SUPPLEMENTARY APPENDIX

Dietary nitrate supplementation to improve exercise capacity in hypoxic COPD

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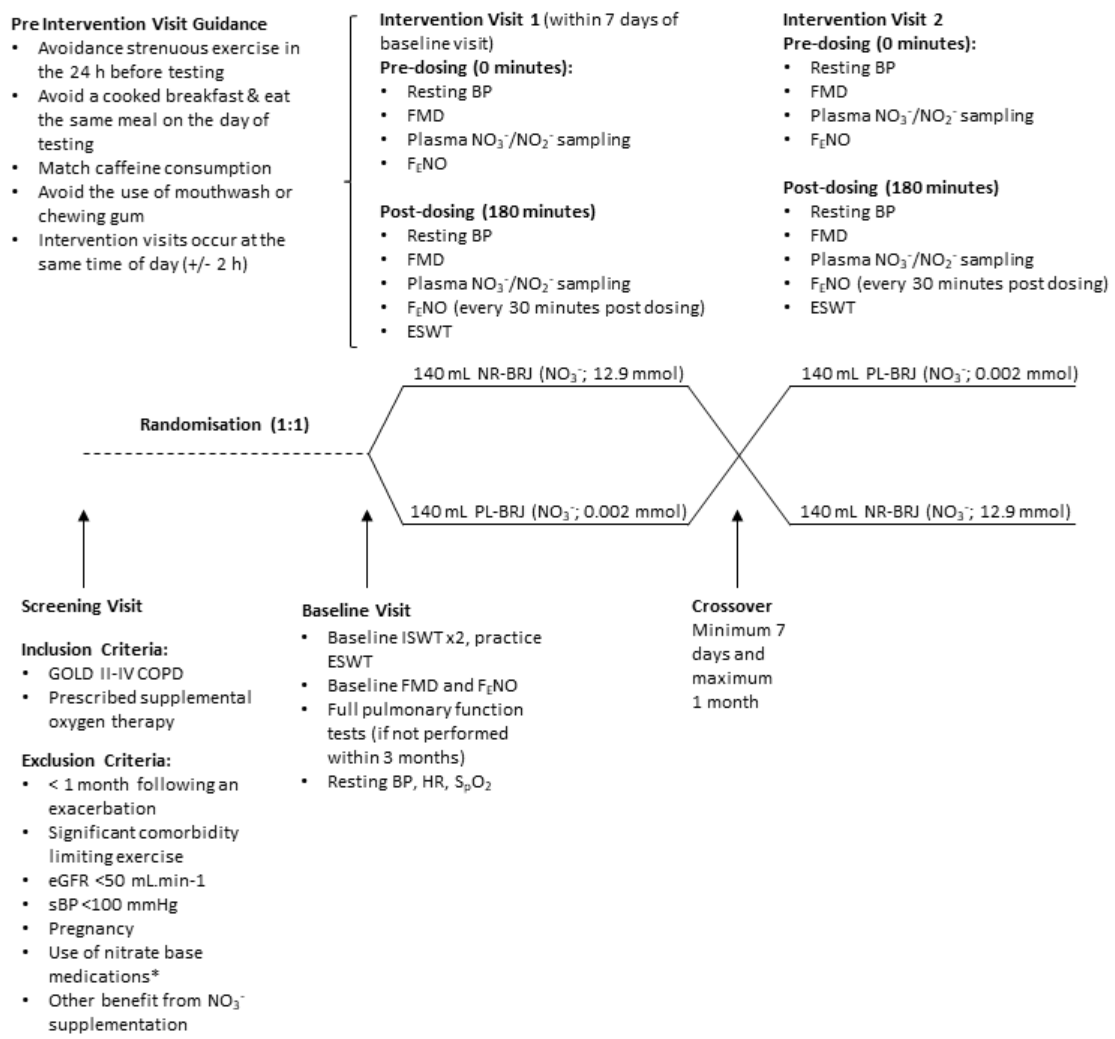

Figure E1. Study flow diagram

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 -80ºC. 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 37ºC 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

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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

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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].

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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)

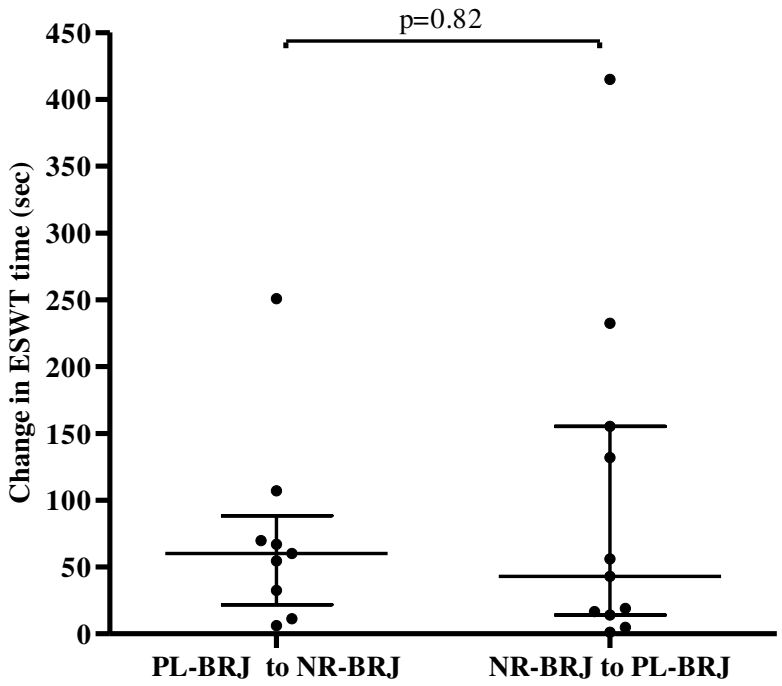
	F_ENO post NR-BRJ (ppb)	**FeNo post PL-BRJ (ppb)**
Baseline	18.5 (15.0, 21.5)	18.0 (14.0, 22.5)
0 Minutes	19.5 (16.0, 22.5)	19.0 (15.0, 22.5)
30 Minutes	44.5 (27.0, 63.0)	21.0 (17.0, 25.5)
60 Minutes	49.5 (33.5, 78.5)	21.5 (17.0, 29.0)
90 Minutes	54.0 (26.5, 90.0)	19.0 (15.0, 27.5)
120 Minutes	49.0 (32.5, 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.

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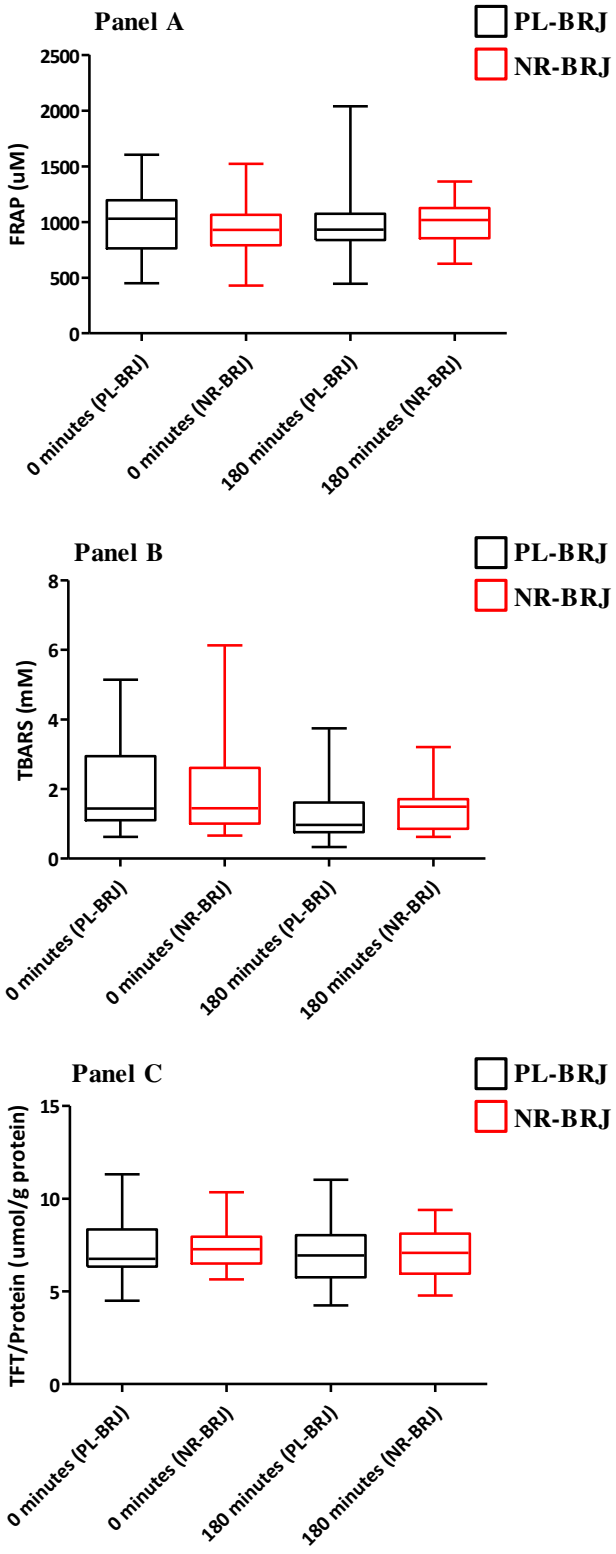
Figure E3. Measures of oxidative stress



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

REFERENCES

1. Cumpstey AF, Hennis PJ, Gilbert-Kawai ET, Fernandez BO, Poudevigne M, Cobb A, Meale P, Mitchell K, Moyses H, Pöhnl H, Mythen MG, Grocott MPW, Feelisch M, Martin DS. Effects of dietary nitrate on respiratory physiology at high altitude - Results from the Xtreme Alps study. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 2017: 71: 57-68.
2. Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, Scheeren T, Godecke A, Schrader J, Schulz R, Heusch G, Schaub GA, Bryan NS, Feelisch M, Kelm M. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free radical biology & medicine* 2003: 35(7): 790-796.
3. Rassaf T, Bryan NS, Kelm M, Feelisch M. Concomitant presence of N-nitroso and S-nitroso proteins in human plasma. *Free radical biology & medicine* 2002: 33(11): 1590-1596.
4. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical biochemistry* 1996: 239(1): 70-76.
5. Zeb A, Ullah F. A Simple Spectrophotometric Method for the Determination of Thiobarbituric Acid Reactive Substances in Fried Fast Foods. *Journal of analytical methods in chemistry* 2016: 2016: 9412767.
6. Banne AF, Amiri A, Pero RW. Reduced level of serum thiols in patients with a diagnosis of active disease. *Journal of anti-aging medicine* 2003: 6(4): 327-334.
7. Frenay AS, de Borst MH, Bachtler M, Tschopp N, Keyzer CA, van den Berg E, Bakker SJL, Feelisch M, Pasch A, van Goor H. Serum free sulfhydryl status is associated with patient and graft survival in renal transplant recipients. *Free radical biology & medicine* 2016: 99: 345-351.
8. Koning AM, Meijers WC, Pasch A, Leuvenink HGD, Frenay AS, Dekker MM, Feelisch M, de Boer RA, van Goor H. Serum free thiols in chronic heart failure. *Pharmacological research* 2016: 111: 452-458.
9. Thijssen DH, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, Parker B, Widlansky ME, Tschakovsky ME, Green DJ. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *Am J Physiol Heart Circ Physiol* 2011: 300(1): H2-12.
10. Rodriguez-Miguelez P, Seigler N, Harris RA. Ultrasound Assessment of Endothelial Function: A Technical Guideline of the Flow-mediated Dilation Test. *Journal of visualized experiments : JoVE* 2016(110).
11. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 2012: 9(7): 676-682.