**Dietary nitrate supplementation to enhance exercise capacity in hypoxic COPD: EDEN-OX a double-blind, placebo-controlled, randomised crossover study**

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## ABSTRACT

**Rationale** Dietary nitrate supplementation improves skeletal muscle oxygen utilisation and vascular endothelial function. We hypothesised that these effects might be sufficient to improve exercise performance in patients with COPD and hypoxia severe enough to require supplemental oxygen.

**Methods** We conducted a single-centre, double-blind, placebo-controlled, cross-over study, enrolling adults with COPD who were established users of long-term oxygen therapy. Participants performed an endurance shuttle walk test, using their prescribed oxygen, three hours after consuming either 140 mL of nitrate-rich beetroot juice (BRJ) (12.9 mmol nitrate), or placebo (nitrate-depleted BRJ). Treatment order was allocated (1:1) by computer generated block randomisation.

**Measurements** The primary outcome was endurance shuttle walk test time. Secondary outcomes included area under the curve to isotime for fingertip oxygen saturation and heart rate parameters during the test, blood pressure and endothelial function assessed using flow mediated dilatation. Plasma nitrate and nitrite levels as well as fraction of exhaled nitric oxide were also measured.

**Main Results** 20 participants were recruited, and all completed the study. Nitrate-rich BRJ supplementation prolonged exercise endurance time in all participants as compared to placebo; median (IQR) 194.6 (147.5, 411.7) seconds vs 159.1 (121.9, 298.5) seconds; estimated treatment effect 62 (33 to 106) seconds ($p<0.0001$); and improved endothelial function; NR-BRJ group +4.1 (-1.1, 14.8)% vs PL-BRJ group -5.0 (-10.6, -0.6)%; $p = 0.0003$.

**Conclusion** Acute dietary nitrate supplementation increases exercise endurance in COPD patients who require supplemental oxygen.

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## Key Messages

### What is the key question?

- Can dietary nitrate supplementation enhance exercise performance in individuals with a hypoxic COPD phenotype?

### What is the bottom line?

- In a double-blind, placebo-controlled, randomised crossover study, an acute dose of dietary nitrate increased endurance shuttle walk time in individuals with a hypoxic COPD phenotype.

### Why read on?

- As COPD becomes more severe, hypoxaemia may develop which impacts on the ability to perform day to day activities. Interventions which improve endothelial function as demonstrated here, and increase the efficiency of oxygen use may help to address this.


### Keywords

Dietary nitrate supplementation, nitric oxide, chronic obstructive pulmonary disease, exercise, nitrate, nitrite, beetroot juice

## INTRODUCTION

People with chronic obstructive pulmonary disease (COPD) may develop hypoxaemia as the condition becomes more severe, impacting on their ability to perform day to day activities. Mechanisms include ventilation perfusion mismatch, reduced cardiac output due to hyperinflation and pulmonary vascular limitation as well as reduced muscle efficiency [1-5]. In individuals who are sufficiently hypoxaemic long-term oxygen therapy (LTOT) improves survival and in many individuals ambulatory oxygen (AOT) improves exercise performance [6].

Nitric oxide (NO) has potential as a modulator of exercise performance. A ubiquitous signalling molecule, NO is involved in a number of processes at a tissue and cellular level including; mitochondrial and cellular respiration [7,8], glucose uptake into skeletal muscle [9], skeletal muscle contraction [10] [11], neurotransmission [12] and fatigue development [13]. NO is produced both by oxygen-dependant nitric oxide synthases catalysing its production from L-arginine and an alternative Nitrate($NO_3^-$)-Nitrite($NO_2^-$)-NO pathway [14]. The latter can be influenced by supplementation with exogenous dietary $NO_3^-$ and is enhanced in conditions of hypoxia and low pH as found in exercising skeletal muscle [15].

Dietary $NO_3^-$ supplementation has been shown to reduce the oxygen cost of exercise in healthy individuals in normoxic conditions [16,17] and in conditions of hypoxaemia [18-23]. Recently our research group has shown that it augments the improvements in exercise capacity seen in people with COPD following pulmonary rehabilitation [24,25]. We have also previously shown that dietary nitrate supplementation reduces the oxygen cost of exercise during endurance cycle ergometry in COPD [26]. However, that study, which excluded patients who required supplemental oxygen, did not demonstrate an improvement in exercise capacity.

The aim of the present study was therefore to assess the acute effect of dietary supplementation in the form of nitrate-rich beetroot juice (NR-BRJ) on exercise performance in individuals with COPD who require supplemental oxygen on exertion, hypothesising that this would increase exercise capacity, measured as endurance shuttle walk time (ESWT), as well as improving endothelial function in people with this specific phenotype.

## MATERIALS AND METHODS

### Study design

The effect of dietary nitrate supplementation on exercise performance in hypoxia (EDEN-OX) study was a single-centre, double-blind, placebo controlled, randomised cross-over trial comparing the effects of dietary nitrate supplementation to a matched placebo in individuals with COPD who require long-term oxygen therapy (LTOT) and use ambulatory oxygen therapy (AOT) during exercise. All participants provided informed consent and the study was approved by the London Chelsea Research and Ethics Committee (Ref: 15/LO/0975) and conducted in line with the principles of the Declaration of Helsinki. The study was registered prospectively on a publicly accessible database (ISRCTN14888729). The data presented here relate only to the planned COPD cohort in that study.

People with GOLD grade II-IV COPD [27] who were established users of LTOT, in accordance with NICE guidelines [28] were recruited from outpatient clinical services at Royal Brompton and Harefield NHS Foundation Trust (NW London), between 4th November 2016 and the 8th August 2017, with the last participant's final visit completed on the 15th of January 2018.

Exclusion criteria for the study included clinical instability (i.e. less than one month after an exacerbation), significant comorbidity limiting exercise tolerance, significant renal impairment (estimated glomerular filtration rate <50mL.min$^{-1}$), hypotension (systolic blood pressure <100mmHg), pregnancy, use of $NO_3^-$-based medicine or phosphodiesterase V inhibitors or the presence of other conditions that might be influenced by nitrate supplementation (i.e., ischaemic heart disease or peripheral vascular disease). These conditions were assessed at the screening visit through review of the clinical history and assessing relevant clinical data.

### Methods

### Interventions

The intervention was a commercially available concentrated $NO_3^-$-rich BRJ (NR-BRJ) (98%) drink cut with organic lemon juice (2%), containing 0.8 g, 12.9 mmol of $NO_3^-$ (140mL Beet-It® SPORT shot, James White Drinks, Ipswich, UK). The placebo beetroot juice (PL-BRJ) produced by James White Ltd was 140mL of the same beverage in which nitrate was removed

by a standardised method of passing the juice, prior to pasteurisation, through an ion exchange column, containing Purolite A520E which exchanges $NO_3^-$ against chloride [29]. The placebo-BRJ (PL-BRJ) is identical in appearance, packaging, taste and smell, and also causes beeturia (orange to red discolouration of urine).

**Study conduct**

At an initial baseline visit the COPD assessment test (CAT), hospital anxiety and depression (HAD) and MRC dyspnoea scores were recorded as well as measurement of body composition by bioelectrical impedance analysis using a Bodystat 4000 device (Bodystat, Isle of Man, UK). Participants then performed two incremental shuttle walk tests (ISWT) to determine the walking speed to be used for the ESWT [30] and then a practice ESWT. All walking tests throughout the study were performed on the participant's usual AOT flow rate and the method for carrying the AOT was recorded to ensure the same method was always used (Supplementary Appendix Study conduct. Figure E1. Study flow diagram).

Prior to the two subsequent intervention visits and throughout the study period, participants were asked to avoid the use of antimicrobial mouthwash and chewing gum, as this has been shown to reduce the oral facultative bacteria whose nitrate reductase activity is essential for the metabolism of an oral nitrate load [31]. They were asked to consume the same meal on the morning of each study assessment. This was to create as standardised conditions as possible, reducing differing levels of dietary $NO_3^-$ consumption as a source of variation within individuals, whilst not altering their usual diet greatly. They were also asked to match caffeine consumption to standardise any ergogenic effect arising from it [32] and to avoid strenuous exercise in the 24h period prior to the intervention visits.

The two intervention visits began at the same time of day (+/- 2h), with a minimum of a seven-day washout period and a maximum one-month gap between them. Participants were randomly assigned to the order in which they received NR-BRJ or PL-BRJ using a computer-generated block randomisation list, block size 10, produced by an independent statistician. The researchers responsible for enrolment and outcome measurements remained blinded throughout the study and during data analysis. Following their arrival, after a 10-minute rest period, participants were observed consuming either the NR-BRJ or the PL-BRJ, empty bottles

were collected and recorded. All outcome measures were undertaken three hours after ingestion of either NR-BRJ or PL-BRJ.

## Outcomes

### Exercise capacity

The primary outcome was the ESWT time compared between treatment conditions. Given the cross-over design and taking 65 seconds (95%CI 45-85) to be the minimal clinically significant difference in the ESWT [30] and a pooled mean difference within individuals of 26 seconds for repeat testing, to have an 80% statistical power, with a significance level of 0.05, 16 participants would be required to reject the null hypothesis that the active intervention was not superior to placebo. To allow for a 25% withdrawal rate a sample size of 20 was chosen.

### Plasma nitrate/nitrite levels and markers of oxidative stress

Plasma $NO_3^-$ and $NO_2^-$ levels were used as a combined biomarker of $NO_3^-$ ingestion, metabolism and nitric oxide availability [33,34]. Plasma samples were obtained on arrival and three hours after consumption of NR-BRJ or PL-BRJ (see Supplementary Appendix for full details).

Oxidative stress biomarkers were assessed in plasma samples by a combination of three distinct readouts including antioxidant potential, i.e. measurement of the ferric-reducing ability of plasma (FRAP) [35], lipid oxidation products by thiobarbituric acid-reactive substances (TBARS) [36] and total free thiols with normalisation for protein [37] (see Supplementary Appendix for full details).

### Fractional exhaled nitric oxide ($F_ENO$)

$F_ENO$ was measured as a steady exhalation rate of 50 $mL.sec^{-1}$ with a NIOX Mino (Aerocrine Systems, Solna, Sweden) at the screening visit and then at the intervention visits at baseline prior to NR-BRJ/PL-BRJ consumption and then at six further intervals (30, 60, 90, 120, 150 and 180 minutes). Both the study participant and researchers were blinded to the results and an independent researcher, not directly involved with the trial, uploaded the data into a password

protected database. These data were only available to the researchers following the unblinding of the study.

**Endothelial function**

Endothelial function was assessed by flow medicated dilatation (FMD) of the brachial artery three hours after NR-BRJ/PL-BRJ consumption [38] using a high-resolution Doppler ultrasound to measure at baseline and sequentially over a period of 120 seconds after release of circulatory arrest of the upper arm [39]. All measurements were performed by a single trained operator (see Supplementary Appendix for full details).

**Continuous oxygen saturations and heart rate analysis**

For each ESWT performed, pulse oximetry was recorded (Pulsox 300i Pulse Oximeter, Konica Minolta, Tokyo, Japan) throughout until the participant had recovered (recovery was defined by return of Borg dyspnoea scale to that recorded prior to the ESWT). To maintain blinding, the pulse oximeter display was covered throughout the testing and the data downloaded by an independent researcher, not directly involved with the trial, who uploaded the data to a password protected database. These data were only available to the researchers following unblinding of the trial.

**Statistical analysis**

Data are presented as mean (SD) or if not normally distributed as median and interquartile range (IQR). Differences in response between treatment conditions were assessed using a paired T-test or a Wilcoxon signed-rank test as appropriate. Treatment effect was estimated using the Hodges-Lehman estimate of shift parameters. The process of determining the Hodges-Lehman estimator entails estimating the average difference in outcomes (x-y) for every possible n(n+1)/2 pair and then deriving the overall median of all averages (the Hodges-Lehmann estimator). A distribution-free confidence interval is estimated using large-sample approximation. Analysis was performed using SPSS version 24 for Windows (SPSS Software, Chicago, Illinois, USA) and Stata version 16.1 for Windows (StataCorp 2019, Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC).

To perform the comparison of continuous oxygen saturations ($S_pO_2$) and heart rate (HR) between the two treatment conditions, individual ESWT data periods were subjected to a 30 second rolling average using MATLAB (MATLAB and Statistics Toolbox Release 2017a, The MathWorks, Inc., Natick, Massachusetts, USA) and then expressed as percentages of isotime (defined as the duration of the shortest of the two ESWT). These individual responses were then grouped to allow analysis of heart rate and $S_pO_2$ against percentage of isotime (plotted at the midpoint of each $10^{th}$ percentile of isotime). The area under the curve (AUC) was assessed for each individual participant and the two treatment conditions compared using a Wilcoxon signed-rank test. Figures were prepared using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, California, USA). A p value of $< 0.05$ was considered to be statistically significant.

## RESULTS

We screened 67 people for eligibility (Figure 1); 31 declined to participate, seven had a comorbidity precluding participation and nine were not using supplementary $O_2$. Of twenty participants enrolled in the study, ten were randomised to receive PL-BRJ first and ten NR-BRJ first. All participants completed the study. Table 1 shows their baseline characteristics which were well-matched between the two order allocation groups. There were no serious adverse effects reported, though all participants reported beeturia. The average time between each intervention visit was seven days.

**Table 1. Characteristics of cross-over allocation groups: NR-BRJ or PL-BRJ received first**

| Measurement | NR-BRJ First (n=10) | PL-BRJ First (n=10) | p value | Whole Group (n=20) |
|---|---|---|---|---|
| **Sex (% Female:Male)** | 30:70 | 50:50 | 1.0 | 40:60 |
| **Age (Years)** | 68 (62, 73) | 67 (64, 76) | 0.7 | 67.6 (8.5) |
| **Caucasian (%)** | 100 | 100 | 1 | 100 |
| **Smoking (Pack Years)** | 28 (14, 50) | 64 (57, 96) | 0.006 | 52 (21.6) |
| **BMI ($kg.m^{-2}$)** | 24.3 (20.9, 29.0) | 26.2 (21.4, 30.1) | 0.5 | 25.2 (4.7) |
| **FFMI ($kg.m^{-2}$)** | 18.4 (15.8, 20.1) | 18.0 (14.0, 20.2) | 0.8 | 18.1 (15.8, 19.9) |
| **Inhaled Medications** | | | | |
| **SABA (%)** | 91 | 100 | 0.4 | 95 |
| **LABA-ICS (%)** | 91 | 78 | 0.4 | 95 |
| **LAMA (%)** | 91 | 100 | 0.4 | 85 |
| **Baseline Resting $O_2$ Saturations $F_iO_2$ 0.21 (%)** | 92 (89, 94) | 92 (89, 93) | 0.8 | 92 |
| **Pre-LTOT Prescription Baseline $P_aO_2$ (kPa)** | 6.9 (6.0, 7.3) | 6.6 (6.4, 7.2) | 0.6 | 6.8 |
| **Oxygen Prescription ($L.min^{-1}$)** | 4 (2, 6) | 2 (2, 4) | 0.2 | 3.0 (2.0, 6.0) |
| **CAT Score** | 20 (18, 29) | 19 (15, 28) | 0.6 | 21 (8.0) |
| **MRC Dyspnoea Score** | 4 (4, 4) | 4 (4, 4) | 1.0 | 4 (4, 4) |
| **HAD Score A** | 4 (3, 7) | 7 (2, 10) | 0.3 | 4.0 (2.3, 8.8) |
| **HAD Score D** | 4 (4, 5) | 5 (4, 7) | 0.7 | 4.5 (4.0, 5.6) |
| **Systolic BP (mmHg)** | 139 (123, 149) | 135 (115, 139) | 0.1 | 137 (121, 143) |
| **Diastolic BP (mmHg)** | 76 (66, 83) | 70 (65, 80) | 0.4 | 73 (65, 82) |
| **MAP (mmHg)** | 94 (91, 104) | 91 (85, 94) | 0.2 | 92 (80, 100) |
| **Lung Function** | | | | |
| **FEV1 (L)** | 0.7 (0.6, 1.0) | 0.7 (0.3, 1.0) | 0.3 | 0.7 (0.6, 1.0) |
| **FVC (L)** | 2.7 (1.9, 3.1) | 1.6 (1.4, 3.2) | 0.3 | 2.7 (1.6, 3.1) |
| **FEV1/FVC Ratio** | 0.3 (0.3, 0.3) | 0.3 (0.2, 0.4) | 1.0 | 0.3 (03, 0.3) |
| **RV %Predicted** | 211 (181, 235) | 212 (188, 233) | 0.8 | 212 (186, 233) |
| **TLco %Predicted** | 33 (19, 45) | 36 (28, 44) | 0.9 | 32 (19. 44) |
| **GOLD Stage** | | | | |
| **III (%)** | 22 | 45 | 1.0 | 35 |
| **IV (%)** | 78 | 55 | 1.0 | 65 |
| **ISWT Distance (meters)** | 300 (280, 360) | 370 (220, 280) | 0.04 | 279 (70) |
| **ESWT Time (secs)** | 172 (137, 267) | 181 (158, 193) | 0.6 | 179 (152, 193) |

Data in order of intervention, either NR-BRJ or PL-BRJ first. Data shown are median (IQR), mean (SD) or percentage (%). P value is for independent t-test, or Mann-Whitney test comparing groups.

*Abbreviations: NR – Nitrate-rich; PL – Placebo; BRJ – Beetroot Juice; BMI – Body Mass Index; FFM – Fat Free Mass; FFMI – Fat Free Mass Index: SABA – Short Acting Beta Agonist; LABA – Long Acting Beta Agonist; ICS – Inhaled Corticosteroid; LAMA – Long Acting Muscarinic Agonist; $F_iO_2$ – Fraction of Inspired Oxygen, LTOT – Long Term Oxygen Therapy; CAT – COPD Assessment Test; MRC – Medical Research Council; HADS A – Hospital Anxiety Depression Scale Anxiety; HADS D – Hospital Anxiety Depression Scale Depression; BP – Blood Pressure; MAP – Mean Arterial Pressure; $FEV_1$ – Forced Expiratory Volume in 1 Second; FVC – Forced Vital Capacity; RV – Residual Volume; $TLco_c$ – Transfer Factor for Carbon Monoxide Corrected for haemoglobin; $PaO_2$ – Partial Pressure of Oxygen; GOLD – Global Initiative for Chronic Obstructive Lung Disease; ISWT – Incremental Shuttle Walk Test; ESWT – Endurance Shuttle Walk Test*

**Exercise outcomes**

Exercise endurance time was longer for all study participants after NR-BRJ compared to PL-BRJ (Figure 2); median (IQR) ESWT time; NR-BRJ 194.6 (147.5, 411.7) seconds vs PL-BRJ 159.1 (121.9, 298.5) seconds, estimated treatment effect 62.5 (95% CI 33 to 106) seconds; p 0.000089. There was no evidence of an intervention order effect (Supplementary Appendix Figure E2). There was one individual who was a clear outlier for exercise endurance response. However, a sensitivity analysis, removing their data led to a slight change in primary study outcome but not the overall statistical significance with median (IQR) ESWT time; NR-BRJ 193.8 (145, 389.6) seconds vs PL-BRJ 158.2 (121.6, 236.6) seconds, estimated treatment effect 56.5 (95% CI 30 to 88) seconds indicating a significant increase in the ESWT time with NR-BRJ, p=0.0001 (Supplementary Appendix Results E1).

Pulse oximetry data were available for only 18 participants, because recording failed for two of them. The average area under the curve for oxygen saturations was higher in the NR-BRJ group compared to PL-BRJ. These differences were more apparent at isotime and peak exercise, with no difference at rest, during warm up or recovery (Figure 3 Panel A and Supplementary

Table E1). The estimated treatment effect was also statistically significant 43.69 (29.09 to 58.28), p < 0.0001- The area under the curve for HR response to NR-BRJ or PL-BRJ did not show any difference. The estimated treatment effect was also not statistically significant (-41.17 (-116.74 to 34.40), p = 0.27). (Figure 3 Panel B and Supplementary Appendix Table E1).

**Endothelial function and blood pressure**

Two participants declined to have the FMD assessment, therefore data were available for 18 participants. At 180 minutes following dosing FMD increased with NR-BRJ 4.1 (-1.1 to 14.8) % compared to placebo -5.0 (-10.6 to -0.6) %, estimated treatment effect -11.9 (95%CI -18.9 to -7.15) %; p = 0.0003 (Figure 4).

There was no statistically significant difference in the change in blood pressure parameters from pre-dosing levels between NR-BRJ and PL-BRJ (Figure 5); median (IQR) ΔsBP: NR-BRJ -1.5 (15.0, 10.8) mmHg vs PL-BRJ -0.5 (-10.5, 6.8) mmHg, estimated treatment effect 1 (95%CI -5.5 to 7.0) mmHg; p = 1.0; ΔdBP: NR-BRJ -4.0 (-14.0, 7.0) mmHg vs PL-BRJ -1.0 (-9.3, 5.0) mmHg, estimated treatment effect 1 (95%CI -3 to 5) mmHg, p = 0.481; MAP: NR-BRJ -5.0 (-15.3, 6.0) mmHg vs PL-BRJ -2.5 (-13.5, 7) mmHg, estimated treatment effect 1.5 (95%CI -3.5 to 5) mmHg; p = 0.359.

**Plasma nitrate and nitrite levels and oxidative stress markers**

Paired data on plasma $NO_2^-$ and $NO_3^-$ concentrations were available for 19 participants, as one individual declined sampling. Following supplementation with NR-BRJ there was an 84% increase in plasma $NO_2^-$ and an 887% increase in plasma $NO_3^-$ at 180 minutes post supplementation, but no change with placebo (Figure 6 and Supplementary Appendix Table E2). Both, the NR-BRJ and PL-BRJ supplements were analysed for $NO_2^-$ and $NO_3^-$ content as well (Supplementary Appendix Table E3). The change in plasma $NO_3^-$ and $NO_2^-$ from baseline to 180 seconds was calculated and used to estimate the treatment effect of NR-BRJ. The treatment effect of $NO_3^-$ was 550 (461 to 639) µM. The results suggest that this was higher for NR-BRJ than PL-BRJ and this change was statistically significant, p=0.0003. The treatment effect of NO2- was 0.248 (0.138 to 0.408) µM. The results suggest that this was higher for NR-BRJ than PL-BRJ and this change was statistically significant, p=0.0011.

There was no statistically significant difference in measures of oxidative stress following acute consumption of either supplement; FRAP: NR-BRJ 1018 (853.0, 1125) µM vs PL-BRJ 930.2 (836.8, 1073) µM; p = 1.0; TBARS: NR-BRJ 1.499 (0.855, 3.209) mM vs PL-BRJ 0.971 (0.766, 1.614) mM; p = 0.4; total free thiol per protein: NR-BRJ 7.079 (5.961, 8.115) $\mu mol.g^{-1}$ protein vs PL-BRJ 6.942 (5.768, 8.026) $\mu mol.g^{-1}$ protein; p = 0.5) (Supplementary Appendix Figure E3).

Paired measures of $F_ENO$ were available for 16 participants at all seven time points (Figure 7, and Supplementary Appendix Table E5). For four participants, there was device failure resulting in no data being recorded. The median (IQR) AUC for when the subjects were on PL-BRJ was 3622.5 (3181.9, 4796.9) and the corresponding results for when the subjects were on NR-BRJ was 9440.6 (6273.8, 11831.3) and the treatment effect with its 95% CI was 5407 (3096 to 7576), p=0.0011. The results suggest that the $F_ENO$ levels while the subjects were on NR-BRJ were significantly higher than when they were on PL-BRJ. Post-acute (zero minutes) supplementation with NR-BRJ $F_ENO$ increased by 184% at 180 minutes post supplementation.

## DISCUSSION

The major finding of this study was that, in people with COPD who are hypoxic to the extent that they meet criteria for long term oxygen therapy, dietary $NO_3^-$ supplementation improves exercise capacity compared to placebo. The improvement in ESWT time that we observed was accompanied by less desaturation during exercise. Supplementation also improved endothelial function assessed using FMD. In line with previous observations [24,25], blood pressure was numerically lower as well but the difference in response was not statistically significant.

### Significance of findings

Although studies have previously considered dietary $NO_3^-$ supplementation in COPD with inconsistent results [24-26,40], this is the first stratified medicine approach focusing on the specific phenotype of individuals with COPD with hypoxaemia requiring LTOT. Our previous study, in non-hypoxaemic COPD patients, found that there was a reduction in the oxygen cost of exercise during cycle ergometry yet no improvement in exercise capacity [26]. In conditions of

hypoxia, the L-arginine-NOS pathway is compromised while the $NO_3^-$-$NO_2^-$-NO pathway is facilitated due to a lesser inhibition of $NO_2^-$ bioactivation by oxygen [14,20,22]. As such, dietary $NO_3^-$ supplementation could be expected to have more impact in hypoxic rather than normoxic individuals, both through effects in skeletal muscle and impacts on the pulmonary vasculature.

The mechanism by which ESWT lengthened is likely to involve multiple synergistic pathways. The finding of relatively preserved oxygen saturation during exercise in the $NO_3^-$-supplemented condition could reflect either more efficient oxygen utilisation peripherally or a beneficial impact on central haemodynamics associated with/related to reduced hypoxia induced pulmonary vasoconstriction, or a combination of the two. Despite each participant using their prescribed oxygen for each walk test, there was an observed desaturation in the placebo arm. This could mean that their oxygen prescription may have been insufficient and that a higher flow rates might also have increased exercise capacity. This finding of the attenuation of desaturation by NR-BRJ may well be explained by the enhancement of the $NO_3^-$-$NO_2^-$-NO pathway in conditions of hypoxia. The observation that $NO_3^-$ supplementation was associated with improvements in endothelial function assessed using FMD, is likely to be relevant to the acute mechanism of benefit from $NO_3^-$ supplementation, but also raises the possibility that longer-term dosing might reduce the risk of vascular events which are common in COPD. The effects seen are almost certainly not COPD-specific and work is needed to investigate possible benefits in other long-term lung conditions associated with hypoxia, including interstitial lung diseases and the various categories of pulmonary hypertension.

The estimated treatment effect of dietary $NO_3^-$ supplementation on ESWT time found in this study was 62.5 seconds, which falls fractionally short of the MID defined in pharmacotherapy trials as 65 seconds [30]. However, in pharmacological trials where the ESWT time is the outcome, interventions are typically administered over weeks or months. The demonstration of an effect of similar magnitude in a single dose study is therefore encouraging though further studies of longer-term use will be needed before any clinical recommendations can be made.

**Study limitations**

The use of a robust placebo strengthens the reliability of the findings, as does the fact that the improvement in walking time was accompanied by an appropriate physiological response

(lower heart rate and higher oxygen saturation). An additional strength was the use of a walking rather than cycling test, which is of clinical relevance to patients as it reflects most individuals' main form of exercise and daily physical activity. This was a single dose study and therefore questions remain as to the impact that regular dosing might have and whether this would translate into meaningful clinical effects. The dose used was selected based on previous studies but future work should investigate whether there is a dose response or ceiling effect. We have also shown that the NR-BRJ does indeed contain a higher quantity of $NO_3^-$ and provide independent confirmation that $NO_3^-$ is only present at very low levels in the placebo juice used in our study.

## Conclusion

Beetroot juice is cheap and readily accessible and has the potential to be used widely as a dietary supplement if effective in specific patient groups. Its beneficial effects appear to be mediated by inorganic nitrate without affecting plasma redox status, upon acute administration. Further mechanistic work is needed to work out the relative impact of the possible mechanisms, in particular the impact of muscle vs pulmonary or cardiac/systemic circulation effects, and longer-term studies will be needed to establish if the effects on exercise performance and endothelial function observed here translate into clinically meaningful benefits.

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## CONTRIBUTIONS

Professors Hopkinson and Polkey developed the original idea for the research study. Professor Hopkinson and Dr Pavitt designed and wrote the study protocol. Mr Banya designed the statistical analysis plan. Dr Pavitt, Dr Lewis and Ms Buttery undertook patient visits and collected trial data. Professor Feelisch, Dr Fernandez and Miss Mikus-Lelinska undertook plasma analysis. Dr Pavitt analysed the data and wrote the first draft of the manuscript. All authors edited and contributed to the final manuscript.

## DATA SHARING STATEMENT

Individual participant data that underlie the results in the article, after de-identification (text, tables, figures and appendices) will be made available from the corresponding author upon request. The study protocol and statistical analysis plan will also be available. Data will be made immediately available to anyone who wishes to access the data, for any purpose, following publication. Data will be available indefinitely.

## TRANSPARENCY STATEMENT

Professor Hopkinson affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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## FIGURE LEGENDS

**Figure 1. CONSORT diagram for recruitment and trial completion**

*Abbreviations: NR-BRJ – Nitrate-rich Beetroot Juice; PL-BRJ – Placebo Beetroot Juice*

**Figure 2. Effect of dietary nitrate supplementation on endurance shuttle walk time**

ESWT time (seconds) for PL-BRJ and NR-BRJ dosing conditions. Data presented as individual ESWT times (seconds) in both dosing conditions. Wilcoxon signed-rank test was used to compare ESWT time between the different dosing conditions. NR-BRJ 194.6 (147.5, 411.7) seconds vs PL-BRJ 159.1 (121.9, 298.5) seconds, estimated treatment effect 62 seconds (95% CI 33 to 106); p = 0.000089.

*Abbreviations: ESWT – Endurance Shuttle Walk Test; PL-BRJ – Placebo Beetroot Juice; NR-BRJ – Nitrate-rich Beetroot Juice*

**Figure 3. Effect of dietary nitrate supplementation on isotime $O_2$ saturation and heart rate during endurance shuttle walk test**

**Panel A.** $O_2$ saturation analysis in the NR-BRJ (red) and PL-BRJ (black) dosing conditions at $10^{th}$ percentiles of isotime and at rest, warm-up, peak exercise and recovery. Data presented as median and IQR. The area under the curve for each treatment group was estimated and reported as mean (SD). The results for Saturations for when the subjects were on PL-BRJ were 1161.85 (47.59) and the results for when the subjects on NR-BRJ were 1205.54 (46.39). The treatment effect was estimated to be 43.69 (29.09 to 58.28) $p < 0.0001$. The results suggest that on the average the area under the curve for saturations was higher when on NR-BRJ than when on PL-BRJ. These differences tended to show more during the isotime and peak periods

**Panel B.** Heart rate (HR) analysis in the NR-BRJ (red) and PL-BRJ (black) dosing conditions at $10^{th}$ percentiles of isotime and at rest, warm-up, peak exercise and recovery. Data presented as median and IQR. The area under the curve for each treatment group was estimated and reported as mean (SD). The mean (SD) area under the curve for the HR data when the subjects were on PL-BRJ was 1299.93 (186.05) for when the subjects were on NR-BRJ results was

1258.76 (174.01). The estimated treatment effect was -41.17 (-116.74 to 34.40), p=0.27. The results show that while at individual time points the HR was higher for when the subjects were on PL-BRJ, there was no statistically significant difference in the area under the curve.

*Abbreviations: % - percentage, bpm – beats per minute, NR-BRJ – Nitrate-rich Beetroot Juice; PL-BRJ – Placebo Beetroot Juice.*

**Figure 4. Effect of dietary nitrate supplementation on endothelial function**

Percentage change in FMD from baseline and 180 minutes after supplementation with NR-BRJ or PL-BRJ. Data presented ate 25$^{th}$ and 75$^{th}$ percentile boxes with the solid line representing the median value, and the whiskers the minimum and maximum values. Wilcoxon sign-rank test was used to compare the percentage change in FMD in the NR-BRJ (red) and PL-BRJ (black) dosing conditions. There was a statistically significant difference in the FMD percentage change with an increase in the NR-BRJ group 4.1 (-1.1, 14.8) vs a reduction in the PL-BRJ group -5.0 (-10.6, -0.6) %; p = 0.0003.

*Abbreviations: FMD – Flow mediated dilatation; NR-BRJ – Nitrate-rich Beetroot Juice; PL-BRJ – Placebo Beetroot Juice*

**Figure 5. Effect of dietary nitrate supplementation on blood pressure parameters**

Change in blood pressure parameters (sBP, dBP and MAP) relative to baseline blood pressure three hours prior to dosing with either NR-BRJ or PL-BRJ. Data presented as 25$^{th}$ to 75$^{th}$ percentile with the solid line representing the median value, and the whiskers the minimum to maximum values. Wilcoxon signed rank test was used to compare blood pressure parameters. The median (IQR) change in sBP: NR-BRJ -1.5 (15.0, 10.8) mmHg vs PL-BRJ -0.5 (-10.5, 6.8) mmHg; p = 1.0. The median (IQR) in dBP: NR-BRJ 4.0 (-14.0, 7.0) mmHg vs PL-BRJ -1.0 (-9.3, 5.0) mmHg; p = 0.481. The median (IQR) change in MAP: NR-BRJ -5.0 (-15.3, 6) mmHg vs PL-BRJ -2.5 (-13.5, 7) mmHg; p = 0.359.

*Abbreviations: BP – Blood Pressure; sBP – systolic blood pressure; dBP – diastolic blood pressure; MAP – mean arterial pressure; PL-BRJ – placebo beetroot juice; NR-BRJ – nitrate-rich beetroot juice*

**Figure 6. Plasma nitrite and nitrate levels**

Data presented are median (IQR) with whiskers representing minimum to maximum values. Plasma $NO_2^-$ and $NO_3^-$ concentrations were measured at baseline (zero minutes) and 180minutes after dosing with the interventions. Wilcoxon sign-rank test was used to compare change in plasma $NO_2^-$ and $NO_3^-$ concentrations between intervention groups. Mann-Whitney U test was used to compare change in plasma $NO_2^-$ and $NO_3^-$ concentrations between treatment conditions.

**Panel A. Changes in plasma $NO_2^-$ concentrations**

There was a statistically significant difference between baseline plasma $NO_2^-$ concentration and post dosing with NR-BRJ for plasma $NO_2^-$: pre-dosing plasma $NO_2^-$ concentration 0.306 (0.227, 0.402) µM vs post-dosing 0.620 (0.488, 0.673) µM; **** p = 0.000076). There was also a statistically significant difference between post dosing plasma $NO_2^-$ concentration between NR-BRJ and PL-BRJ dosing conditions: post dose of NR-BRJ $NO_2^-$ concentration 0.620 (0.488, 0.673) µM vs post dose of PL-BRJ $NO_2^-$ concentration 0.306 (0.227, 0.402) µM; †††† p = 0.000009.

**Panel B. Changes in plasma $NO_3^-$ levels**

There was a statistically significant difference between baseline plasma $NO_3^-$ concentration and post dosing with NR-BRJ for plasma $NO_3^-$: pre-dosing plasma $NO_3^-$ concentration 62.59 (41.68, 77.29) µM vs post-dosing 617 (556.25, 725.88) µM; **** p = 0.00004. There was also a statistically significant difference between post dosing plasma $NO_3^-$ concentration between NR-BRJ and PL-BRJ dosing conditions: post dose NR-BRJ $NO_3^-$ plasma concentration 617.71 (556.25, 725.88) µM vs PL-BRJ plasma $NO_3^-$ concentration 45.31 (31.39, 58.84) µM; †††† p = 5.66 x10-11).

*Abbreviations: NR-BRJ – nitrate-rich beetroot juice, PL-BRJ – placebo beetroot juice, µM - micromole*

**Figure 7. Exhaled Nitric Oxide**

Data presented are median (dot) with whiskers representing interquartile range. Red dot and line representing NR-BRJ and black dot and line representing PL-BRJ. $F_ENO$ was measured at baseline (study visit 1) and subsequently at intervention visits at seven time points (0minutes, 30minutes, 60minutes, 90minutes, 120minutes 150minutes and 180 minutes) dosing with either NR-BRJ or PL-BRJ. Kruskal-Wallis H Test was used to assess the effect of either NR-BRJ or PL-BRJ on $F_ENO$.

In both intervention groups there was no statistical difference between $F_ENO$ at baseline (measured at study visit 1) and time point zero minutes (measured at intervention visits prior to supplementation with intervention beverage). There was a statistically significant difference between measured $F_ENO$ at all subsequent time points post intervention consumption. 30minutes $p = 0.0011$, 60minutes $p=0.0001$, 90minutes $p=0.0006$, 120minutes $p=0.0002$, 150minutes $p=0.0002$, 180miniutes $p=0.0024$ (** $p \leq 0.01$, *** $p \leq 0.001$) (See Supplementary Appendix 7).

*Abbreviations: $F_ENO$ – Fractional Exhaled Nitric Oxide; ppb – Parts Per Billion; NR-BRJ – Nitrate rich beetroot Juice; PL-BRJ – Placebo beetroot juice.*

**Figure 1**

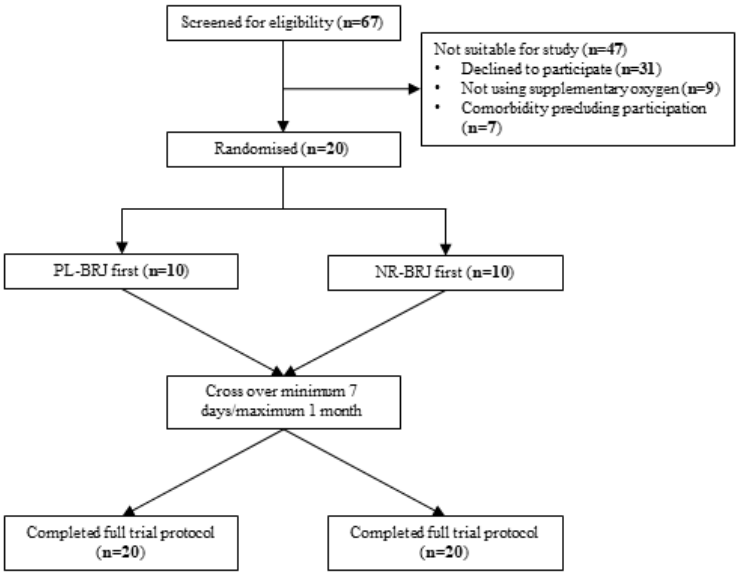



**Figure 2**

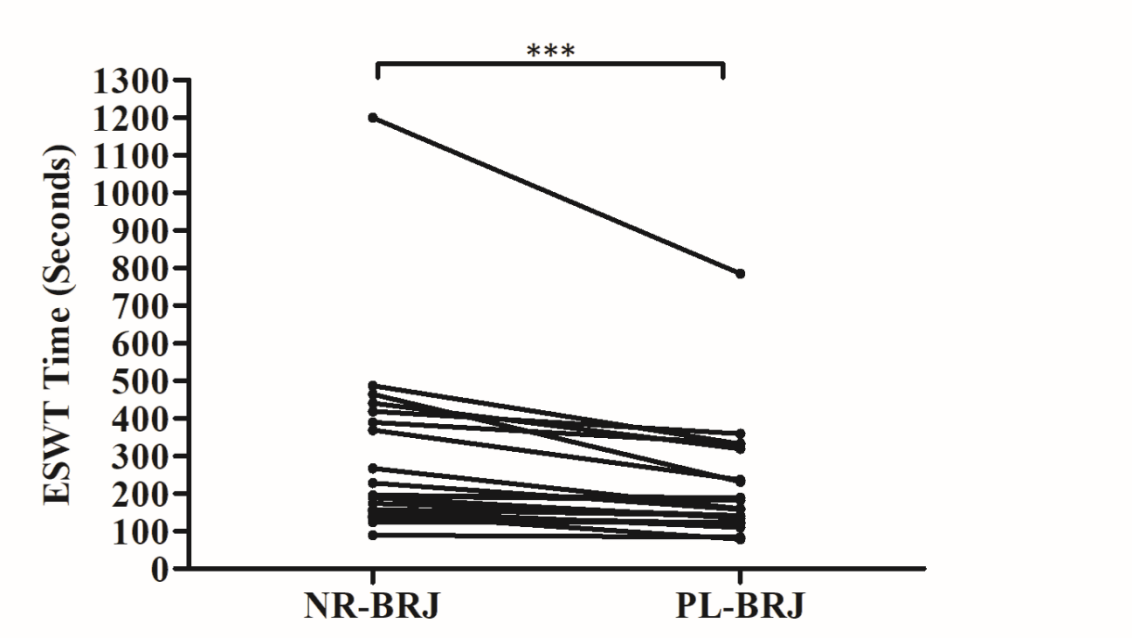


**Figure 3**

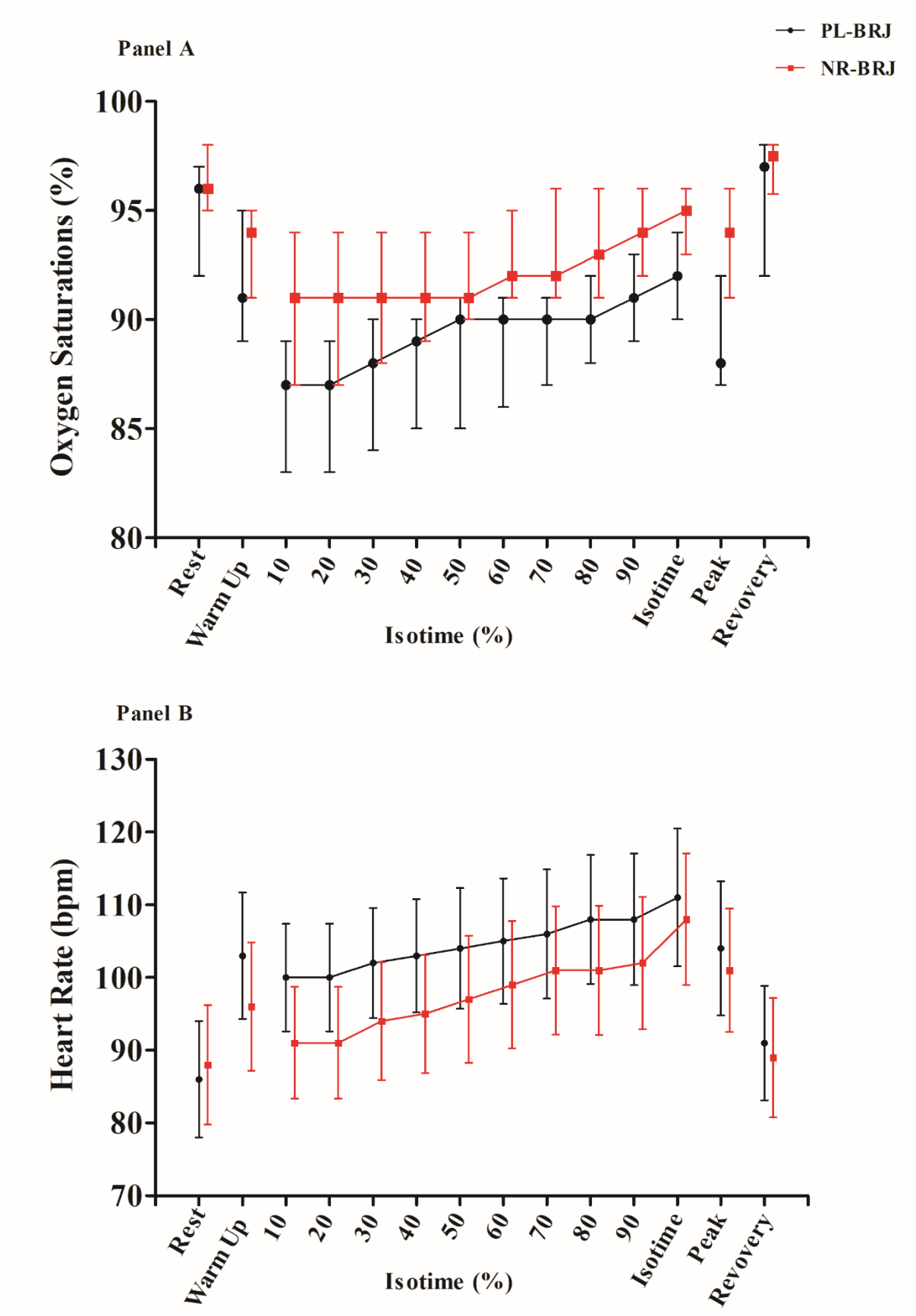

Panel A
PL-BRJ
NR-BRJ
Oxygen Saturations (%)
100
95
90
85
80
Rest
Warm Up
10
20
30
40
50
60
70
80
90
Isotime
Peak
Revovery
Isotime (%)
Panel B
Heart Rate (bpm)
130
120
110
100
90
80
70
Rest
Warm Up
10
20
30
40
50
60
70
80
90
Isotime
Peak
Revovery
Isotime (%)

**Figure 4**

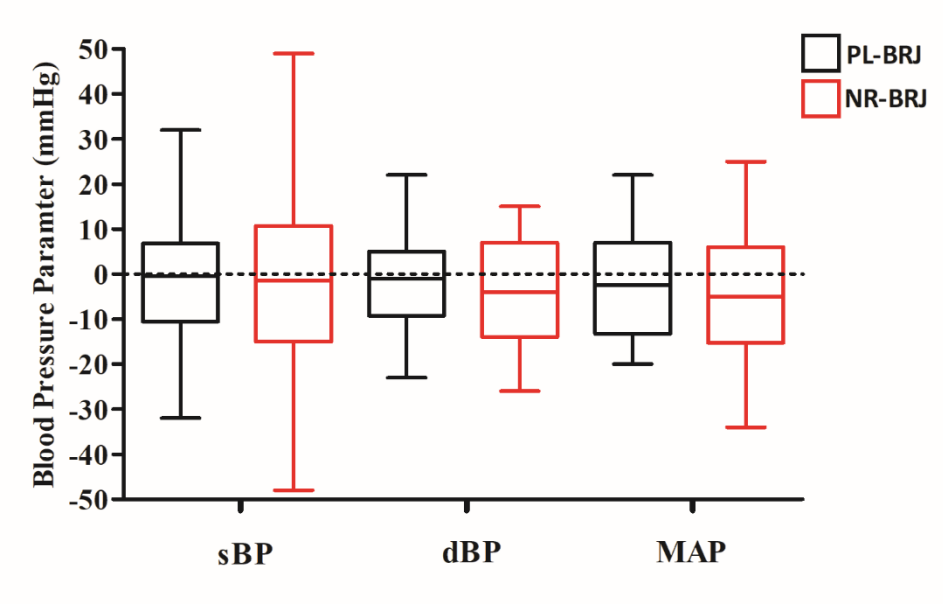



**Figure 5**

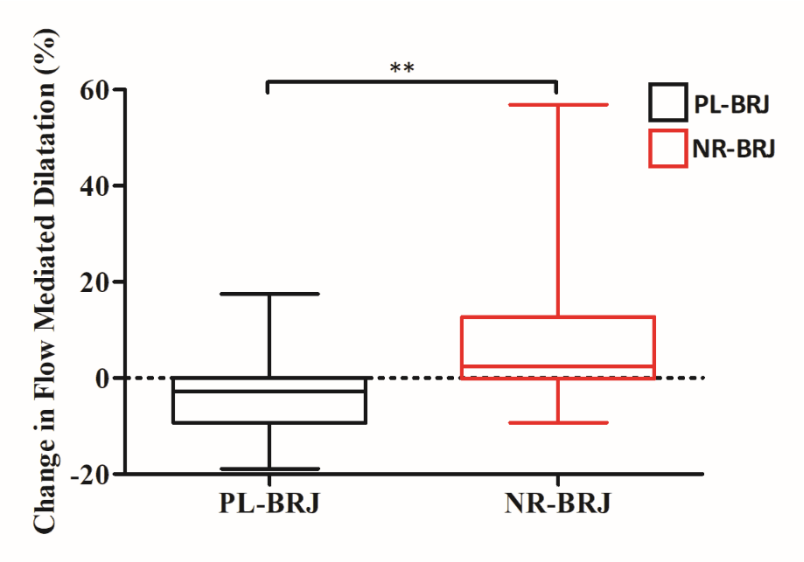


**Figure 6**

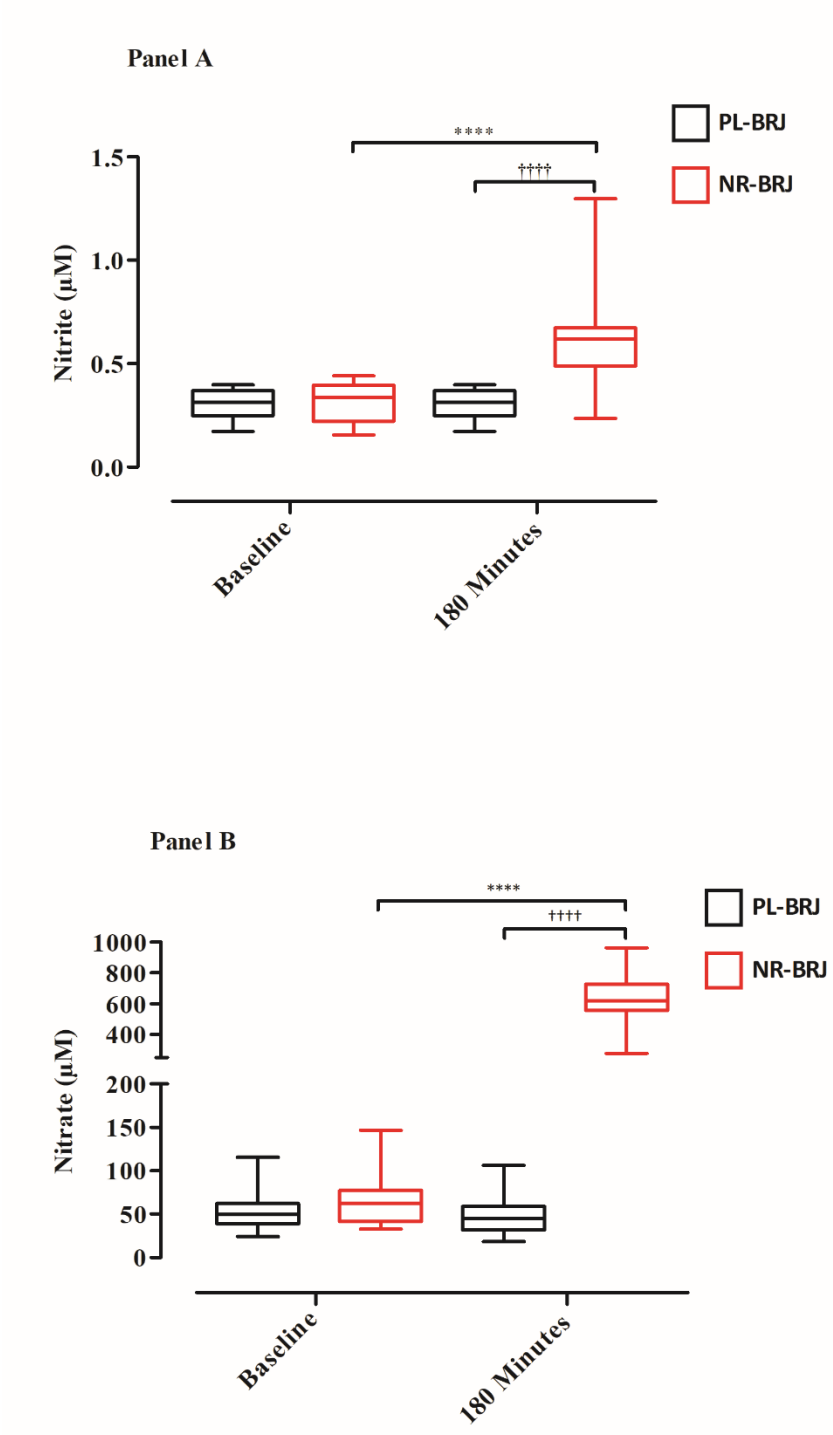
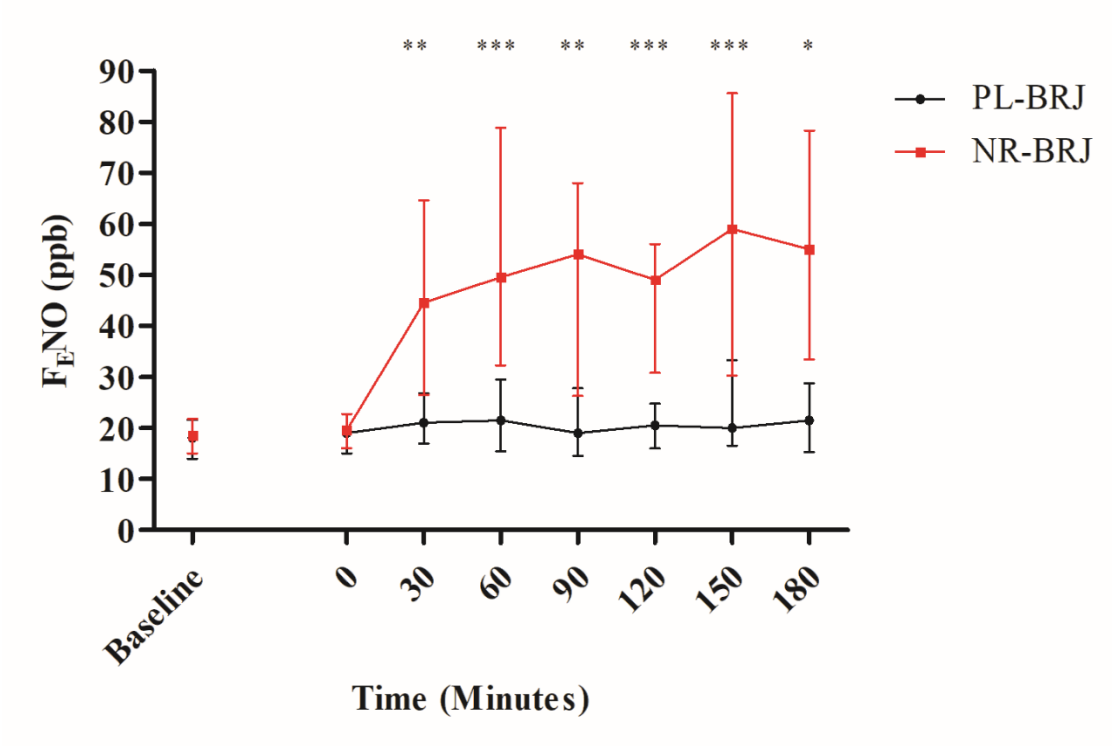

Panel A
PL-BRJ
NR-BRJ
****
††††
Nitrite (µM)
1.5
1.0
0.5
0.0
Baseline
180 Minutes
Panel B
PL-BRJ
NR-BRJ
****
††††
Nitrate (µM)
1000
800
600
400
200
150
100
50
0
Baseline
180 Minutes

**Figure 7**


**
***
**
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*
90
80
70
60
50
40
30
20
10
0
FENO (ppb)
PL-BRJ
NR-BRJ
Baseline
0
30
60
90
120
150
180
Time (Minutes)

**SUPPLEMENTARY APPENDIX**

**Dietary nitrate supplementation to improve exercise capacity in hypoxic COPD**

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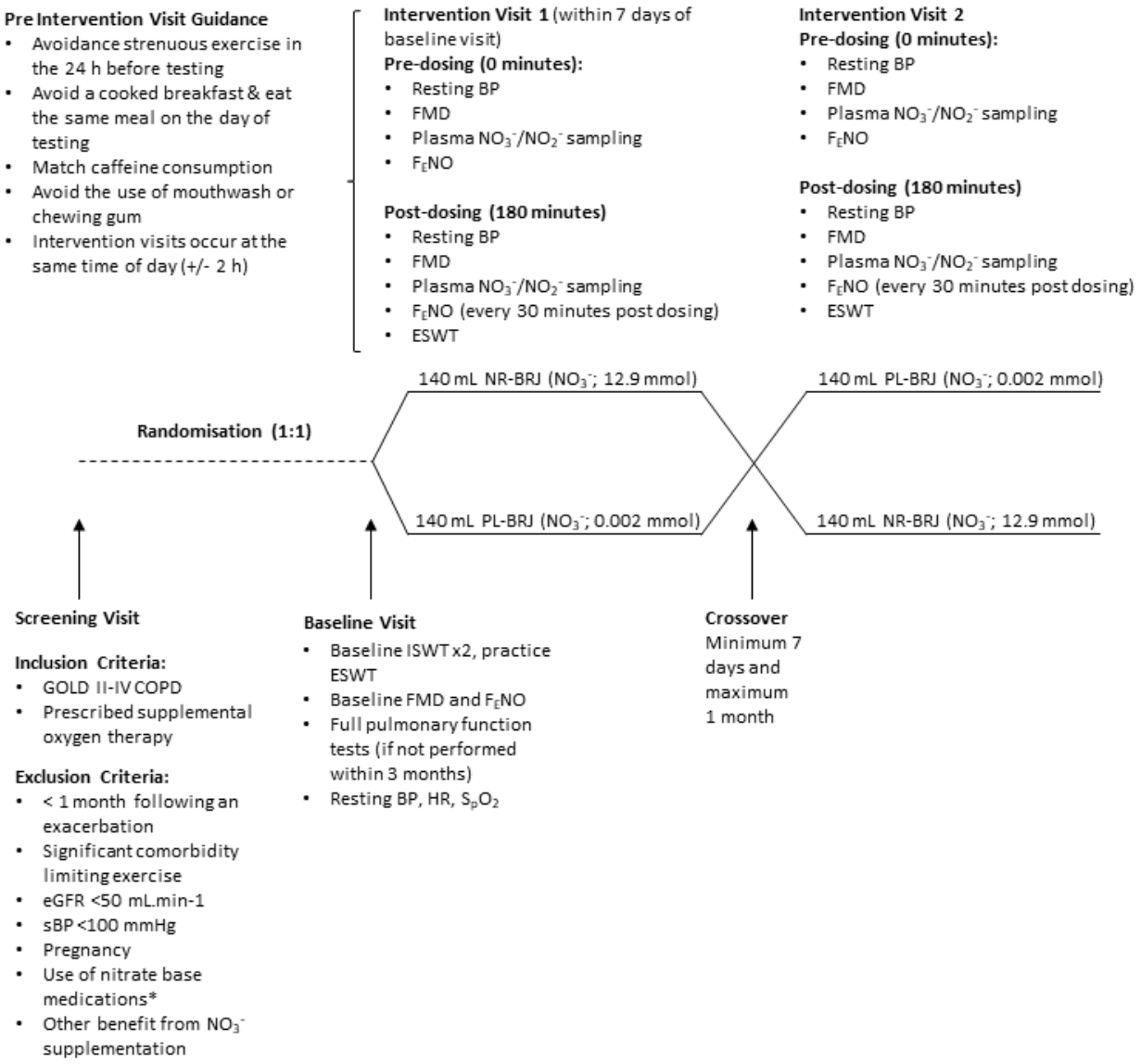



**Figure E1. Study flow diagram**

*Abbreviations: $NO_3^-$ - Nitrate; BP – Blood Pressure; FMD – Flow Mediated Dilatation; $NO_2^-$ - Nitrite; $F_ENO$ – Fractional Exhaled Nitric Oxide; ESWT – Endurance Shuttle Walk Test; BRJ – Beetroot Juice; PL - Placebo; GOLD – Global Initiative for Chronic Obstructive Lung Disease; COPD – Chronic Obstructive Pulmonary Disease; eGFR – Estimated Glomerular Filtration Rate; sBP – Systolic Blood Pressure; ISWT – Incremental Shuttle Walk Test; HR – Heart Rate; $S_pO_2$ – Oxygen Saturations*

## SUPPLEMENTARY METHODS

### Plasma nitrate/nitrite levels - additional methods

Plasma $NO_3^-$ and $NO_2^-$ levels were used as a combined biomarker of $NO_3^-$ ingestion, metabolism and nitric oxide availability [1, 2]. Plasma samples were obtained on arrival and three hours after consumption of either NR-BRJ or PL-BRJ. Samples were obtained by venesection of 6 mL of venous blood into lithium heparin tubes. Within five minutes of collection the vials were split into 3 mL aliquots, with one mixed with 300 µL of 100 mM stock of N-ethylmaleimide (NEM) solution (final concentration 10 mM). The samples were then centrifuged at 1,000 g for eight minutes at room temperature. Subsequently, 1 mL of the supernatant was aliquoted into 2 mL polypropylene cryotubes, snap frozen with liquid nitrogen and stored at -80$^o$C. Plasma nitrate and nitrite concentrations were measured following protein precipitation with methanol (1:1 v/v) by a dedicated high-performance liquid chromatography (HPLC) system equipped with an anion-exchange column, an in-line Cd/Cu reduction column and a post-column diazo coupling reactor coil (Griess reaction) (Eicom NOx analyser, ENO-20, San Diego, USA) [3].

### Oxidative stress – additional methods

### Ferric-reducing ability of plasma (FRAP)

The FRAP assay is a measure of the antioxidant potential in the extracellular compartment [4]. The same plasma samples used for nitrate/nitrate measurement were also used to process this assay. Briefly, 150 µl of FRAP reagent (containing 300 mM acetate buffer at pH 3.6, 10 mM TPTZ [2,4,6-Tris(2-pyridyl)s-triazine], 20 mM $FeCl_3$ at a ratio of 10:1:1 (v:v:v)) was added to 5 µl of diluted plasma (1:3, v:v) into a 96-well plate containing 15 µl of MQ water in each well. The plate was incubated at 37$^o$C for 30 minutes. The absorbance at 593 nm was taken immediately after incubation using a microplate reader (Spectramax M5, Molecular Devices, California USA). FRAP values for the samples were obtained by comparing the absorbance at 593 nm with the known concentrations in the standards ($FeSO_4 \cdot 7H_2O$).

### Thiobarbituric acid-reactive substance (TBARS)

TBARS is a measure of lipid oxidation and is measured using a TBARS assays [5]. The same plasma samples used for nitrate/nitrate measurement were also used to process this assay. In

brief the TBARS assay incorporated the use of an malodialdehyde (MDA) source such as 1,1,3,3 Tetramethoxypropane after hydrolysis as standard, 0.6N tricholoroacetic acid as the acid reagent and thiobarbituric acid (0.26g in 50 mL glacial acetic acid) as colour reagent. Prior to analysis, samples were deproteinised by acid precipitation by taking 300 uL samples and adding an equal volume of acid reagent, mixed and incubated for 15 min at room temperature. The supernatant was isolated by 4 min centrifugation at > 12,000 x *g*. The resulting supernatant was further treated with colour reagent (2:1, v:v), incubated for 1h at 100°C and immediately cooled on ice for 10 min. Treated samples were plated into 96-well microplates and absorbance readings were read at 532 nm using a microplate reader (Spectramax M5, Molecular Devices, California USA). TBARS values for the samples were obtained by comparing the absorbance with known concentrations of MDA standards.

**Total free thiols per protein**

Systemic oxidative stress can be measured as the depletion of the free thiol pool in plasma [6]. The same plasma samples used for nitrate/nitrate measurement were also used to process this assay. Thiol groups were measured as previously described [7, 8]. In brief, 75 µl plasma samples were diluted 1:4 (v:v) with a 0.1 M Tris buffer (pH 8.2) and transferred 90 uL of diluted sample to a 96-well microplate. Using a microplate reader (Molecular Devices Spectramax M5, California, USA), background absorption was measured at 412 nm with a reading at 630 nm for baseline correction. Subsequently, 20 µl 1.9 mM 5,5-Dithio-bis(2-nitrobenzoic acid) [DTNB] in 0.1 M phosphate buffer (pH 7) was added to the samples and standards. Following 20 minutes of incubation at room temperature while mixing, absorption was remeasured at 412 and 630 nm. The concentration of total free thiols in the samples was determined by comparing their absorbance reading to that of an L-cysteine standard before and after addition of DTNB to samples/satandards.

**Endothelial function - additional methods**

Endothelial function was assessed using flow medicated dilatation (FMD) of the brachial artery [9] using a high-resolution doppler ultrasound (GE Logiq 3, GE Medical Systems, Milwaukee, Wisconsin, USA) and a 10 MHz multi-frequency linear array probe were used in B-mode. Brachial artery diameter was measured at baseline and sequentially after release of circulatory arrest of the upper arm over a period of 120 seconds [10], three hours after NR-BRJ/PL-BRJ

consumption. All measurements were performed by a single trained operator. Circulatory arrest was generated via a rapid cuff inflation system (Hokanson, Bellevue, WA, USA), which was positioned proximal to the brachial artery and rapidly inflated to 250 mmHg for five minutes. Data were saved for off-line analysis using ImageJ2 software [11].

## SUPPLEMENTARY RESULTS

**Table E1. Exercise oxygen saturations and heart rate analysis**

| Measure | PL-BRJ (n=18) | NR-BRJ (n=18) |
|---|---|---|
| **Saturations (%)** | | |
| **Rest** | 96 (90, 97) | 96 (92, 97) |
| **Warm-up** | 91 (89, 95) | 94 (90, 95) |
| **Isotime** | 92 (89, 94) | 96 (93, 97) |
| **Peak** | 88 (86, 92) | 94 (91, 96) |
| **Recovery** | 97 (92, 98) | 98 (96, 98) |
| **Heart Rate (bpm)** | | |
| **Rest** | 86 (74, 88) | 88 (78, 91) |
| **Warm-up** | 103 (88, 108) | 96 (88, 102) |
| **Isotime** | 111 (103, 123) | 109 (96, 116) |
| **Peak** | 104 (96, 111) | 101 (112) |
| **Recovery** | 91 (79, 101) | 89 (81, 98) |

The area under the curve for each treatment group was estimated and reported as mean (SD). The results for Saturations for when the subjects were on placebo beetroot juice were 1161.85 (47.59) and the results for when the subjects on Nitrate-rich beetroot juice were 1205.54 (46.39). The treatment effect was estimated to be 43.69 (29.09 to 58.28) $p < 0.0001$. The results suggest that on the average the area under the curve for saturations was higher when on Nitrate-rich beetroot juice than when on placebo. These differences tended to show more during the Isotime and peak periods.

The mean (SD) area under the curve for the HR data when the subjects were on placebo beetroot juice was 1299.93 (186.05) for when the subjects were on Nitrate-rich beetroot juice results was 1258.76 (174.01). The estimated treatment effect was -41.17 (-116.74 to 34.40), $p=0.27$. The results show that while at individual time points the HR was higher for when the subjects were on Placebo, there was no statistically significant difference in the area under the curve.

*Abbreviations; bpm – Beats Per Minute; PL-BRJ – Placebo Beetroot Juice; Nitrate-rich Beetroot Juice*

**Table E2. Between intervention analysis of plasma nitrite and nitrate**

| Measurement | PL-BRJ (n=19) | NR-BRJ (n=19) |
|---|---|---|
| **Baseline Nitrite (µM)** | 0.32 (0.25, 0.37) | 0.34 (0.22, 0.39) |
| **Baseline Nitrate (µM)** | 51.89 (38.98, 62.28) | 62.59 (41.68, 77.29) |
| **180 Minute Nitrite (µM)** | 0.31 (0.23, 0.47) | 0.60 (0.48, 0.67) |
| **180 Minute Nitrate (µM)** | 45.31 (31.39, 58.84) | 617.71 (508.6, 725.88) |
| **Difference in Nitrite (µM) from baseline to 180 minutes** | 0.023 (-0.044, 0.079) | 0.276 (0.144, 0.463) |
| **Difference in Nitrate (µM) from baseline to 180 minutes** | -4.61 (-9.63, 6.23) | 543.25 (441.78, 674.23) |

Results are reported as median (IQR).

The treatment effect of Nitrate was estimated with the Hodges-Lehman estimate and it was 550 (461 to 639) µM. The results suggest that the was a higher for when the subjects were on Nitrate-rich beetroot juice than when they were on placebo and this change was statistically significant, p=0.0003.

The treatment effect of Nitrite was estimated with the Hodges-Lehman estimate and it was 0.248 (0.138 to 0.408) µM. The results suggest that the was a higher for when the subjects were on Nitrate-rich beetroot juice than when they were on placebo and this change was statistically significant, p=0.0011.

*Abbreviations: PL BRJ – Placebo Beetroot Juice; NR-BRJ – Nitrate-rich Beetroot Juice*

**Table E3. Nitrate levels in active and placebo juice and analytic materials**

| Samples | Nitrite | | | | Nitrate | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean (µM) | SD | SEM | %CV | Mean (µM) | SD | SEM | %CV |
| **NEM Stock Solution** | 0.07 | 0.03 | 0.02 | 45.41 | 22.37 | 2.33 | 1.34 | 10.40 |
| **Cryotube** | 0.09 | 0.02 | 0.01 | 17.15 | 2.44 | 0.53 | 0.31 | 22.33 |
| **PL-BRJ** | 195.86 | 2.12 | 1.22 | 1.08 | 55.05 | 0.68 | 0.39 | 1.24 |
| **NR-BRJ** | 10.75 | 0.20 | 0.11 | 1.83 | 120411.03 | 5267.10 | 3040.96 | 4.37 |

Concentration of $NO_2^-$ and $NO_3^-$ in NEM Stock Solution, Cryotubes, Cryotubes with 0.9% Sodium Chloride, PL-BRJ and NR-BRJ.

*Abbreviations: NEM – N-Ethylmaleimide; SD – Standard Deviation; SEM – Standard Error Mean; %CV – Percentage Coefficient of Variation; PL-BRJ – Placebo Beetroot Juice; NR-BRJ – Nitrate Rich Beetroot Juice; µM – micromole.*

**Table E4. Fractional Exhaled Nitric Oxide (FeNO)**

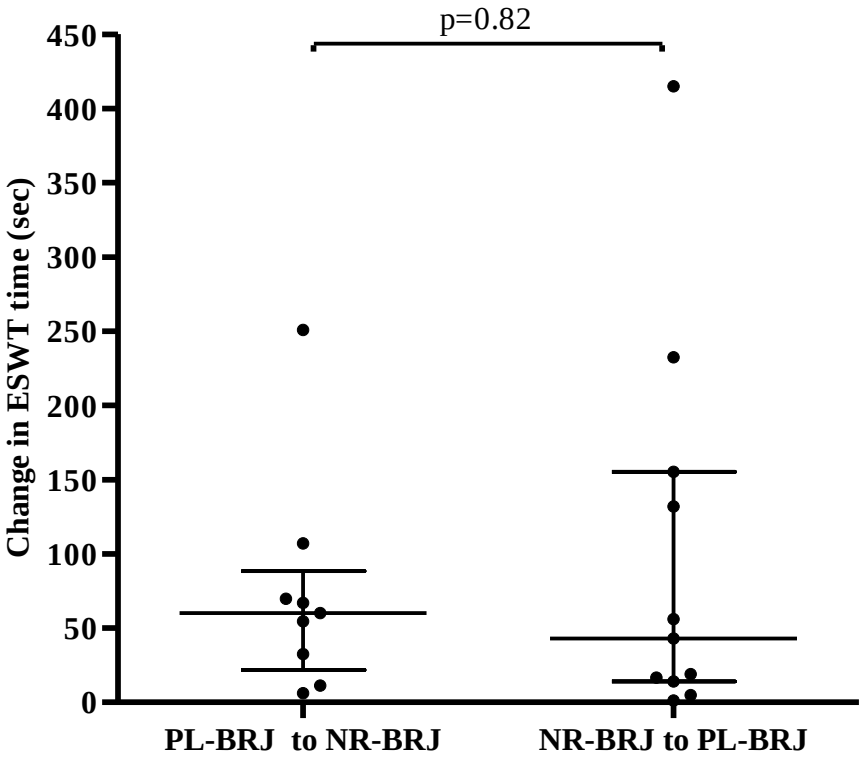
| | $F_ENO$ post NR-BRJ (ppb) | FeNo post PL-BRJ (ppb) |
|---|---|---|
| **Baseline** | 18.5 (15.0, 21.5) | 18.0 (14.0, 22.5) |
| **0 Minutes** | 19.5 (16.0, 22.5) | 19.0 (15.0, 22.5) |
| **30 Minutes** | 44.5 (27.0, 63.0) | 21.0 (17.0, 25.5) |
| **60 Minutes** | 49.5 (33.5, 78.5) | 21.5 (17.0, 29.0) |
| **90 Minutes** | 54.0 (26.5, 90.0) | 19.0 (15.0, 27.5) |
| **120 Minutes** | 49.0 (32.5, 56.0) | 20.5 (16.0, 24.5) |
| **150 Minutes** | 59.0 (33.5, 84.0) | 20.0 (17.0, 32.5) |
| **100 Minutes** | 55.0 (35.0, 76.5) | 21.5 (15.5, 27.5) |

$F_ENO$ levels measured at baseline (visit 1) and subsequently at intervention visits (visits 3 and 4) at time point zero minutes prior to dosing with either PL-BRJ or NR-BRJ and subsequently every 30 minutes until 180 minutes post dosing. Data presented: median (IQR).

The AUC was calculated for each treatment group and compared to estimate the treatment effect using the Hodges-Lehman estimate. The median (IQR) AUC for when the subjects were on placebo was 3622.5 (3181.9, 4796.9) and the corresponding results for when the subjects were on Nitrate-rich beetroot juice was 9440.6 (6273.8, 11831.3) and the treatment effect with its 95% CI was 5407 (3096 to 7576), p=0.0011. The results suggest that the FeNO levels while the subjects were on Nitrate-rich beetroot juice were significantly higher than when they were on placebo

*Abbreviations: $F_ENO$ – Fractional Exhaled nitric Oxide; IQR – Interquartile Range; AUC – area under the curve; ppb – Parts Per Billion*

**Figure E2. Primary Outcome Order Effect**



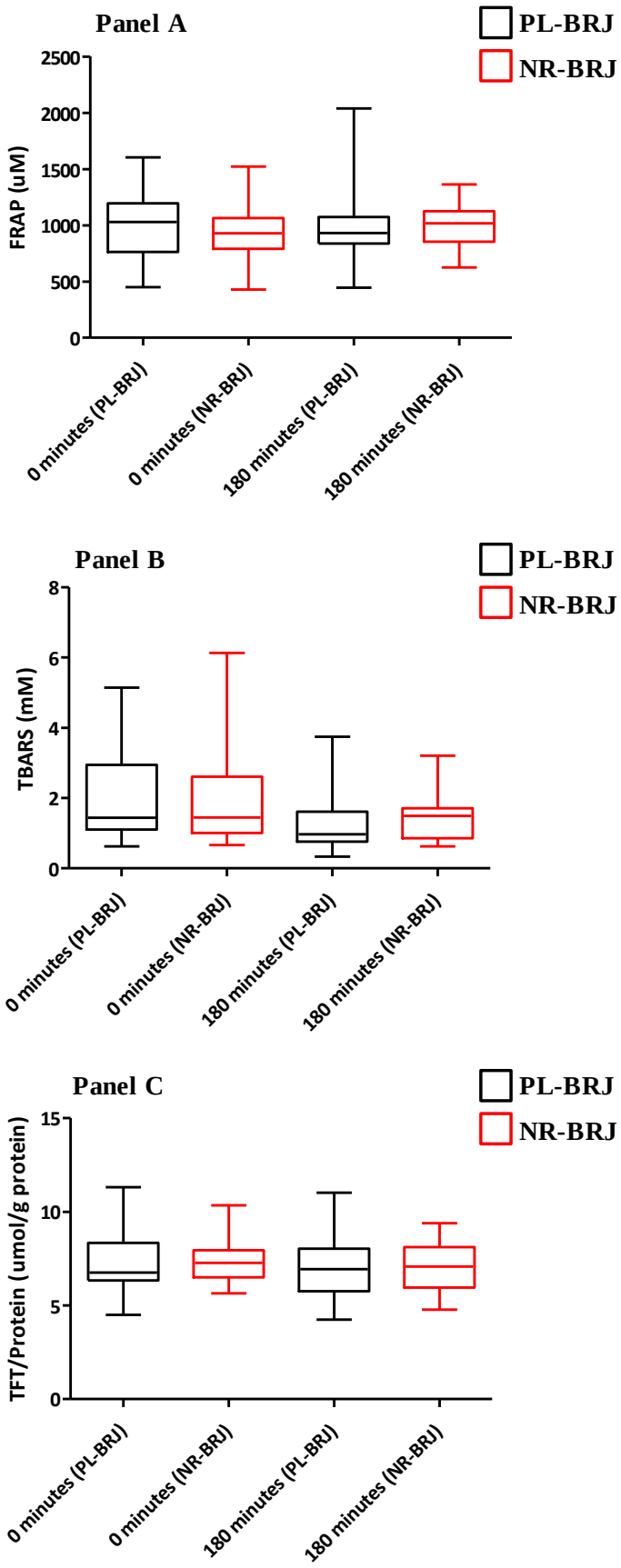

Change in ESWT time (seconds) when testing for intervention order effect, if PL-BRJ was applied first or NR-BRJ. Data presented median (line) and interquartile range (whiskers) with as individual data points (dots). Mann-Whitney U test, the median (IQR) change in ESWT time if PL-BRJ was applied first was 60.0 (21.8, 88.4) seconds, compared to 43.1 (14.03, 155.3) seconds, if NR-BRJ was applied first; p = 0.82.

*Abbreviations: ESWT – Endurance Shuttle Walk Test; PL-BRJ – Placebo Beetroot Juice; NR-BRJ – Nitrate Rich Beetroot Juice*

**Results E1. Effect of dietary nitrate supplementation on endurance shuttle walk time**

There is a clear outlier in this dataset. When this individual's data was removed from analysis, all individuals still walked further following consumption the $NO_3^-$-rich BRJ. There was a statistically significant difference between the median (IQR) ESWT time with the outlier removed; $NO_3^-$-rich BRJ 193.8 (145.5, 389.6) seconds vs PL 158.2 (121.6, 236.6) seconds; p = 0.0001. Regarding this specific individual at baseline assessment their best ISWT distance was 370 meters, using the ESWT conversion table the ESWT speed was calculated as 4.65 km/h which equates to ESWT level 11. All individuals undertook a practice ESWT, this individual's practice ESWT time was 599 seconds. The ESWT time that this individual achieved following consumption of the placebo beverage was 785 seconds. For both ESWT this individual reported peak Borg Dyspnoea scale of 8. This individual's data was included in the full analysis as it is felt to be a true representation of this individuals exercise endurance.

**Figure E3. Measures of oxidative stress**



Measures of oxidative stress for PL-BRJ and NR-BRJ Dosing conditions. Data presented as Data presented are median and interquartile range (box) with whiskers representing minimum to maximum values. Plasma samples were measured at baseline (zero minutes) and 180 minutes after dosing. Wilcoxon sign-rank test was used to compare change in measures of oxidative stress between intervention groups. Mann-Whitney U test was used to compare change in measures of oxidative stress between treatment conditions.

**Panel A. Ferric reducing ability of plasma (FRAP)**

There was no statistically significant difference between interventions for baseline and post intervention FRAP. Baseline FRAP PL-BRJ: 1028 (762.9, 1195) µM vs FRAP NR-BRJ: 927.7 (790.2, 1064) µM; p = 0.7. Post intervention FRAP PL-BRJ: 930.2 (836.8, 1073) µM vs FRAP NR-BRJ: 1018 (853.0, 1125) µM; p = 1.0. (Wilcoxon sign-rank test)

There was no statistically significant difference between baseline FRAP levels and post dosing levels with either PL-BRJ and NR-BRJ. FRAP PL-BRJ: 1028 (762.9, 1195) µM vs post dosing 930.2 (836.8, 1073) µM; p = 0.9. FRAP NR-BRJ: 927.7 (790.2, 1064) µM vs post dosing 1018 (853.0, 1125) µM; p = 0.3. (Mann-Whitney U test).

**Panel B. Thiobarbituric acid-reactive substance (TBARS)**

There was no statistically significant difference between interventions for baseline and post intervention TBARS. Baseline TBARS PL-BRJ: 1.443 (1.102, 2.940) mM vs TBARS NR-BRJ: 1.450 (1.007, 2.613) mM; p = 0.8. Post intervention TBARS PL-BRJ: 0.971 (0.766, 1.614) mM vs TBARS NR-BRJ: 1.499 (0.855, 3.209) mM; p = 0.4. (Wilcoxon sign-rank test)

There was no statistically significant difference between baseline TBARS levels and post dosing levels with either PL-BRJ and NR-BRJ. TBARS PL-BRJ: 1.443 (1.102, 2.940) mM vs post dosing 0.971 (0.766, 1.614) mM; p = 0.8. TBARS NR-BRJ: 1.450 (1.007, 2.613) mM vs post dosing 1.499 (0.855, 3.209) mM; p = 0.3. (Mann-Whitney U test).

**Panel C. Total free thiols (TFT) per protein**

There was no statistically significant difference between interventions for baseline and post intervention TFT per protein. Baseline TFT per protein PL-BRJ: 6.754 (6.328, 8.342) µmol.g$^{-1}$ protein vs TFT per protein NR-BRJ: 7.284 (6.508, 7.960) µmol.g$^{-1}$ protein; p = 0.9. Post intervention TFT per protein PL-BRJ: 6.942 (5.768, 8.026) µmol.g$^{-1}$ protein vs TFT per protein NR-BRJ: 7.079 (5.961, 8.115) µmol.g$^{-1}$ protein; p = 0.5. (Wilcoxon sign-rank test)

There was no statistically significant difference between baseline TFT per protein levels and post dosing levels with either PL-BRJ and NR-BRJ. TFT per protein PL-BRJ: 6.754 (6.328, 8.342) µmol.g$^{-1}$ protein vs post dosing 6.942 (5.768, 8.026) µmol.g$^{-1}$ protein; p = 0.1. TFT per protein NR-BRJ: 7.284 (6.508, 7.960) µmol.g$^{-1}$ vs post dosing 7.079 (5.961, 8.115) µmol.g$^{-1}$ protein; p = 0.4. (Mann-Whitney U test).

*Abbreviations: FRAP - Ferric Reducing Ability of Plasma; TBARS - Thiobarbituric Acid-Reactive Substance; TFT – Total Free Thiols; PL-BRJ – Placebo Beetroot Juice; NR-BRJ – Nitrate-rich Beetroot Juice; mM - millimole*

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