

30 Abstract

31 Raynaud's phenomenon (RP) is characterised by recurrent transient peripheral vasospasm
32 and lower nitric oxide (NO) bioavailability in the cold. We investigated the effect of nitrate-
33 rich beetroot juice (BJ) supplementation on *i*) NO-mediated vasodilation, *ii*) cutaneous
34 vascular conductance (CVC) and skin temperature (T_{sk}) following local cooling and *iii*)
35 systemic anti-inflammatory status.

36 Following baseline testing, twenty-three individuals with RP attended four times, in a double-
37 blind, randomized crossover design, following acute and chronic (14 days) BJ and nitrate-
38 depleted beetroot juice (NDBJ) supplementation. Peripheral T_{sk} and CVC were measured
39 during and after mild hand and foot cooling, and during transdermal delivery of acetylcholine
40 and sodium nitroprusside. Markers of anti-inflammatory status were also measured.

41 Plasma [nitrite] was increased in the BJ conditions ($P < 0.001$). Compared to the baseline
42 visit, thumb CVC was greater following chronic-BJ ($\Delta 2.0 \text{ flux} \cdot \text{mmHg}^{-1}$, $P = 0.02$) and
43 chronic-NDBJ ($\Delta 1.45 \text{ flux} \cdot \text{mmHg}^{-1}$, $P = 0.01$) supplementation; however, no changes in T_{sk}
44 were observed ($P > 0.05$). Plasma [interleukin-10] was greater, pan endothelin and systolic
45 and diastolic blood pressure (BP) were reduced, and forearm endothelial function was
46 improved, by both BR and NDBJ supplementation ($P < 0.05$).

47 Acute and chronic BJ and NDBJ supplementation improved anti-inflammatory status,
48 endothelial function and BP. CVC following cooling increased post chronic-BJ and chronic-
49 NDBJ supplementation, but no effect on T_{sk} was observed.

50 Key points

51 Beetroot supplementation improves

- 52 1) thumb blood flow,
- 53 2) anti-inflammatory status,
- 54 3) endothelial function and
- 55 4) reduces BP

56 in people with Raynaud's

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60 New and noteworthy

61 This is the first study to examine the effect of dietary nitrate supplementation in individuals
62 with Raynaud's phenomenon. The principal novel findings from this study were that both
63 beetroot juice and nitrate depleted beetroot juice: 1) increased blood flow in the thumb
64 following a cold challenge; 2) enhanced endothelium-dependant and -independent
65 vasodilation in the forearm; 3) reduced systolic and diastolic blood pressure, and [pan-
66 endothelin]; and 4) improved inflammatory status in comparison to baseline.

67

68 **1.0 Introduction**

69 Raynaud's phenomenon (RP) is characterized by recurrent transient vasospasm of the fingers
70 and/or toes in response to a cold or stressful stimulus (66) which causes discomfort and pain.
71 Administration of NO donors, such as the organic nitrate, Glyceryl Trinitrate (GTN), can
72 improve blood flow in those with cold sensitivity (32) and RP (1). Reduced NO
73 bioavailability has been implicated in the aetiology of RP. Although GTN can improve blood
74 flow in RP (1), chronic GTN administration produces a tolerance and diminishing
75 vasodilatory effect (54). Moreover, organic nitrates (i.e. GTN and isosorbide mononitrate)
76 can provoke deleterious side effects, such as headaches (66). Alternative long-term therapies
77 to improve blood flow in RP warrant investigation.

78 Topical application of inorganic nitrate on the forearm and fingers of individuals with RP can
79 increase blood flow (64) and may counter the vasoconstrictor effects of the endothelins which
80 are elevated in RP (10). Leafy green vegetables and beetroot have a particularly high
81 concentration of inorganic nitrate (8) and their vasodilatory effects can improve
82 cardiovascular health (24). Dietary inorganic nitrate supplementation has been shown to
83 improve skin blood flow (48) and microvascular function (39), and to lower blood pressure
84 (BP) in healthy individuals (68) and those with hypertension (39), peripheral arterial disease
85 (41) and heart failure (69). Promisingly, oral ingestion of inorganic nitrate does not appear to
86 cause the same side effects or tachyphylaxis (57) reported for organic nitrates.

87 The efficacy of dietary inorganic nitrate supplementation is considered NO-mediated and
88 evoked through the stepwise reduction of nitrate to nitrite, and finally, nitrite to NO (52).
89 After oral consumption dietary inorganic nitrate is absorbed into the circulation, concentrated
90 in the saliva and converted to nitrite via anaerobic bacteria on the dorsum of the tongue (15).

91 This nitrite is then swallowed (4) and absorbed into the circulation, where it acts as a storage
92 pool for subsequent NO production. The reduction of nitrite to NO is expedited in conditions
93 of acidosis and hypoxia (13), as seen in the digital vasculature in RP (66). The entero-salivary
94 pathway is considered a complementary system for NO synthesis (50), which becomes
95 increasingly important when the nitric oxide synthase (NOS) system is deficient, such as
96 when individuals with RP are exposed to the cold (62).

97 Consumption of beetroot juice (BJ) can lower BP to a greater extent than an equimolar dose
98 of an inorganic nitrate salt (36), suggesting that other components of BJ might interact with
99 nitrate to elicit additive or synergistic effects on vascular function. The antioxidant (67)
100 properties of BJ may be important, as attenuated NO-mediated vasodilation in inflammatory
101 conditions is partly due to elevated oxidative stress (21, 49, 65). As RP is characterized by
102 systemic oxidative stress and inflammation (6, 29), BJ may have a beneficial effect by
103 increasing both NO and antioxidants. (21). BJ may therefore offer an inexpensive and safe
104 intervention to reduce oxidative stress and inflammation, to enhance peripheral blood flow
105 and rewarming, and to mitigate pain following a local cold challenge in RP.

106 This study investigated the effects of acute and chronic BJ and nitrate-depleted BJ (NDBJ)
107 supplementation on 1) cutaneous blood flow, rewarming and pain sensation following a local
108 cold challenge, 2) inflammatory biomarkers and, 3) endothelium-dependant and -independent
109 vasodilation and BP in individuals with RP. We hypothesised that, compared to NDBJ, BJ
110 would increase plasma [nitrate] and [nitrite], lower inflammation, and improve BP,
111 endothelium-dependant and -independent vasodilation, cutaneous vascular conductance
112 (CVC) and peripheral skin rewarming following a local cold challenge.

113

114 **2.0 Methods**

115 **2.1 Participants**

116 Individuals were recruited if they had primary or secondary RP, were at least 18 years old
117 and were willing and able to provide consent for participation in the study. Individuals were
118 excluded if they had known renal impairment (estimate of glomerular filtration rate < 30);
119 uncontrolled hypertension; were taking organic nitrates, nicorandil, or thiazolidinidiones; had
120 experienced a myocardial or cerebrovascular event in the previous 3 months; were a current

121 smoker (any smoking event within the last 3 months) or if they had any other serious medical
122 condition which would interfere with data interpretation or participant safety. Participants
123 taking phosphodiesterase inhibitors were asked to refrain from using them for the duration of
124 the study. Mouthwash was prohibited for the duration of the study and for at least 1 week
125 prior to the first visit as reduction in the oral microflora is known to alter nitrate metabolism
126 (27). Additionally, participants were asked to avoid caffeine and alcohol for 3 and 24 hours
127 prior to testing, respectively. Finally, participants were asked to record what they ate prior to
128 each visit and replicate this where possible for the 24 hours before arriving at the laboratory
129 for subsequent testing.

130 Twenty-seven participants were recruited (see Table 1) from a clinical database of individuals
131 with RP from the Rheumatology Department, Queen Alexandra Hospital (Portsmouth, UK).
132 Posters, word of mouth and local interest groups were also targeted for recruitment. All
133 participants provided written informed consent and their flow through the trial is shown in
134 Figure 1. A favourable ethics opinion was granted by the Hampshire B NRES Committee
135 (17/SC/0148) and this double-blind, randomised, cross-over trial was registered on the
136 ClinicalTrials.gov website, ID # NCT03129178.

137

138 **2.2 Pre-experimental tests**

139 Following enrolment into the study, a letter was sent to each participant's General
140 Practitioner (GP), to inform them of their participation within the trial. Participants who did
141 not want their GP informed ($n = 1$) were provided with the GP letter. Participants were given
142 the opportunity to ask any questions they may have after reading the participant information
143 sheet. A standard medical history and clinical examination was undertaken, which included
144 height, body mass, ankle-brachial pressure index and venous blood samples. Seated resting
145 BPs (6 on each arm, mean of last 3 recorded) were performed using an automated BP monitor
146 (Omron M5, Omron, Milton Keynes, UK). Participants then undertook a test of
147 microvascular endothelial function (iontophoresis) in an ambient temperature of 23°C,
148 followed by a cold challenge in an ambient temperature of 30°C (both described below) as
149 baseline measures.

150 During the first visit, concealed allocation was used by an independent researcher to
151 randomise participants to begin either the BJ or NDBJ arm of the study. Specifically, a
152 computer programme (www.randomizer.org) was utilised to randomly allocate study numbers
153 to treatment order. The appropriate bottles of BJ were placed in a sealed opaque envelope.

154 Participants were then provided with instructions and their first acute dose to take away with
155 them. It was estimated that twenty-five individuals with RP were needed to achieve a
156 moderate to large effect size in a pilot study (30). Therefore, we aimed to recruit 30
157 individuals with RP to account for a 15% drop-out rate.

158

159 **2.3 Protocol and outcome measures**

160 For visits 2 and 4 (the acute supplementation visits), participants were instructed to ingest an
161 acute dose of 140 mL of either BJ (delivering 12.4 mmol of inorganic nitrate) or NDBJ
162 (NDBJ; delivering 0.1 mmol of inorganic nitrate (Beet It, James Whites Drinks Ltd.) one and
163 a half hours before arriving at the laboratory. We previously reported that both the BJ and
164 NDBJ have similar antioxidants and polyphenol content (59). On arrival at the laboratory,
165 participants rested for 10 minutes. Resting seated BP was then measured 5 times, with the
166 mean of the last 3 recorded. A ~ 20 mL venous blood sample was then drawn from the
167 antecubital fossa. The iontophoresis derived measures of microvascular endothelial function
168 conducted at baseline were then repeated in ambient conditions (23°C), followed by a cold
169 challenge in an ambient temperature of 30°C (both described below). Visits 3 and 4 (after the
170 crossover period) were separated by at least 7 days to allow washout and all visits were
171 conducted at the same time of day (\pm 1 hour) in a counter-balanced order.

172 Visits 3 and 5 (the chronic supplementation visits) were identical in nature to visits 2 and 4,
173 however followed chronic supplementation of 70 mL·day⁻¹ of either BJ or NDBJ for 13 days
174 with 140 mL consumed on the day of testing. Participants, if requested, were reminded to
175 take the juice via text message or voice mails.

176 **2.4 Outcome measures**

177 Our primary outcome measure was change in cutaneous blood flow and rewarming following
178 a local cold challenge. Secondary outcome measures included, endothelium-dependant and -
179 independent vasodilation, pain, inflammatory biomarkers and BP.

180 *Microvascular endothelial function test*

181 Individuals were acclimated for a minimum of 30 minutes in an ambient temperature of 23.2
182 \pm 0.4°C prior to acetylcholine (ACh) and sodium nitroprusside (SNP) being delivered
183 transdermally via iontophoresis to three sites in the following order: 1) volar aspect of the left

184 forearm, 2) middle phalanx of the middle finger of the left hand, and 3) dorsal aspect of the
185 left foot as previously described (17).

186 Briefly, following cleaning of the skin surface with water for injection, two perspex rings
187 were attached to the skin with one acting as an anode, and the other as the cathode. These
188 electrodes were connected to the iontophoresis controller (MIC 2, Moor Instruments, UK).
189 Both chambers had an 8 mm inner diameter. The anode chamber was filled with ~ 0.5 mL of
190 ACh (Braun, Melsungen, Germany), with a 1% concentration dissolved in water for injection.
191 The cathode chamber was filled with ~ 0.5 mL of SNP (Sigma-aldrich, Missouri, USA) with
192 a 0.01% concentration dissolved in water for injection. The protocol for electrical pulses
193 included: four at 25 μ A, followed by a single pulse of 50 μ A, 100 μ A, 150 μ A and 200 μ A.
194 These pulses lasted for 20 s with 120 s intervals between each pulse where no current was
195 applied. An interval of five minutes was given between testing each site (forearm, finger and
196 foot).

197 Laser doppler probes (VP1T / 7, Moor Instruments, UK), connected to a perfusion monitor
198 (moor VMS-LDF, Moor Instruments, UK) were used to assess skin blood flow. Data were
199 recorded using an acquisition system (Powerlab, AD Instruments, Australia) and software
200 (LabChart 7, AD Instruments, Australia). The laser doppler probes were secured in the
201 Perspex rings prior to the iontophoresis protocol on the forearm, finger, dorsal foot and on the
202 corresponding site on the contra-lateral limb (to differentiate between local and systemic
203 responses). Skin blood flow responses were expressed as CVC ($CVC = \text{skin flux}/MAP$;
204 $\text{flux}\cdot\text{mmHg}^{-1}$). The average skin blood flow for both ACh and SNP was calculated over the
205 final 20 s of the intervals between each successful pulse (i.e. 100-120 s post each pulse) (17).
206 Maximal skin blood flow, taken at the highest point which was not always following the final
207 pulse and area under the curve (AUC) were calculated for each participant. Skin temperature
208 (T_{sk}) was recorded with skin thermistors (Grants Instruments, Cambridge) placed next to the
209 Perspex chambers. BP was measured on the contra-lateral arm to the site of iontophoresis
210 using an automated BP monitor (Omron M5, Omron, Milton Keynes, UK) before and after
211 each iontophoresis protocol to calculate mean arterial pressure (MAP).

212 *Cold sensitivity test*

213 The cold sensitivity test used in this study has been comprehensively described elsewhere
214 (17). Testing took place in a climatic controlled chamber at an air temperature of $29.6 \pm$

215 0.87°C. Participants were asked to remove their shoes and socks, and rest in a semi-
216 recumbent position for 15 minutes. Those capable of cycling ($n = 20$) were asked to cycle on
217 an ergometer (Tunturi, T6, Turku, Finland) between 20 and 50 W for 12 minutes as this has
218 been shown to improve the reliability of the test by removing central vasoconstrictor tone
219 (18). Participants were then asked to rest in a semi-recumbent position for a minimum of 5
220 minutes while resting toe temperature and blood flow were recorded.

221 The participants placed their foot ($n=21/23$ right foot) into a plastic bag (to keep it dry) and
222 immersed into $15.02 \pm 0.01^\circ\text{C}$ water to the point of their mid-malleoli for 2 minutes.
223 Following the immersion period, the bag was removed and the rate of toe skin rewarming and
224 blood flow were recorded while the participant was semi-recumbent. This procedure was then
225 repeated on the hand (mean temperature; $14.95 \pm 0.02^\circ\text{C}$; $n = 22/23$ right hand), following 5
226 minutes seated rest. During the rewarming the arm was supported.

227 Skin blood flow was assessed using a laser Doppler probe (VP1T / 7, Moor Instruments, UK)
228 secured to the Great toe pads during foot immersion and on the pads of the thumbs during
229 hand immersion. Analysis of skin blood flow was conducted using minute averages before,
230 during and after immersion (i.e. rewarm period) and expressed as CVC. CVC was analysed
231 between conditions at the following time points: pre immersion, and during 5 and 10 minutes
232 of rewarming following removal from the water.

233 T_{sk} was measured using an infrared camera (A320G, FLIR systems, UK) and in accordance
234 with the protocol described by Moreira, Costello, Brito, Adamczyk, Ammer, Bach, Costa,
235 Eglin, Fernandes and Fernández-Cuevas (53). The camera lens was positioned 1.0 m away
236 from the sole of the participant's foot and the palm of the hand and the spot analysis function
237 on the FLIR software (FLIR systems, UK) was used to analyse the surface temperature on the
238 pads of the toes / fingers immediately prior to immersion and at the end of each minute
239 during the 10 minute re-warm. The thumb, mean finger (mean of all 5), great toe, mean toe
240 and coldest toe T_{sk} were analysed between conditions and across time at multiple time points:
241 pre-immersion and 5 and 10 minute into the rewarming period. Within our laboratory, the
242 coefficient of variation for the cold sensitivity test for finger and toe T_{sk} is 2.7% and 8.7%,
243 respectively (17). MAP was calculated from BP measured using an automated blood pressure
244 monitor (Omron M5, Milton Keynes, UK) on the left arm prior to each immersion and
245 following both rewarming periods.

246 Both thermal comfort and sensation were measured using a 20 cm scale (0 = very
247 cold/uncomfortable; 10 = neutral; 20 = very hot/comfortable; modified from Zhang,
248 Huizenga, Arens and Wang (70)) and recorded prior to immersion, during immersion and
249 every 2 minutes of the rewarming period. Pain sensation was assessed using a numerical
250 rating scale for pain (0 no pain, 10 unimaginable, unspeakable pain; (19)) at the same time
251 points.

252 **2.5 Biochemical analysis**

253 Venous blood (and saliva where blood could not be taken, $n = 2$) samples were taken and
254 processed prior to testing on each study visit. Blood samples for plasma [nitrate] and [nitrite]
255 were taken in Lithium Heparin tubes and ethylenediaminetetraacetic acid tubes for
256 assessment of oxidative stress and inflammatory markers. The blood and saliva samples were
257 placed in a chilled (4°C) centrifuge and spun at 4500 g for 10 minutes immediately following
258 collection. Once spun, the plasma and saliva was pipetted into aliquots with a link
259 anonymised code. The samples were then placed in a -80°C freezer until subsequent analysis.
260 Plasma and saliva samples were analysed for [nitrate] and [nitrite] using a Sievers NO analyser
261 (Sievers NOA 280i, Analytix Ltd. Durham, UK), via a modification of the ozone
262 chemiluminescence technique previously described by Bateman, Ellis and Freeman (3).
263 Plasma [peroxiredoxin-4] and [thioredoxin-1] were quantified using in-house ELISAs
264 developed using commercially available antigens and antibodies (Abcam, Cambridge). The
265 human SOD3 antigen and rabbit antiserum directed against human [superoxide dismutase-3]
266 were developed as previously described (26). A cytometric bead array technique was used to
267 quantify plasma interleukin (IL)-6 and IL-10 on a BD C6 Accuri Flow Cytometer (BD
268 Biosciences, Berkshire, UK). [Pan endothelin] was quantified using commercially available
269 DuoSet ELISA kits (R&D Systems, Abingdon, UK).

270 **2.6 Qualitative analysis**

271 Semi-structured interviews were conducted to examine the acceptability of the supplement
272 and the testing procedures. Specifically, semi-structured interviews explored participants'
273 experiences of the study procedures and consumption of BJ. At the time of the interviews,
274 both the interviewer and participants were still blinded to the treatment order. A total of ten
275 semi-structured interviews were necessary to reach a point of data saturation (i.e., where no
276 new information was provided by the participants). Interviews were conducted by a
277 researcher with experience in qualitative research methods. Interviews were recorded,

278 transcribed verbatim, and analysed through thematic analysis as outlined by Braun and
279 Clarke (7). A deductive process was used throughout the analysis, where transcripts were
280 reviewed and direct quotations were used to establish initial codes for each question posed.
281 Codes were then grouped together to create themes based on identified similarities. Coding of
282 transcripts relied on a reflexive process, where themes were constantly compared to initial
283 codes and the data set as a whole.

284 **2.7 Data analysis**

285 As previously reported with iontophoresis (17), some individuals had high skin resistance
286 which meant that not all pulses could be delivered to the forearm, fingers and feet. Where
287 participants had incomplete data sets (current response curves), the number of pulses
288 analysed was the same within individual for each visit (17).

289 The distribution of data was assessed using descriptive methods (skewness, outliers, and
290 distribution plots) and inferential statistics (Shapiro–Wilk test). Where normal distribution
291 was violated non parametric analyses were performed. For the cold sensitivity test, statistical
292 differences were assessed using 5×3 repeated-measures ANOVAs (condition [baseline,
293 acute BJ, chronic BJ, acute NDBJ and chronic NDBJ supplementation] * time [pre
294 immersion, 5 min, 10 min]) for mean toe, coldest toe, great toe, mean finger, coldest finger
295 and thumb T_{sk} , thumb and great toe skin blood flows. For the endothelial function test,
296 maximum CVC, AUC and T_{sk} were analysed using repeated-measures ANOVAs (baseline,
297 acute BJ, chronic BJ, acute NDBJ and chronic NDBJ supplementation). Plasma [nitrate] and
298 [nitrite] were analysed using repeated measures ANOVA and all other biomarkers were
299 analysed using Friedman tests. Where appropriate, post-hoc tests were conducted using
300 pairwise comparisons with least significant differences. Where data were not normally
301 distributed Friedman tests were used with Wilcoxon follow ups. Data are presented as mean
302 (SD) or as median and 25th and 75th percentiles unless otherwise stated. Statistical analysis
303 was performed on SPSS version 24 (Chicago, IL) and statistical difference and trends
304 towards significance were accepted as 2-tailed $P < 0.05$ and $P < 0.1$ respectively. Interviews
305 were transcribed verbatim and analysed thematically.

306

307 **3.0 Results**

308 Twenty-seven individuals consented to take part in the trial and twenty-three completed the
309 study. A detailed analysis of participant recruitment and withdrawal, is shown in Figure 1.
310 We report 5 adverse events. Of the 4 participants who withdrew, 1 withdrew to relocate for
311 work, 1 reported hot flushes and 2 reported nausea and sickness (all outside of the
312 laboratory). One of these adverse events was related to the intervention and the other two
313 may have been related to the intervention. Two participants reported gastrointestinal distress
314 which was tolerable for the duration of the study. Participant-reported adherence to the
315 supplementation protocol was excellent, with only one participant reporting missing one day
316 during the chronic supplementation period. All participants reported avoiding mouthwash
317 during the testing periods. Numerous participants reported red stools and beeturia as with
318 previous studies (17, 58-60). The full data set for this trial has been made available on our
319 University repository (<https://doi.org/10.17029/f9c6af22-d8f5-4989-9cf1-1422b9467010>).

320 **3.1 Plasma [nitrate] and [nitrite]**

321 Supplementation with BJ significantly increased plasma [nitrate] ($P < 0.001$; Figure 2), and
322 [nitrite] ($P < 0.001$; Figure 2). Post-hoc analysis revealed a statistically significant rise in
323 plasma [nitrate] between acute NDBJ and acute BJ supplementation ($43 \pm 25 \mu\text{M}$; 339 ± 146
324 μM ; $P < 0.001$, respectively) and chronic NDBJ and chronic BJ supplementation (52 ± 29
325 μM ; $397 \pm 96 \mu\text{M}$; $P < 0.001$, respectively) and plasma [nitrite] between acute NDBJ and
326 acute BJ supplementation ($69 \pm 23 \text{ nM}$; $428 \pm 187 \text{ nM}$; $P < 0.001$, respectively) and chronic
327 NDBJ and chronic BJ supplementation ($87 \pm 31 \text{ nM}$; $428 \pm 117 \text{ nM}$; $P < 0.001$, respectively)
328 (Figure 2). There were no differences between acute and chronic NDBJ ($P > 0.05$) and acute
329 and chronic BJ ($P > 0.05$).

330 **3.2 Cold sensitivity test**

331 A significant difference in CVC was observed between the visits for supplement ($P = 0.01$),
332 time ($P = 0.01$), but not their interaction ($P = 0.52$) in the thumb (see figure 3, B). Follow-up
333 tests revealed increased thumb CVC between baseline and chronic NDBJ ($2.0 \text{ flux.mmHg}^{-1}$,
334 $P = 0.02$) and chronic BJ ($1.45 \text{ flux.mmHg}^{-1}$, $P = 0.01$). Chronic supplementation resulted in
335 a greater thumb CVC than acute supplementation for both the NDBJ ($1.9 \text{ flux.mmHg}^{-1}$, $P =$
336 0.03) and BJ ($1.3 \text{ flux.mmHg}^{-1}$, $P = 0.01$; see figure 3, B) conditions. No differences were
337 seen in the great toe CVC (all $P \geq 0.05$; see figure 3, A).

338 T_{sk} of the toes (great toe, coldest toe and mean toe temperature) and fingers (thumb, coldest
339 finger and mean finger temperature) was not altered by acute or chronic supplementation
340 with BJ or NDBJ ($P > 0.05$ for all comparisons; Figures 3C and 3D). T_{sk} of the toes and
341 fingers were not different when split for disease type (data not shown, $P > 0.05$).

342 *Thermal comfort, sensation and pain*

343 There were no differences in thermal sensation, thermal comfort or pain sensation for the
344 hand at any time point during the cold sensitivity test between visits ($P < 0.05$). In the foot,
345 there were no differences between conditions for thermal sensation or pain sensation at any
346 time point. Although thermal comfort was similar prior to immersion, it was perceived
347 differently during immersion ($P < 0.05$), immediately after immersion ($P = 0.004$) and during
348 rewarming ($P = 0.02$). During immersion, participants reported feeling more thermally
349 comfortable in the baseline condition compared to the acute-NDBJ ($P = 0.04$; Table 2) and
350 chronic-BJ supplementation conditions ($P = 0.006$; Table 2). Thermal comfort was also
351 greater following acute- compared to chronic-NDBJ supplementation ($P = 0.001$) during
352 immersion. Immediately after immersion, participants felt less thermal comfort compared to
353 baseline in all the other conditions (acute NDBJ; $P = 0.003$, acute BJ; $P = 0.03$, chronic
354 NDBJ; $P = 0.002$, and chronic BJ; $P = 0.002$; Table 2). During the rewarming phase, the
355 baseline condition was reported as more thermally comfortable than either the acute NDBJ (P
356 $= 0.03$; Table 2) or the chronic BJ ($P = 0.008$; Table 2) conditions.

357 **3.3 Microvascular endothelial function**

358 Endothelial-dependant and -independent function was significantly different between the
359 visits for supplement in the forearm for ACh_{Max} ($P = 0.05$), SNP Max ($P = 0.02$) and SNP
360 AUC ($P = 0.03$) but not ACh AUC ($P = 0.21$). Post-hoc tests revealed that, compared to
361 baseline, acute-BJ increased CVC with ACh (Max, $P = 0.02$) and chronic-BJ increased CVC
362 with SNP (Max, $P = 0.05$). Chronic-BJ supplementation was also found to significantly
363 increase CVC with SNP (Max, $P = 0.001$; and AUC, $P = 0.02$) compared to NDBJ. Trends
364 towards a significant increase in CVC compared to baseline were also seen with chronic-BJ
365 with ACh (max $P = 0.07$) and SNP (AUC, $P = 0.09$) and acute-NDBJ with SNP (AUC, $P =$
366 0.08).

367 The responses to ACh and SNP were similar during all five visits on the finger (ACh Max, P
368 $= 0.67$; ACh AUC, $P = 0.84$; SNP Max, $P = 0.80$; SNP AUC; $P = 0.95$) and foot (ACh Max,

369 $P = 0.10$; ACh AUC, $P = 0.25$; SNP Max, $P = 0.21$; SNP AUC, $P = 0.52$). See figures 4 and
370 5 respectively.

371 **3.4 BP**

372 Systolic and diastolic BP were different across time (SBP; $P < 0.001$, DBP; $P < 0.001$).
373 Compared to acute NDBJ, acute BJ significantly reduced systolic BP (127 ± 16 mmHg vs.
374 121 ± 16 mmHg, $P = 0.01$) and diastolic BP (77 ± 8 mmHg vs. 74 ± 7 mmHg; $P = 0.03$). This
375 effect was not present with chronic supplementation for either systolic BP (NDBJ: 122 ± 15
376 mmHg; BJ: 121 ± 16 mmHg, $P = 0.43$) or diastolic BP (NDBJ: 75 ± 8 mmHg; BJ: 74 ± 8
377 mmHg, $P = 0.49$). Compared to baseline, both NDBJ and BJ reduced systolic BP (acute
378 NDBJ: 8.0 ± 11 mmHg, $P = 0.02$; acute BJ: 13.6 ± 10.8 mmHg, $P < 0.001$; chronic NDBJ, 12
379 ± 14 mmHg, $P < 0.001$; and chronic BJ: 14 ± 11 mmHg, $P < 0.001$) and diastolic BP (acute
380 NDBJ: 6 ± 8 mmHg, $P = 0.02$; acute BJ, 9 ± 7 mmHg, $P < 0.001$; chronic NDBJ: 8 ± 9
381 mmHg, $P < 0.001$; and chronic BJ: 9 ± 7 mmHg, $P < 0.001$) see figure 6.

382 **3.5 Cytokines and redox markers**

383 [Pan endothelin] was reduced after supplementation with BJ ($P = 0.03$; Figure 7A). Acute
384 NDBJ ($P = 0.01$) and BJ ($P = 0.04$) resulted in higher [pan endothelin] compared to baseline.
385 There was a trend for an increase with chronic NDBJ ($P = 0.07$) but not chronic BJ ($P = 0.18$;
386 Figure 7A). BJ supplementation altered plasma concentrations of the anti-inflammatory
387 cytokine, IL-10 ($P < 0.001$; Figure 7B) with IL-10 increasing in all four experimental
388 conditions compared to baseline (acute NDBJ $P < 0.001$; acute BJ, $P < 0.001$; chronic-NDBJ,
389 $P = 0.001$; and chronic-BJ, $P = 0.002$; Figure 7B) but did not alter IL-6 ($P = 0.97$; Figure 5C)
390 between conditions. Plasma [SOD3], ($P = 0.18$; Figure 5D), TRX-1, ($P = 0.11$; Figure 7E)
391 and PRDX-4 ($P = 0.28$; Figure 7F) did not differ between visits. IL-10 was significantly
392 higher than baseline for all time points for primary but not secondary RP ($P < 0.001$) data
393 not shown).

394 **3.6 Qualitative interviews**

395 Semi-structured exit interviews were conducted with 10 participants. Several recruitment
396 strategies were used (as described in the methods section) and participants recommended that
397 similar strategies be used to recruit participants in the future with the additional use of social
398 media suggested for future trials. Most participants ($n = 20$) experienced symptoms of RP in
399 their hands, with three individuals stating that they felt greater discomfort in their feet.

400 Overall, participants said that the study was a positive experience. One participant indicated
401 the BJ made her feel ill. However, this participant decided to continue with the study and did
402 not withdraw. None of the participants were sure they had felt any positive health benefits as
403 a result of the juice, during any of the sessions. One participant indicated she felt as though
404 the juice "...opened up her blood vessels" but this was deemed as neither a positive nor
405 negative reaction to the juice. Most participants did not enjoy drinking the juice, with only
406 one participant indicating that he enjoyed it. Participants mentioned the juice had an
407 unpleasant taste, with some individuals complaining it tasted metallic, too sweet and had a
408 thick composition. Despite the negative reaction to the juice, most individuals indicated they
409 simply adjusted to the juice. Nearly all individuals said they would wait to find out the results
410 of the study before purchasing the juice or discussing it with other individuals. One
411 individual, said he would happily purchase it and recommend it highly to his friends and
412 family.

413

414 **4.0 Discussion**

415 This is the first study to examine the effect of dietary nitrate supplementation in individuals
416 with RP. Specifically, we examined the effects of supplementation with BJ on cutaneous
417 blood flow, rewarming and pain sensation following a local cold challenge, and inflammatory
418 biomarkers, antioxidant enzymes, endothelium-dependant and -independent vasodilation, and
419 BP in individuals with RP compared to baseline and NDBJ. The principal novel findings
420 from this study were that 1) both BJ and NDBJ increased blood flow in the thumb following
421 a cold challenge; 2) enhanced endothelium-dependant and -independent vasodilation in the
422 forearm; 3) reduced systolic BP, diastolic BP and [pan-endothelin]; and 4) improved
423 inflammatory status in comparison to baseline. These findings suggest acute and chronic BJ
424 and NDBJ supplementation have the potential to reduce inflammatory status and improve
425 aspects of vascular function in individuals with RP.

426 **4.1 Plasma [nitrate] and [nitrite]**

427 Plasma [nitrate] and [nitrite] were elevated following acute and chronic BJ supplementation
428 compared to NDBJ. This elevation in circulating plasma [nitrite] represents an increase in the
429 potential for nitric oxide synthase (NOS) independent NO generation, with NOS-dependent
430 NO generation attenuated in conditions of increased oxidative stress (21, 49, 65) such as in

431 RP (6, 29). Hypoxic and acid environments are known to increased conversion of nitrite to
432 NO (13), and since the digital vasculature is more hypoxic and acidic in RP (1) increased
433 plasma [nitrite] following BJ supplementation had the potential to increased blood flow and
434 re-warming compared to NDBJ.

435 **4.2 Cold sensitivity test**

436 In individuals with cold sensitivity, acute BJ does not improve skin temperature or blood flow
437 in the hands or feet (17). However, an increased skin blood flow in the thumb in individuals
438 with RP following chronic supplementation of BJ and NDBJ was observed in the current
439 study. No effect in the feet was observed, which may at least in part be due to divergent
440 mechanisms of vascular control in the hands and feet (55). Thumb skin blood flow was
441 higher prior to immersion and stayed higher during and following immersion in both chronic
442 supplementation protocols indicating this effect is likely due to the antioxidant content of
443 beetroot as opposed nitrate content of BJ and the elevations in [nitrate] and [nitrite] given the
444 comparable responses in the BJ and NDBJ conditions. Indeed, some antioxidant and
445 polyphenol compounds found in the BJ/NDBJ have previously been shown to have
446 vasodilatory properties such as chlorogenic acid (56); quercetin (16); caffeic acid (45) and
447 this may at least in part explain why we see changes in blood flow in both types of juice. The
448 observed improvement in skin blood flow did not however translate into increased skin
449 temperature. Although the increase in skin temperature following the cold challenge is due to
450 cutaneous blood flow in healthy controls, individuals with poor peripheral blood flow rewarm
451 passively in a warm environment (14). Therefore, it could be that, although CVC was
452 increased in the small area under the laser doppler probe, the overall increase in skin blood
453 flow was not large enough to translate into a statistical or clinically meaningful change in
454 skin temperature (i.e. 0.5°C) (2).

455 **4.3 Thermal comfort, sensation and pain**

456 Neither BJ or NDBJ altered thermal sensation or comfort in the hand or feet during the cold
457 sensitivity test, which is similar to previous findings in cold sensitive individuals (17).
458 Thermal comfort of the foot was however, reduced during cooling and in the subsequent
459 rewarming period following BJ supplementation, despite T_{sk} being the same. Since thermal
460 comfort was not altered prior to immersion, BJ may have altered the perception of cooled
461 skin though not to a level where increased pain was reported. Thermal sensation and
462 particularly thermal comfort are subjective and some participants struggled to decide on a

463 number to report, especially when their foot was numb. The apparent decrease in comfort
464 could be a function of being more familiar with the scales and therefore being able to respond
465 more promptly at a time of dynamic activation of the cutaneous thermoreceptors which is
466 critical for perception of comfort (14). Given CVC increased in the thumb and thermal
467 sensation was not worse there could be a site-specific effect that is linked to alterations in
468 blood flow. Most participants reported RP symptoms in the hands and not their feet, so an
469 increased CVC in the hand with no changes in thermal sensation maybe a positive outcome,
470 however this warrants further investigation.

471 **4.4 Endothelial function**

472 Individuals with RP exhibit cutaneous microvascular dysfunction which manifests as reduced
473 finger blood flow in all environmental conditions compared to controls (28). Some of this
474 dysfunction is due to impaired endothelium dependent vasodilation (43). The effect of nitrate
475 supplementation on endothelial function has been examined in numerous cohorts such as
476 healthy individuals (48), cold sensitive (17), obese (37), type 2 diabetes mellitus (25), and has
477 been reviewed elsewhere (46). The dose appears to be important (46), however comparison
478 of acute vs chronic supplementation has yet to be examined in any population. Neither BJ nor
479 NDBJ altered microvascular endothelial function in the fingers or the foot. However,
480 improvements in endothelium-independent (SNP) and -dependent (ACh) vasodilation were
481 observed in the forearm following chronic BJ supplementation. Potential explanations for the
482 change in SNP-induced smooth muscle cell function following chronic supplementation
483 could be due to a suppression in eNOS derived NO (11) and or nitrite mediated inhibition of
484 NADPH derived superoxide, leading to increased bioavailability of NO (22). Collectively,
485 such effects might explain the enhanced responsiveness to the exogenous NO donor, SNP.
486 Collectively, such effects might explain the enhanced responsiveness to an exogenous NO
487 donor. Chronic BJ also increased cutaneous vasodilation in the forearm to ACh
488 administration when compared to chronic NDBJ, suggesting that the elevated nitrite, NO
489 and/or their intermediates may have evoked additional vasodilatory effects from those
490 elicited by the antioxidants in BJ. Improvements in forearm arm blood flow suggest that the
491 effects of BJ and NDBJ are systemic (31). Iontophoresis on the foot and fingers was not
492 always possible due to high skin resistance meaning that the sample size was compromised,
493 which may explain why no effect was observed at these sites. Conversely, it may be that
494 nitrite levels were not increased sufficiently to improve digital microvascular function.
495 However, given the increased blood flow following hand cooling this seems implausible.

496 Endothelins are a family of peptides, which cause vasoconstriction and thus antagonise the
497 actions of NO. Although acute BJ and NDBJ reduced plasma concentrations of pan-
498 endothelin compared to baseline (Figure 5) chronic BJ and NDBJ did not alter plasma [pan-
499 endothelin] which suggests that divergent mechanisms may underpin changes in endothelial
500 function after acute and chronic BJ and NDBJ supplementation. RP is associated with
501 elevated [endothelin-1] (10), which can lead to chronic pain (61) and may also explain why
502 endothelial function is impaired (38). However, our cohort do did not appear to have
503 impaired endothelial function at baseline compared to healthy controls (17). Therefore, we
504 cannot preclude a larger effect in individuals with overt endothelial dysfunction. Our cohort
505 however, self-reported high levels of physical activity (4.3 ± 2.5 bouts of >30 min per week).
506 Regular exercise which is a key stimulus for promoting endogenous NO production via shear
507 stress induced activation of endothelial NOS (9). This may explain, in part, why additional
508 nitrate via the entero-salivary pathway failed to show an additional benefit between BJ vs.
509 NDBJ, and perhaps a more sedentary cohort of participants may have benefited. IL-10, on the
510 other hand, increased across all time points and, therefore, may play a larger role in mediating
511 the changes in vascular function after acute and chronic BJ and NDBJ supplementation in
512 individuals with RP.

513 **4.5 Blood pressure**

514 The present data also demonstrated a reduction in both systolic and diastolic BP in all
515 conditions compared to baseline. To our knowledge, this is the first study to demonstrate that
516 NDBJ can reduce BP. Explanations for this reduction in BP are multifactorial. A well-
517 recognised phenomenon of BP trials is that BP appears to fall across time, and as such
518 caution should be taken with interpreting these findings. However, there are other active
519 ingredients in BJ, including antioxidants and polyphenols (59, 67) such as, batalins, gallic
520 acid, chlorogenic acid and quercetin (see Shepherd, Wilkerson, Dobson, Kelly, Winyard,
521 Jones, Benjamin, Shore and Gilchrist (59) for further detail) of which some are vasoactive
522 (16, 45, 56). Supplements rich in antioxidants have also been shown to improve redox
523 balance and improve vascular function (44). The reported reduction in [pan-endothelin]
524 (Figure 5a), with and without elevations in plasma [nitrite] (Figure 2b), may have contributed
525 to the improved endothelial function and reduced systolic and diastolic BP observed in the
526 current study. There was also a small additional reduction in both systolic and diastolic BP
527 after acute BJ compared to acute NDBJ supplementation. This can be explained, at least in
528 part, by shifts in the oral microbiome reducing capacity following nitrate supplementation

529 (33), meaning that the effect could be larger with acute vs. chronic supplementation. This
530 effect may also have been larger if BP was measured at 2.5 hours after ingestion, to match
531 peak plasma [nitrite] (68). Previously published studies in clinical populations that have
532 observed a reduction in BP after BJ supplementation have also not used a true placebo,
533 meaning that the antioxidant content was unlikely matched between conditions (5, 42), as
534 was the case in the present study, and thus the effect of antioxidant / polyphenol rich BJ on
535 BP needs to be examined in more detail and rigour.

536 **4.6 Cytokines and antioxidant enzymes**

537 BJ is rich in antioxidants and polyphenols (58), however the effect of BJ supplementation on
538 systemic redox balance and inflammation have not been reported. There is some evidence to
539 suggest that individuals suffering with Raynaud's phenomenon have elevated systemic levels
540 of inflammation and oxidative stress (6, 29). One previous study used exercise as a model to
541 induce acute oxidative stress and inflammation, and reported no effect of 3 days of BJ (~ 210
542 mg) on [ROS] or [IL-10] versus an isocaloric placebo in healthy individuals (12). In the
543 current study, we report that both one day and 2 weeks of supplementation with BJ and
544 NDBJ increased plasma [IL-10], but did not alter [IL-6]. An increase in plasma [IL-10]
545 suggests that both BJ and NDBJ induced an anti-inflammatory effect in this population. The
546 mechanism/s for this effect and whether it would remain after supplementation ceased or if
547 individuals would need to continue supplementing daily is unclear. Post hoc analysis, in our
548 study revealed statistical differences in [IL-10] for primary but not secondary RP. Although
549 we are not powered to detect these changes, future studies should examine the potential
550 ergogenic of beetroot juice supplementation at different levels of disease severity and in
551 particular, inflammatory status. Two weeks of supplementation with BJ did not alter the
552 concentration of markers of oxidative stress (PRDX-4, TRX-1 and SOD3). PRDX-4 and
553 TRX-1 are endogenous antioxidant enzymes, which are secreted into the extracellular
554 environment (i.e. plasma) in response to elevated intracellular oxidative stress; whilst SOD3
555 is an extracellular antioxidant enzyme anchored to the membrane of cells to eliminate
556 superoxide anions (i.e. ROS) directly within the extracellular space. A growing body of
557 evidence now supports a role for PRDX, TRX and SOD3 in regulating the inflammatory
558 response (23, 34, 35). Although these markers were unaltered following 2 weeks of
559 supplementation in the present study, this does not preclude the possibility that longer-term
560 supplementation with BJ or NDBJ may reduce these and other markers of oxidative stress
561 and inflammation in this population.

562 **4.7 Qualitative interviews**

563 Participants' accounts support the statistical findings from this study that blood flow
564 increased in the hands, with one participants stating they felt their "blood vessels open up".
565 One participant suggested that they felt ill, which coincides with the reported adverse events
566 including headaches and hot flushes. It is theoretically possible this may be due to increased
567 blood flow to the brain (40) and increased skin perfusion (47), which may lead to side effects
568 in a similar way to organic nitrates (66). Conversely, one participant was happy to
569 recommend the juice and enjoyed the taste. Two individuals with scleroderma reported
570 gastrointestinal distress however this may be related to their condition (20). Larger trials are
571 needed to establish rates of adverse effects following BJ supplementation.

572 **4.8 Strengths, limitations and future work**

573 A strength of this research was its robust experimental design (double-blind, randomised,
574 crossover trial). Several limitations do, however, warrant discussion. Firstly, the qualitative
575 interviews were conducted between 2 and 12 weeks following participants' completion of the
576 study, which may limit the accuracy of recall in some cases. We may also have missed the
577 peak BP effects as BP was taken at 1.5 hours after ingestion. The sample size was relatively
578 small and non-homogenous, including a spread of primary and secondary conditions. As a
579 consequence, it was not possible to examine differences between these groups to determine
580 whether one group may potentially benefit more from BJ supplementation. NO
581 responsiveness and metabolism is known to be reduced in older individuals (51, 63), we
582 therefore cannot preclude that the effect seen in this study would be larger in a group of
583 younger individuals with primary RP. A larger definitive trial, examining the efficacy of BJ
584 supplementation, is therefore needed in individuals with RP. Given these results it appears
585 that BJ supplementation may indeed offer an inexpensive intervention to improve endothelial
586 (dys)function in individuals with RP.

587

588 **5.0 Conclusion**

589 This is the first study to examine the effect of dietary nitrate supplementation on extremity
590 rewarming, endothelial function and BP in individuals with RP. We show that both BJ and
591 NDBJ increased blood flow in the thumb following a cold challenge; improved endothelium
592 dependant and independent vasodilation in the forearm; and reduced systolic and diastolic BP

593 in comparison to baseline. These effects appear to be linked, at least in part, to reduced
594 inflammatory markers. Efficacy trials are warranted to verify these findings.

595

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608

609 **7.0 Contribution Statement**

610 AS, HM, JC, ZS, SB, MG, SY and CE designed the research. CE, HM, JC, PG, HM, DW,
611 AW and AS conducted research. SB, NB and AW provided essential reagents. AS, HM, JC,
612 CE, SB, NB, AW, PG, HM, HM, DW, analysed data or performed statistical analysis. AS,
613 CE, SB and ZS wrote the paper. AS had primary responsibility for final content. All authors
614 read and approved the final manuscript.

615

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Figure legends

Figure 1. Participant flow through the trial.

Figure 2. Mean (SD) plasma [nitrate] (A) and [nitrite] (B) in the baseline, acute nitrate-depleted (acute-NDBJ), nitrate-rich (acute-BJ) beetroot juice, and chronic nitrate-depleted (chronic-NDBJ) and nitrate-rich (chronic-BJ) beetroot juice supplementation conditions ($n = 21$). * $P < 0.001$ significantly different from acute-NDBJ and chronic-NDBJ and baseline. Plasma [nitrate] and [nitrite] were analysed using repeated measures ANOVA. **Figure 3.** Mean \pm SD great toe CVC (A), thumb CVC (B), great toe skin temperature (C) and thumb skin temperature (D) for baseline (open squares \square), acute nitrate-depleted beetroot juice (NDBJ) (open circles \circ), acute (beetroot juice) BJ (closed circles \bullet), chronic NDBJ (closed triangles \blacktriangledown) and chronic BJ (closed diamonds \blacklozenge) supplementation conditions. * $P < 0.05$ significant difference in four places within panel B, 1) baseline to chronic-NDBJ 2) baseline to chronic-BJ, 3) acute-NDBJ and chronic-NDBJ, and 4) acute-BJ and chronic-BJ ($n = 23$). Data was analyzed using 5×3 repeated-measures ANOVAs (condition [baseline, acute-BJ, chronic-BJ, acute-NDBJ and chronic-NDBJ supplementation] * time [pre immersion, 5 min, 10 min]) for mean toe, coldest toe, great toe, mean finger, coldest finger and thumb T_{sk} , thumb and great toe skin blood flows. **Figure 4.** Data are presented as median and interquartile range (25 and 75 percentiles) for maximum CVC, area under the curve (AUC). Significant difference is depicted with a * (< 0.05) and trends with a # (< 0.10). For the endothelial function test, maximum CVC and AUC were analysed using repeated-measures ANOVAs (baseline, acute BJ, chronic BJ, acute PL and chronic PL supplementation). Where data were not normally distributed Friedman tests were used with Wilcoxon follow ups.

Figure 5. Data are presented as median and interquartile range (25 and 75 percentiles) for maximum CVC, area under the curve (AUC). Significant difference is depicted with a * (< 0.05) and trends with a # (< 0.10). For the endothelial function test, maximum CVC and AUC were analysed using repeated-measures ANOVAs (baseline, acute BJ, chronic BJ, acute PL and chronic PL supplementation). Where data were not normally distributed Friedman tests were used with Wilcoxon follow ups.

Figure 6. Systolic (A) and diastolic (B) diastolic blood pressure for baseline, acute (acute-NDBJ) and chronic (chronic-PL) nitrate-depleted beetroot juice (NDBJ) and acute (acute-BJ) and chronic (acute-NDBJ) nitrate-rich

beetroot juice (BJ) supplementation conditions. Data are presented as mean \pm SD * $P < 0.05$ significantly different from corresponding brackets ($n = 23$). Data were analysed using repeated measures ANOVA.

Figure 7. Median and IQR (25th and 75th percentiles) for Pan endothelin (A), IL-10 (B), IL-6 (C), SOD3 (D), TRX-1 (E) and PRDX-4 (F) at baseline, acute (acute-NDBJ) and chronic (chronic-NDBJ) nitrate-depleted beetroot juice (NDBJ) and acute (acute-BJ) and chronic (chronic-BJ) nitrate-rich beetroot juice (BJ) supplementation conditions. * $P < 0.05$ significantly different from baseline, # = trending towards significance ($P = 0.07$) ($n = 21$). Friedman tests were used to assess main effects and where appropriate, post-hoc tests were conducted using pairwise comparisons with least significant differences.

Table 1. Participant characteristics ($n=23$).

Characteristics	Mean or %
Age	64.3 \pm 15.3 y
Female	83%
Raynaud's duration	29.8 \pm 23.9 y
Secondary Raynaud's	39.1%
Scleroderma	17.4%
Sjögren's syndrome	17.4%
Systemic lupus	8.7%
Mixed connective tissues disorder	4.3%
Rheumatoid arthritis	4.3%
Height	1.7 \pm 0.1 m
Mass	64.3 \pm 10.5 kg
Baseline systolic BP	134.0 \pm 18.6 mmHg
Baseline diastolic BP	83.2 \pm 10.2 mmHg
Number of 30+ minutes of exercise per week	4.3 \pm 2.5
Portions of fruit and vegetables per day	5.6 \pm 2.7
Mini mental state exam	28.9 \pm 0.9

Data presented as mean \pm SD or as a % unless otherwise stated from participants who completed the trial. N.B. A number of individuals presented with two diseases specifically relating to secondary Raynaud's Phenomenon. Mini mental state exam ranges from 0-30, which we used a cut of 18 to determine capacity to consent.

Table. 2. Thermal sensation, thermal comfort and pain in the foot and hand during the cold sensitivity test at baseline, nitrate-depleted (PL) and nitrate-rich (BJ) beetroot juice supplementation conditions. Average rewarm is the mean over the last 8 minutes of rewarming.

	Condition	<i>n</i>	Pre-immersion	During immersion	Immediately after immersion	Average rewarm	
Foot	Thermal sensation	Baseline	21	11.8 ± 4.0	4.4 ± 2.6	7.6 ± 4.3	11.0 ± 2.9
		Acute NDBJ	21	12.7 ± 4.2	3.8 ± 2.2	5.4 ± 2.9	9.7 ± 3.1
		Acute BJ	21	12.4 ± 4.8	3.7 ± 2.6	5.6 ± 2.4	10.5 ± 3.2
		Chronic NDBJ	21	13.3 ± 3.8	4.6 ± 3.2	5.5 ± 3.2	10.5 ± 2.7
		Chronic BJ	21	11.9 ± 4.2	3.9 ± 2.3	5.2 ± 2.6	9.7 ± 3.4
	Thermal comfort	Baseline	21	13.0 ± 5.3	9.1 ± 5.0	11.2 ± 4.1	13.2 ± 3.6
		Acute NDBJ	21	12.6 ± 4.2	7.1 ± 5.4 ¹	7.5 ± 4.0 ¹	11.3 ± 3.8 ¹
		Acute BJ	21	13.0 ± 5.2	7.6 ± 4.4	9.2 ± 3.9 ¹	12.1 ± 3.7
		Chronic NDBJ	21	13.1 ± 5.1	7.5 ± 4.3	7.4 ± 3.0 ¹	12.2 ± 3.5
		Chronic BJ	21	12.7 ± 5.0	6.6 ± 4.6 ^{1,2}	8.2 ± 4.5 ¹	11.1 ± 3.6 ¹
Hand	Pain	Baseline	20	0.2 ± 0.8	1.0 ± 1.7	1.0 ± 1.7	0.2 ± 0.6
		Acute NDBJ	20	0.0 ± 0.0	0.7 ± 1.3	0.7 ± 1.3	0.2 ± 0.6
		Acute BJ	20	0.2 ± 0.8	0.7 ± 1.3	0.7 ± 1.3	0.3 ± 0.8
		Chronic NDBJ	20	0.0 ± 0.0	0.8 ± 1.7	0.8 ± 1.7	0.9 ± 2.7
		Chronic BJ	20	0.1 ± 0.4	0.6 ± 1.2	0.6 ± 1.2	0.2 ± 0.6
	Thermal sensation	Baseline	21	13.2 ± 2.4	4.1 ± 2.9	6.0 ± 3.6	11.5 ± 2.8
		Acute NDBJ	21	13.8 ± 3.3	3.6 ± 2.2	5.6 ± 2.9	12.1 ± 3.2
		Acute BJ	21	13.6 ± 2.5	3.4 ± 2.3	5.8 ± 2.7	12.1 ± 3.9
		Chronic NDBJ	21	13.9 ± 3.4	3.9 ± 3.0	5.1 ± 3.7	11.9 ± 3.2
		Chronic BJ	21	13.7 ± 2.4	3.6 ± 2.2	5.8 ± 2.7	12.2 ± 3.2
Thermal comfort	Baseline	21	13.7 ± 4.5	6.2 ± 4.5	9.3 ± 4.7	13.4 ± 3.8	
	Acute NDBJ	21	15.1 ± 4.0	6.8 ± 5.3	8.0 ± 4.3	12.6 ± 3.7	
	Acute BJ	21	14.8 ± 3.9	7.3 ± 4.4	8.4 ± 4.1	13.1 ± 3.2	
	Chronic NDBJ	21	14.6 ± 4.1	7.2 ± 4.3	6.7 ± 3.9	12.9 ± 3.0	
	Chronic BJ	21	15.6 ± 3.5	6.4 ± 4.5	8.4 ± 3.9	13.5 ± 3.5	
Pain	Baseline	20	0.1 ± 0.4	0.6 ± 1.2	0.2 ± 0.6	0.2 ± 0.6	
	Acute NDBJ	20	0.0 ± 0.0	0.7 ± 1.3	0.4 ± 1.0	0.2 ± 0.6	
	Acute NDBJ	20	0.2 ± 0.7	0.7 ± 1.3	0.4 ± 1.0	0.3 ± 0.8	
	Chronic PL	20	0.0 ± 0.0	0.8 ± 1.7	0.5 ± 1.0	0.9 ± 2.7	
	Chronic BJ	20	0.1 ± 0.4	0.6 ± 1.2	0.3 ± 0.8	0.2 ± 0.6	

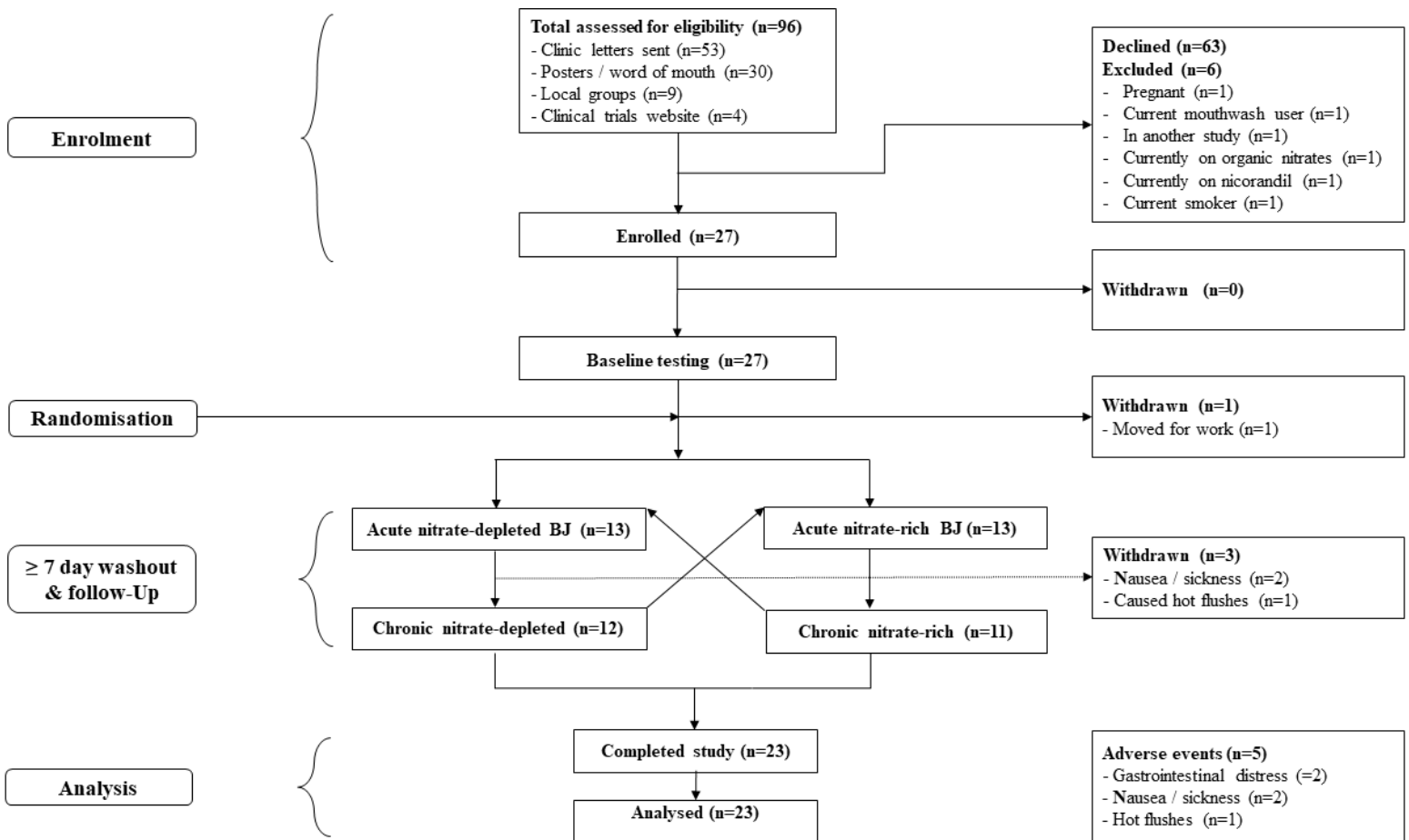
Data are presented as means ± SD. ¹ = significantly different from baseline ($P < 0.05$), ² acute placebo and chronic beetroot ($P < 0.05$).

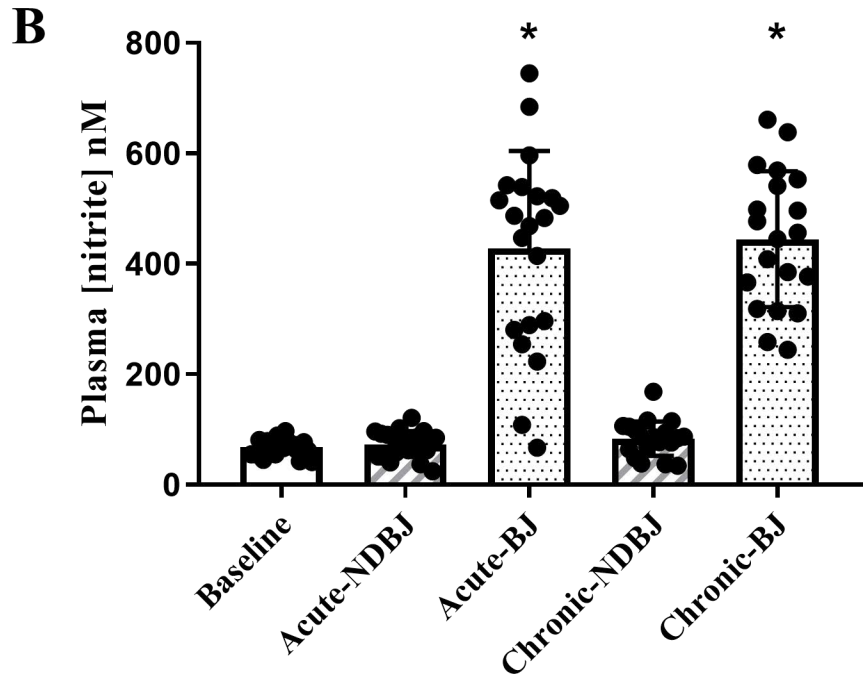
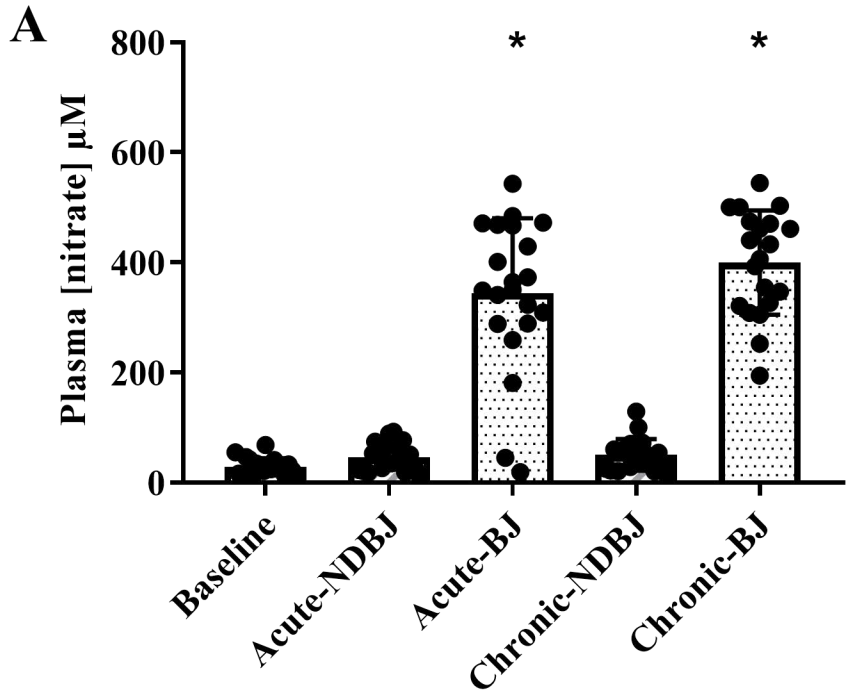
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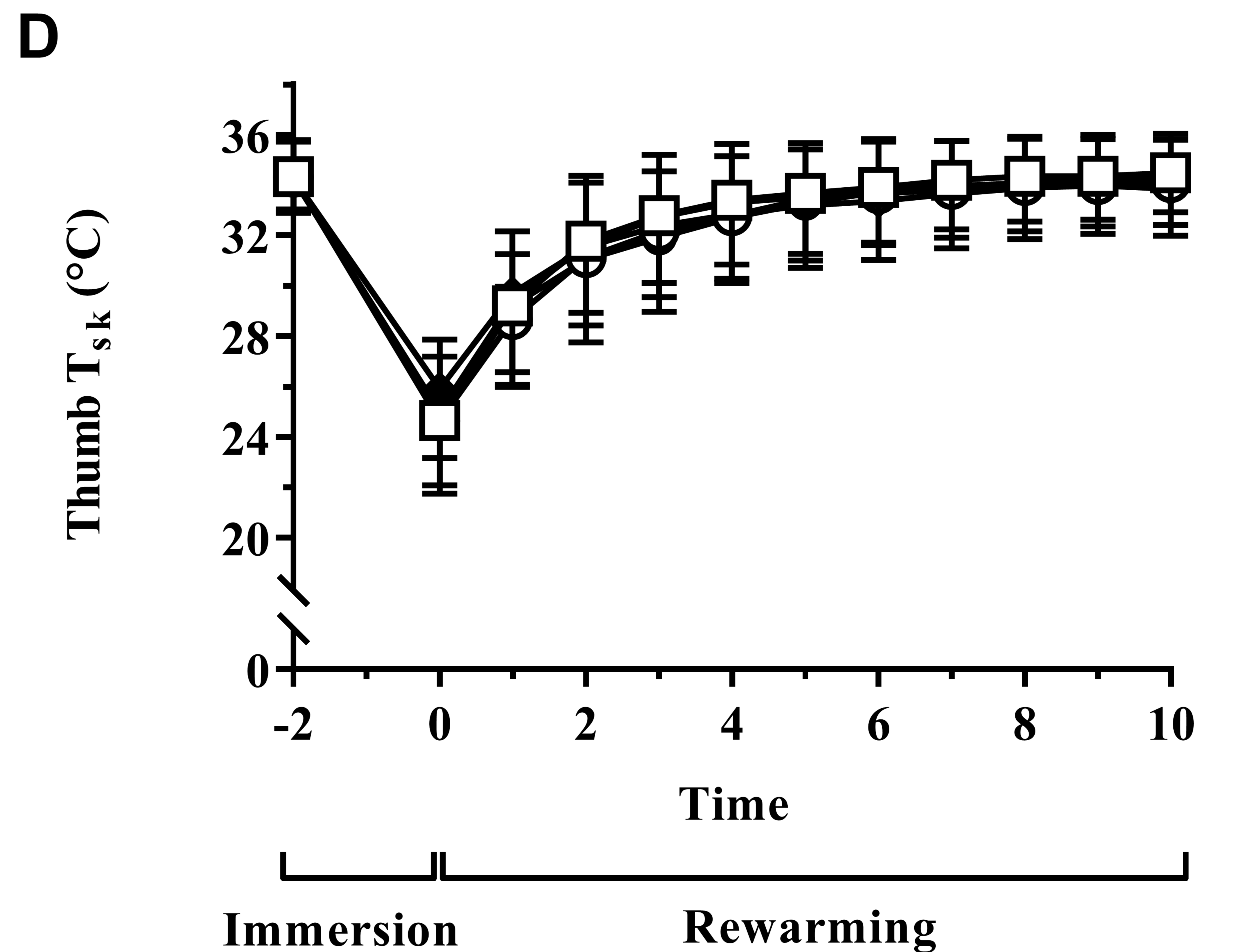
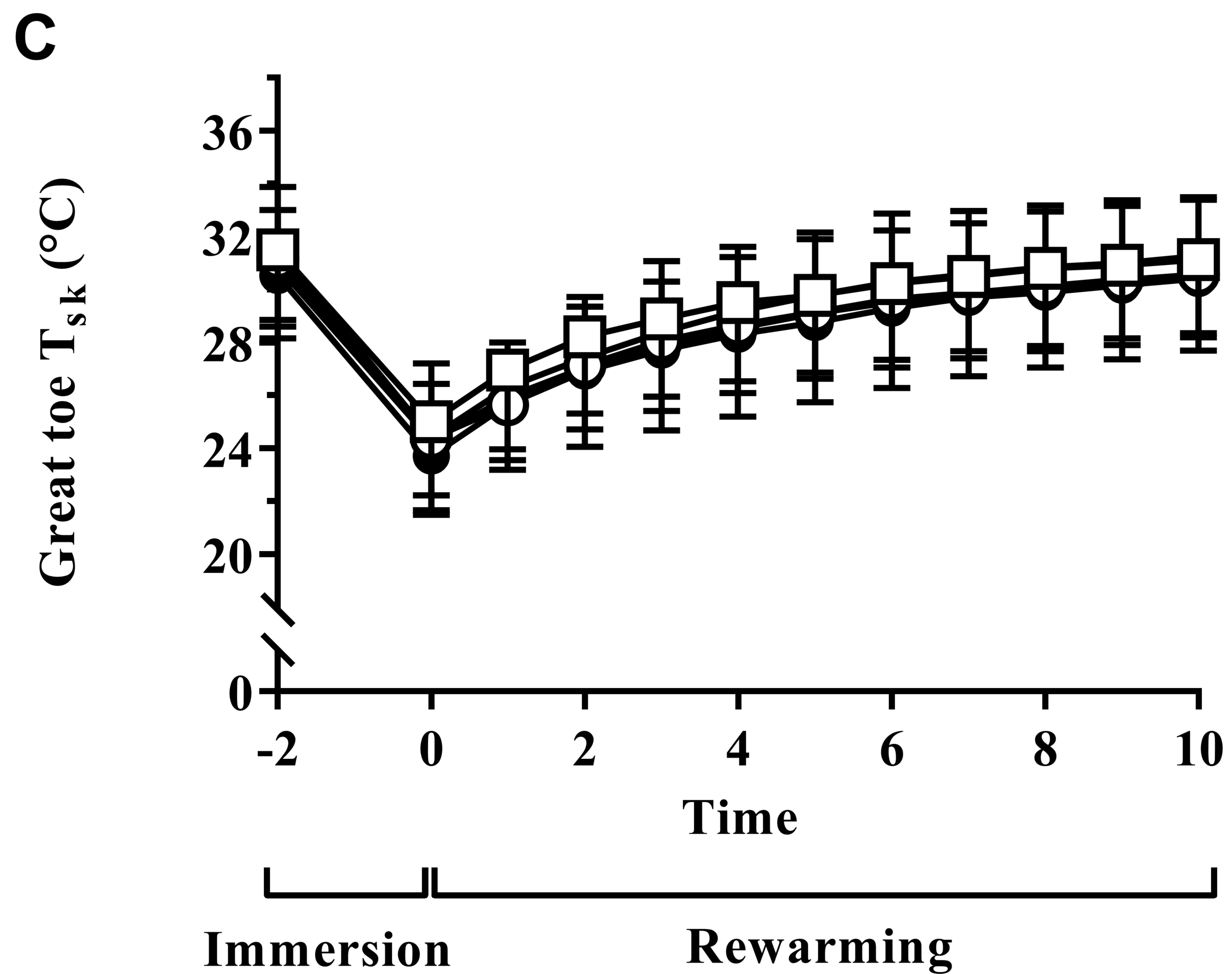
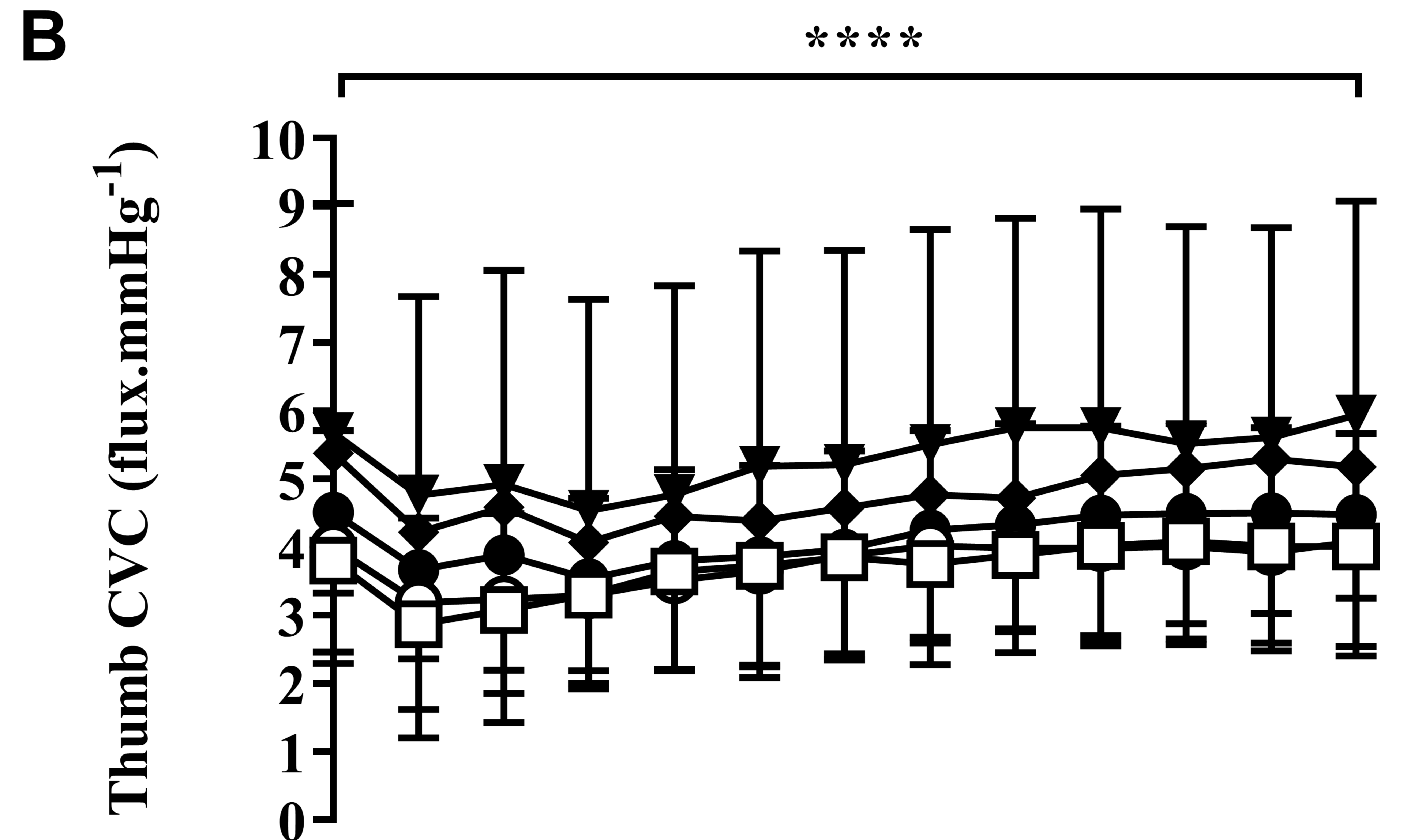
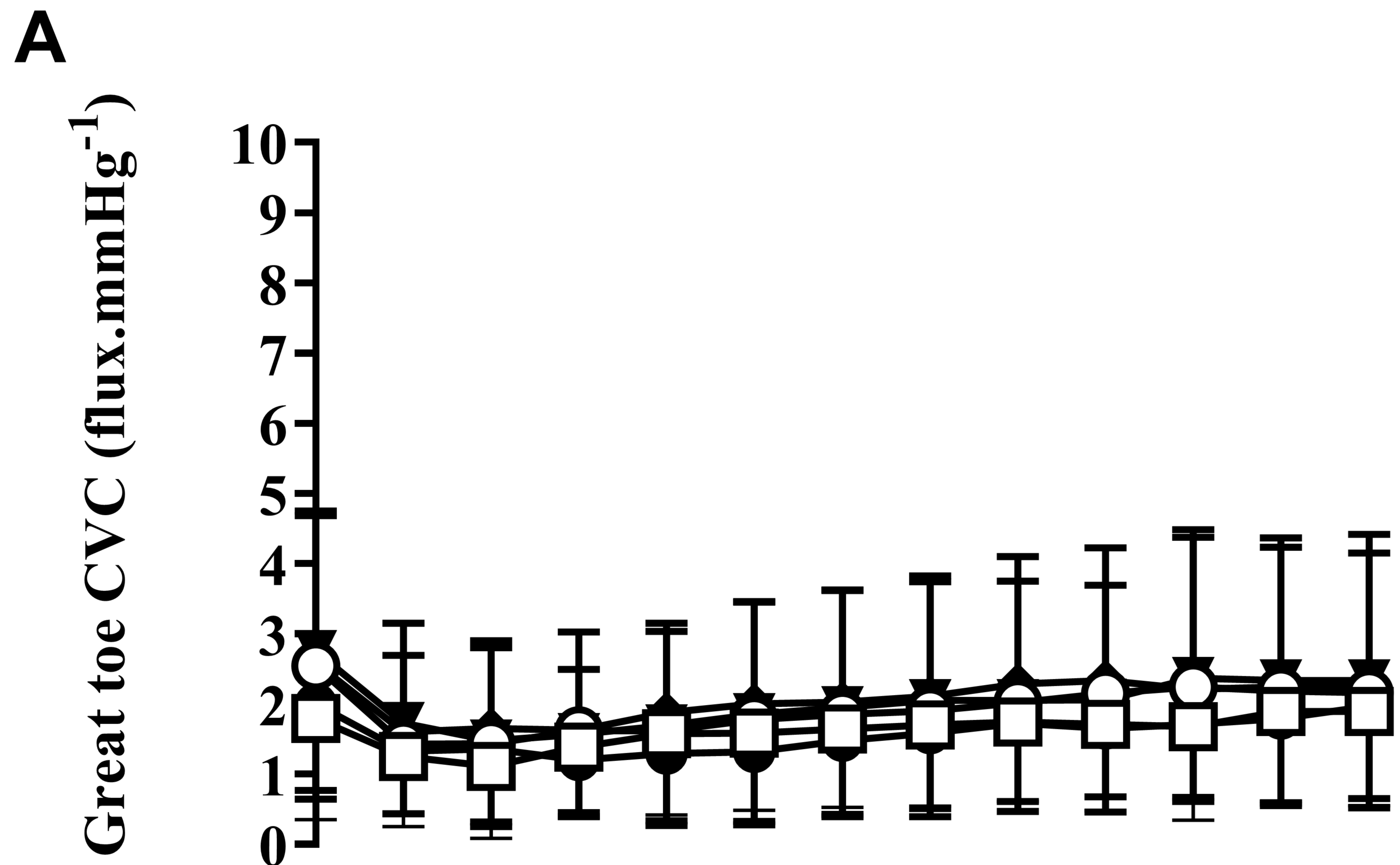
CVC response to iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) on the forearm, finger and foot in the baseline, acute and chronic nitrate rich (BJ) and nitrate depleted (PL) beetroot juice supplementation conditions.

		<i>n</i>	ACh Max	ACh AUC	<i>n</i>	SNP Max	SNP AUC	T _{sk} (°C)
Forearm	Baseline	18	3.1 ± 1.7	15.3 ± 10.2	16	3.3 ± 1.5	12.7 ± 7.0	29.4 ± 0.3
	Acute NDBJ	17	4.4 ± 1.3	20.8 ± 7.9	16	4.1 ± 1.5	17.5 ± 8.2 ³	29.0 ± 0.4
	Acute BJ	17	4.9 ± 1.7 ²	21.9 ± 9.4	16	4.0 ± 1.3	14.7 ± 6.3	29.0 ± 0.4
	Chronic NDBJ	17	3.7 ± 2.0	15.8 ± 8.1	16	3.0 ± 2.0	11.5 ± 8.4	28.9 ± 0.6
	Chronic BJ	17	4.3 ± 1.7 ³	18.8 ± 11.1	16	4.7 ± 1.9 ^{1,3}	17.6 ± 8.0 ^{1,2}	29.0 ± 0.4
	Baseline	15	2.5 ± 1.9	10.6 ± 9.5	15	1.6 ± 1.0	6.7 ± 4.8	28.4 ± 0.3
Finger	Acute NDBJ	15	1.3 (0.8, 2.6)	6.8 (2.8, 9.4)	15	1.2 (0.4, 3.4)	5.7 (1.5, 14.7)	27.1 ± 0.5
	Acute BJ	15	1.9 (0.6, 6.1)	6.4 (2.0, 29.5)	15	1.6 (0.7, 4.0)	5.1 (2.6, 16.0)	27.3 ± 0.6
	Chronic NDBJ	15	2.9 ± 2.7	13.9 ± 19.1	15	2.2 ± 2.4	9.9 ± 12.3	27.2 ± 0.4
	Chronic BJ	15	2.7 ± 2.2	11.4 ± 11.1	15	2.1 ± 1.5	9.2 ± 7.8	27.4 ± 0.4
	Baseline	15	1.7 ± 1.7	6.2 ± 6.9	15	1.1 ± 0.9	3.8 ± 3.2	27.4 ± 0.6
Foot	Acute NDBJ	15	3.4 ± 2.8	11.3 ± 9.8	15	1.7 ± 1.8	5.8 ± 4.6	27.0 ± 0.52
	Acute BJ	15	2.7 ± 1.1	8.2 ± 4.3	15	1.6 ± 1.2	4.4 ± 3.5	26.7 ± 0.8
	Chronic NDBJ	15	2.2 ± 2.1	5.0 (1.4, 12.7)	15	1.8 ± 1.4	5.4 (2.3, 7.4)	26.6 ± 0.6
	Chronic BJ	15	3.4 ± 2.6	11.2 (6.0, 19.3)	15	2.5 ± 1.9	6.7 (2.9, 10.3)	26.6 ± 0.6

Data are presented as means ± SD or median and interquartile range (25 and 75 percentiles) for maximum CVC, area under the curve (AUC) and skin temperature (T_{sk}). ¹ = different to nitrate-depleted beetroot juice (PL) at the same time point and ² = different from baseline, ³ = trend (< 0.10) towards difference with baseline. For the endothelial function test, maximum CVC, AUC and T_{sk} were analysed using repeated-measures ANOVAs (baseline, acute BJ, chronic BJ, acute PL and chronic PL supplementation). Where data were not normally distributed Friedman tests were used with Wilcoxon follow ups.

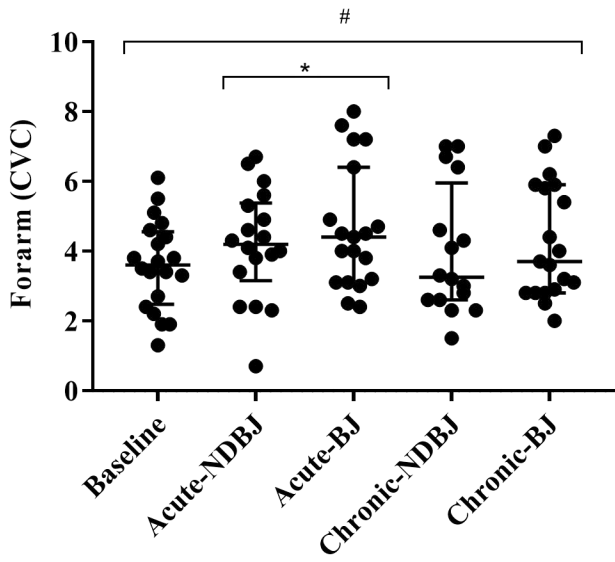






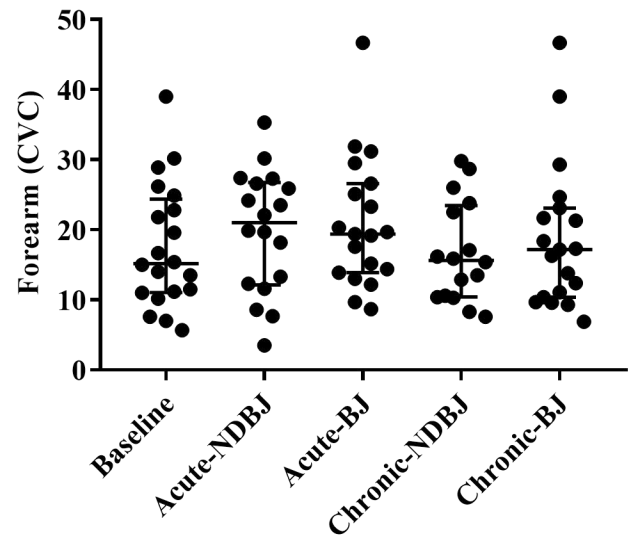
ACh Max

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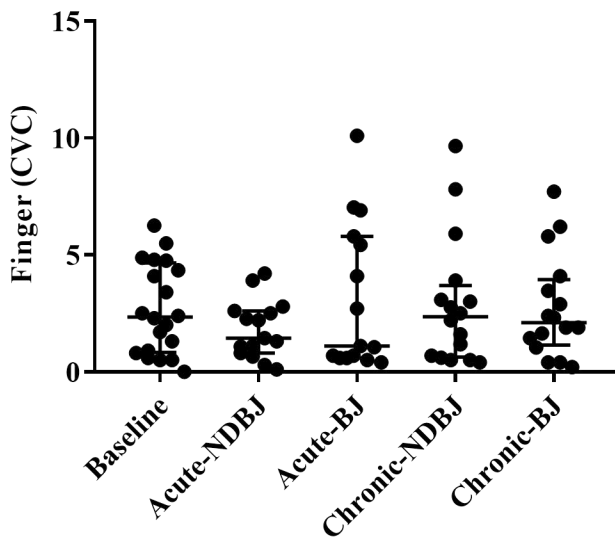


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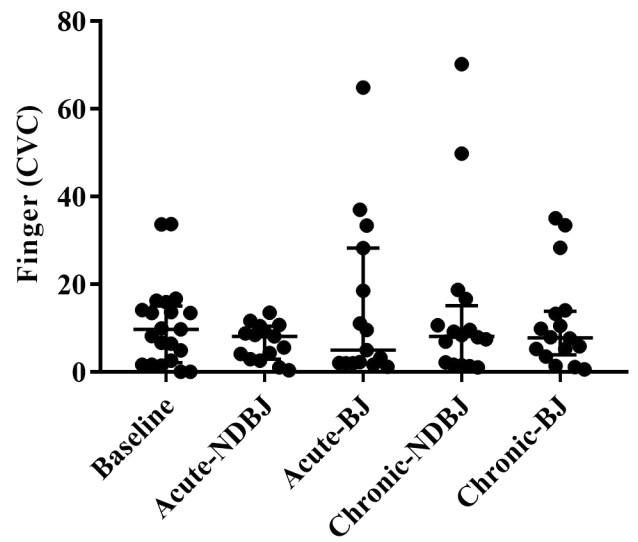
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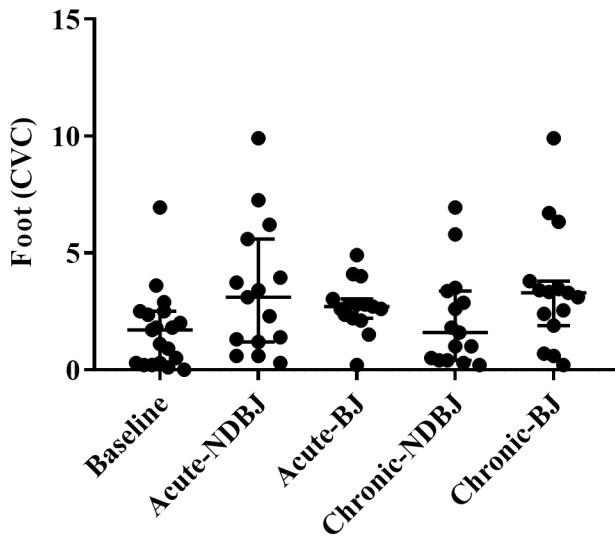
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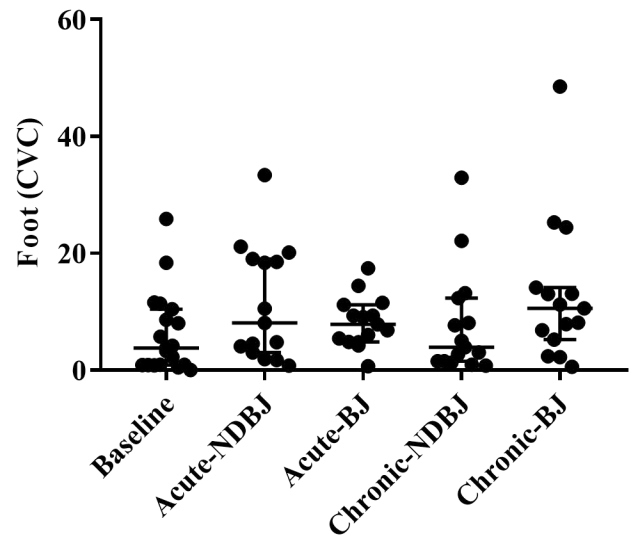
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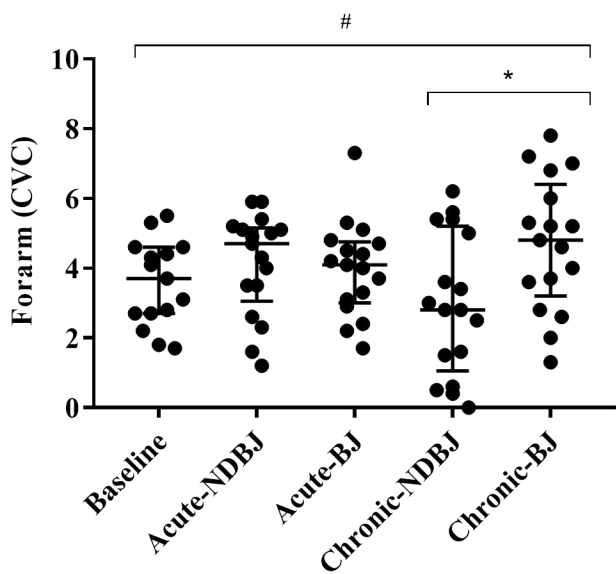


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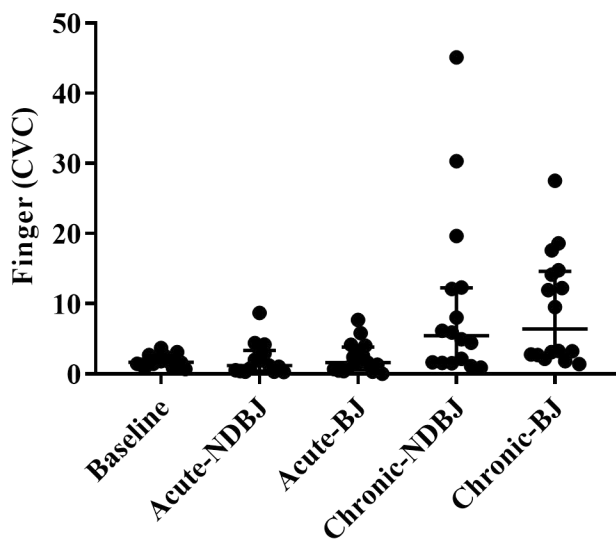


SNP Max

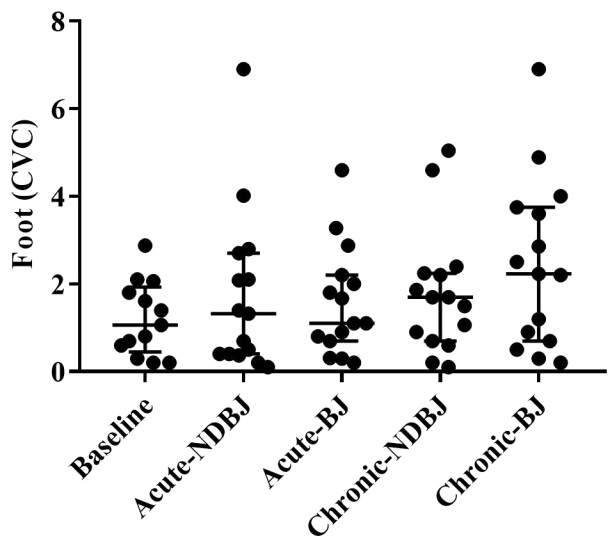
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C

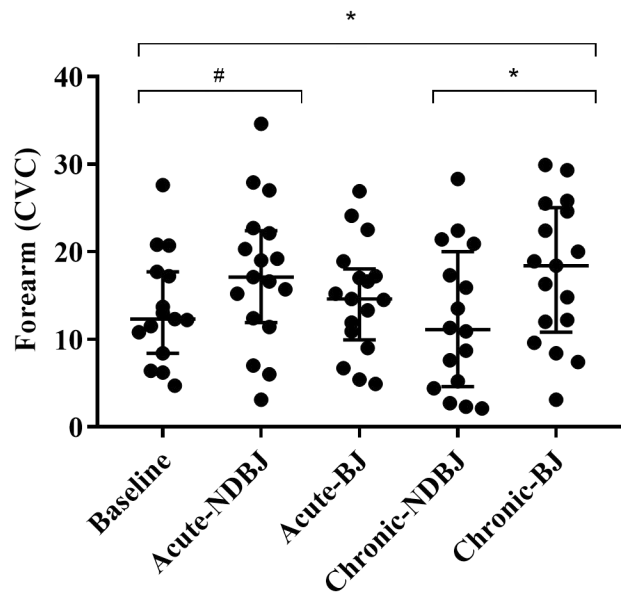


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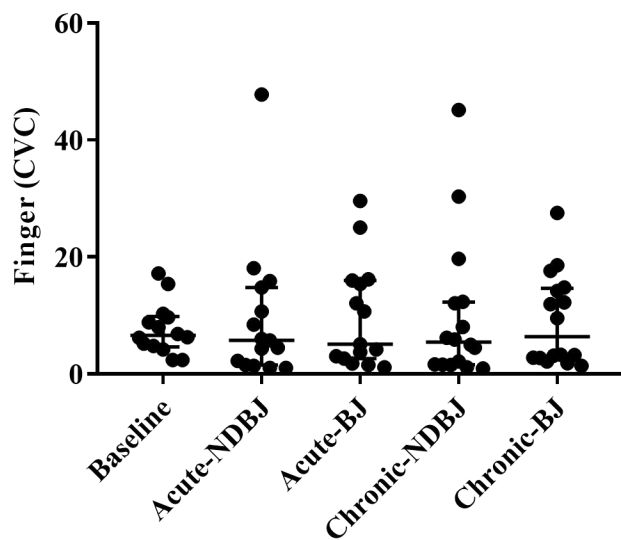


SNP AUC

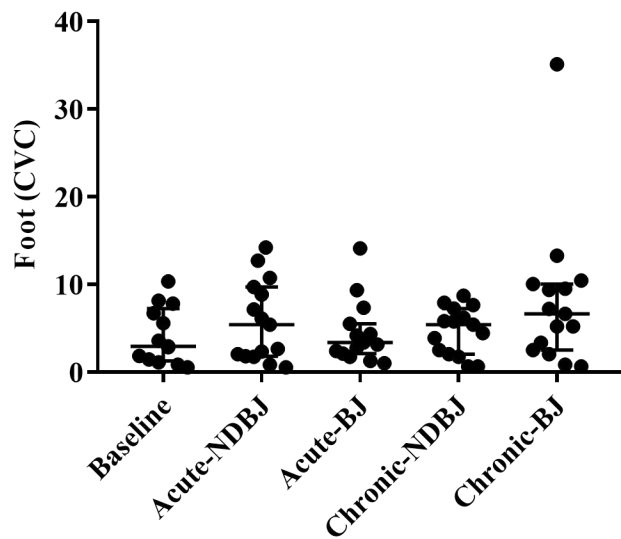
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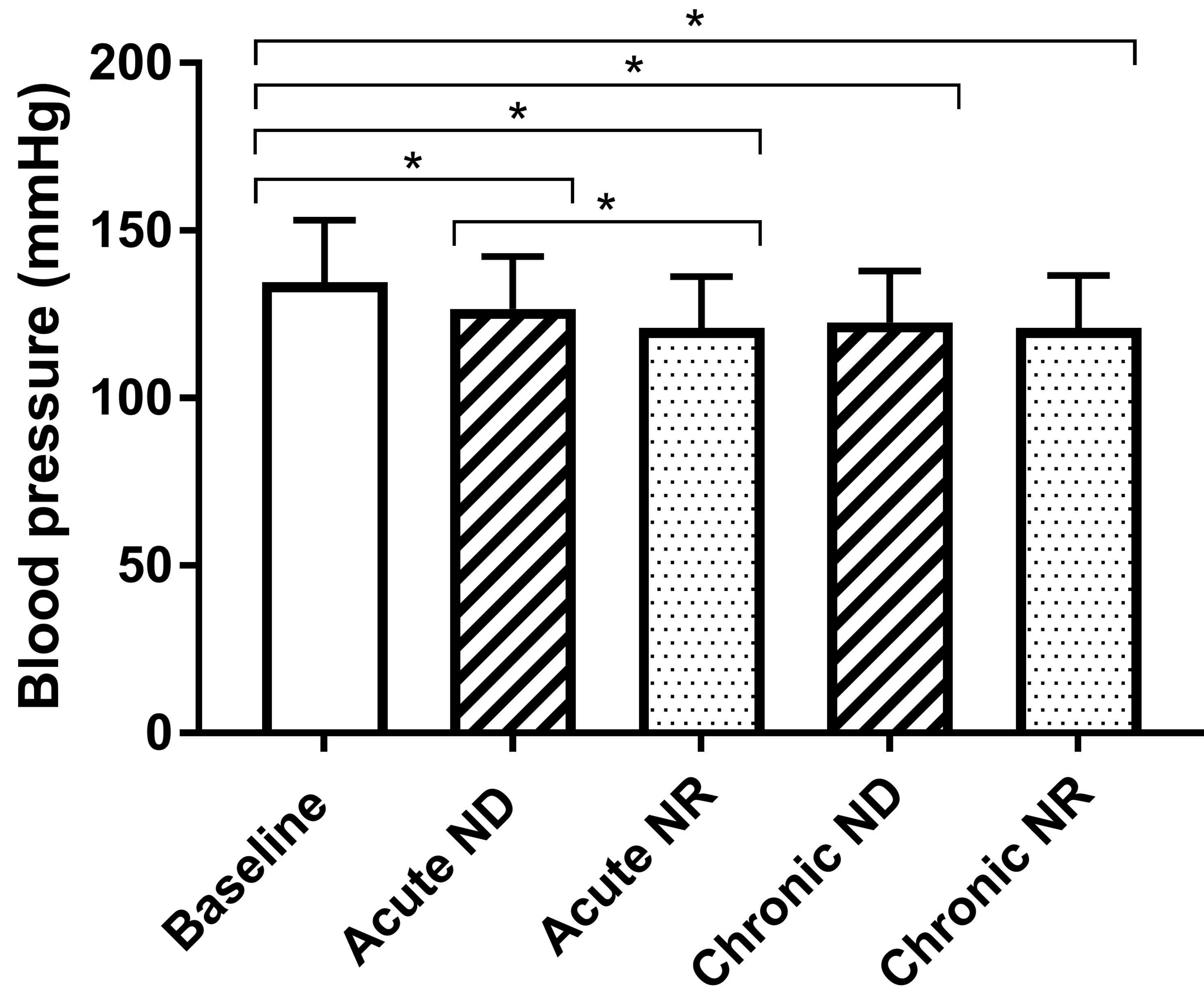


D



F



A**B**