Beet the cold: Beetroot juice supplementation improves peripheral blood flow, endothelial function and anti-inflammatory status in individuals with Raynaud's phenomenon.

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Abstract

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

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

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

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

Key points

Beetroot supplementation improves

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

in people with Raynaud's

New and noteworthy

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

1.0 Introduction

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

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

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

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

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

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

2.0 Methods

2.1 Participants

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

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

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

2.2 Pre-experimental tests

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

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

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

2.3 Protocol and outcome measures

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

Visits 3 and 5 (the chronic supplementation visits) were identical in nature to visits 2 and 4, however followed chronic supplementation of 70 $mL \cdot day^{-1}$ of either BJ or NDBJ for 13 days with 140 mL consumed on the day of testing. Participants, if requested, were reminded to take the juice via text message or voice mails.

2.4 Outcome measures

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

Microvascular endothelial function test

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

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

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

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

Cold sensitivity test

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

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

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

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

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

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

2.5 Biochemical analysis

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

2.6 Qualitative analysis

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

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

2.7 *Data analysis*

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

The distribution of data was assessed using descriptive methods (skewness, outliers, and distribution plots) and inferential statistics (Shapiro–Wilk test). Where normal distribution was violated non parametric analyses were performed. For the cold sensitivity test, statistical differences were assessed 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. For the endothelial function test, maximum CVC, AUC and T_{sk} were analysed using repeated-measures ANOVAs (baseline, acute BJ, chronic BJ, acute NDBJ and chronic NDBJ supplementation). Plasma [nitrate] and [nitrite] were analysed using repeated measures ANOVA and all other biomarkers were analysed using Friedman tests. Where appropriate, post-hoc tests were conducted using pairwise comparisons with least significant differences. Where data were not normally distributed Friedman tests were used with Wilcoxon follow ups. Data are presented as mean (SD) or as median and 25^{th} and 75^{th} percentiles unless otherwise stated. Statistical analysis was performed on SPSS version 24 (Chicago, IL) and statistical difference and trends towards significance were accepted as 2-tailed $P < 0.05$ and $P < 0.1$ respectively. Interviews were transcribed verbatim and analysed thematically.

3.0 Results

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

3.1 Plasma [nitrate] and [nitrite]

Supplementation with BJ significantly increased plasma [nitrate] ($P < 0.001$; Figure 2), and [nitrite] ($P < 0.001$; Figure 2). Post-hoc analysis revealed a statistically significant rise in plasma [nitrate] between acute NDBJ and acute BJ supplementation (43 ± 25 μM; 339 ± 146 μM; $P < 0.001$, respectively) and chronic NDBJ and chronic BJ supplementation (52 ± 29 μM; 397 ± 96 μM; $P < 0.001$, respectively) and plasma [nitrite] between acute NDBJ and acute BJ supplementation (69 ± 23 nM; 428 ± 187 nM; $P < 0.001$, respectively) and chronic NDBJ and chronic BJ supplementation (87 ± 31 nM; 428 ± 117 nM; $P < 0.001$, respectively) (Figure 2). There were no differences between acute and chronic NDBJ ($P > 0.05$) and acute and chronic BJ ($P > 0.05$).

3.2 Cold sensitivity test

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

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

3.4 BP

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

3.5 Cytokines and redox markers

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

3.6 Qualitative interviews

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

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

4.0 Discussion

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

4.1 Plasma [nitrate] and [nitrite]

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

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

4.2 Cold sensitivity test

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

4.3 Thermal comfort, sensation and pain

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

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

4.4 Endothelial function

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

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

4.5 Blood pressure

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

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

4.6 Cytokines and antioxidant enzymes

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

4.7 Qualitative interviews

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

4.8 Strengths, limitations and future work

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

5.0 Conclusion

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

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

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7.0 Contribution Statement

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

8.0 References

1. **Anderson M, Moore T, Hollis S, Jayson M, King T, and Herrick A**. Digital vascular response to topical glyceryl trinitrate, as measured by laser Doppler imaging, in primary Raynaud's phenomenon and systemic sclerosis. *Rheumatology* 41: 324-328, 2002.

2. **Bach AJ, Stewart IB, Minett GM, and Costello JT**. Does the technique employed for skin temperature assessment alter outcomes? A systematic review. *Physiological measurement* 36: R27, 2015.

3. **Bateman RM, Ellis CG, and Freeman DJ**. Optimization of Nitric Oxide Chemiluminescence Operating Conditions for Measurement of Plasma Nitrite and Nitrate. *Clinical Chemistry* 48: 570-573, 2002.

4. **Benjamin N, O'Driscoll F, Dougall H, Duncan C, Smith S, Golden M, and McKenzie H**. Stomach NO synthesis. *Nature* 368: 502, 1994.

5. **Berry MJ, Justus NW, Hauser JI, Case AH, Helms CC, Basu S, Rogers Z, Lewis MT, and Miller GD**. Dietary nitrate supplementation improves exercise performance and decreases blood pressure in COPD patients. *Nitric Oxide* 2014.

6. **Biondi R, Coaccioli S, Lattanzi S, Puxeddu A, and Papini M**. Oxidant/antioxidant status in patients with Raynaud's disease. *La Clinica terapeutica* 159: 77-81, 2008.

7. **Braun V, and Clarke V**. Using thematic analysis in psychology. *Qualitative research in psychology* 3: 77-101, 2006.

8. **Bryan N, S, and Hord N, G**. *Dietary nitrate and nitrites: The physiological context for potential health benefits/* Food, nutrition and the nitric oxide pathway: DEStech Publications, 2010, p. pp. 59-78.

9. **Buga GM, Gold ME, Fukuto JM, and Ignarro LJ**. Shear stress-induced release of nitric oxide from endothelial cells grown on beads. *Hypertension* 17: 187-193, 1991.

10. **Bunker C, Goldsmith P, Leslie T, Hayes N, Foreman J, and Dowd P**. Calcitonin gene-related peptide, endothelin-1, the cutaneous microvasculature and Raynaud's phenomenon. *British Journal of dermatology* 134: 399-406, 1996.

11. **Carlström M, Liu M, Yang T, Zollbrecht C, Huang L, Peleli M, Borniquel S, Kishikawa H, Hezel M, Persson AEG, Weitzberg E, and Lundberg JO**. Cross-talk Between Nitrate-Nitrite-NO and NO Synthase Pathways in Control of Vascular NO Homeostasis. *Antioxidants & redox signaling* 23: 295-306, 2015.

12. **Clifford T, Bowman A, Capper T, Allerton DM, Foster E, Birch-Machin M, Lietz G, Howatson G, and Stevenson EJ**. A pilot study investigating reactive oxygen species production in capillary blood after a marathon and the influence of an antioxidant-rich beetroot juice. *Applied Physiology, Nutrition, and Metabolism* 43: 303-306, 2017.

13. **Cosby K, Partovi K, Crawford J, H, Patel R, P, Reiter C, D, Martyr S, Yang B, K, Waclawiw M, A, Zalos G, Xu X, Huang K, T, Shields H, Kim-Shapiro D, B, Schechter A, N, Cannon III R, O, and Gladwin M, T**. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodiltaes the human circulation *Nature Medicine* 9: 1498-1505, 2003.

14. **Davey M, Eglin C, House J, and Tipton M**. The contribution of blood flow to the skin temperature responses during a cold sensitivity test. *European journal of applied physiology* 113: 2411-2417, 2013.

15. **Duncan C, Dougall H, Johnston P, Green S, Brogan R, Smith L, Golden M, and Benjamin N**. Chemical generation of nitric oxide in the mouth from the entrosalivery circulation of dietary nitrate. *Nature Medicine* 1: 546-551, 1995.

16. **Egert S, Bosy-Westphal A, Seiberl J, Kürbitz C, Settler U, Plachta-Danielzik S, Wagner AE, Frank J, Schrezenmeir J, and Rimbach G**. Quercetin reduces systolic blood pressure and plasma oxidised low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype: a double-blinded, placebo-controlled cross-over study. *British Journal of Nutrition* 102: 1065-1074, 2009.

17. **Eglin CM, Costello JT, Bailey SJ, Gilchrist M, Massey H, and Shepherd AI**. Effects of dietary nitrate supplementation on the response to extremity cooling and endothelial function in individuals with cold sensitivity. A double blind, placebo controlled, crossover, randomised control trial. *Nitric Oxide* 70: 76-85, 2017.

18. **Eglin CM, Golden FS, and Tipton MJ**. Cold sensitivity test for individuals with non-freezing cold injury: the effect of prior exercise. *Extreme Physiology & Medicine* 2: 1, 2013.

19. **Ferreira-Valente MA, Pais-Ribeiro JL, and Jensen MP**. Validity of four pain intensity rating scales. *PAIN®* 152: 2399-2404, 2011.

20. **Forbes A, and Marie I**. Gastrointestinal complications: the most frequent internal complications of systemic sclerosis. *Rheumatology* 48: iii36-iii39, 2006.
21. **Förstermann U, and Sessa WC**. Nitric oxide synthases: regulation and function. *European Heart Journal* 33: 829-837, 2012.
22. **Gao X, Yang T, Liu M, Peleli M, Zollbrecht C, Weitzberg E, Lundberg JO, Persson AEG, and Carlström M**. NADPH oxidase in the renal microvasculature is a primary target for blood pressure–lowering effects by inorganic nitrate and nitrite. *Hypertension* 65: 161-170, 2015.
23. **Gerrits EG, Alkhalaf A, Landman GW, van Hateren KJ, Groenier KH, Struck J, Schulte J, Gans RO, Bakker SJ, and Kleefstra N**. Serum peroxiredoxin 4: a marker of oxidative stress associated with mortality in type 2 diabetes (ZODIAC-28). *PloS one* 9: e89719, 2014.
24. **Gilchrist M, Winyard P, and Benjamin N**. Dietary Nitrate - Good or bad? *Nitric Oxide* 22: 104-109, 2010.
25. **Gilchrist M, Winyard PG, Aizawa K, Anning C, Shore A, and Benjamin N**. Effect of dietary nitrate on blood pressure, endothelial function, and insulin sensitivity in type 2 diabetes. *Free Radical Biology and Medicine* 60: 89-97, 2013.
26. **Gottfredsen RH, Tran SM-H, Larsen UG, Madsen P, Nielsen MS, Enghild JJ, and Petersen SV**. The C-terminal proteolytic processing of extracellular superoxide dismutase is redox regulated. *Free Radical Biology and Medicine* 52: 191-197, 2012.
27. **Govoni M, Jansson EÅ, Weitzberg E, and Lundberg JO**. The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide* 19: 333-337, 2008.
28. **Greenstein D, Gupta N, Martin P, Walker D, and Kester R**. Impaired thermoregulation in Raynaud's phenomenon. *Angiology* 46: 603-611, 1995.
29. **Gualtierotti R, Ingegnoli F, Griffini S, Grovetti E, Borghi MO, Bucciarelli P, Meroni PL, and Cugno M**. Detection of early endothelial damage in patients with Raynaud's phenomenon. *Microvascular research* 113: 22-28, 2017.
30. **Hertzog MA**. Considerations in determining sample size for pilot studies. *Research in nursing & health* 31: 180-191, 2008.
31. **Holowatz L, Thompson-Torgerson C, and Kenney W**. Viewpoint: The human cutaneous circulation as a model of generalized microvascular function. *J Appl Physiol*.
32. **Hope K, Eglin C, Golden F, and Tipton M**. Sublingual glyceryl trinitrate and the peripheral thermal responses in normal and cold-sensitive individuals. *Microvascular research* 91: 84-89, 2014.
33. **Hyde ER, Luk B, Cron S, Kusic L, McCue T, Bauch T, Kaplan H, Tribble G, Petrosino JF, and Bryan NS**. Characterization of the rat oral microbiome and the effects of dietary nitrate. *Free Radical Biology and Medicine* 77: 249-257, 2014.
34. **Iversen MB, Gottfredsen RH, Larsen UG, Enghild JJ, Praetorius J, Borregaard N, and Petersen SV**. Extracellular superoxide dismutase is present in secretory vesicles of human neutrophils and released upon stimulation. *Free Radical Biology and Medicine* 97: 478-488, 2016.
35. **Jin D-Y, Chae HZ, Rhee SG, and Jeang K-T**. Regulatory role for a novel human thioredoxin peroxidase in NF-κB activation. *Journal of Biological Chemistry* 272: 30952-30961, 1997.
36. **Jonvik KL, Nyakayiru J, Pinckaers PJ, Senden JM, van Loon LJ, and Verdijk LB**. Nitrate-Rich Vegetables Increase Plasma Nitrate and Nitrite Concentrations and Lower Blood Pressure in Healthy Adults. *The Journal of nutrition* 146: 986-993, 2016.
37. **Joris PJ, and Mensink RP**. Beetroot juice improves in overweight and slightly obese men postprandial endothelial function after consumption of a mixed meal. *Atherosclerosis* 231: 78-83, 2013.
38. **Kahaleh MB, and LeRoy EC**. Vascular factors in the pathogenesis of systemic sclerosis. *Systemic sclerosis: scleroderma Chichester: John Wiley & Sons* 107-118, 1988.
39. **Kapil V, Khambata RS, Robertson A, Caulfield MJ, and Ahluwalia A**. Dietary Nitrate Provides Sustained Blood Pressure Lowering in Hypertensive Patients: A Randomized, Phase 2, Double-Blind, Placebo-Controlled Study. *Hypertension* 65: 320-327, 2015.

40. **Kelly J, Fulford J, Vanhatalo A, Blackwell JR, French O, Bailey SJ, Gilchrist M, Winyard PG, and Jones AM**. Effects of short-term dietary nitrate supplementation on blood pressure, O2 uptake kinetics, and muscle and cognitive function in older adults. *American journal of physiology Regulatory, integrative and comparative physiology* 304: 21, 2013.

41. **Kenjale AA, Ham KL, Stabler T, Robbins JL, Johnson JL, VanBruggen M, Privette G, Yim E, Kraus WE, and Allen JD**. Dietary nitrate supplementation enhances exercise performance in peripheral arterial disease. *Journal of Applied Physiology* 110: 1582-1591, 2011.

42. **Kerley CP, Cahill K, Bolger K, McGowan A, Burke C, Faul J, and Cormican L**. Dietary nitrate supplementation in COPD: An acute, double-blind, randomized, placebo-controlled, crossover trial☆. *Nitric Oxide* 44: 105-111, 2015.

43. **Khan F, and Belch J**. Skin blood flow in patients with systemic sclerosis and Raynaud's phenomenon: effects of oral L-arginine supplementation. *The Journal of rheumatology* 26: 2389-2394, 1999.

44. **Khan F, Ray S, Craigie AM, Kennedy G, Hill A, Barton KL, Broughton J, and Belch JJ**. Lowering of oxidative stress improves endothelial function in healthy subjects with habitually low intake of fruit and vegetables: A randomized controlled trial of antioxidant-and polyphenol-rich blackcurrant juice. *Free Radical Biology and Medicine* 72: 232-237, 2014.

45. **Koltuksuz U, Özen S, Uz E, Aydinç M, Karaman A, Gültek A, Akyol Ö, Gürsoy MH, and Aydin E**. Caffeic acid phenethyl ester prevents intestinal reperfusion injury in rats. *Journal of Pediatric Surgery* 34: 1458-1462, 1999.

46. **Lara J, Ashor AW, Oggioni C, Ahluwalia A, Mathers JC, and Siervo M**. Effects of inorganic nitrate and beetroot supplementation on endothelial function: a systematic review and meta-analysis. *European journal of nutrition* 55: 451-459, 2016.

47. **Levitt EL, Keen JT, and Wong BJ**. Augmented reflex cutaneous vasodilatation following short-term dietary nitrate supplementation in humans. *Experimental physiology* 100: 708-718, 2015.

48. **Levitt EL, Keen JT, and Wong BJ**. Augmented reflex cutaneous vasodilatation following short-term dietary nitrate supplementation in humans. *Experimental physiology* 2015.

49. **Li H, and Forstermann U**. Uncoupling of endothelial NO synthase in atherosclerosis and vascular disease. *Curr Opin Pharmacol* 13: 161-167, 2013.

50. **Lundberg J, Weitzberg E, and Gladwin M, T**. The nitrate-nitrite-nitric oxide pathway in physiology and theraputics. *Nature Reviews Drug Discovery* 7: 156-167, 2008.

51. **Minson CT, Holowatz LA, Wong BJ, Kenney WL, and Wilkins BW**. Decreased nitric oxide-and axon reflex-mediated cutaneous vasodilation with age during local heating. *Journal of applied physiology* 93: 1644-1649, 2002.

52. **Moncada S, and Higgs A**. The L-Argenine-nitric oxide pathway. *New England Journal of Medicine* 329: 2002-2012, 1993.

53. **Moreira DG, Costello JT, Brito CJ, Adamczyk JG, Ammer K, Bach AJ, Costa CM, Eglin C, Fernandes AA, and Fernández-Cuevas I**. Thermographic imaging in sports and exercise medicine: a Delphi study and consensus statement on the measurement of human skin temperature. *Journal of Thermal Biology* 2017.

54. **Needleman P, and Johnson EM**. Mechanism of tolerance development to organic nitrates *Journal of Pharmacology and Experimental Therapeutics* 184: 709, 1973.

55. **Norrbrand L, Kölegård R, Keramidas ME, Mekjavic IB, and Eiken O**. No association between hand and foot temperature responses during local cold stress and rewarming. *European Journal of Applied Physiology* 1-13, 2017.

56. **Ochiai R, Jokura H, Suzuki A, Tokimitsu I, Ohishi M, Komai N, Rakugi H, and Ogihara T**. Green coffee bean extract improves human vasoreactivity. *Hypertension Research* 27: 731-737, 2004.

57. **Omar SA, Artime E, and Webb AJ**. A comparison of organic and inorganic nitrates/nitrites. *Nitric Oxide* 26: 229-240, 2012.

775 58. **Shepherd AI, Gilchrist M, Winyard PG, Jones AM, Hallmann E, Kazimierczak R,**
776 **Rembialkowska E, Benjamin N, Shore AC, and Wilkerson DP**. Effects of dietary nitrate
777 supplementation on the oxygen cost of exercise and walking performance in individuals with type 2
778 diabetes: a randomized, double-blind, placebo-controlled crossover trial. *Free Radical Biology and*
779 *Medicine* 86: 200-208, 2015.
780 59. **Shepherd AI, Wilkerson DP, Dobson L, Kelly J, Winyard PG, Jones AM, Benjamin N, Shore**
781 **AC, and Gilchrist M**. The effect of dietary nitrate supplementation on the oxygen cost of cycling,
782 walking performance and resting blood pressure in individuals with chronic obstructive pulmonary
783 disease: a double blind placebo controlled, randomised control trial. *Nitric Oxide* 2015.
784 60. **Shepherd AI, Wilkerson DP, Fulford J, Winyard PG, Benjamin N, Shore AC, and Gilchrist M**.
785 Effect of nitrate supplementation on hepatic blood flow and glucose homeostasis: A double-blind,
786 placebo controlled, randomised control trial. *American Journal of Physiology-Gastrointestinal and*
787 *Liver Physiology* ajpgi. 00203.02016, 2016.
788 61. **Smith TP, Haymond T, Smith SN, and Sweitzer SM**. Evidence for the endothelin system as an
789 emerging therapeutic target for the treatment of chronic pain. *Journal of pain research* 7: 531, 2014.
790 62. **Tanaka A, Yamazaki M, Saito M, OH-I T, Watanabe Y, and Tsuboi R**. Highly sensitive high-
791 pressure liquid chromatography with ultraviolet light method detected the reduction of serum
792 nitrite/nitrate levels after cold exposure in patients with Raynaud's phenomenon. *The Journal of*
793 *dermatology* 39: 889-890, 2012.
794 63. **Tschudi MR, Barton M, Bersinger NA, Moreau P, Cosentino F, Noll G, Malinski T, and**
795 **Lüscher TF**. Effect of age on kinetics of nitric oxide release in rat aorta and pulmonary artery. *The*
796 *Journal of Clinical Investigation* 98: 899-905, 1996.
797 64. **Tucker A, Pearson R, Cooke E, and Benjamin N**. Effect of nitric-oxide-generating system on
798 microcirculatory blood flow in skin of patients with severe Raynaud's syndrome: a randomised trial.
799 *The Lancet* 354: 1670-1675, 1999.
800 65. **Wadley AJ, Veldhuijzen van Zanten JJCS, and Aldred S**. The interactions of oxidative stress
801 and inflammation with vascular dysfunction in ageing: the vascular health triad. *Age (Dordrecht,*
802 *Netherlands)* 35: 705-718, 2013.
803 66. **Wigley FM**. Raynaud's Phenomenon. *New England Journal of Medicine* 347: 1001-1008,
804 2002.
805 67. **Wootton-Beard PC, and Ryan L**. Combined use of multiple methodologies for the
806 measurement of total antioxidant capacity in UK commercially available vegetable juices. *Plant foods*
807 *for human nutrition (Dordrecht, Netherlands)* 67: 142-147, 2012.
808 68. **Wylie LJ, Kelly J, Bailey SJ, Blackwell JR, Skiba PF, Winyard PG, Jeukendrup AE, Vanhatalo**
809 **A, and Jones AM**. Beetroot juice and exercise: pharmacodynamic and dose-response relationships. *J*
810 *Appl Physiol* 115: 325-336, 2013.
811 69. **Zamani P, Rawat D, Shiva-Kumar P, Geraci S, Bhuva R, Konda P, Doulias PT, Ischiropoulos**
812 **H, Townsend RR, Margulies KB, Cappola TP, Poole DC, and Chirinos JA**. Effect of inorganic nitrate on
813 exercise capacity in heart failure with preserved ejection fraction. *Circulation* 131: 371-380, 2015.
814 70. **Zhang H, Huizenga C, Arens E, and Wang D**. Thermal sensation and comfort in transient
815 non-uniform thermal environments. *European journal of applied physiology* 92: 728-733, 2004.

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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 ± SD great toe CVC (A), thumb CVC (B), great toe skin temperature (C) and thumb skin temperature (D) for baseline (open squares □), acute nitrate-depleted beetroot juice (NDBJ) (open circles ○), acute (beetroot juice) BJ (closed circles ●), chronic NDBJ (closed triangles ▼) and chronic BJ (closed diamonds ♦) 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 ± SD * $P < 0.05$ significantly different from corresponding brackets ($n = 23$). Data were analysed using repeated measures ANOVA.

Figure 7. Median and IQR (25^{th} and 75^{th} 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.

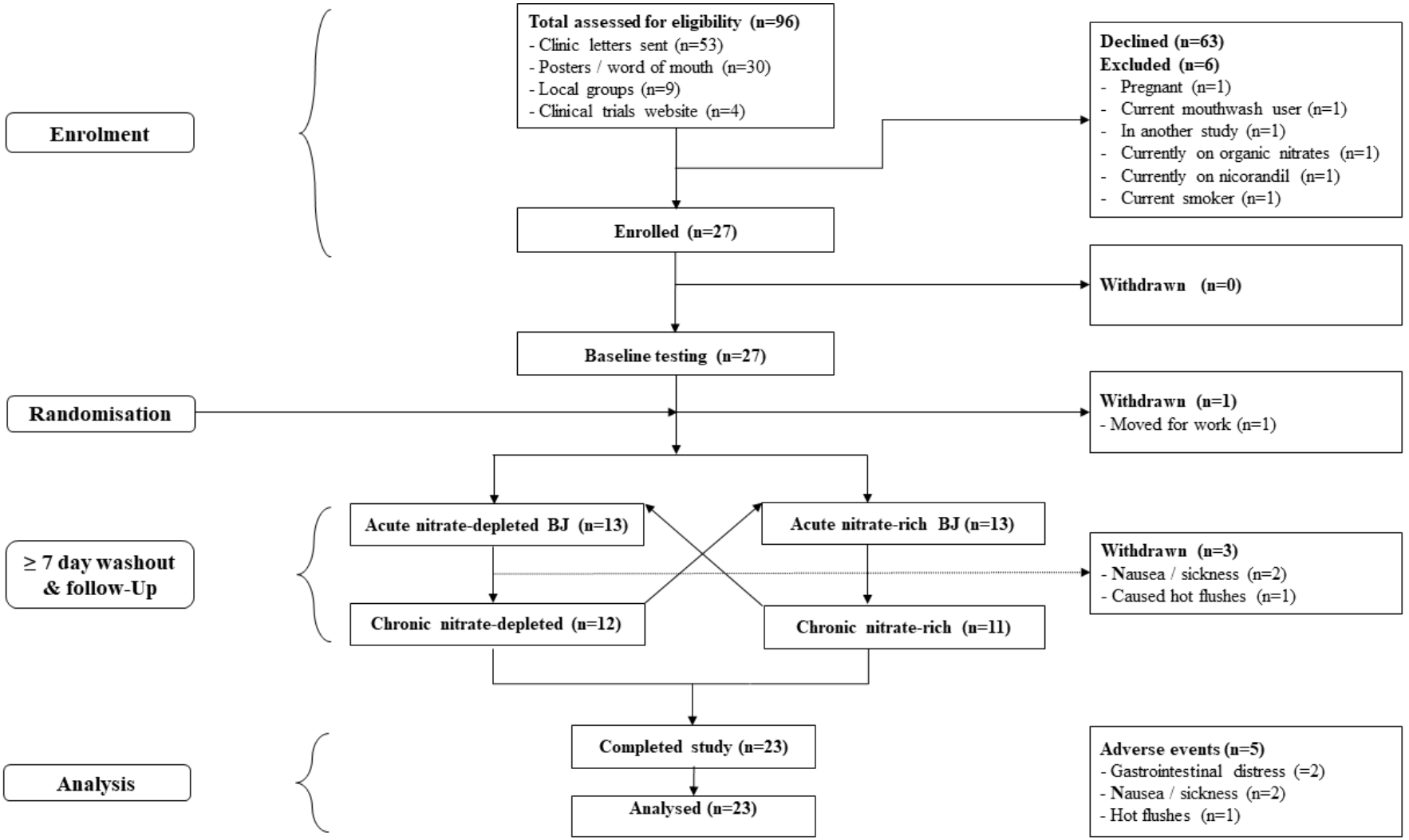


Figure 1. Participant flow through the trial.

Figure 2.

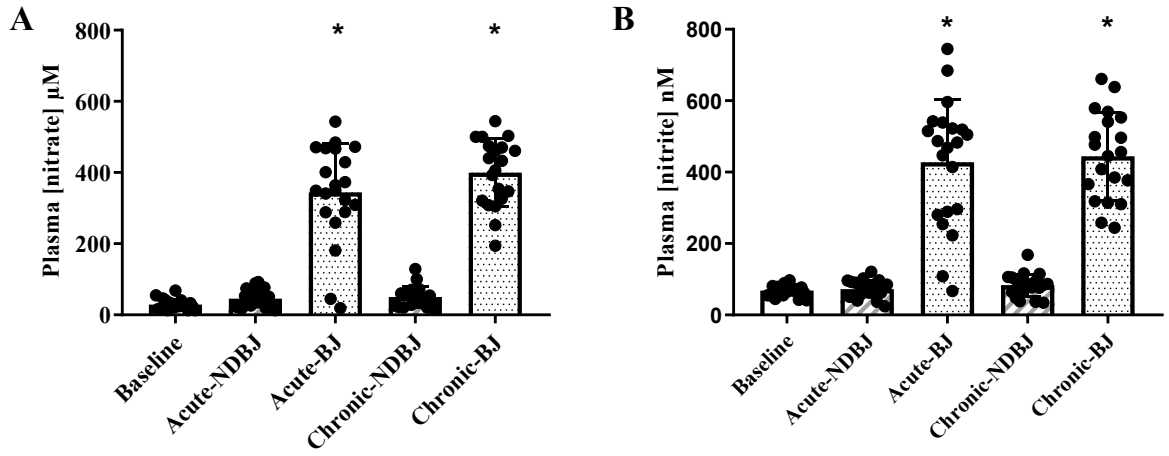

A
Plasma [nitrate] µM
800
600
400
200
0
*
*
Baseline
Acute-NDBJ
Acute-BJ
Chronic-NDBJ
Chronic-BJ
B
Plasma [nitrite] nM
800
600
400
200
0
*
*
Baseline
Acute-NDBJ
Acute-BJ
Chronic-NDBJ
Chronic-BJ

Figure 3.

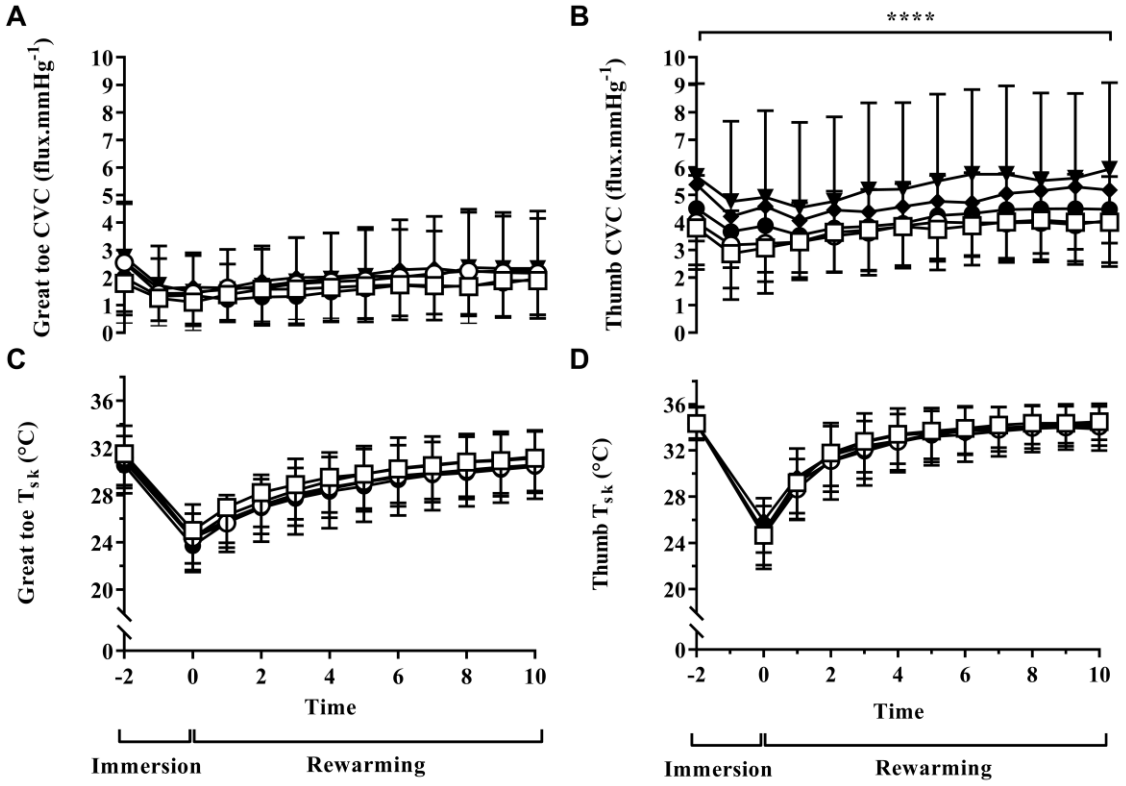

A
B
C
D
****
Great toe CVC (flux.mmHg-1)
Thumb CVC (flux.mmHg-1)
Great toe Tsk (°C)
Thumb Tsk (°C)
Time
Immersion
Rewarming

Figure 4.

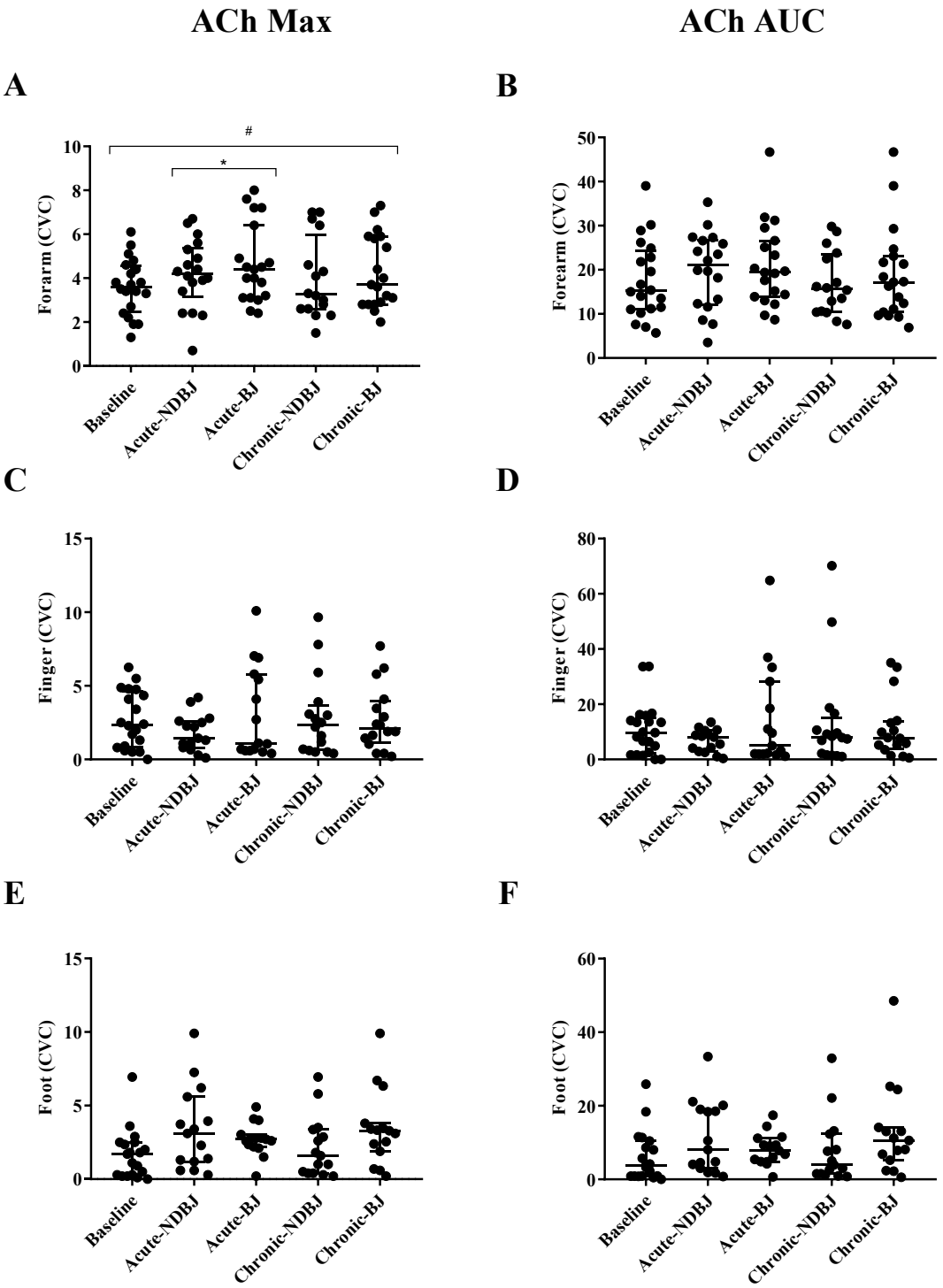
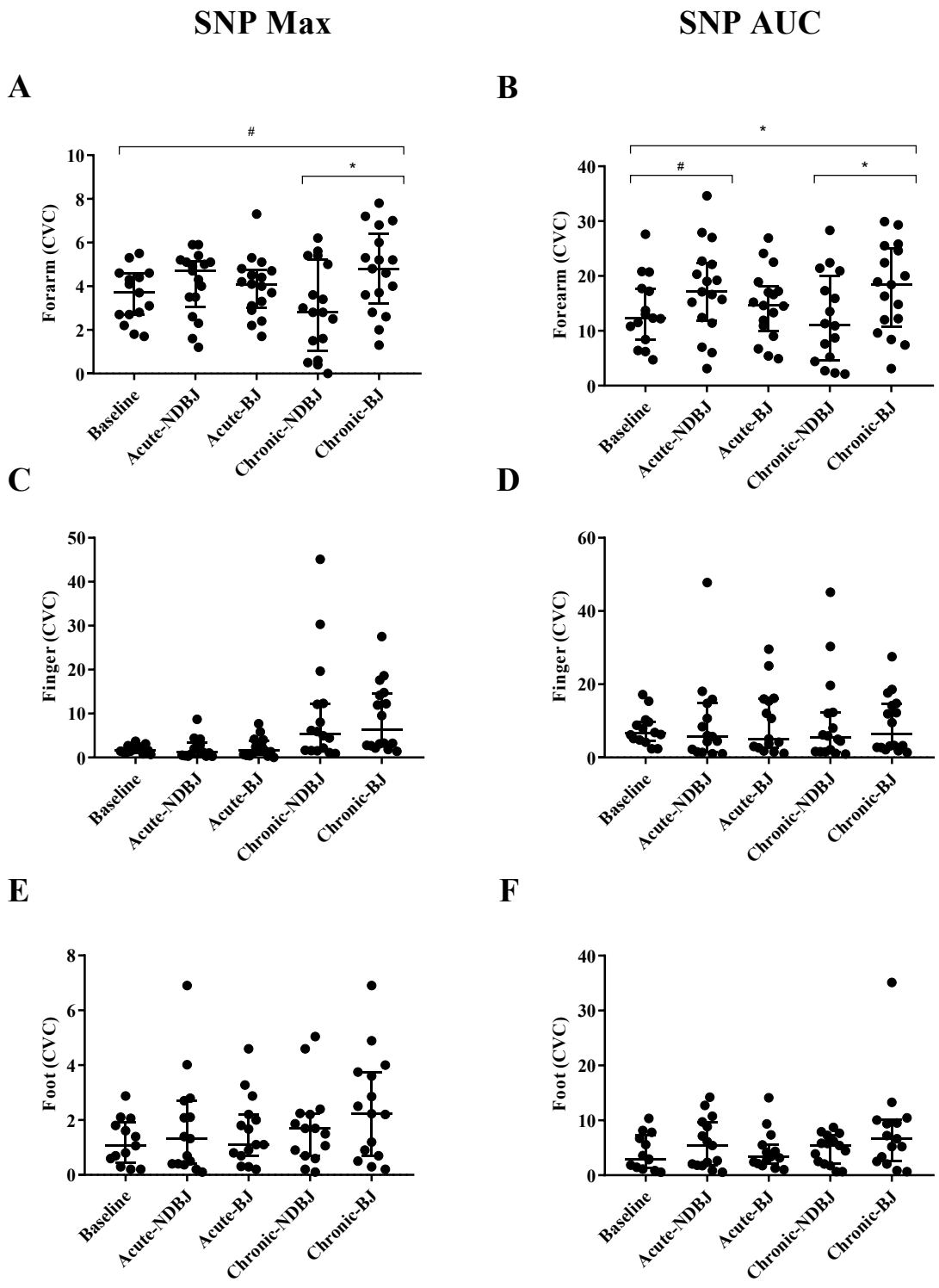

ACh Max
ACh AUC
A
B
C
D
E
F
Forarm (CVC)
Finger (CVC)
Foot (CVC)
#
*
Baseline
Acute-NDBJ
Acute-BJ
Chronic-NDBJ
Chronic-BJ

Figure 5.


SNP Max
SNP AUC
A
B
C
D
E
F
Forarm (CVC)
Forearm (CVC)
Finger (CVC)
Foot (CVC)
Baseline
Acute-NDBJ
Acute-BJ
Chronic-NDBJ
Chronic-BJ
#
*

817

Figure 6.

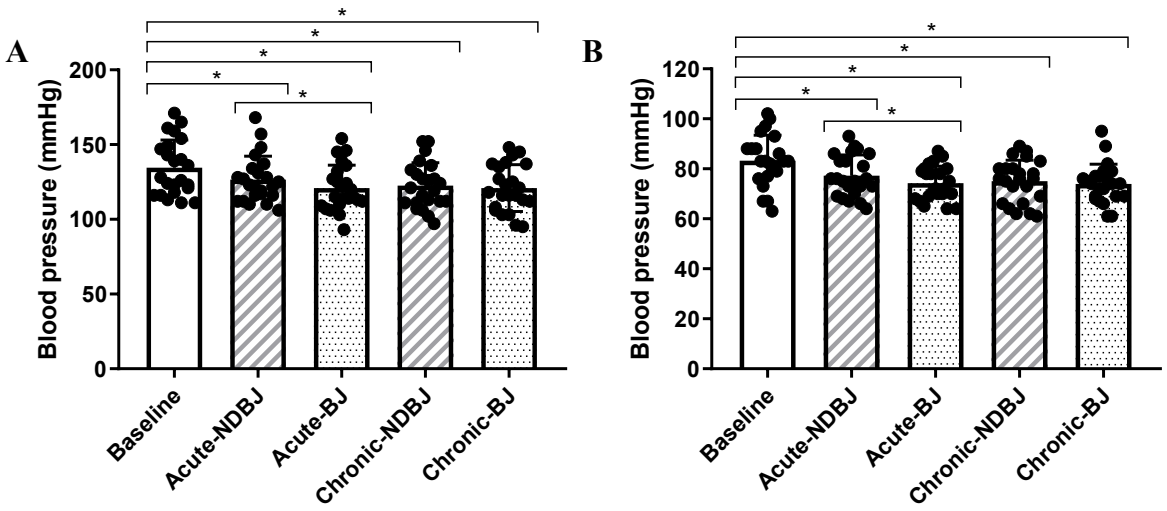

A
Blood pressure (mmHg)
200
150
100
50
0
Baseline
Acute-NDBJ
Acute-BJ
Chronic-NDBJ
Chronic-BJ
B
Blood pressure (mmHg)
120
100
80
60
40
20
0
Baseline
Acute-NDBJ
Acute-BJ
Chronic-NDBJ
Chronic-BJ

Figure 7.

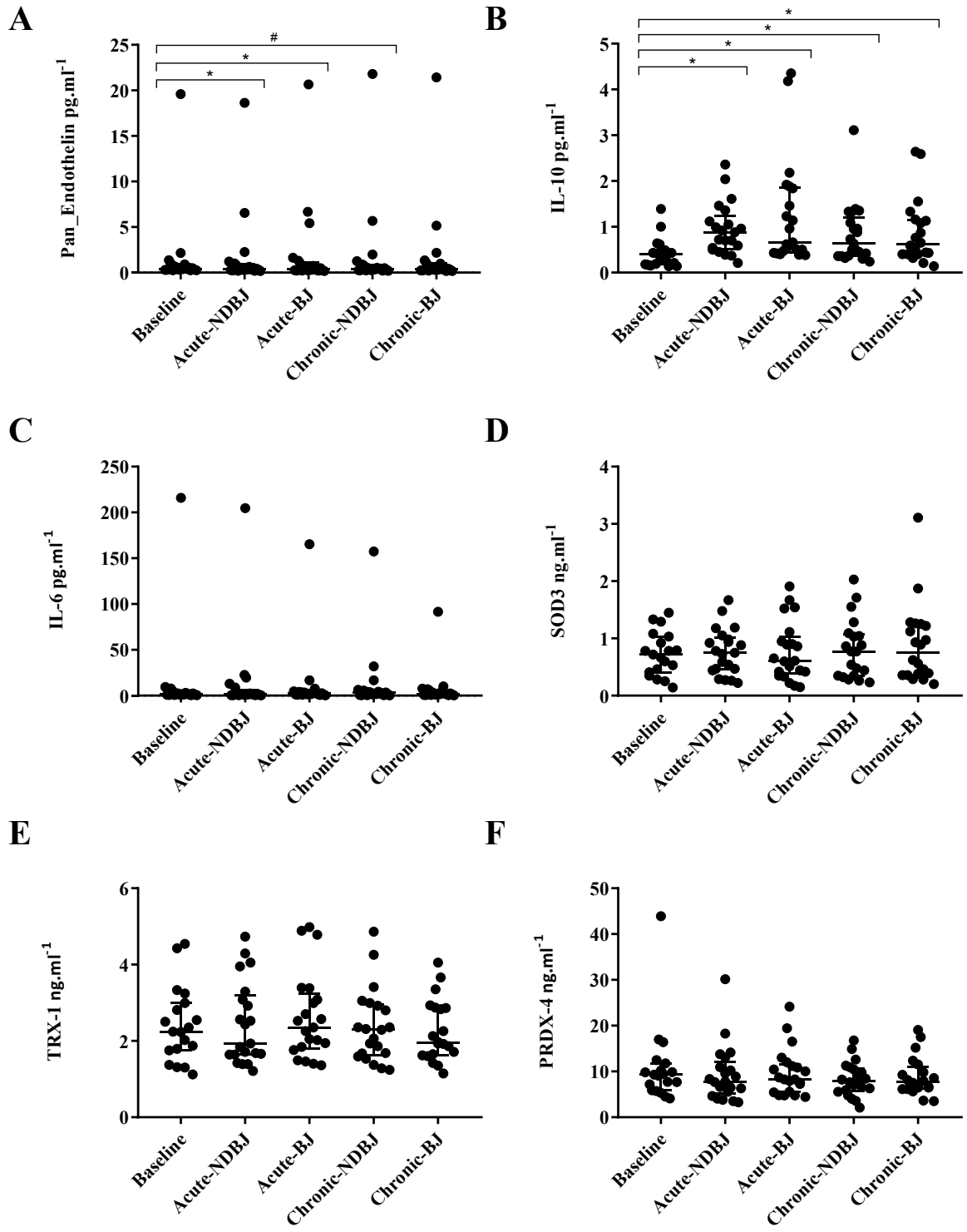

A
Pan_Endothelin pg.ml-1
25
20
15
10
5
0
#
*
*
Baseline
Acute-NDBJ
Acute-BJ
Chronic-NDBJ
Chronic-BJ
B
IL-10 pg.ml-1
5
4
3
2
1
0
*
*
*
*
Baseline
Acute-NDBJ
Acute-BJ
Chronic-NDBJ
Chronic-BJ
C
IL-6 pg.ml-1
250
200
150
100
50
0
Baseline
Acute-NDBJ
Acute-BJ
Chronic-NDBJ
Chronic-BJ
D
SOD3 ng.ml-1
4
3
2
1
0
Baseline
Acute-NDBJ
Acute-BJ
Chronic-NDBJ
Chronic-BJ
E
TRX-1 ng.ml-1
6
4
2
0
Baseline
Acute-NDBJ
Acute-BJ
Chronic-NDBJ
Chronic-BJ
F
PRDX-4 ng.ml-1
50
40
30
20
10
0
Baseline
Acute-NDBJ
Acute-BJ
Chronic-NDBJ
Chronic-BJ

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

Characteristics		Mean or %
Age		64.3 ± 15.3 y
Female		83%
Raynaud's duration		29.8 ± 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 ± 0.1 m
Mass		64.3 ± 10.5 kg
Baseline systolic BP		134.0 ± 18.6 mmHg
Baseline diastolic BP		83.2 ± 10.2 mmHg
Number of 30+ minutes of exercise per week		4.3 ± 2.5
Portions of fruit and vegetables per day		5.6 ± 2.7
Mini mental state exam		28.9 ± 0.9

Data presented as mean ± 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

		Condition	*n*	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]
	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
Hand	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

is the mean over the last 8 minutes of rewarming.

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

Online supplement.

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.

		n	ACh Max	ACh AUC	*n*	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
Finger	Baseline	15	2.5 ± 1.9	10.6 ± 9.5	15	1.6 ± 1.0	6.7 ± 4.8	28.4 ± 0.3
	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
Foot	Baseline	15	1.7 ± 1.7	6.2 ± 6.9	15	1.1 ± 0.9	3.8 ± 3.2	27.4 ± 0.6
	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.