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1 **Changes in body posture alter plasma nitrite but not nitrate concentration in humans**

2

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28 **Abstract**

29 **PURPOSE:** This study evaluated the change (Δ) in plasma volume (PV), nitrate [NO_3^-], and
30 nitrite [NO_2^-] concentration following changes in posture in the presence and absence of
31 elevated plasma [NO_3^-] and [NO_2^-]. **METHODS:** Fourteen healthy participants completed two
32 trials that were preceded by either supplementation with NO_3^- -rich beetroot juice (BR; total of
33 ~ 31 mmol NO_3^-) or no supplementation (CON). Both trials comprised 30 min of lying supine
34 followed by 2 min of standing, 2 min of sitting and 5 min of sub-maximal cycling.
35 Measurements of plasma [NO_3^-] and [NO_2^-] were made by gas-phase chemiluminescence and
36 Δ PV was estimated using the Dill and Costill method. **RESULTS:** Plasma [NO_2^-] decreased
37 from baseline (CON: 120 ± 49 nM, BR: 357 ± 129 nM) after lying supine for 30 min (CON 77
38 ± 30 nM; BR 231 ± 92 nM, both $P < 0.01$) before increasing during standing (CON 109 ± 42
39 nM; BR 297 ± 105 nM, both $P < 0.01$) and sitting (CON 131 ± 43 nM; BR 385 ± 125 nM, both
40 $P < 0.01$). Plasma [NO_2^-] remained elevated following exercise only in CON (125 ± 61 nM
41 $P = 0.02$). Plasma [NO_3^-] was not different between measurement points in either condition
42 ($P > 0.05$). PV increased from baseline during the supine phase before decreasing upon standing,
43 sitting, and exercise in both trials (all $P < 0.05$). **CONCLUSIONS:** Changing body posture
44 causes rapid and consistent alterations in plasma [NO_2^-]. Researchers should therefore carefully
45 consider the effect of posture when measuring this variable.

46

47 **Key Words:** beetroot juice, exercise, plasma volume

48 **1. Introduction**

49 Nitric oxide (NO) is a ubiquitous signalling molecule which is synthesised endogenously from
50 L-arginine by NO synthases (NOS) in the presence of oxygen (O₂) and regulates a multitude of
51 biological processes (Forstermann and Sessa 2012). For example, NO is transiently released
52 from endothelial cells, diffusing from the production site to smooth muscle cells, resulting in
53 dilation of the vasculature (Ignarro et al. 1987). Nevertheless, the majority of NO does not
54 reach its target cells due to rapid oxidation to nitrite (NO₂⁻) and nitrate (NO₃⁻) (Kelm 1999).

55 However, when pO₂ is reduced endogenous NO synthesis is diminished and can result in a
56 reduced blood flow to the periphery (Lundberg et al. 2008). Under these conditions it has been
57 shown that NO metabolites can be utilised as a reservoir for NO production via the NO₃⁻ - NO₂⁻
58 - NO pathway. Firstly, NO₃⁻ can be reduced to NO₂⁻ by facultative bacteria on the tongue
59 (Duncan et al. 1995) via the entero-salivary system (Lundberg and Govoni 2004). The resultant
60 NO₂⁻ is ingested and absorbed into the blood plasma where it may be reduced to NO under
61 certain local physiological conditions (Lundberg et al. 1994; Millar et al. 1998; Modin et al.
62 2001; Castello et al. 2006). However, due to the short half-life of NO, plasma [NO₂⁻] is still
63 considered to provide the best approximation of vascular NO bioavailability (Kelm 1999;
64 Lauer et al. 2001). Increased plasma [NO₂⁻] is also associated with improved endothelial
65 function (Rassaf et al. 2006) and superior exercise capacity (Totzeck et al. 2012) and is
66 therefore routinely measured in cardiovascular and exercise science research.

67

68 NO₃⁻ is also readily available within our diet and its consumption has been shown to
69 significantly increase plasma [NO₃⁻], and thus [NO₂⁻]. There is now an abundance of research
70 which has explored the potential therapeutic and ergogenic benefits of supplementation with
71 dietary NO₃⁻ (Siervo and Lara 2013; Pawlak-Chaouch et al. 2016; McMahon et al. 2017). This

72 research typically encapsulates repeated measurements of physiological variables during
73 periods of rest and exercise. During the experimental protocols, blood samples may be
74 routinely collected while participants are either supine (Muggeridge et al. 2015), seated
75 (Sandbakk et al. 2015), or standing (Wylie et al. 2013b). However, it is well-established that
76 alterations in posture lead to marked changes in plasma volume (PV) that can alter the
77 molecular concentrations of common biochemical analytes (Thompson et al. 1928; Fawcett
78 and Wynn 1960; Hagan et al. 1978; Hagan et al. 1980; Lippi et al. 2015). A probable
79 explanation is that postural-induced alterations in hydrostatic pressure force fluid and protein
80 to shift between the blood and interstitial space (Cohn 1966; Stokke et al. 1986). Lippi and
81 colleagues (2015) report that PV was decreased by 3% and 14% when moving from a supine
82 posture to sitting and standing, respectively. Furthermore, short duration high-intensity
83 exercise has been shown to decrease PV between 5-22 % in various exercise modalities
84 (Kargotich et al. 1998).

85

86 Previous research has shown considerable variability in the values of basal and supplemented
87 plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ (Wylie et al. 2013b; Muggeridge et al. 2015; Sandbakk et al. 2015;
88 McMahon et al. 2017). Given that NO_3^- is measured in the micromolar range and NO_2^- in the
89 nanomolar range, the measured concentration of these variables may be subject to postural-
90 induced changes. Indeed, we have recently shown that plasma $[\text{NO}_2^-]$ declined significantly
91 over a 30 min period while participants lay supine (Muggeridge et al. 2015), suggesting that
92 posture and PV shifts may alter plasma $[\text{NO}_2^-]$. However, several factors exist which may
93 account for these observations. For example, prolonged sitting is known to decrease shear
94 stress, whereas standing may increase shear stress and endogenous NO production (Uematsu
95 et al. 1995; Sessa 2004; Hsieh et al. 2014; Restaino et al. 2016; Morishima et al. 2017). This

96 demonstrates that further research is required to determine the impact of posture on NO
97 metabolites.

98

99 No study has explored the effects of postural changes on the measured concentrations of plasma
100 $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$. The aim of the present study, therefore, was to determine the magnitude of
101 the postural-induced changes in plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ and PV at rest and following short-
102 duration exercise. The experiment was conducted both with and without prior dietary NO_3^-
103 supplementation. We hypothesised that postural changes would alter plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$
104] which would be inversely associated with the change in PV.

105

106 **2. Methods**

107 2.1. Participants

108 Fourteen healthy and recreationally active participants (9 males and 5 females, age 27 ± 4 years,
109 stature 176 ± 7 cm, and body mass 71 ± 11 kg) volunteered to participate in the study. Written
110 informed consent was obtained from all individual participants included in the study. The study
111 was approved by the School of Science and Sport Ethics Committee at The University of the
112 West of Scotland and all procedures were performed in accordance with the 1964 Declaration
113 of Helsinki and its later amendments.

114

115 2.2. Study design

116 Each participant attended the laboratory on two separate occasions with a minimum of six days
117 between each visit. The experimental conditions were identical in each visit with the exception
118 that the first trial was conducted with no dietary intervention (control; CON). The second was

119 preceded by ingestion of 3 x 70 ml of NO₃⁻-rich beetroot juice (Beet it, James White Drinks,
120 UK) the day before and 2 x 70 ml, 2 h before the first blood sample (BR; total of ~31 mmol
121 NO₃⁻). Participants recorded their diet 24 h prior to CON and were asked to repeat this as
122 closely as possible prior to BR. All trials were completed before 11 a.m. at the same time of
123 day for each participant and following an overnight fast. Participants were instructed to avoid
124 caffeine, foods high in NO₂⁻ and NO₃⁻ (e.g. green leafy vegetables and cured meats), alcohol,
125 mouthwash, and strenuous exercise 24 h prior to the experiment. Participants were provided
126 with one bottle of drinking water (Harrogate, UK) prior to CON trial and given instructions to
127 arrive at the lab well hydrated. Participants recorded the volume of water ingested prior to CON
128 and matched the volume prior to BR.

129

130 2.3. Procedures

131 Following standard anthropometric measurements (stature and body mass), participants lay in
132 a supine posture to allow for the insertion of a cannula into the antecubital vein. Following
133 cannulation, participants stood up for 10 min prior to lying supine to start the experimental
134 trial. Baseline measurements (0 min) of venous blood, blood pressure (BP) and heart rate (HR)
135 were recorded immediately. The measurement of BP was conducted using an automated
136 sphygmomanometer (Omron M10, Kyoto, Japan) in triplicate during supine measures and in
137 duplicate for the standing and seated measures. Mean arterial pressure (MAP) was calculated
138 by the following equation:

139

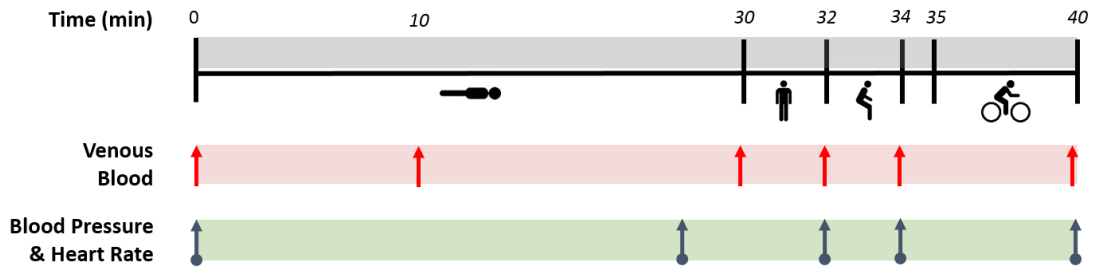
$$140 \text{ MAP} = (2 \times \text{diastolic BP} + \text{systolic BP}) / 3$$

141

142 Continuous measurement of HR was conducted using telemetry (Polar electro, Oy, Finland).
143 Participants lay supine for a total of 30 min followed immediately by 2 min of standing, 2 min
144 of sitting, and then 5 min of cycling at 60% of the age-predicted maximal heart rate. The
145 duration of the standing, sitting, and exercise phases was kept brief to minimise
146 pharmacokinetic alterations in plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ in the BR trial. Plasma $[\text{NO}_2^-]$ and
147 $[\text{NO}_3^-]$ are known to peak at ~2.5 h and ~1.5 h, respectively (Webb et al. 2008; Lundberg and
148 Weitzberg 2009; Larsen et al. 2010; Wylie et al. 2013a; McIlvenna et al. 2017). Therefore, the
149 end of the supine phase was designed to coincide with the peak in plasma $[\text{NO}_2^-]$. The
150 experimental protocol reflects many exercise physiology research studies that incorporate both
151 resting and exercise phases (Wylie et al. 2013b; Muggeridge et al. 2015; Sandbakk et al. 2015).

152

153 Collection of venous blood, BP and HR were repeated throughout the experiment as detailed
154 in Figure 1. The measurement of BP was not made during exercise due to difficulties in
155 obtaining a stable measurement. Venous blood was collected in 10 ml aliquots and the cannula
156 flushed with sterile 0.9% saline solution between samples to keep the line patent. Whole blood
157 was initially separated into EDTA vacutainers (BD Vacutainer). One vacutainer was
158 refrigerated at 4°C for the later analysis of haemoglobin concentration and haematocrit. All
159 samples were analysed within 6 h. The other vacutainer was centrifuged at 4000 rpm and 4°C
160 for 10 min within 3 min of collection (Pelletier et al. 2006; Bailey et al. 2009). The plasma was
161 then separated, frozen at -80 °C, and analysed within 4 months of initial collection for
162 determination of $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$.



163

164 Fig. 1. Schematic of measurement time points for CON and BR trials.

165

166 2.4. Plasma Nitrite and Nitrate analysis

167 Measurements of $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ were made using ozone-based chemiluminescence
 168 (Rogers et al. 2005). For the measurement of $[\text{NO}_2^-]$, tri-iodide reagent (2.5 ml glacial acetic
 169 acid, 0.5 ml of 18 Ω deionised water and 25 mg sodium iodide) and 100 μL of anti-foaming
 170 agent were placed into a customised glass purge vessel infused with nitrogen and inlet that was
 171 heated to 50 $^\circ\text{C}$. This purge vessel was further connected to an NO analyser (Sievers NOA
 172 280i, Analytix, UK). A standard curve was produced by injecting 100 μL of NO_2^- solutions
 173 (1000 nM, 500 nM, 250 nM, 125 nM, and 62.5 nM) and control sample containing deionised
 174 water. The area under the curve (AUC) for the latter was subtracted from the NO_2^- solutions to
 175 account for NO_2^- in the water used for dilutions. Following this, plasma samples were thawed
 176 in a water bath at 37 $^\circ\text{C}$ for 3 min and 100 μL of the sample was injected into the purge vessel
 177 in duplicate. The concentration of NO cleaved during the reaction was then measured by the
 178 NO analyser. The AUC was calculated using Origin software (version 7) and divided by the
 179 gradient of the slope. The coefficient of variation for the measurement of $[\text{NO}_2^-]$ in the current
 180 study was 3%.

181

182 For the measurement of plasma $[\text{NO}_3^-]$, vanadium reagent (32 mg of vanadium tri-chloride, 4
183 ml of 1M hydrochloric acid and 500 μL of water) and 100 μL of anti-foaming agent were placed
184 into the glass purge vessel and heated to 95 °C. A standard curve was produced by injecting
185 25-50 μL of NO_3^- solutions (100 μM , 50 μM , 25 μM , 12.5 μM , and 6.25 μM) and a control
186 sample containing deionised water. Plasma samples were thawed and de-proteinised (200 μL
187 of sample, 400 μL of zinc sulphate in deionised water at 10% weight/volume and 400 μL of
188 sodium hydroxide in deionised water at ratio of 1:1). Subsequently, 15-25 μL of the sample
189 was injected into the purge vessel in duplicate and plasma $[\text{NO}_3^-]$ calculated as previously
190 described for the NO_2^- assay. The coefficient of variation for the measurement of $[\text{NO}_3^-]$ in the
191 current study was 6%.

192

193 2.5. Determination of Plasma Volume Change

194 To determine the haematocrit, a small volume of venous blood was extracted into heparinised
195 capillary tubes that were sealed at the distal end with a wax seal. The capillary tubes were then
196 spun for 8 min at 15,000 revolutions/min in a micro-haematocrit centrifuge before the
197 haematocrit was measured in triplicate using a Hawksey haematocrit reader. The coefficient of
198 variation for the measurement of haematocrit in the current study was 0.4%. Haemoglobin
199 concentration was determined using the Randox colorimetric method (RX Monza, Randox
200 Laboratories, UK). Briefly, 20 μL of whole blood was mixed in a cuvette with 2.5 ml of
201 haemoglobin reagent before being incubated for 3 min at 25 °C. The haemoglobin
202 concentration was determined by measuring absorbance when light at a wavelength of 546 nm
203 was passed through the cuvette. The coefficient of variation for the measurement of
204 haemoglobin in the current study was 2%. Total blood volume (TBV) and total PV (TPV) at
205 baseline were estimated using the Nadler equations (Nadler et al. 1962):

206

207 Males TBV = (0.3669 x height in meters³) + (0.03219 x body mass in kilograms) + 0.6041

208

209 Females TBV = (0.3561 x height in meters³) + (0.03308 x body mass in kilograms) + 0.1833

210

211
$$PV = TBV * (1 - \text{Haematocrit})$$

212

213 The percentage change (Δ) in PV was estimated using the change in haematocrit and
214 haemoglobin values using the method described by Dill and Costill (1974). The PV values for
215 each time point are expressed as estimated TPV and the percentage Δ from the baseline (0 min)
216 sample (Fig. 4).

217

218 2.6. Statistical Analysis

219 All analyses were carried out using the Statistical Package for Social Sciences, Version 22
220 (SPSS Inc., Chicago, IL, USA). GraphPad Prism version 7 (GraphPad Software Inc., San
221 Diego, USA) was used to create the figures. Data are expressed as the mean \pm standard
222 deviation unless otherwise stated. The distribution of the data was tested using the Shapiro-
223 Wilk test. A one-way repeated-measures ANOVA was used to examine the differences
224 between measurement points [NO₃⁻], [NO₂⁻], PV, HR and BP. Post-hoc analysis was used to
225 determine the difference from the baseline and all other time points using a paired samples t-
226 tests with Bonferroni correction for multiple pairwise comparisons. The association between
227 absolute and Δ plasma [NO₃⁻], [NO₂⁻], and TPV values was determined using Pearson's

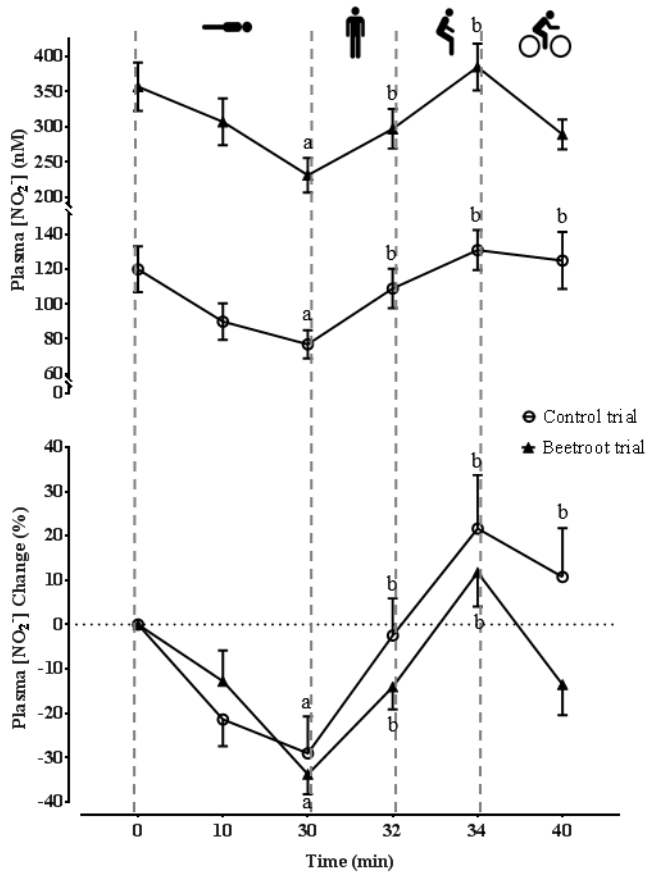
228 correlation coefficient. Statistical significance was declared when $P < 0.05$. Probability values
229 are expressed with 95% confidence intervals (95% CI) where appropriate.

230

231 **3. Results**

232 3.1. Nitrite

233 Baseline values of $[\text{NO}_2^-]$ were significantly elevated in the BR trial compared to the CON trial
234 ($P < 0.01$, 95% CI 154-320 nM, Fig. 2) and at all other time points. There was a significant main
235 effect of measurement point on plasma $[\text{NO}_2^-]$ in both trials ($P < 0.001$). Plasma $[\text{NO}_2^-]$ was not
236 different to baseline after 10 min of lying supine in either condition (both $P > 0.05$) but
237 decreased significantly after 30 min (CON: $P = 0.02$, 95% CI 5-82 nM; BR: $P < 0.01$, 95% CI
238 39-213 nM). In the CON trial, plasma $[\text{NO}_2^-]$ increased from the 30 min supine time point upon
239 standing ($P < 0.01$, 95% CI 15-51 nM) and sitting ($P < 0.01$, 95% CI 32-76 nM) and remained
240 higher following exercise ($P < 0.05$, 95% CI 7-89 nM). In the BR trial, plasma $[\text{NO}_2^-]$ increased
241 from 30 min supine upon standing ($P < 0.01$, 95% CI 35-98 nM) and sitting ($P < 0.01$, 95% CI
242 58-251 nM). Following exercise, plasma $[\text{NO}_2^-]$ was significantly reduced compared to sitting
243 ($P = 0.02$, 95% CI 13-180 nM) but was not different to the 30 min supine time point ($P = 0.14$).
244 Plasma $[\text{NO}_2^-]$ was not correlated with TPV either for absolute (CON, $R = 0.04$, $P = 0.74$; BR,
245 $R = 0.03$, $P = 0.78$) or Δ values (CON, $R = -0.12$, $P = 0.31$; BR $R = -0.11$, $P = 0.39$).



246

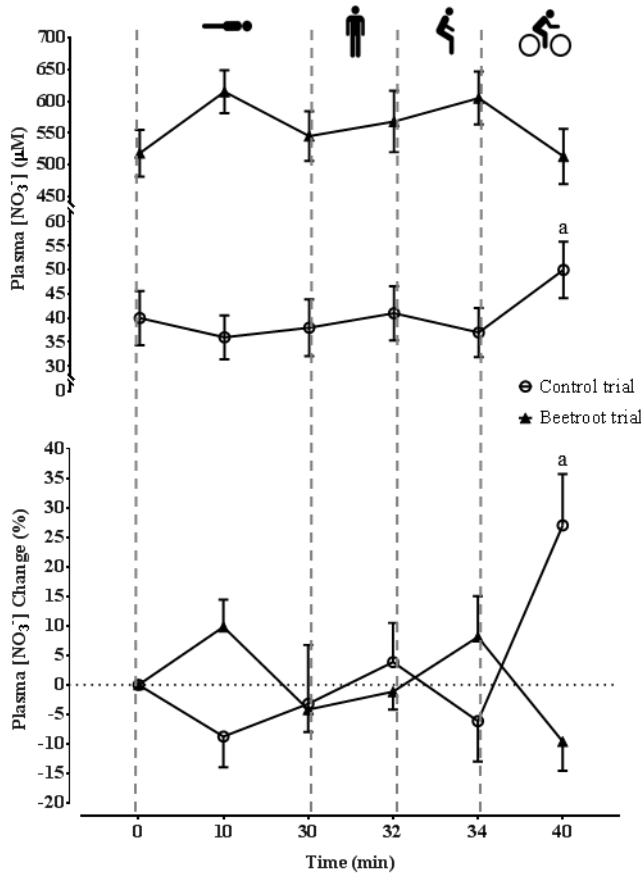
247 Fig. 2. Changes in mean \pm SEM plasma [NO₂⁻] expressed as absolute values (top) and
 248 percentage change from baseline (bottom). The graphical images at the top of the figure denote
 249 the supine, standing, seated, and exercise phases of the trial. a denotes a significant decrease
 250 compared to baseline ($P < 0.05$). b denotes a significant increase compared to 30 min time point
 251 ($P < 0.05$). All time points in the BR trial were significantly higher than the CON trial ($P < 0.01$).

252

253 3.2. Nitrate

254 Plasma [NO₃⁻] at baseline was higher in the BR trial compared to CON ($P < 0.01$, 95% CI 395-
 255 561 μ M, Fig. 3) and at all other time points. There was a significant main effect of measurement
 256 point on plasma [NO₃⁻] in the CON trial ($P < 0.01$) but not the BR trial ($P = 0.20$). In the CON
 257 trial, plasma [NO₃⁻] was higher after exercise compared to 10 min of lying supine ($P < 0.01$,

258 95% CI 4-24 μM) but was not different between any other measurement points. Plasma $[\text{NO}_3^-]$
 259] was not correlated with TPV either for absolute (CON, $R=-0.10$, $P=0.37$; BR, $R=-0.18$,
 260 $P=0.10$) or Δ values (CON, $R=-0.21$, $P=0.08$; BR $R=-0.02$, $P=0.86$).



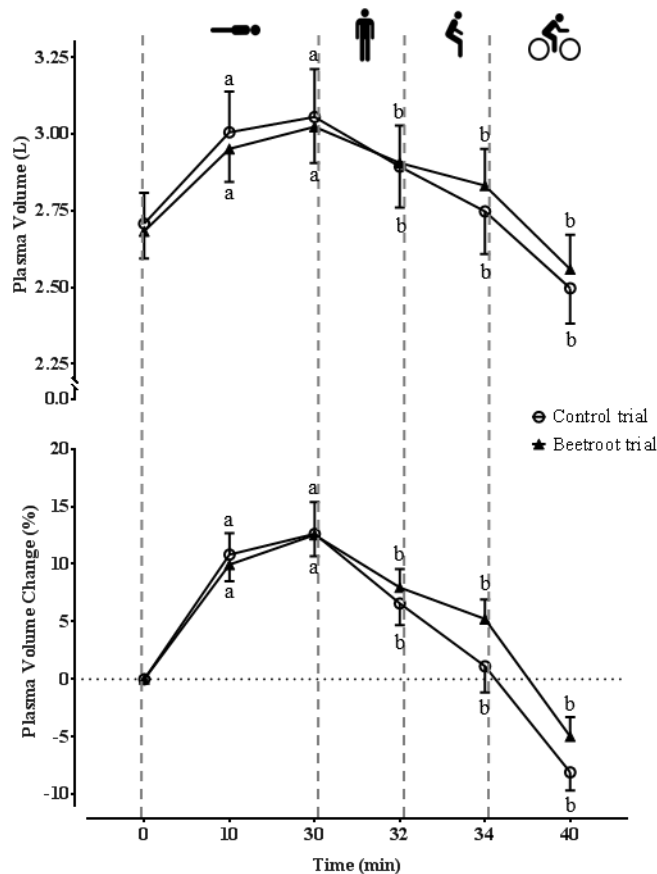
261

262 Fig. 3. Changes in mean \pm SEM plasma $[\text{NO}_3^-]$ expressed as absolute values (top) and
 263 percentage change from baseline (bottom). The graphical images at the top of the figure denote
 264 the supine, standing, seated, and exercise phases of the trial. a denotes a significant increase
 265 compared to 10 min time point ($P<0.01$). All time points in the BR trial were significantly
 266 higher than the CON trial ($P<0.01$).

267

268 3.3. Plasma Volume

269 After 10 min of lying supine, PV increased from baseline in both conditions (all $P < 0.01$, CON
 270 95% CI 0.09-0.51 L, BR 95% CI 0.12-0.42 L, Fig. 4) and remained elevated at the 30 min
 271 measurement point (all $P < 0.05$, CON 95% CI 0.04-0.63 L, BR 95% CI 0.15-0.53 L). PV then
 272 declined significantly from the 30 min supine measurement upon standing, sitting, and exercise
 273 in both conditions (all $P < 0.05$).



274
 275 Fig. 4. Changes in mean \pm SEM plasma volume expressed as absolute values (top) and
 276 percentage change from baseline (bottom). The graphical images at the top of the figure denote
 277 the supine, standing, seated, and exercise phases of the trial. a denotes a significant increase
 278 compared to baseline ($P < 0.01$). b denotes a significant decrease compared to 30 min of laying
 279 supine ($P < 0.05$).

280

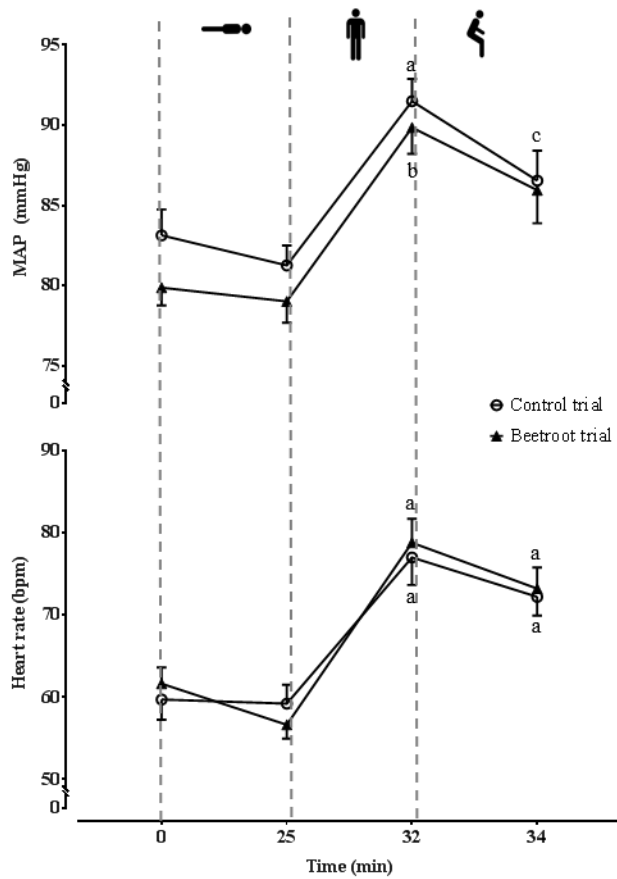
281 3.4. Blood Pressure

282 Baseline measurements of MAP were significantly lower in the BR trial compared to CON
283 ($P < 0.01$, 95% CI 1-5 mmHg, Fig. 5) but was not different at any other time point. There was a
284 significant main effect of time on MAP in both conditions ($P < 0.01$). MAP was higher during
285 standing compared to baseline (CON, $P < 0.01$, 95% CI 4-13 mmHg; BR, $P < 0.01$, 95% CI 4-16
286 mmHg) and following 25 min lying supine (CON, $P < 0.01$, 95% CI 6-14 mmHg; BR, $P < 0.01$,
287 95% CI 4-17 mmHg). In the CON trial, MAP was higher during sitting compared to lying
288 supine for 25 min ($P = 0.04$, 95% CI 0-10 mmHg). In the BR trial, MAP was higher during
289 standing compared to sitting ($P = 0.03$, 95% CI 0-7 mmHg).

290

291 3.5 Heart Rate

292 There was a significant main effect of time ($P < 0.01$) on HR in both the CON and BR trials.
293 HR was typically higher during the standing and sitting phases compared to the supine time
294 points (all $P < 0.01$). There was no difference in HR between sitting and standing measurements
295 (both $P > 0.05$). In both conditions, post-exercise HR was higher than all other time points (all
296 $P < 0.01$).



297

298 Fig. 5. Changes in mean \pm SEM mean arterial pressure (top) and heart rate (bottom) expressed
 299 as absolute values. The graphical images at the top of the figure denote the supine, standing,
 300 seated, and exercise phases of the trial. a denotes a significant increase compared to baseline
 301 and 25 min time point ($P < 0.01$). b denotes a significant increase compared to all other time
 302 points ($P < 0.05$). c denotes significant increase compared to 25 min time point ($P < 0.05$).
 303 Baseline measurements of mean arterial pressure were lower in the BR trial compared to the
 304 CON trial ($P < 0.01$).

305

306 4. Discussion

307 To our knowledge, this study is the first to report that plasma $[\text{NO}_2^-]$ is substantially altered by
 308 varying body posture while these changes have minimal impact on plasma $[\text{NO}_3^-]$. Here, we

309 report that plasma $[\text{NO}_2^-]$ is increased during sitting and standing compared to lying supine
310 which substantially extends our previous findings that plasma $[\text{NO}_2^-]$ declines during a period
311 of lying supine (Muggeridge et al. 2015).

312

313 As expected, moving between different body postures resulted in consistent, marked and rapid
314 changes in PV. Following a period of standing upright, PV increased by ~10% after 10 min in
315 the supine posture. There was a further increase in PV at the 30 min time point, although this
316 was of a much smaller magnitude (~13% from baseline). Previous data suggests that PV
317 stabilises approximately 20 min in the supine posture (Hagan et al. 1978) which is pertinent
318 when measuring the concentration of blood metabolites in exercise studies. On the other hand,
319 moving from supine to standing resulted in an almost immediate (~2 min) reduction in PV
320 which reduced further as participants continued to the seated posture. Again, the magnitude of
321 the response was profound, with PV dropping by 6-10% following brief periods of standing
322 and sitting from the end of the supine phase. A short period of exercise caused a further large
323 reduction in PV which corresponded to a decline of ~19% from the end of the supine phase.
324 These data are broadly in line with values reported elsewhere in the literature (Hagan et al.
325 1978) although others (Hansen 1968; Lippi et al. 2015) have reported larger declines in PV
326 during standing (~14%), likely due to longer period of time in this posture.

327

328 These postural-induced alterations in PV are readily explainable and the likely mechanisms
329 have been known for some time (Thompson et al. 1928). Adopting a standing posture increases
330 local hydrostatic pressure, particularly in the lower limbs, which forces fluid and some
331 molecules from the intravascular to the interstitial space (Krogh et al. 1932). The augmented
332 reduction in PV during exercise is most likely caused by an increased intra-capillary pressure

333 in the contracting muscles (Hansen 1968). These fluid shifts do eventually stabilise due to
334 counter pressure exerted by the tissue and an increase in the intravascular oncotic pressure
335 (Youmans et al. 1934). As evidenced in the present study, the reduction in circulating blood
336 volume and venous return can lead to an increase in HR and BP. It is important to note,
337 however, that standing may initially reduce BP and transient changes in this response may vary
338 between individuals (Eşer et al. 2007).

339

340 For the first time, we demonstrate that plasma $[\text{NO}_2^-]$ changes substantially between supine,
341 standing and sitting phases. Where PV increases during the supine phase, $[\text{NO}_2^-]$ decreases
342 during lying supine and increases on standing and sitting. These data are perhaps not surprising
343 given postural-induced PV shifts have previously been reported to alter the concentration of
344 other constituents in the blood in a similar fashion (Thompson et al. 1928; Fawcett and Wynn
345 1960; Lippi et al. 2015). Consequently, it might be expected that a considerable proportion of
346 the change in plasma $[\text{NO}_2^-]$ can be accounted for by a “dilution effect” where, for example,
347 an increased PV reduces the measured concentration of the number of NO_2^- particles. However,
348 there was no correlation between either absolute or Δ plasma $[\text{NO}_2^-]$ and PV values suggesting
349 plasma fluid shifts account for only a small proportion of the variance in $[\text{NO}_2^-]$ during postural
350 changes. Furthermore, plasma $[\text{NO}_3^-]$ does not change uniformly as posture is altered and was
351 not correlated with PV. Instead, it seems probable that postural-induced alterations in NO
352 metabolism may account for these findings.

353

354 In line with previous research, both plasma $[\text{NO}_2^-]$ and $[\text{NO}_3^-]$ were considerably elevated by
355 ingestion of NO_3^- -rich beetroot juice (198% and 1163%, respectively). However, given plasma
356 $[\text{NO}_2^-]$ is reported to peak ~2.5 h after acute ingestion of beetroot juice (Webb et al. 2008), it

357 is perhaps surprising that plasma $[\text{NO}_2^-]$ in the present study declined during the supine phase
358 of the BR trial to the same extent as CON (i.e. 2 – 2.5 h after ingestion). Nevertheless, we
359 (McIlvenna et al. 2017) and others (James et al. 2015) have previously demonstrated that
360 plasma NO_2^- pharmacokinetics following dietary NO_3^- ingestion appear to vary substantially
361 between individuals. For example, Wylie and colleagues (2013a) reported that time taken for
362 plasma $[\text{NO}_2^-]$ to peak following administration of a similar dose of NO_3^- -rich beetroot juice
363 ranged between 77 and 213 min. Therefore, it is plausible that while dietary-derived NO_2^- was
364 still increasing in the plasma in some participants, it was declining in others.

365

366 Measurement of plasma $[\text{NO}_2^-]$ and $[\text{NO}_3^-]$ is further complicated by the fact that changes in
367 posture also alter the rate of endogenous NO production. Shear stress is a frictional force
368 exerted by blood moving across the endothelium and is reported to increase during standing
369 compared to sitting (Morishima et al. 2017). Endothelial cells rapidly respond to shear stress
370 with an acute increase in intracellular calcium that enhances the binding of calmodulin to eNOS
371 and increases eNOS activity and NO production (Boo and Jo 2003; Rassaf et al. 2006). On the
372 contrary, moving from a supine to a seated position has been demonstrated to reduce shear rate
373 in young but not old participants (Trinity et al. 2015). Given that shear rate was not measured
374 in the current study, we can only speculate as to how this may have impacted endogenous
375 synthesis of NO and related metabolites. Conversion of NO to NO_3^- by heme proteins in the
376 blood and tissues occurs fairly rapidly (Shiva et al. 2006), such that NO_3^- is considered to be
377 the major breakdown product in the presence of sufficient amounts of O_2 (Kelm 1999).
378 Conversely, NO can be oxidised to NO_2^- via various oxidants in plasma and tissues (Shiva et
379 al. 2006). It should also be noted that a considerable portion of NO_2^- and NO_3^- are stored in
380 tissues. In rodents, the liver, blood, and skeletal muscle contain equivalent amounts of NO_2^-
381 ($\sim 0.5 - 0.7$ nmol/g) whereas NO_3^- is considerably higher in muscle (~ 200 nmol/g) compared

382 to blood (~80 nmol/g) and the liver (~10 nmol/g) (Piknova et al. 2015). Furthermore,
383 Nyakayiru et al. (2017) have recently shown that ingestion of sodium NO_3^- results in a
384 substantial and sustained increase in muscle $[\text{NO}_3^-]$, with reported values exceeding those in
385 plasma. However, muscle $[\text{NO}_2^-]$ was below the detection limit both before and after NO_3^-
386 supplementation. Therefore, when considering the impact of postural-induced fluid shifts in
387 the context of measuring $[\text{NO}_2^-]$ and $[\text{NO}_3^-]$, we must also factor in the change in endogenous
388 NO production, the oxidation of NO to various metabolic endpoints, and the transfer of these
389 metabolites to and from different tissues.

390

391 Data from this study also demonstrates that short-duration sub-maximal cycling exercise leads
392 to a reduction in plasma $[\text{NO}_2^-]$ in both conditions but a variable response in plasma $[\text{NO}_3^-]$.
393 Although plasma $[\text{NO}_3^-]$ did not differ statistically between time points overall, there was a
394 substantial increase from pre- to post-exercise in the CON trial and a reduction in the BR trial.
395 The reduction in plasma $[\text{NO}_2^-]$ is consistent with some (Larsen et al. 2007; Kelly et al. 2014),
396 but not all (Larsen et al. 2010) previous studies and potentially results from an increased
397 conversion of NO_2^- to NO during exercise. Differences between study cohorts and the intensity
398 and duration of the exercise protocols may explain the inconsistencies in these data. Cosby and
399 colleagues (2003) suggest that NO_2^- is a major bioavailable pool of NO and present data
400 demonstrating an increased reduction of NO_2^- to NO by deoxyhemoglobin during exercise.
401 Furthermore, in animal studies, the initiation of exercise has been shown to increase the
402 demand for NO and upregulate eNOS activity (Maiorana et al. 2003). Therefore, post-exercise
403 changes in plasma $[\text{NO}_2^-]$ and $[\text{NO}_3^-]$ in dietary NO_3^- supplementation studies must be
404 interpreted cautiously due to the aforementioned pharmacokinetics of these metabolites and the
405 individual variability in the response (James et al. 2015; McIlvenna et al. 2017).

406

407 Although the precise mechanisms explaining the alterations in plasma $[\text{NO}_2^-]$ remain unclear,
408 the magnitude of the change in this outcome during the adoption of different postures highlights
409 the importance of standardising posture in experimental trials where this outcome is important.
410 Indeed, an inconsistent approach to the posture of participants during blood collection may at
411 least partly explain why measurements of plasma $[\text{NO}_3^-]$ are comparable between different
412 studies in healthy participants while $[\text{NO}_2^-]$ varies considerably. The present study is not
413 without limitations as the phases of sitting, standing, and exercise were very brief and the fate
414 of the ingested NO_3^- is impossible to determine without more advanced measurement methods.
415 Furthermore, the order of the trials was not randomised and nor was there inclusion of a placebo
416 condition that required the ingestion of a matched volume of NO_3^- -depleted beetroot juice.
417 However, our data demonstrates the proportional change in PV, $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ were
418 consistent between BR and CON conditions suggesting these experimental limitations do not
419 diminish confidence in the findings. Notwithstanding, there are two primary recommendations
420 that emanate from this work. Firstly, if differences in $[\text{NO}_2^-]$ are to be compared either within
421 or between participants in an experimental trial, participants should be lying supine for a
422 standardised period of time before blood collection. For baseline measurements the supine
423 period should be a minimum of 20 – 30 min. For post-exercise measurements the supine period
424 should be brief but standardised. Secondly, the posture of participants during blood collection
425 and the duration that this posture was maintained before blood collection should be clearly
426 documented in research manuscripts to allow better comparison of data between studies.

427

428 **5. Conclusion**

429 The principal finding from this study is that posture has a profound impact on the concentration
430 of plasma NO_2^- regardless of whether $[\text{NO}_2^-]$ was normal or elevated by dietary NO_3^-
431 supplementation. The lack of correlation between PV and $[\text{NO}_2^-]$ suggests that fluid shifts
432 cannot solely account for this response. While postural alterations in shear stress and
433 endogenous NO production may be contributing factors, we do not have experimental data to
434 support this notion. Nevertheless, researchers should standardise the posture of participants at
435 rest and post exercise when multiple blood samples are to be collected and fully document
436 these procedures during dissemination of their data.

437

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442

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