endothelial function or blood
65 pressure in individuals with cold sensitivity.

66

67 **1.0 Introduction**

68 Non-freezing cold injury (NFCI) is caused by prolonged exposure to cold, and often cold and
69 wet, conditions. NFCI most commonly affects the feet, although the hands can also be
70 affected (Eglin et al. 2013). Chronic NFCI, which may last for many years, is characterised (in
71 variable combination and severity) by cold sensitivity, numbness, hyperhidrosis and
72 persistent pain which can significantly affect an individual's quality of life (Golden et al.
73 2013). NFCI has been reported in individuals following exposure to cold environments such
74 as: mountaineering and hill walking (Imray et al., 2009), diving (Laden et al., 2007), cycling
75 (Fraser and Loftus, 1979), in homeless individuals (Wrenn, 1991) and the elderly (Williams et
76 al., 2005) as well as in individuals working in cold environments, (Mills and Mills, 1993,
77 Cattermole, 1999, Golden et al., 2013).

78 Although severe NFCI can be debilitating, its pathophysiology is not fully understood and
79 therefore a definitive diagnostic tool is not available (Eglin et al., 2013). Sub-clinical forms of
80 NFCI have also been characterised in individuals frequently exposed to cold conditions for
81 short durations during recreational activities such as windsurfing, surfing and open water
82 swimming. These individuals are cold sensitive (their hands and feet are cooler than
83 "normal" individuals and they take longer to rewarm following cold exposure) but are not
84 considered to have a cold injury (Eglin, 2011). As with NFCI and primary Raynaud's
85 (Gardner-medwin et al., 2001, O'Reilly et al., 1992), this cold sensitivity is associated with a

86 reduced basal skin blood flow and a smaller increase in skin blood flow upon rewarming
87 (Davey et al., 2013, Hope et al., 2014). The resultant cooler peripheral skin temperature will
88 result in reduced skin oxygen tension (Sheffield et al., 1996, Montgomery and Horwitz,
89 1950) and may put these individuals at greater risk of cold injury on subsequent cold
90 exposure (Cattermole, 1999).

91 Animal models of NFCI have shown reduced levels of oxygen in the cooled tissues
92 (Montgomery et al., 1954) and that NFCI may be associated with a pro oxidant state (Geng
93 et al., 2015). Local cooling has been shown to inhibit endothelial nitric oxide synthase
94 (eNOS) as well as increase noradrenaline release (Hodges et al., 2006). In addition, eNOS
95 activity has been shown to be positively associated with temperature (Kellogg et al., 2008).
96 Nitric oxide (NO•) is a known vasodilator and plays a fundamental role in the control of skin
97 blood flow (Hodges et al., 2006, Minson et al., 2001). Moreover, NO• released from S-
98 nitrosohemoglobin (Stamler et al., 1997) in hypoxic environments plays a key role in
99 regulating the physiological oxygen gradient. We have previously shown that glyceryl
100 trinitrate, a NO• donor, increases the rate of rewarming following foot cooling in individuals
101 with cold sensitivity (Hope et al., 2014). However, individuals develop a tolerance to GTN
102 and show diminishing vasodilatory effects with chronic treatment (Needleman and Johnson,
103 1973). In addition, the deleterious side effects such as headaches (Hsi et al., 2005) suggests
104 that organic nitrates are not optimal long-term therapies for individuals with cold sensitivity.

105 Leafy green vegetables and particularly beetroot have a high concentration of inorganic
106 nitrate (Bryan and Hord, 2010). These vegetables are thought to be beneficial to
107 cardiovascular health due to their vasodilatory effects (Gilchrist et al., 2010) with recent
108 reports suggesting that tolerance to inorganic nitrate does not occur (as inferred by blood
109 pressure responses) for at least 28 days (Thompson et al., 2017). Inorganic nitrate can act as
110 a source of systemic NO• generation (Lundberg and Govoni, 2004). Briefly, inorganic nitrate
111 is converted to nitrite by facultative anaerobic bacteria on the dorsum of the tongue
112 (Duncan et al., 1995) with small quantities of this nitrite being converted to NO• and other
113 nitrogen oxides such as S-Nitrosothiols (RSNO) by the acidic environment of the stomach
114 (Benjamin et al., 1994). The remaining nitrite and RSNO are then absorbed into the
115 circulation where they act as a storage pool for subsequent NO• production (Lundberg and
116 Weitzberg, 2005), which is expedited in hypoxaemia (Cosby et al., 2003), such as that

117 observed in cold sensitivity (Davey et al., 2013, Montgomery and Horwitz, 1950, Sheffield et
118 al., 1996, Hope et al., 2014). This enterosalivary pathway and its purported therapeutic
119 effects have been reviewed elsewhere (Lundberg et al., 2008). Inorganic nitrate, in the form
120 of beetroot juice, improves skin blood flow (Levitt et al., 2015), microvascular function (Keen
121 et al., 2014) and lowers blood pressure (BP) in healthy individuals (Webb et al., 2008) and in
122 individuals with hypertension (Kapil et al., 2015) and peripheral arterial disease (Kenjale et
123 al., 2011). In contrast, some studies have shown no effect of nitrate supplementation on
124 vascular health markers despite increases in circulating NO• intermediates in healthy (Bahra
125 et al., 2012, Shepherd et al., 2016) and clinical populations (Gilchrist et al., 2013). However,
126 the potential for beetroot juice to offer an inexpensive, safe and potentially effective
127 intervention to improve peripheral circulation in individuals with cold sensitivity has not
128 been studied. Recently, nitrate supplementation has also been shown to lower sympathetic
129 nerve activity (Notay et al., 2017) and nitrate/nitrite has been shown to restore vascular
130 function when NOS is inhibited (Ferguson et al., 2016, Carlström et al., 2010). Therefore, as
131 cold sensitive individuals exhibit impaired vascular function possibly due to lower eNOS
132 activity and increased sympathetic drive, nitrate supplementation, and the associated
133 increase in the circulating NO• pool, might help alleviate the associated detrimental effects
134 as shown with organic nitrates (Hope et al., 2014, Anderson et al., 2002).

135 We hypothesised that compared to baseline and placebo, nitrate supplementation would
136 increase plasma nitrite concentration, the rate of cutaneous rewarming following a local
137 cold challenge and augment endothelium-dependant vasodilation in individuals with cold
138 sensitivity.

139

140 **2.0 Methods**

141 All procedures for this randomised placebo-controlled, double-blind, cross-over designed
142 trial were approved by the University of Portsmouth Science Faculty Research Ethics
143 committee (2016-107A) and all volunteers provided written informed consent prior to
144 participation. All testing took place at the Department of Sport and Exercise Science,
145 University of Portsmouth between January and March 2017 when the outdoor air
146 temperature averaged $5.8 \pm 2.9^{\circ}\text{C}$ at the time of testing (range 0°C to 10°C).

147 **2.1 Participants**

148 Participants were recruited based on their self-reported frequent exposure to cold
149 environments (e.g. winter sea swimming, sailing etc.) or often having cold hands and feet. A
150 baseline cold sensitivity test (described below) was conducted to determine whether the
151 participants had cold sensitive feet or hands. This was defined as a toe or finger skin
152 temperature less than 32°C prior to the cold water immersion and after 5 minutes of
153 rewarming in 30°C air (House et al., 2015, Eglin et al., 2013). Exclusion criteria included;
154 diagnosis of a prior freezing injury, peripheral vascular disease, thalassaemias affecting
155 haemoglobin and / or hepatitis B.

156 All participants were non-smokers (for at least 1 year). Participants refrained from
157 consuming food high in nitrate the day before testing and were asked to keep a food diary
158 for the 24 hours before their first visit to the laboratory and to replicate this prior to each
159 visit. Participants abstained from alcohol for 24 hours and caffeine for 3 hours prior to
160 testing. The participants also refrained from using any antibacterial mouth wash for 7 days
161 prior to each test as this has been shown to reduce the concentration of oral bacterial that
162 are responsible for the reduction of nitrate to nitrite (Govoni et al., 2008). Female
163 volunteers were asked about their menstrual cycle to determine whether they were in the
164 follicular or luteal phase or whether they were peri- or post-menopausal. However, the
165 phase of the menstrual cycle was not controlled for since reproductive hormone status does
166 not affect the responses to local cooling (Lunt and Tipton, 2014), thermal perception (Lunt
167 and Tipton, 2014) or iontophoresis of acetylcholine (Ketel et al., 2009).

168 **2.2 Protocol**

169 The participants attended the laboratory on three separate occasions at the same time of
170 day to reduce any circadian effects. On arrival at the laboratory, resting seated blood
171 pressure was measured using an automated blood pressure monitor (Omron HEM-705C,
172 Omron, Milton Keynes, UK) with the average of the final three measurements from the
173 brachial artery being recorded and used to calculate mean arterial pressure (MAP). On the
174 first visit (Baseline) the participants then undertook a cold sensitivity test (see below) and if
175 they were classified as cold sensitive, a venous blood sample was taken for measurement of

176 plasma nitrate and nitrite using the ozone chemiluminescence technique (Sievers NOA 280;
177 Analytix Ltd, Durham, UK) using a protocol adapted from Bateman et al. (2002).

178 One and a half hours before arriving at the laboratory for the second and third visit, which
179 were separated by a wash-out period of at least 7 days, the participants consumed either
180 140 ml of concentrated beetroot juice (nitrate supplementation; 11.9 mmol nitrate; Beet it,
181 James White Drinks Ltd., Ashbocking, UK) or nitrate-depleted concentrated beetroot juice as
182 a placebo (Placebo; 0.02 mmol nitrate; Beet it, James White Drinks Ltd., Ashbocking, UK).
183 This dose has been used in previous studies (Shepherd et al., 2016) and results in peak
184 nitrite concentrations between two and three hours (Wylie et al., 2013) coinciding with the
185 testing. The placebo was similar to the nitrate-rich beetroot juice in taste, colour, texture,
186 appearance, odour and packaging (Gilchrist et al., 2014). On arrival at the laboratory the
187 participants' blood pressure was measured, as described above, and a venous blood sample
188 was taken for measurement of plasma nitrate and nitrite. Following this, participants
189 undertook an endothelium function test followed by a cold sensitivity test of their foot and
190 hand (both described below).

191 **2.3 Cold sensitivity test**

192 The cold sensitivity test used in this study has been described in detail elsewhere (Eglin et
193 al., 2013, Hope et al., 2014). Participants entered a climatic chamber controlled at an air
194 temperature of 30.3 (0.4) °C, removed their shoes and socks, and rested in a semi-
195 recumbent position for 15 minutes. They then exercised on a cycle ergometer (874E,
196 Monark, Sweden) for 12 minutes at an external work rate of 50 W (previously shown to
197 improve the reliability of the test, (Eglin et al., 2013). Following the 12 minutes cycling, the
198 participant then rested in a semi-recumbent position for 5 minutes whilst resting toe skin
199 temperature and blood flow were recorded.

200 The participant's left foot was then placed in a plastic bag and immersed in a water bath
201 stirred and maintained at 15.1 ± 0.2 °C to the point of their mid-malleoli for 2 minutes.
202 After the immersion period, the plastic bag was removed and rewarming monitored for 10
203 minutes whilst the participant remained resting in a semi-recumbent position.

204 The participant then rested in a seated position for 5 minutes whilst finger skin temperature
205 and blood flow was recorded. The participant, still seated, placed their left hand in a plastic
206 bag and immersed it to the level of the wrist in water at 15.0 ± 0.2 °C for 2 minutes. After
207 the immersion period, the plastic bag was removed and rewarming monitored for 10
208 minutes whilst the participant remained resting in a seated position with their arm
209 supported by an arm rest.

210 2.3.1 *Measurements*

211 Skin blood flow was measured using a laser Doppler probe (VP1T / 7, Moor Instruments, UK)
212 placed on the Great toe pads during foot immersion and on the pads of the thumbs during
213 hand immersion. Skin blood flow was analysed using minute averages before, during and
214 after immersion (rewarm period) and expressed as cutaneous vascular conductance (CVC =
215 skin flux/MAP; flux:mmHg⁻¹). CVC was analysed between conditions at the following time
216 points: pre immersion, 5 and 10 min of rewarming.

217 Skin temperature was measured using an infrared camera (A320G, FLIR systems, UK)
218 according to the guidelines described in (Moreira et al., 2017). The camera was positioned
219 1.0 m away from the sole of the foot and 0.7 m away from the palm of the hand and
220 recorded using the spot analysis function on the FLIR software (FLIR systems, UK) prior to
221 immersion and every minute during the 10 minute rewarming periods. Great toe, coldest
222 toe, mean toe, thumb and mean finger skin temperature were analysed between conditions
223 at the following time points: pre immersion, 5 and 10 min of rewarming. The coefficient of
224 variation in our laboratory for the cold sensitivity test is 2.7% for the finger skin
225 temperatures and 8.7% for the toe skin temperatures (unpublished data, $n=13$).

226 Blood pressure was measured on the right arm prior to foot and hand immersion and at the
227 end of both rewarming periods using an automated blood pressure monitor (Omron HEM-
228 705C, Omron, Milton Keynes, UK) for calculation of MAP.

229 Thermal sensation and comfort were measured using 20 cm scales (0 = very
230 cold/uncomfortable; 10 = neutral; 20 = very hot/comfortable; modified from Zhang et al.
231 (2004)) and recorded prior to immersion, during immersion and every 2 minutes of the
232 rewarming period. Pain sensation was recorded using a subjective numerical rating scale

233 (NRS) for pain (0 no pain, 10 unimaginable, unspeakable pain; (Ferreira-Valente et al., 2011))
234 at the same time points.

235 **2.4 Endothelium function test**

236 Following an acclimation period of at least 20 minutes, acetylcholine (ACh) was delivered
237 transdermally using iontophoresis to three sites in the following order: volar aspect of the
238 left forearm, middle phalanx of the middle finger of the left hand and dorsal aspect of the
239 left foot as previously described (Maley et al. 2017). Briefly, a perspex ring containing the
240 anode was attached to the skin site with the cathode attached using a gel pad at the wrist or
241 ankle. Both electrodes were connected to a battery powered iontophoresis controller (MIC
242 2, Moor Instruments, UK). The anode chamber (8 mm inner diameter) was filled with
243 approximately 0.5 mL of ACh. ACh was dissolved into sterile water for injection (Braun,
244 Melsungen, Germany) to yield a concentration of 1%. The protocol consisted of eight pulses:
245 four pulses of 25 μ A followed by a single pulse of 50 μ A, 100 μ A, 150 μ A and a final pulse of
246 200 μ A applied for 20 seconds with 60 second intervals between each pulse where no
247 current was applied. After an interval of 5 minutes the protocol was repeated on the next
248 skin site. The tests were conducted at a room temperature of 23.2 (0.4) °C.

249 *2.4.1 Measurements*

250 Skin blood flow was measured using a laser Doppler probe (VP1T / 7, Moor Instruments, UK)
251 connected to a laser Doppler perfusion monitor (moor VMS-LDF, Moor Instruments, UK).
252 Flux data from the laser Doppler and iontophoresis controller was recorded using a data
253 acquisition system and software (Powerlab and LabChart 7, AD Instruments, Australia). The
254 laser Doppler probe was placed into the perspex ring used for iontophoresis on the forearm,
255 finger, dorsal foot and on the corresponding site on the contra-lateral limb. Skin blood flow
256 responses were expressed as CVC.

257 Average skin blood flow in response to iontophoresis of ACh was calculated over the final 20
258 seconds of the interval between successive pulses and between 40 to 60 seconds after the
259 final pulse (Maley et al., 2017). These responses were expressed as absolute CVC as baseline
260 CVC did not differ between conditions for any site. ED₅₀, expressed as 95 % confidence
261 intervals, was calculated using GraphPad (Version 5, USA). Maximum skin blood flow and

262 area under the curve (AUC) were calculated for each participant. The point at which the skin
263 blood flow was at a maximum point was not always identified following the final pulse
264 therefore maximum skin blood flow was taken from wherever it was highest. Skin
265 temperature was measured adjacent to the iontophoresis site using a skin thermistor
266 (Grants Instruments, Cambridge) and recorded on a data logger (Grants Instruments,
267 Cambridge). Blood pressure was measured on the contralateral arm to iontophoresis using
268 an automated blood pressure monitor (Omron HEM-705C, Omron, Milton Keynes, UK)
269 before and after each ACh dose response curve for calculation of MAP.

270 **2.5 Sample size and randomisation**

271 An *a priori* sample size calculation was performed based on data from our previous study on
272 the effect of GTN in cold sensitive individuals (Hope et al. 2013) which showed a 4.2 ± 3.0 °C
273 change in skin temperature 10 minutes post immersion during the cold sensitivity test. For
274 90% power and an α -level of 5% (two tailed) it was calculated that 13 participants were
275 required.

276 A computer programme generated a random sequence that was used to assign each
277 participant to begin the trial in one of two arms. Participants were then supplied with the
278 requisite juice (in a sealed, opaque envelope prepared by individuals not involved in the
279 trial) which was counter-balanced and blinded from the participants and researchers.

280 **2.6 Data analysis**

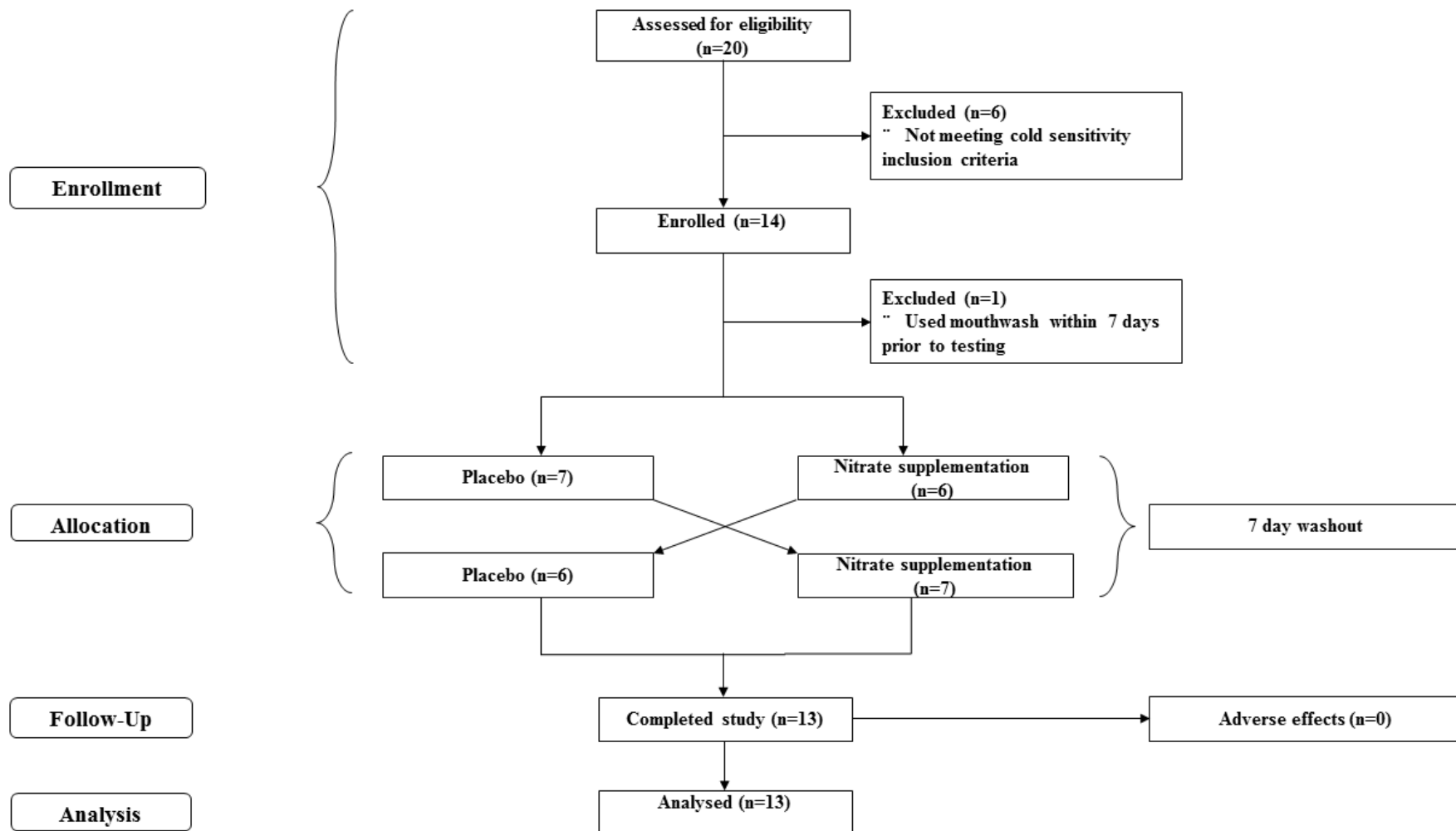
281 As with other studies using this technique (Maley et al., 2017) some individuals had high
282 skin resistance which meant that it was not possible to deliver all of the current pulses in
283 each skin site for all participants. Therefore, only those who were able to receive the first
284 pulse of iontophoresis were included in analyses. Where an incomplete current response
285 curve was delivered (due to high skin resistance at the higher currents), the number of
286 pulses used in the analysis was the same within individual for both conditions. A blood
287 sample was not obtained from two participants due to technical difficulties and therefore
288 $n=11$ for Baseline and $n=12$ for Placebo and nitrate supplementation for plasma nitrate and
289 nitrite concentrations.

290 Assumption of normal distribution of data was assessed using descriptive methods
291 (skewness, outliers, and distribution plots) and inferential statistics (Shapiro–Wilk test).
292 Where normality was not met, nonparametric tests were performed. For the cold sensitivity
293 test, statistical differences were assessed using repeated measures ANOVAs (condition
294 [Baseline, Placebo, nitrate supplementation] * time [pre immersion, 5 min, 10 min]) for toe
295 skin temperatures, thumb and Great toe skin blood flows. Finger skin temperatures, thermal
296 comfort, thermal sensation and pain were analysed using Friedman tests. For the
297 endothelial function test, maximum CVC, AUC and skin temperature were analysed using
298 ANOVAs (condition [Baseline, Placebo, nitrate supplementation] * skin site [forearm, finger,
299 foot]). ED₅₀ values were analysed using paired samples t-test. Plasma nitrate and nitrite
300 concentrations were analysed using a one way ANOVA. Where appropriate, post-hoc tests
301 were conducted using pairwise comparisons with Bonferroni corrections. Data are
302 presented as mean (SD), unless otherwise stated. Statistical analysis was performed on SPSS
303 version 22 (Chicago, IL) and statistical difference was accepted as $P < 0.05$.

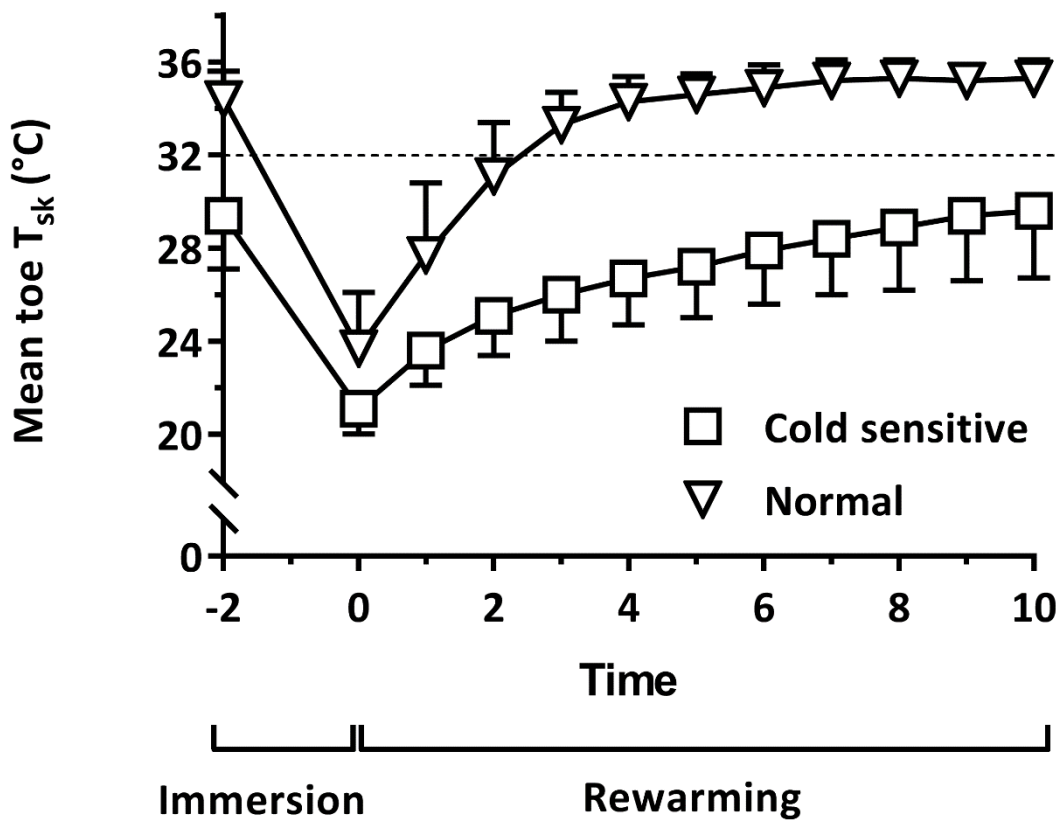
304 **3.0 Results**

305 Twenty volunteers (10 women, 10 men) gave written informed consent to participate in this
306 trial. Following the baseline cold sensitivity test, 6 individuals (5 women, 1 man; age $25.8 \pm$
307 4.2 y; height 1.71 ± 0.13 m; body mass 77.7 ± 17.7 kg) were withdrawn from the trial as they
308 did not meet the skin temperature requirements to be classified as cold sensitive (House et
309 al., 2015) (Figure 1). Fourteen individuals (Figure 1a) were randomised to start in either the
310 nitrate supplementation arm or the placebo arm. One individual was subsequently
311 withdrawn following randomisation (nitrate supplementation first, placebo second) due to
312 use of mouthwash during the intervention period. Therefore, thirteen cold sensitive
313 individuals completed the study (4 women, 9 men; age 34.5 ± 13.2 y; height 1.77 ± 0.07 m;
314 body mass 85.0 ± 15.9 kg). Consistent with our previous findings (Shepherd et al., 2016) the
315 beetroot juice was well tolerated and no adverse events (other than beeturia and
316 discoloured stools) were reported, neither could the participants identify whether they had
317 ingested the placebo or beetroot juice.

318



320 Figure 1A



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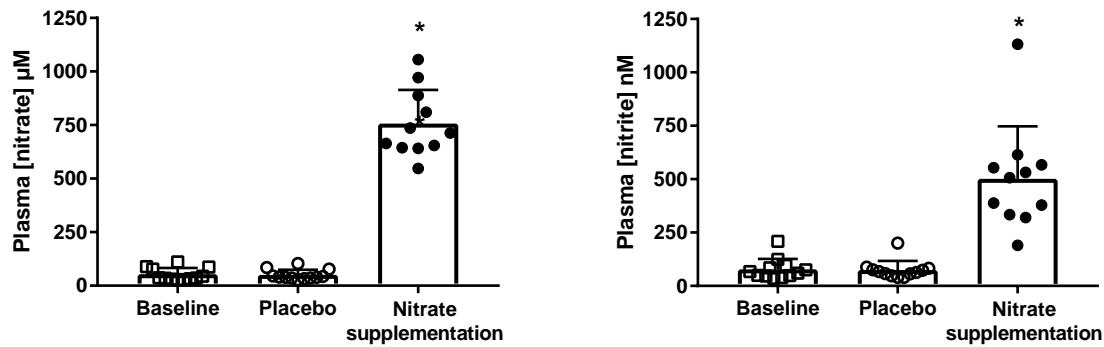
322 Figure 1B

323 **Figure 1.** Participant flow through the trial (A) and average (SD) mean toe skin temperature
324 during the Baseline cold sensitivity test in individuals with ($n=13$) and without cold
325 sensitivity ($n=6$) (B). The dotted line represents the cut off skin temperature (T_{sk}) pre-
326 immersion (-2 minutes) and at 5 minutes of rewarming used for determining cold sensitivity.

327 *4.1 Plasma nitrate and nitrite concentrations*

328 There was a statistically significant rise in plasma nitrate (Baseline, $53.1 \pm 29.4 \mu\text{M}$; Placebo,
329 $50.5 \pm 24.3 \mu\text{M}$; nitrate supplementation, $756.8 \pm 175.2 \mu\text{M}$; $F_{(2, 20)} = 198.1$, $P < 0.001$) and
330 nitrite (Baseline, $73.7 \pm 48.8 \text{ nM}$; Placebo, $73.5 \pm 44.5 \text{ nM}$; nitrate supplementation, $501.5 \pm$
331 245.8 nM ; $F_{(2, 20)} = 33.6$, $P < 0.001$) following nitrate supplementation when compared to
332 Placebo and Baseline (Figure 2).

333



334

335 **Figure 2.** Mean (SD) and individual plasma nitrate (A) and nitrite (B) concentrations in the
 336 Baseline, Placebo and nitrate supplementation conditions (n=11). * $P < 0.001$ significantly
 337 different from Placebo and Baseline.

338

339 4.2 Cold sensitivity test

340 There was no effect of nitrate supplementation on skin blood flow in the Great toe ($F_{(2, 24)} =$
 341 1.31, $P = 0.289$), or thumb ($F_{(2, 24)} = 0.42$, $P = 0.660$; Figure 3) when compared to Placebo or
 342 Baseline.

343 Skin blood flow was significantly different across time for the Great toe ($F_{(2, 24)} = 6.79$, $P =$
 344 0.005) with a lower CVC observed at 5 min rewarming compared to pre-immersion ($P =$
 345 0.002; Figure 3A). Thumb skin blood flow also differed across time ($F_{(2, 24)} = 9.39$, $P = 0.001$)
 346 with CVC being lower at 5 and 10 min of rewarming compared to pre-immersion ($P = 0.025$
 347 and $P = 0.016$ respectively; Figure 3C).

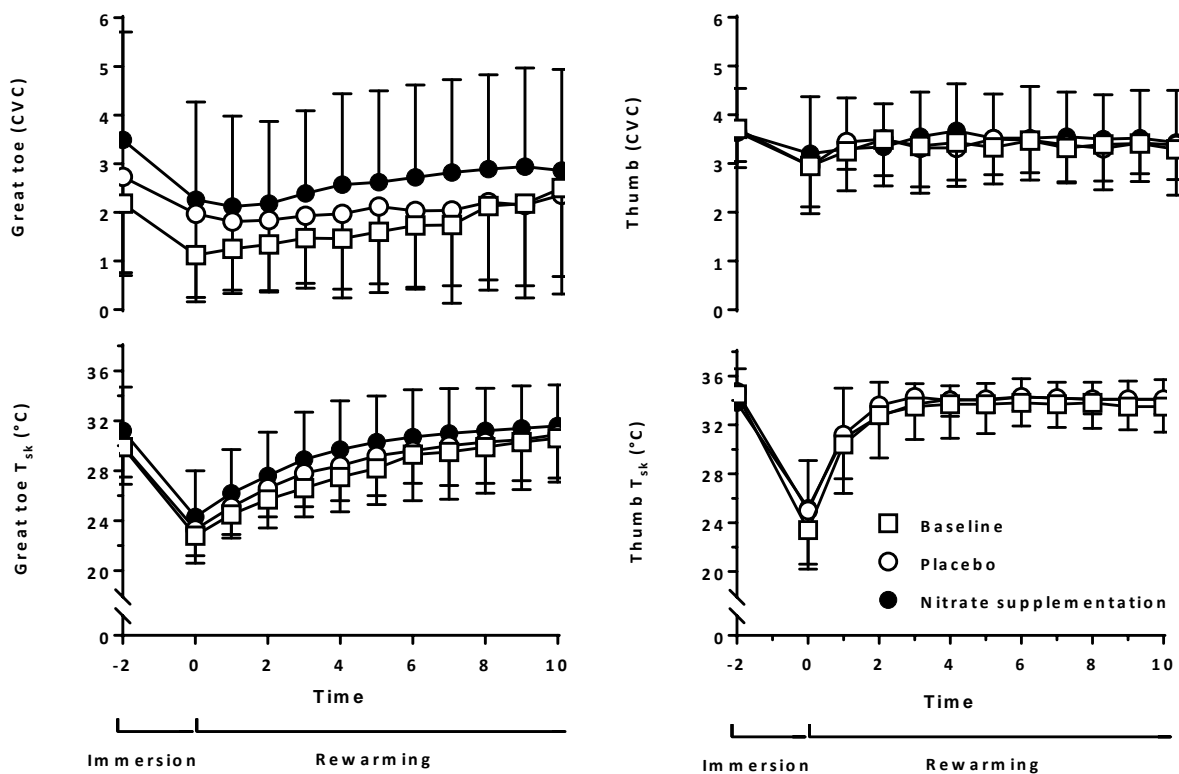
348 There was no interaction between time and condition for skin blood flow in either the Great
 349 toe ($F_{(4, 48)} = 1.58$, $P = 0.195$) or thumb ($F_{(4, 48)} = 0.62$, $P = 0.650$) when nitrate
 350 supplementation was compared to Placebo and Baseline (Figure 3 and Table 1).

351 There was no effect of nitrate supplementation on skin temperature of the Great toe ($F_{(2, 24)}$
 352 $= 0.70$, $P = 0.51$), coldest toe ($F_{(2, 24)} = 0.81$, $P = 0.46$), mean toe ($F_{(2, 24)} = 0.61$, $P = 0.55$), or
 353 mean finger (pre immersion: $X^2_{(2)} = 2.57$, $P = 0.27$, 5 minutes rewarming: $X^2_{(2)} = 2.63$, $P =$
 354 0.27, or 10 minutes rewarming: $X^2_{(2)} = 2.47$, $P = 0.29$).

355 Skin temperature was significantly different across time for the Great toe ($F_{(2, 24)} = 14.8$, $P <$
 356 0.001), coldest toe ($F_{(2, 24)} = 18.8$, $P < 0.001$) and mean toe ($F_{(2, 24)} = 35.2$, $P < 0.001$). Skin

357 temperature after 5 minutes of rewarming was colder than pre immersion ($P = 0.028$; $P =$
 358 0.001 $P < 0.001$ respectively) but had returned to pre-immersion temperatures after 10
 359 minutes ($P = 0.318$; $P = 1.00$; $P = 1.00$ respectively).

360 There was no interaction between time and condition for skin temperature (Great toe: $F_{(4,$
 361 $48) = 1.60$, $P = 0.19$; coldest toe $F_{(4, 48) = 1.81$, $P = 0.14$; mean toe $F_{(4, 48) = 0.81$, $P = 0.52$)
 362 when nitrate supplementation was compared to Placebo and Baseline (Figure 3 and Table



363 1).

364

365 **Figure 3.** Skin blood flow and temperature during the cold sensitivity test of the foot and
 366 hand in the Baseline, Placebo and nitrate supplementation conditions. Mean (SD) cutaneous
 367 vascular conductance (CVC) in the Great toe (A) and thumb (B) and skin temperature (T_{sk}) in
 368 the Great toe (C) and thumb (D) are shown ($n = 13$).

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Table 1. Mean (SD) toe, coldest toe and mean finger skin temperature (T_{sk}) during the cold sensitivity test in the Baseline, Placebo and nitrate supplementation conditions ($n = 13$).

		Rewarming		
		Pre immersion	5 minutes	10 minutes
Mean toe T_{sk} (°C)	Baseline	29.4 ± 2.4	27.2 ± 2.3	29.6 ± 3.1
	Placebo	30.0 ± 3.2	28.7 ± 4.0	30.3 ± 3.9
	Nitrate supplementation	30.8 ± 3.5	29.0 ± 3.7	30.7 ± 3.4
Coldest toe T_{sk} (°C)	Baseline	28.2 ± 2.3	25.8 ± 1.9	28.5 ± 3.2
	Placebo	28.6 ± 3.3	27.6 ± 4.2	29.2 ± 4.2
	Nitrate supplementation	29.6 ± 3.6	28.1 ± 3.4	29.6 ± 3.4
Mean finger T_{sk} (°C)	Baseline	34.3 ± 1.7	33.1 ± 3.3	33.0 ± 3.0
	Placebo	34.6 ± 1.4	33.2 ± 3.1	33.6 ± 2.3
	Nitrate supplementation	34.5 ± 1.2	33.7 ± 1.8	34.0 ± 1.9

There were no differences in thermal sensation, thermal comfort or pain votes at any time point during the cold sensitivity test between conditions (Table 2).

387 **Table 2.** Thermal sensation, thermal comfort and pain in the foot and hand during the cold
 388 sensitivity test in the Baseline, Placebo and nitrate supplementation conditions (n=13).
 389 Average rewarm is the mean vote over the last 8 minutes of rewarming.

390

		Condition	Pre immersion	During immersion	Immediately after immersion	Average rewarm
Foot	Thermal sensation	Baseline	12.0 ± 2.2	4.9 ± 2.0	6.8 ± 2.7	10.0 ± 2.1
		Placebo	12.2 ± 2.5	4.5 ± 1.5	6.3 ± 1.9	10.3 ± 1.9
		Nitrate supplementation	13.3 ± 2.1	4.8 ± 1.9	6.5 ± 1.7	10.0 ± 1.4
	Thermal comfort	Baseline	14.7 ± 1.9	9.5 ± 4.6	10.9 ± 4.4	13.1 ± 2.9
		Placebo	13.4 ± 3.3	8.5 ± 3.0	10.9 ± 4.1	13.6 ± 3.1
		Nitrate supplementation	13.4 ± 3.7	8.5 ± 3.8	9.9 ± 3.8	12.8 ± 3.0
	Pain	Baseline	0.8 ± 1.0	0.4 ± 0.7	0.1 ± 0.3	0 ± 0
		Placebo	0.5 ± 0.8	0.2 ± 0.6	0 ± 0	0 ± 0
		Nitrate supplementation	0.9 ± 1.1	0.5 ± 0.8	0.1 ± 0.3	0 ± 0
Hand	Thermal sensation	Baseline	12.7 ± 2.4	4.5 ± 1.6	6.3 ± 2.9	11.0 ± 2.7
		Placebo	12.7 ± 2.0	4.4 ± 1.5	5.9 ± 1.9	11.0 ± 2.1
		Nitrate supplementation	11.8 ± 2.0	5.2 ± 1.8	6.5 ± 2.4	11.5 ± 2.1
	Thermal comfort	Baseline	14.6 ± 2.3	7.8 ± 3.8	8.5 ± 4.6	12.8 ± 2.3
		Placebo	14.0 ± 3.2	8.7 ± 3.7	10.3 ± 4.0	13.5 ± 3.0
		Nitrate supplementation	14.3 ± 3.1	7.9 ± 3.9	9.7 ± 4.1	13.2 ± 2.5
	Pain	Baseline	0.9 ± 0.9	0.7 ± 0.9	0.1 ± 0.3	0 ± 0
		Placebo	0.9 ± 1.2	0.3 ± 0.6	0.0 ± 0.8	0 ± 0
		Nitrate supplementation	0.8 ± 1.1	0.5 ± 1.0	0.1 ± 0.3	0 ± 0

391 *4.3 Endothelial function*

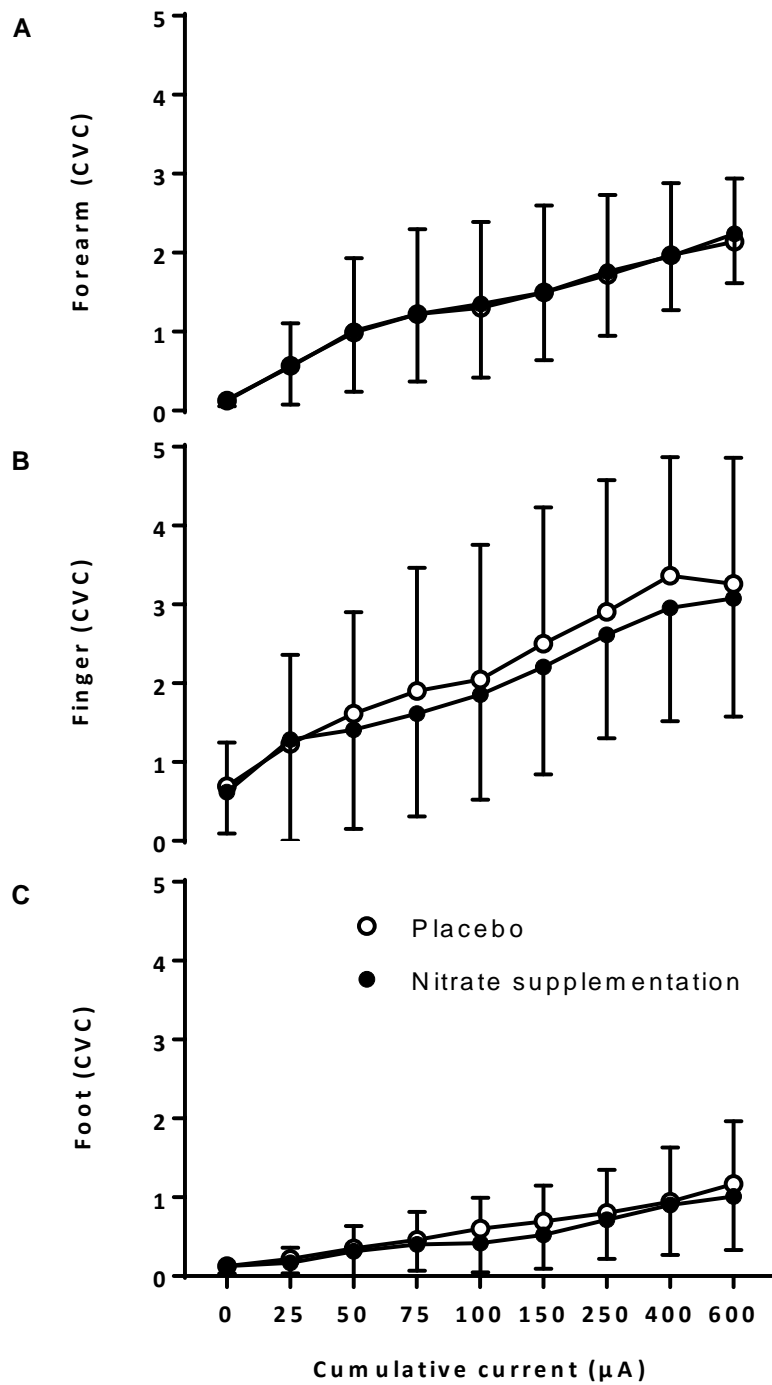
392 Skin temperature during ACh iontophoresis was similar in both the Placebo and nitrate
 393 supplementation conditions ($F_{(1, 11)} = 0.30, P = 0.59$; $F_{(2, 22)} = 0.54, P = 0.58$; Table 3). As

394 expected skin temperature was different between skin sites, with the foot being 3.6°C
 395 colder than the forearm and 2.6°C colder than the finger ($F_{(2, 22)} = 14.7, P < 0.001$; Table 3).

396 Average CVC prior to iontophoresis was similar between conditions, for the forearm (n=12,
 397 0.13 ± 0.08 flux.mmHg⁻¹, $P = 0.59$), the finger (n=12, 0.66 ± 0.53 flux.mmHg⁻¹, $P = 0.60$) and
 398 the foot (n = 13, 0.13 ± 0.09 flux.mmHg⁻¹, $P = 0.99$). There was no effect of nitrate
 399 supplementation on the vasodilatory dose response to transdermal delivery of ACh in the
 400 forearm, finger or foot (Figure 4). Maximum skin blood flow ($F_{(1, 11)} = 0.01, P = 0.92$) and
 401 AUC ($F_{(1, 11)} = 0.32, P = 0.59$) were similar between Placebo and nitrate supplementation
 402 (Table 3) and no significant interactions were observed (maximum blood flow: $F_{(2, 22)} = 1.84,$
 403 $P = 0.18$; AUC: $F_{(2, 22)} = 0.66, P = 0.53$). No differences were seen in ED₅₀ for the forearm ($P =$
 404 0.63), finger ($P = 0.62$) or foot ($P = 0.30$) between Placebo and nitrate supplementation
 405 (Table 3). However there was a difference between skin sites in the magnitude of the
 406 vasodilation to ACh (maximum skin blood flow: $F_{(2, 22)} = 19.7, P < 0.001$; AUC: $F_{(2, 22)} = 14.3, P$
 407 < 0.001 ; Figure 4 and Table 3).

408 **Table 3.** Skin blood flow response to iontophoresis of ACh on the forearm, finger and foot in
 409 the Placebo and nitrate supplementation conditions. Data are given as mean \pm SD for
 410 maximum CVC, area under the curve (AUC) and skin temperature (T_{sk}) and 95% confidence
 411 intervals are given for the ED₅₀.

		<i>n</i>	Maximum (CVC)	ED ₅₀ (μA)	AUC	T _{sk} (°C)
Forearm	Placebo	13	2.0 \pm 1.0	75.9 to 118.8	9.6 \pm 7.2	30.2 \pm 1.2
	Nitrate supplementation	13	2.3 \pm 0.7	69.2 to 111.1	10.6 \pm 5.3	30.1 \pm 1.0
Finger	Placebo	12	3.3 \pm 1.6	73.4 to 109.3	16.8 \pm 11.1	29.5 \pm 3.3
	Nitrate supplementation	12	3.1 \pm 1.5	67.3 to 102.8	15.1 \pm 9.3	28.8 \pm 3.3
Foot	Placebo	13	1.2 \pm 0.8	105.0 to 147.6	4.7 \pm 3.1	26.5 \pm 1.9
	Nitrate supplementation	13	1.0 \pm 0.7	119.3 to 167.7	4.0 \pm 2.8	26.6 \pm 2.0



412

413

414 **Figure 4.** Cutaneous vascular conductance following iontophoresis of ACh on the forearm
 415 (A), finger (B) and foot (C) in the Placebo and nitrate supplementation conditions ($n = 13$
 416 participants), for finger CVC, $n = 12$ at cumulative current 400 and 600 μA).

417 *4.4 Resting blood pressure*

418 There was no effect of nitrate supplementation on systolic blood pressure (Baseline, $123 \pm$
419 12 mmHg, Placebo 121 ± 9 mmHg, nitrate supplementation 124 ± 13 mmHg; $F_{(2, 22)} = 0.87$, P
420 $= 0.43$), diastolic blood pressure (Baseline, 79 ± 10 mmHg, Placebo 74 ± 10 mmHg, nitrate
421 supplementation 76 ± 11 mmHg; $F_{(2, 22)} = 2.81$, $P = 0.08$) or mean arterial blood pressure
422 (Baseline, 94 ± 11 mmHg, Placebo 90 ± 9 mmHg, nitrate supplementation 92 ± 11 mmHg; F
423 $_{(2, 22)} = 2.29$, $P = 0.13$) when compared to Placebo and Baseline.

424 **5.0 Discussion**

425 This is the first study to examine the effects of nitrate supplementation in the form of
426 concentrated beetroot juice on extremity rewarming following a cold stimulus in individuals
427 with cold sensitivity. Contrary to our hypotheses, acute nitrate supplementation did not
428 alter extremity rewarming, endothelial function, blood pressure, pain or thermal comfort
429 and sensation. This was despite a physiologically meaningful rise in plasma nitrite
430 concentrations in the nitrate supplementation condition (Figure 2).

431 Plasma nitrate and nitrite increased by $703.6 \mu\text{M}$ and 423.5 nM respectively following
432 ingestion of beetroot juice containing 11.9 mmol of nitrate. This change from baseline in
433 plasma nitrite concentration following nitrate supplementation is similar to that reported in
434 other studies with similar supplementation regimes such as Webb et al. (2008) and Wylie et
435 al. (2013) who report changes of 180 nM and 395 nM respectively. We would note that due
436 to our protocol design (venous blood samples were taken 1.5 hours post ingestion which is
437 approximately 1 hour prior to peak plasma nitrate and nitrite concentrations (Wylie et al.,
438 2013)) we cannot preclude that nitrate and nitrate values maybe higher than what we have
439 reported. This was done in order to enable the primary outcomes to be measured at the
440 time of peak plasma nitrite concentration.

441 There was no effect of nitrate supplementation on peripheral blood flow and skin
442 temperature following exposure to a cold stimulus when compared to Placebo or Baseline
443 (Figure 3 and Table 1). As a consequence nitrate supplementation did not improve thermal
444 sensation, thermal comfort or reduce the associated pain with the cold stimulus when
445 compared to Placebo or Baseline (Table 2). These findings were in contrast to our previous
446 study which showed that organic nitrate (GTN spray) increased peripheral blood flow and
447 skin temperature in drug naive individuals with cold sensitivity (Hope et al., 2014). The

448 disparity between the findings in our two studies is likely due to any combination of the
449 differences between inorganic nitrate and organic nitrates, such as their pharmacokinetics,
450 pharmacodynamics and bio-activation (Janero et al., 2004, Gilchrist et al., 2011, Omar et al.,
451 2012). Elucidating which of these differences were responsible for the lack of effect in
452 peripheral blood flow in the current study requires further investigation. Irrespective of
453 which of these differences are responsible for the lack of effect seen in this trial, it is difficult
454 to compare organic and inorganic nitrates as NO• donors. For instance, 80-90% of GTN is
455 metabolised by liver (Münzel et al., 2005) whereas, the oral microflora within the mouth are
456 an essential component for the processing of inorganic nitrite production and subsequent
457 NO• production (Duncan et al., 1995). These differences, among others are reviewed
458 elsewhere by Münzel et al. (2005) and Omar et al. (2012).

459 Elevations in reactive oxygen species have been shown in rat models of NFCI (Geng et al.,
460 2015) but have yet to be shown in individuals with cold sensitivity. However, individuals
461 with cold sensitivity have significantly impaired microvasculature (see Figure 1 for
462 comparison to the “normal” response) and therefore redox balance may be altered in
463 favour of a pro-oxidant state (Bertuglia and Giusti, 2003). This would result in a reduced
464 bioavailability of NO•, principally via uncoupling of endothelial nitric oxide synthase and
465 reduction of tetrahydrobiopterin (Landmesser et al., 2003) which leads to an increase of
466 superoxide’s and peroxynitrite. Further research is required to determine whether this sub-
467 clinical population have elevated levels of oxidative stress and whether chronic, rather than
468 acute, beetroot juice supplementation would alter this redox balance thus restoring
469 endothelial function. As individuals with cold sensitivity have cooler extremities they are
470 likely to have diminished eNOS activity reducing NO• production (Kellogg et al., 2008).
471 Longer term nitrate supplementation may alleviate these reductions in the NO• pool.
472 Moreover, recent data have shown that nitrate supplementation may reduce sympathetic
473 nervous activity (Notay et al., 2017) which may also be beneficial to individuals with cold
474 sensitivity.

475 Skin blood flow and temperature were lower in the toes than fingers during the cold
476 sensitivity test (Figure 3; Table 1) and whilst all participants had cold sensitive feet, only one
477 participant had cold sensitive hands. This is despite their self-reported cold exposures to
478 involve the hands and feet to similar degrees. This supports a recent study which reported a

479 heterogeneous response of the hands and feet to a cold challenge (Norrbrand et al., 2017)
480 and that the feet appear to be more susceptible to cold injury than the hands (DeGroot et
481 al., 2003, Golden et al., 2013).

482 There was no effect of nitrate supplementation on the skin blood flow response to
483 transdermal delivery of ACh in the forearm, finger or foot when compared to Placebo. A
484 previous study in patients with type 2 diabetes also showed no effect of nitrate
485 supplementation on the response to ACh (Gilchrist et al. 2013). Other studies have reported
486 nitrate supplementation resulted in an increase in CVC in the response to local (Keen et al.,
487 2014) and whole body (Levitt et al., 2015) heating in healthy individuals. The increase in CVC
488 in these two studies was a function of a decreased MAP since raw flux data was not altered
489 by nitrate supplementation, as in the current study. Differences in nitrate supplementation
490 regime (a 3 day [~ 5 mmol/day] supplementation vs an acute dose 1.5 hours [~ 12 mmol]
491 prior to testing) and the lack of a placebo in the other studies could explain the lack of effect
492 of nitrate supplementation on MAP in the current study. It is possible that a longer
493 supplementation period may augment ACh-induced vasodilation in the peripheral
494 microvasculature. This study was designed to have peak plasma nitrite concentrations (~ 3
495 hours post ingestion) during our primary outcome testing (cold sensitivity test) which means
496 that the iontophoresis (endothelial function test) was likely to have been conducted at
497 lower concentrations of plasma nitrite. We therefore, cannot exclude that, if the
498 iontophoresis was conducted at peak plasma nitrite concentrations that nitrate
499 supplementation could have been beneficial.

500 There was no difference in systolic, diastolic or mean arterial BP following acute nitrate
501 supplementation compared to Baseline or Placebo. Studies investigating short term nitrate
502 supplementation and its effects on blood pressure in clinical populations with endothelial
503 dysfunction have resulted in conflicting reports. For example, reductions in systolic blood
504 pressure have been shown in individuals with chronic obstructive pulmonary disease (COPD)
505 (Berry et al., 2014, Leong et al., 2015, Kerley et al., 2015) and hypertension (Kapil et al.,
506 2015) whilst in contrast, other studies have found no effect in individuals with COPD
507 (Shepherd et al., 2015b, Curtis et al., 2014), type 2 diabetes (Gilchrist et al., 2013, Shepherd
508 et al., 2015a) and heart failure (Zamani et al., 2015). Differences between these studies
509 include, dosing strategies (~ 5 vs ~ 12 mmol), timing of the supplement (ranging from 1.5

510 hours to 4 days which may affect peak plasma nitrite concentrations (Leong et al., 2015))
511 and importantly, some studies did not include a true placebo (Berry et al., 2014, Kerley et
512 al., 2015). Peak plasma nitrite and corresponding BP reductions typically occur at 2.5 to 4
513 hours post nitrate ingestion (Wylie et al., 2013). We measured BP indices at 1.5 hours post
514 ingestion for pragmatic reasons, therefore we are likely to have missed the peak in plasma
515 nitrite concentration and cannot exclude that the lack of effect on BP maybe due to timing.
516 Larger, well controlled studies are needed in clinical cohorts to elucidate if any clinically
517 meaningful benefits of short term dietary nitrate supplementation exist as the evidence to
518 date is equivocal.

519 The main strength to this study is the rigorous experimental design (a double blind, placebo
520 controlled, crossover, randomised control trial). As the current study examined acute
521 supplementation, future research should explore the potential benefits of longer
522 supplementation periods and examine if increasing the bioavailability of nitric oxide could
523 improve endothelial function and microvascular responses to a cold challenge in individuals
524 with impaired peripheral blood flow (e.g. non-freezing cold injury, Raynaud's phenomenon
525 and Scleroderma). A limitation to this study is that we did not measure change in redox
526 balance. Antioxidants and polyphenols in the beetroot juice have the potential to alter NOx
527 bioavailability. Given that the antioxidant and polyphenol content are the same for the
528 nitrate enriched (nitrate supplementation) and depleted (Placebo) beetroot juices
529 (Shepherd et al., 2015a) and that we showed no difference between baseline, placebo or
530 nitrate supplementation conditions for any variable, it is unlikely that the acute
531 consumption of beetroot and associated antioxidant / polyphenol content will have had any
532 effect.

533 **6.0 Conclusion**

534 This is the first study to examine the effects of inorganic nitrate supplementation in the
535 form of concentrated beetroot juice ingestion on rewarming following a cold stimulus in
536 individuals with cold sensitivity. Despite a physiologically meaningful rise in plasma nitrite
537 concentrations, acute nitrate supplementation did not alter extremity rewarming,
538 endothelial function, blood pressure, pain or thermal comfort and sensation. Although we
539 cannot preclude the possibility that a chronic nitrate supplementation regime could improve
540 extremity rewarming and microvascular function in cold sensitive individuals, acute nitrate

541 supplementation does not appear to improve vascular function in this sub-clinical
542 population following acute cold exposure.

543 **7.0 Acknowledgments:**

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545 from the technicians and Daniel Bailey during data collection. We gratefully acknowledge
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547 **8.0 Contribution Statement**

548 CE, JC, SB, MG, HM, and AS were involved in the conception of this work. CE, JC, HM, and AS
549 were involved in the acquisition of data. All authors have been involved in the drafting of
550 this work and revisions for intellectually important content.

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