Effect of inorganic nitrate on exercise capacity, mitochondria respiration, and vascular function in heart failure with reduced ejection fraction

Effect of inorganic nitrate on exercise capacity, mitochondria respiration and vascular function in heart failure reduced ejection fraction

Mary N Woessner[a,b], Christopher Neil[a,b], Nicholas J Saner[a], Craig A Goodman[ab], Luke C McIlvenna[a], Joaquin Ortiz de Zevallos[a,d], Andrew Garnham[a,] Itamar Levinger[a,c] ,and Jason D Allen[a, d]

a. Institute for Health and Sport (IHES), Victoria University, Melbourne, Australia.
b. Western Health, St Albans, Victoria Australia.
c. Australian Institute for Musculoskeletal Science (AIMSS), University of Melbourne and Western Health, St Albans, VIC Australia
d. Department of Kinesiology & Division of Cardiovascular Medicine, University of Virginia, Charlottesville, VA, USA

Corresponding Author:

Dr. Mary Woessner

Institute for Health and Sport (iHeS), Victoria University

PO Box 14428, Melbourne, Australia

Phone: +61 0421 692 161

Email: mary.woessner@vu.edu.au

Supplementary Figure 1: 10.6084/m9.figshare.11307308
Supplementary Table 1: 10.6084/m9.figshare.11307311

ABSTRACT

Background: Chronic under perfusion of the skeletal muscle tissues is a contributor to a decrease in exercise capacity in patients with heart failure reduced ejection fraction (HFrEF). This under perfusion is due, at least in part, to impaired nitric oxide (NO) bioavailability. Oral inorganic nitrate supplementation increases NO bioavailability and may be used to improve exercise capacity, vascular function and mitochondrial respiration.

Methods: Sixteen patients with HFrEF (15 men, 63 ± 4 y, BMI: 31.8 ± 2.1 $kg \cdot m^{-2}$) participated in a randomised, double-blind, crossover design study. Following consumption of either nitrate rich beetroot juice (16 mmol nitrate/day), or a nitrate-depleted placebo for five days participants completed separate visits for assessment of exercise capacity, endothelial function and muscle mitochondrial respiration. Participants then had a two week washout prior to completion of the same protocol with the other intervention. Statistical significance was set *a priori* at $p<0.05$ and between treatment differences were analysed via paired- t-test analysis.

Results: Following nitrate supplementation both plasma nitrate and nitrite increased (933%, $p<0.001$ and 94%, $p< 0.05$, respectively). No differences were observed for VO_{2peak} (nitrate 18.5 ± 5.7ml·kg-1·min-1, placebo: 19.3 ± 1.4$ml \cdot kg^{-1} \cdot min^{-1}$; $p=0.13$) or time to exhaustion (nitrate: 1165 ± 92sec, placebo: 1207 ± 96sec, $p=0.16$) following supplementation. There were no differences between interventions for measures of vascular function, mitochondrial respiratory function or protein expression (all $p>0.05$).

Conclusions: Inorganic nitrate supplementation did not improve exercise capacity and skeletal muscle mitochondrial respiratory function in HFrEF. Future studies should explore alternative interventions to improve peripheral muscle tissue function in HFrEF.

NEW AND NOTEWORTHY

This is the largest study to date to examine the effects of inorganic nitrate supplementation in patients with HFrEF and the first to include measures of vascular function and mitochondrial respiration. While daily supplementation increased plasma nitrite, our data indicates that supplementation with inorganic nitrate as a standalone treatment is ineffective at improving exercise capacity, vascular function or mitochondrial respiration in patients with HFrEF.

Key Words: Nitric Oxide, Beetroot Juice, Exercise Capacity, Nitrate-Nitrite-NO pathway

INTRODUCTION

Patients with chronic heart failure (CHF) are characterised by reduced aerobic capacity (VO_{2peak}) and early fatigue (6). Improving VO_{2peak} is an important clinical goal in CHF as it is correlated with reduced mortality rate and increased quality of life (13, 29).

It is well accepted that impairments in peripheral tissues have a significant contribution to the reduced exercise capacity in patients with CHF (3). Nitric oxide (NO), a free radical released by the endothelium in response to shear stress, is a key regulator of peripheral tissue blood flow and has been linked to vascular function, mitochondrial function and tissue perfusion (24, 25). Reductions in NO bioavailability and impaired mitochondrial function play a critical role in limiting exercise capacity in patients with CHF and are associated with the development and progression of the syndrome (8, 25). As such, it is important to uncover whether increasing NO bioavailability through exogenous NO precursors can improve peripheral function and exercise capacity in patients with CHF.

Inorganic nitrate supplementation increases NO bioavailability, via the nitrate-nitrite-NO reduction pathway (32). Nitrate supplementation has been shown to modify exercise capacity in patients with peripheral arterial disease and in some forms of CHF (16, 17, 34, 36). While previous studies demonstrate the efficacy of oral nitrate supplementation to increase exercise capacity in patients with preserved ejection fraction (HFpEF), the potential of the intervention in those with a reduced ejection fraction (HFrEF) is poorly understood due to limited few studies (4, 10, 17, 41). Additionally, no previous nitrate supplementation studies in CHF populations have explored the potential effects on vascular function and mitochondrial respiratory function. As both have been previously identified as mediators of health and exercise capacity, exploring the efficacy of nitrate for improving these outcomes

could be of significant clinical value. To date, oral inorganic nitrate studies in HFrEF have had small sample sizes and heterogeneous ejection fraction (EF) inclusion criteria.

Therefore, the primary aim of this study was to test the hypothesis that chronic oral inorganic nitrate supplementation will improve VO_{2peak} during treadmill exercise in patients with HFrEF. Secondary aims were to determine the effects on vascular function and skeletal muscle mitochondrial respiratory function in this population.

METHODOLOGY

The full protocol for this clinical trial was previously published (35). The study was a randomized, placebo-controlled, double blind crossover study. It was approved by the Melbourne Health and by Victoria University Human Ethics Committees and has been registered in the Australian New Zealand Clinical Trials Registry [ACTRN12615000906550].

The study design is illustrated in Figure 1. In brief, following a screening visit, participants were randomised to consume either nitrate-rich beetroot juice (210 ml, 16 mmol nitrate) or a nitrate-depleted placebo for five days (210 ml, <0.1 mmol nitrate) (James White Drinks, Ipswich, UK). Following this five-day loading, the participants continued daily dosing until the completion of the three testing visits (average days dosing prior to CPX=7, vascular= 10, biopsy= 15). The total days of supplementation and testing order were matched for each participant for both treatments and all participants had a two-week washout period between treatments.

Recruitment and eligibility

Participants were identified through medical chart reviews and interested individuals were provided a detailed description of the nature of the study and, if interested, were invited to sign an informed consent and complete a screening cardiopulmonary exercise test that also served as a familiarisation visit. Participants were screened either over the phone or in person to ensure they met all inclusion criteria. The key criteria were for participants to have an EF <40%, be on stable medications (for 3 months), and to have no existing injuries. While individuals with comorbidities were invited to participate, CHF had to be considered their primary condition (see Figure 2). In total, 882 medical charts were reviewed, nineteen participants were recruited and sixteen individuals (62.6 ± 3.6 years) with diagnosed HFrEF (EF 30.4 ± 1.8 %) completed the study.

Supplementation

Participants consumed a total of 210 ml (16 mmol nitrate) per day. They were asked to consume one 70 ml bottle with each meal. However, on testing days they were requested to consume the morning dose exactly 2.5 hours prior to the appointment time (15, 32). Compliance to supplementation and conversion of nitrate to nitrite was confirmed by a blood draw on each of the two interventional CPX testing visits. For the duration of the trial, all participants were asked to refrain from the use of any type of mouthwash due to demonstrated reductions in conversion of nitrate to nitrite via oral bacteria (33). They were also asked to maintain their normal dietary and exercise patterns for the duration of the study. While diet was not specifically monitored throughout the study, participants were given instructions on certain high nitrate food items to avoid.

Aerobic capacity assessment

The CPX tests utilised a two-step treadmill protocol whereby all participants first completed six minutes of low-intensity walking at 1.4 km/hour at a 4% grade. The protocol then increased in speed or incline (in an individualised manner, with intensities replicated at subsequent visits) every two minutes. All tests were continued until the participant reached volitional exhaustion. The total time to exhaustion was recorded as the total exercise duration. Expired respiratory gases were collected breath-by-breath via a facemask attached to a gas analyser (Medgraphics, cardio2 and CPX/D System – Utilising Breezeex Software, 142090-001, Revia, Minnesota, USA) and heart rate (HR) was monitored continuously via a 12-lead ECG (Mortara, X-Scribe II, Milwaukee, WI, USA). The gas exchange threshold was calculated via the V-slope method (2). VO_{2peak} was recorded as the average VO_2 over the final 30 seconds of exercise. Tissue oxygenation was captured noninvasively using a near-infrared spectrometry (NIRS, PortaMon, Artinis Medical Systems B.V., The Netherlands) device positioned on the medial side of the gastrocnemius muscle of the participant. Prior to placement of the device, a skinfold assessment was performed. Individuals with a reading >200mm did not have the NIRS device placed as the adipose tissue thickness in these individuals would interfere with the NIRS interpretation.

Plasma nitrate and nitrite concentrations

Venous blood draws were taken at each of the testing CPX visits to confirm supplementation adherence and conversion of nitrate to nitrite. Following five minutes of seated rest, a venous blood sample was drawn from the antecubital vein, immediately transferred into five 1 ml microtubes containing 5 μL heparin (1 to 1000 μ/ml) and centrifuged at 3°C for 3 minutes at 5,000 g). The plasma was removed, snap frozen in liquid nitrogen and transferred to a -80°C freezer for storage until subsequent analysis. Analysis of plasma nitrite and nitrate

concentrations was performed utilising Ozone-based chemiluminescence using a Sievers NOA model 280i (GE Analytical Instruments) in conjunction with a custom-designed reaction chamber (28).

Vascular function

Participants were asked to hold all morning medications until vascular post-testing. Following 10 minutes of supine rest, endothelial function was assessed via brachial artery flow mediated dilation (FMD) using a high-resolution ultrasound (Terason, LifeHealthcare, New South Wales, Australia) with R wave trigger (35). Ten-second video clips were captured in duplicate at baseline and during forearm occlusion and a continuous two-minute video was captured after the occlusion cuff release (reactive hyperaemia). Peak change following reactive hyperaemia was calculated as the percentage change in brachial artery diameter from baseline to immediately following peak hyperaemia.

For all BP measurements, the non-invasive SphygomoCor® (AtCor Medical, Sydney, NSW, Australia) diagnostic system was utilised (12). A SphygomoCor® brachial blood pressure (BP) cuff was fitted on the upper arm. The system recorded pulsations at the brachial artery and produced (via a generalised transfer function) aortic pressure waveforms and predicted central systolic BP, diastolic BP, mean arterial pressure, pulse pressure, augmentation index and aortic pressure. Two measurements were captured, with the lower of the two readings recorded. If the two blood pressure readings were >6 mmHg apart, a third measure was recorded to ensure a true resting value and the average of the two lowest BP were recorded.

Muscle biopsies

Muscle biopsy samples were collected from the vastus lateralis, using a Bergström biopsy needle with manual suction, as previously described (20). Biopsies were performed in the

morning with the participant in a fasted state, with the exception of the beetroot juice supplementation. Individuals who were taking a prescribed blood thinner (n=4), if approved by the doctor, were asked to withhold this medication for the 48hours prior to the muscle biopsy for each intervention arm of the trial. One portion (10-20 mg) was immediately immersed in a 5 ml tube containing ~3 ml of biopsy preserving solution kept on ice and used for in-situ measurements of mitochondrial respiratory function, while the other portion was immediately frozen in liquid nitrogen and stored at -80°C for subsequent analyses.

Fibre preparation and high-resolution respirometry

Procedures for the following protocol have been previously published (27). Muscle fibres were separated with forceps and immediately placed in ice-cold preserving solution BioPS. The plasma membrane was permeabilised by agitation for 30 min at 4°C in BioPS containing 50 µg/ml saponin and subsequently washed in the respiration medium MIR05. Mitochondrial respiration was measured in duplicate (from 2–4 mg wet weight of muscle fibres) in MiR05 at 37°C, using a high resolution respirometer (Oxygraph-2k, Oroboros, Innsbruck, Austria). A substrate-uncoupler-inhibitor titration (SUIT) protocol was utilised (27). The SUIT sequence was as follows: malate (2 mM) and pyruvate (5 mM) in the absence of adenylates were added for measurement of leak respiration $(CI)_{L.}$ ADP (5 mM) was added for measurement of oxidative phosphorylation capacity$(CI)_{P}$. Succinate (10 mM) was added for the measurement of p through complex 1 and 2 combined $(CI+II)_{P}$. Cytochrome c (10 mM) was then added to test for outer mitochondrial membrane integrity (an oxygen flux increase of <15% from (CI+II)p was considered acceptable). This was followed by a series of stepwise carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) titrations (0.75–1.5 mM), for measurement of electron transport capacity $(_{E})$ through CI and CII $(CI+II)_{E}$. Rotenone (0.5 mM), an inhibitor of CI, was added to determine E through CII $(CII)_{E}$. Finally,

the addition of antimycin A (2.5 mM), an inhibitor of CIII, allowed measurement and correction of residual oxygen consumption (ROX), indicative of non-mitochondrial oxygen consumption. Reoxygenation during the protocol was by direct syringe injection of O_2 was necessary to maintain O_2 levels between 275 and 450 nmol/ml and to avoid potential oxygen diffusion limitation. Oxygen concentration (in nanomoles per milliliter) and flux (in picomoles per second per milligram) were recorded with DatLab software (Oroboros). Mitochondrial specific respiration (pmol $O_2 \cdot s^{-1} \cdot CS^{-1}$) was calculated by normalising mass-specific respiration (pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$) by the citrate synthase activity (mol$\cdot h^{-1} \cdot$kg protein^{-1}).

Whole-muscle lysates

The protein concentration of muscle sample homogenates was determined in triplicate with a commercial colorimetric assay (Protein Assay kit-II; Bio-Rad, Gladesville, NSW, Australia), against bovine serum albumin standards (BSA, A9647; Sigma-Aldrich).

Western blotting

Protein content of the muscle homogenates were assessed using standard western blot protocol (23). Equal amounts of total protein were loaded into wells on Criterion^{TM} 4-20% TGX Stain-Free^{TM} Precast gels (Bio-Rad) and normalised against mixed homogenate internal standards as previously described (23). The primary antibodies used were from Cell Signaling Technology and included AKT (#9272), p-AKT ser473 (#9271), p38MAPK (#9212), p-p38MAPK Thr180/Tyr182, #9211), mTORC1 (#2983), p-mTORC1 ser2448 (#5586). One antibody from Calbiochem for PGC-1α (#st1202) was also utilized. Following TBST washes, samples were incubated at room temperature with the appropriate host species–specific secondary antibody for 60 min, before being exposed to a

chemiluminescence solution. Images were taken with a ChemiDoc Imaging System fitted (Bio-Rad). Densitometry was performed with Image Lab 5.0 software (Bio-Rad).

Citrate synthase activity analysis

Citrate synthase (CS) activity was determined in triplicate on a 96 well microtiter plate by adding 5 µL of a 6 $mg \cdot ml^{-1}$ muscle homogenate (freeze thawed in liquid nitrogen twice), 40 µL of 3mM acetyl CoA, 25 µL of 1mM 5,59-dithiobis (2-nitrobenzoic acid) (DTNB), 165 µL of 100 mM Tris buffer (pH 8.3, kept at 30 °C). After addition of 15 µL of 10 mM oxaloacetic acid, the plate was immediately placed in an xMark-Microplate spectrophotometer (Bio-Rad) at 30°C, and after 30 s of linear agitation, absorbance at 412 nm was recorded every 15 s for 3 min. CS activity is reported as moles per hour per kilogram protein.

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (version 22 (SPSS Inc. Chicago, IL, USA). Between treatment differences were analysed via paired t-tests. Statistical significance was set *a-priori* at $p < 0.05$. Figures were created utilising GraphPad Prism Version 7.00 for Windows (GraphPad Software, La Jolla, California USA). Unless otherwise indicated, all results are presented as mean ± standard error of the mean (SEM).

Results

Nineteen patients commenced the trial, however, three dropped out prior to completion of both rounds of testing due to reasons unrelated to the study. Anthropometric and clinical characteristics of the 16 who completed the study are described in Table 1. There was a single female participant in the study. Statistical analyses were conducted both including and

295 excluding this participant's data. As the results of this sub analyses did not result in
296 significantly alter the findings (by either including or excluding this data point), the
297 participant was included.
298
299 *Plasma nitrate/nitrite*
300 Adherence to the supplementation was ~98%, as confirmed by dosing logs and bottle cap
301 returns. Plasma nitrate and nitrite concentrations increased following supplementation (933%,
302 $p<0.001$ and 94%, $p< 0.05$) respectively, Figure 3A-D. One participant's plasma nitrite data
303 was excluded from the analysis due to a concentration 4 standard deviations above the mean.
304
305 *Exercise outcomes*
306 There were no differences in VO_{2peak} (Figure 4 A) or TTE (Figure 4 B) between the nitrate
307 and placebo interventions.
308
309 Similarly, there were no differences between the two treatments in deoxygenated or
310 oxygenated haemoglobin at rest or at any stage of the exercise testing (Figure 5). Additional
311 numerical data are displayed for each stage in Supplementary Table 1
312 (https://figshare.com/s/a3f0d84096353204636a).
313
314 *Vascular function*
315 Twelve participants completed the vascular testing (four could not be analysed due to
316 insufficient image quality). There were no significant differences between interventions in
317 the resting brachial BPs (SBP, DBP and MAP) between the placebo and nitrate
318 interventions (Δ= -2, -1, -2 mmHg, all $p>0.30$). There were also no significant differences in
319 the measures of aortic pressure or stiffness (Table 2).
320

Finally, there were no differences in resting brachial artery diameters (nitrate 3.92 ± 0.16 mm and placebo 4.0 ± 0.13 mm, p=0.44) or peak reactive hyperaemic response (nitrate 5.7 ± 1.1 % and placebo 4.1 ± 0.68 %, p=0.06) between interventions.

Mitochondrial respiratory function

Seven patients completed duplicate skeletal muscle biopsies. Absolute values for both mass specific (pmol $O2.s^{-1}\cdot 1mg^{-1}$) and mitochondrial specific (pmol $O2\ s^{-1}\cdot CS^{-1}$) respiration values are presented in Supplementary Table 2 https://figshare.com/s/f169ec7501a557dda895. None of the examined parameters were significantly different between interventions (all p>0.05).

There were no differences noted in maximal oxidative phosphorylation between the nitrate and placebo interventions (Figure 6, p>0.05) and no correlations between any of the mass-specific or mitochondrial-specific respiration values and VO_{2peak} (all correlations p>0.1).

There were no differences between the nitrate and placebo interventions for mTORC1 (Figure 7 A-D) p38MAPK (Figure 7 E; 4 H) Akt (Figure 7 I-L) and PGC-1α (Figure 7 M-N).

Discussion

We report that in patients with HFrEF, chronic oral inorganic nitrate supplementation had no significant effect on aerobic exercise capacity, vascular function, peripheral and central blood pressures or muscle respiration.

Previous studies in both healthy and clinical cohorts have indicated significant increases in plasma nitrate and nitrite following supplementation (1, 7, 10, 21). In the present study, there was a significant increase in plasma nitrate and nitrite following supplementation. However in absolute terms, a 342nM increase in plasma nitrite is relatively low compared to previously

reported levels in HFpEF (795nM) and healthy (580nM) subjects. This is despite the present study utilising a higher dose than the majority of previous clinical trials, (1, 5, 7, 10, 17, 18, 30). This suggests a potential poor conversion of nitrate to nitrite in HFrEF. The oral microbiome has been shown to play a crucial role in the conversion of plasma nitrate to nitrite, and previous studies have shown that even a single dose of mouthwash can entirely inhibit the conversion process due to its effect at neutralizing required nitrate reducing bacteria. While most supplementation studies, including the present one, now restrict mouthwash use, it is possible that the micriobiome of individuals with HFrEF is distinct and that there is an innate disruption in the reduction pathway. Future studies should consider exploring the reduction pathway in HFrEF and HFpEF.

For the main outcomes of the study, there were no differences between peak or submaximal aerobic capacities between treatments. These findings are in agreement with a previous study in HFrEF which reported no improvement in exercise capacity following a smaller (12.9 mmol) chronic dose of inorganic nitrate (10). The present study also showed no differences in gas exchange threshold or VO_2 during recovery. There have been two previous positive findings for aerobic capacity in the HFrEF patient cohort, however, they employed varying cutoffs for EF% (including patients with EF >40% in their samples) and one utilized a recumbent cycle modality which may have increased venous return to the right atrium and influenced central hemodynamics (4, 17). When these factors are controlled for, it appears supplementation has no effect on aerobic exercise capacity in HFrEF.

One of the most reported benefits of nitrate supplementation is a reduction in SBP (14, 32, 37). While previous studies in HFpEF have consistently demonstrated decreases in peripheral BP following supplementation, the data in HFrEF suggest no beneficial effect to blood

pressure. To our knowledge, this was the first study to assess vascular function parameters in HFrEF following nitrate supplementation under controlled conditions including having participants arrived fasted from food, caffeine and medications. We reported no differences in peripheral or central measures of BP nor vascular stiffness between nitrate and placebo interventions. Our results corroborate and expand on the findings of previous smaller trials in HFrEF showing no effect on BP.

No previous studies which have utilised nitrate supplementation with patients with CHF have examined the effects FMD (7, 11, 39, 40). In the present study, the peak percent change in brachial diameter from baseline following nitrate supplementation was 5.7% compared to 4.2% following placebo. This response is similar to another nitrate supplementation study in patients with hypercholesterolemia (nitrate: 6.8%, placebo: 4.9%, p=0.05) (31). FMD is mediated, at least in part, by NO bioavailability and thus it was postulated that supplementation targeting an increase in NO would lead to an increase in FMD response, suggesting improved vascular function (9). While our results suggest that the supplementation could have some beneficial effect on endothelial function, neither of the changes were significant, nor did they translate into improvements in other clinical or functional measures. While improving vascular function remains a critical goal in CHF, improving FMD through nitrate supplementation may not be the best target for improving clinical or functional measures in this population.

While increases in tissue oxygenation have been a demonstrated benefit of nitrate supplementation in patients with peripheral arterial disease and in HFpEF, this has not been seen in HFrEF (10, 34, 40). In the current study, we report no effect of supplementation on tissue oxygenation as measured by NIRS. We also report, for the first time in HFrEF, that

mitochondrial respiration and mitochondrial-related protein expression following supplementation did not change. At the onset of this clinical trial, a previous study in humans had demonstrated that nitrate supplementation could improve mitochondrial efficiency via increasing the capacity for ATP synthesis (19). However, to date these results have yet to be replicated with nitrate or nitrite supplementation in mice nor human models (22, 26). Herein we also confirm no beneficial effect on mitochondrial function. Together these findings suggest that chronic nitrate supplementation alone may not be a sufficient stimulus to elicit increases in muscle tissue oxygenation or respiration in HFrEF. It is possible that nitrate supplementation in HFrEF does not translate to an increase in nitrate/nitrite within the muscle tissues. Researchers have recently demonstrated that in rodents and healthy humans skeletal muscle can act as a reservoir for nitrate that is then reduced following intense exercise (38). This storage mechanism has yet to be demonstrated in the muscle tissue of clinical populations and should be a focus for future studies.

The current study has several potential limitations. While the study is the largest to date in this population, it was still a relatively small sample size. The patient population was also primarily male (n=15). This was not intentional as recruitment was open to both men and women, but the lack of women participants does limit the applicability of the findings. In line with some of previous studies assessing the effects of nitrate supplementation in cohorts of patients with CHF, recruitment in the present study was inclusive of those individuals with diagnosed chronic comorbidities (hypertension, diabetes and COPD). Participants with any comorbidity that was either uncontrolled or that was identified as a primary contributor to reduced exercise capacity or symptomology, however, were excluded. Additionally, dietary logs were not a component of this trial. While participants were asked to maintain their normal dietary habits and were given a list of high nitrate food items to avoid, the diet was

not specifically controlled for beyond these measures. Another limitation of the study is that there was only an assessment for plasma nitrate/nitrite performed during the CPX visit of each interventional arm. We therefore do not know what the nitrate/nitrite values are for individual subjects beyond this visit. While a previous dose response study has indicated that nitrate/nitrite levels are maintained for 15 days with continued supplementation, we did not measure this directly in the current study (37). Finally, the measures for muscle tissue oxygenation were performed in the vastus lateralis whereas the NIRS placement was on the gastrocnemius. The measures being performed in different tissues makes it difficult to draw comparisons, but there were no changes noted in either measure.

In conclusion, increasing NO bioavailability in HFrEF via oral inorganic nitrate supplementation appears to be ineffective at improving aerobic capacity in patients with stable HFrEF. There were also no noted benefits to either vascular function or muscle tissue oxygenation/respiration. These findings are in contrast with the mainly positive effects seen in HFpEF and suggest the potential of a physiological discord between the two HF classifications. This is supported by previous studies suggesting that individuals with HFpEF potentially have higher levels of vascular dysfunction, which may suggest a differentiation in therapeutic target for nitrate/nitrite. Additionally, the relatively poor conversion rate of nitrate to nitrite in HFrEF may be a key limitation in the efficacy of oral inorganic nitrate supplementation treatment approaches. Future studies should characterize the diversity and abundance of the oral microbiome in HFrEF to elucidate approaches that could lead to a potential benefit oral nitrate supplementation.

Acknowledgements:

This work was supported by a National Heart Foundation of Australia Vanguard Grant (Award ID: 101389) and an internal Victoria University Central Grant Research Scheme award. All authors have reported that they have no relationships relevant to the contents of this paper to disclose.

References

1. Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, Dimenna FJ, Wilkerson DP, et al. Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. Journal of applied physiology. 2009;107(4):1144-55.

2. Beaver WL, Wasserman K, Whipp BJ. A new method for detecting anaerobic threshold by gas exchange. J Appl Physiol (1985). 1986;60(6):2020-7.

3. Coats AJ. The "muscle hypothesis" of chronic heart failure. Journal of molecular and cellular cardiology. 1996;28(11):2255-62.

4. Coggan AR, Broadstreet SR, Mahmood K, Mikhalkova D, Madigan M, Bole I, et al. Dietary Nitrate Increases VO2peak and Performance but Does Not Alter Ventilation or Efficiency in Patients With Heart Failure With Reduced Ejection Fraction. J Card Fail. 2018;24(2):65-73.

5. Coggan AR, Leibowitz JL, Spearie CA, Kadkhodayan A, Thomas DP, Ramamurthy S, et al. Acute Dietary Nitrate Intake Improves Muscle Contractile Function in Patients With Heart Failure: A Double-Blind, Placebo-Controlled, Randomized Trial. Circ Heart Fail. 2015;8(5):914-20.

6. Drexler H, Riede U, Munzel T, Konig H, Funke E, Just H. Alterations of skeletal muscle in chronic heart failure. Circulation. 1992;85(5):1751-9.

7. Eggebeen J, Kim-Shapiro DB, Haykowsky M, Morgan TM, Basu S, Brubaker P, et al. One Week of Daily Dosing With Beetroot Juice Improves Submaximal Endurance and Blood Pressure in Older Patients With Heart Failure and Preserved Ejection Fraction. JACC Heart failure. 2016;4(6):428-37.

8. Fischer D, Rossa S, Landmesser U, Spiekermann S, Engberding N, Hornig B, et al. Endothelial dysfunction in patients with chronic heart failure is independently associated with

477 increased incidence of hospitalization, cardiac transplantation, or death. Eur Heart J.
478 2005;26(1):65-9.
479 9. Green DJ, Dawson EA, Groenewoud HM, Jones H, Thijssen DH. Is flow-mediated
480 dilation nitric oxide mediated?: A meta-analysis. Hypertension. 2014;63(2):376-82.
481 10. Hirai, Zelt JT, Jones JH, Castanhas LG, Bentley RF, Earle W, et al. Dietary nitrate
482 supplementation and exercise tolerance in patients with heart failure with reduced ejection
483 fraction. American journal of physiology Regulatory, integrative and comparative
484 physiology. 2017;312(1):R13-r22.
485 11. Hirai DM, Zelt JT, Jones JH, Castanhas LG, Bentley RF, Earle W, et al. Dietary
486 nitrate supplementation and exercise tolerance in patients with heart failure with reduced
487 ejection fraction. Am J Physiol Regul Integr Comp Physiol. 2017;312(1):R13-R22.
488 12. Hwang MH, Yoo JK, Kim HK, Hwang CL, Mackay K, Hemstreet O, et al. Validity
489 and reliability of aortic pulse wave velocity and augmentation index determined by the new
490 cuff-based SphygmoCor Xcel. Journal of human hypertension. 2014;28(8):475-81.
491 13. Jeng C, Yang MH, Chen PL, Ho CH. The influence of exercise tolerance on quality of
492 life among patients with heart failure. Quality of life research : an international journal of
493 quality of life aspects of treatment, care and rehabilitation. 2004;13(5):925-32.
494 14. Kapil V, Khambata RS, Robertson A, Caulfield MJ, Ahluwalia A. Dietary nitrate
495 provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2,
496 double-blind, placebo-controlled study. Hypertension. 2015;65(2):320-7.
497 15. Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, et al.
498 Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived
499 NO. Hypertension. 2010;56(2):274-81.

16. Kenjale AA, Ham KL, Stabler T, Robbins JL, Johnson JL, Vanbruggen M, et al. Dietary nitrate supplementation enhances exercise performance in peripheral arterial disease. Journal of applied physiology. 2011;110(6):1582-91.

17. Kerley CP, O'Neill JO, Reddy Bijjam V, Blaine C, James PE, Cormican L. Dietary nitrate increases exercise tolerance in patients with non-ischemic, dilated cardiomyopathy-a double-blind, randomized, placebo-controlled, crossover trial. J Heart Lung Transplant. 2016;35(7):922-6.

18. Lansley KE, Winyard PG, Bailey SJ, Vanhatalo A, Wilkerson DP, Blackwell JR, et al. Acute Dietary Nitrate Supplementation Improves Cycling Time Trial Performance. Medicine and Science in Sports and Exercise. 2011;43(6):1125-31.

19. Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Ekblom B, Lundberg JO, et al. Dietary inorganic nitrate improves mitochondrial efficiency in humans. Cell Metab. 2011;13(2):149-59.

20. Levinger I, Howlett KF, Peake J, Garnham A, Hare DL, Jerums G, et al. Akt, AS160, metabolic risk factors and aerobic fitness in middle-aged women. Exercise immunology review. 2010;16:98-104.

21. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nature reviews Drug discovery. 2008;7(2):156-67.

22. Monaco CMF, Miotto PM, Huber JS, van Loon LJC, Simpson JA, Holloway GP. Sodium nitrate supplementation alters mitochondrial H2O2 emission but does not improve mitochondrial oxidative metabolism in the heart of healthy rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2018;315(2):R191-R204.

23. Murphy RM, Lamb GD. Important considerations for protein analyses using antibody based techniques: down-sizing Western blotting up-sizes outcomes. The Journal of physiology. 2013;591(23):5823-31.

24. Nakamura M, Ishikawa M, Funakoshi T, Hashimoto K, Chiba M, Hiramori K. Attenuated endothelium-dependent peripheral vasodilation and clinical characteristics in patients with chronic heart failure. American heart journal. 1994;128(6):1164-9.

25. Nisoli E, Falcone S, Tonello C, Cozzi V, Palomba L, Fiorani M, et al. Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. Proc Natl Acad Sci U S A. 2004;101(47):16507-12.

26. Ntessalen M, Procter NEK, Schwarz K, Loudon BL, Minnion M, Fernandez BO, et al. Inorganic nitrate and nitrite supplementation fails to improve skeletal muscle mitochondrial efficiency in mice and humans. The American journal of clinical nutrition. 2020;111(1):79-89.

27. Pesta D, Gnaiger E. High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. Methods in molecular biology (Clifton, NJ). 2012;810:25-58.

28. Pinder AG, Rogers SC, Khalatbari A, Ingram TE, James PE. The measurement of nitric oxide and its metabolites in biological samples by ozone-based chemiluminescence. Methods in molecular biology (Clifton, NJ). 2008;476:11-28.

29. Swank AM, Horton J, Fleg JL, Fonarow GC, Keteyian S, Goldberg L, et al. Modest increase in peak VO2 is related to better clinical outcomes in chronic heart failure patients: results from heart failure and a controlled trial to investigate outcomes of exercise training. Circ Heart Fail. 2012;5(5):579-85.

30. Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Pavey TG, Wilkerson DP, et al. Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. Am J Physiol Regul Integr Comp Physiol. 2010;299(4):R1121-31.

549 31. Velmurugan S, Gan JM, Rathod KS, Khambata RS, Ghosh SM, Hartley A, et al.
550 Dietary nitrate improves vascular function in patients with hypercholesterolemia: a
551 randomized, double-blind, placebo-controlled study. The American journal of clinical
552 nutrition. 2016;103(1):25-38.
553 32. Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, et al. Acute
554 blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via
555 bioconversion to nitrite. Hypertension. 2008;51(3):784-90.
556 33. Woessner M, Smoliga JM, Tarzia B, Stabler T, Van Bruggen M, Allen JD. A stepwise
557 reduction in plasma and salivary nitrite with increasing strengths of mouthwash following a
558 dietary nitrate load. Nitric Oxide. 2016;54:1-7.
559 34. Woessner M, VanBruggen MD, Pieper CF, Sloane R, Kraus WE, Gow AJ, et al. Beet
560 the Best? Circ Res. 2018;123(6):654-9.
561 35. Woessner MN, Levinger I, Neil C, Smith C, Allen JD. Effects of Dietary Inorganic
562 Nitrate Supplementation on Exercise Performance in Patients With Heart Failure: Protocol
563 for a Randomized, Placebo-Controlled, Cross-Over Trial. JMIR Res Protoc. 2018;7(4):e86.
564 36. Woessner MN, McIlvenna LC, Ortiz de Zevallos J, Neil CJ, Allen JD. Dietary nitrate
565 supplementation in cardiovascular health: an ergogenic aid or exercise therapeutic? Am J
566 Physiol Heart Circ Physiol. 2018;314(2):H195-H212.
567 37. Wylie LJ, Kelly J, Bailey SJ, Blackwell JR, Skiba PF, Winyard PG, et al. Beetroot
568 juice and exercise: pharmacodynamic and dose-response relationships. Journal of applied
569 physiology. 2013;115(3):325-36.
570 38. Wylie LJ, Park JW, Vanhatalo A, Kadach S, Black MI, Stoyanov Z, et al. Human
571 skeletal muscle nitrate store: influence of dietary nitrate supplementation and exercise. J
572 Physiol. 2019;597(23):5565-76.

39. Zamani P, French B, Brandimarto JA, Doulias PT, Javaheri A, Chirinos JA, et al. Effect of Heart Failure With Preserved Ejection Fraction on Nitric Oxide Metabolites. Am J Cardiol. 2016;118(12):1855-60.

40. Zamani P, Rawat D, Shiva-Kumar P, Geraci S, Bhuva R, Konda P, et al. Effect of inorganic nitrate on exercise capacity in heart failure with preserved ejection fraction. Circulation. 2015;131(4):371-80; discussion 80.

41. Zamani P, Tan V, Soto-Calderon H, Beraun M, Brandimarto JA, Trieu L, et al. Pharmacokinetics and Pharmacodynamics of Inorganic Nitrate in Heart Failure With Preserved Ejection Fraction. Circ Res. 2017;120(7):1151-61.

Figure Legends

Figure 1 Study design

Adapted from Woessner et al. (35) (https://www.researchprotocols.org/2018/4/e86/) under the terms of Creative Commons Attribution License 4.0. Copyright © Mary N. Woessner, Itamar Levinger, Christopher Neil, Cassandra Smith, Jason D Allen

Figure 2 Participant flow diagram

Abbreviations: EF, ejection fraction, GFR, glomerular filtration rate.

Figure 3 The effect of nitrate supplementation on circulating plasma nitrate and nitrite

Mean plasma nitrate (**A**) and plasma nitrite (**C**) following inorganic nitrate (16 mmol/ day for five days and one acute dose 2.5 hours prior of 6.4 mmol) supplementation. Individual subject responses for nitrate (**B**) and nitrite (**D**). One participant's nitrite data were excluded (n=15, 14 men and 1 woman) due to abnormal levels (4SD above the mean). * indicates $p<0.05$ level, ** indicates $p<0.001$.

Figure 4 The effect of nitrate supplementation on VO_{2peak} and TTE

VO_{2peak} (**A**) and TTE (**B**) during the CPX were not significantly different between the two interventions. Data reported as mean ± standard error of the mean (SEM). Data are displayed for n=16 (15 men and 1 woman). Abbreviations: TTE, time to exhaustion, VO_{2peak}, peak aerobic capacity. No significant differences were noted (all $p>0.05$).

Figure 5 The effect of nitrate supplementation on oxygenated and deoxyengenated haemoglobin

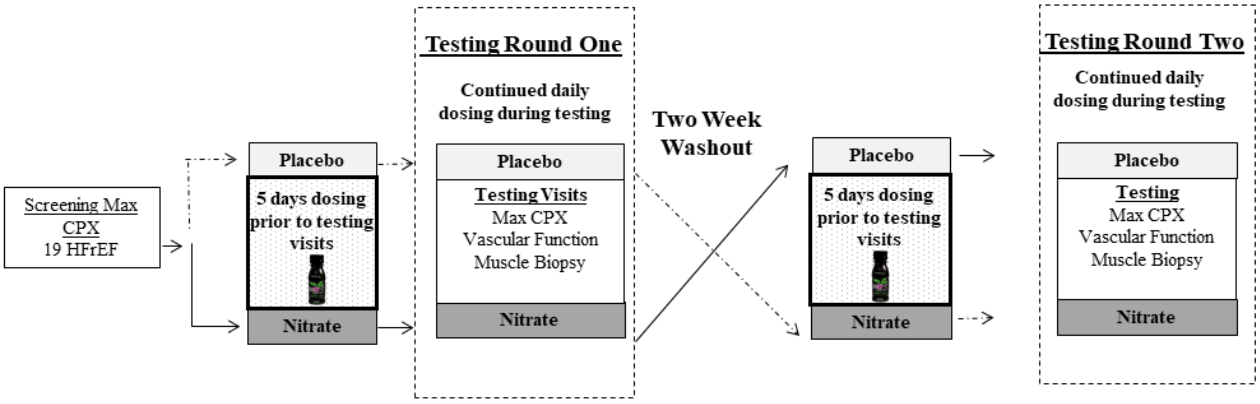
This figure shows group mean differences for HHb (A) and HbO_2 (B) values measured from the NIRS device. The data from the two interventions were matched at specific time points and demonstrate no significant differences between any measured time points for either variable. The zero point on the x-axis is the start of exercise and the vertical dotted line represents the transition between the steady state (first 6 minutes) and the incremental steps of the maximal CPX. To control for the alterations in arterial/venous capacitance during transition from rest to exercise, each NIRS output was individually examined. As the units in NIRS are arbitrary, each participant's baseline value was adjusted to zero point by visually identifying the muscle pump action after onset of exercise and selecting the first point after. This value in AU was then zeroed out and every subsequent point was adjusted by this baseline value. Data are displayed for n=12 men. Four participants were excluded from final NIRS analysis due to poor signal quality. . $p>0.05$ at all timepoints.

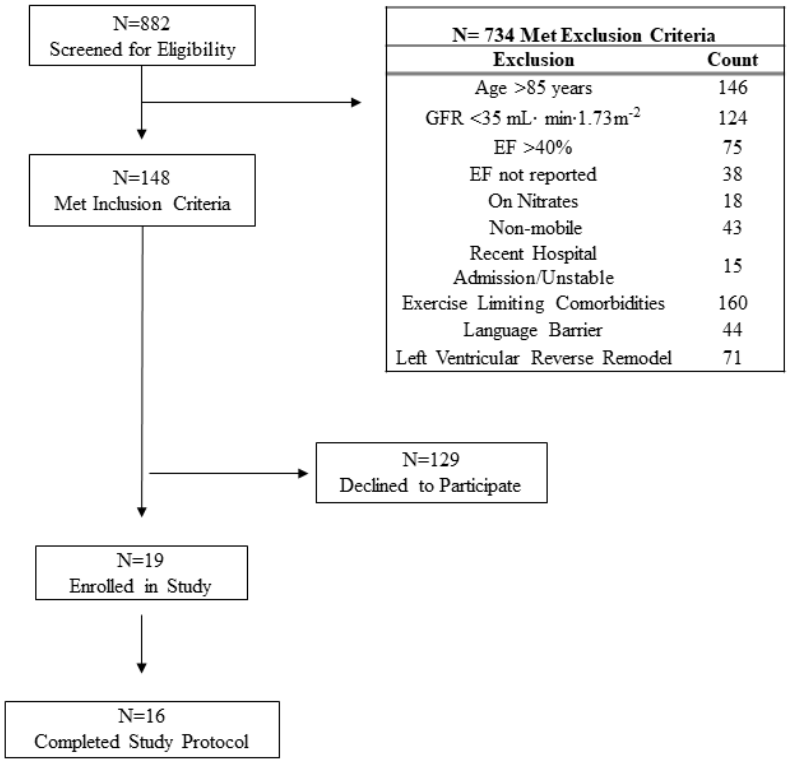
Figure 6 Mass specific and mitochondrial-specific respiratory function for maximal oxidative phosphorylation capacity

Data are displayed as mean ± SEM of complex I and complex II $(CI+CII)_P$ oxidative phosphorylation capacity in both the placebo and nitrate conditions. Data are displayed for n=7 men. $p>0.05$ for all analyses. Abbreviations: mito, mitochondria.

Figure 7 Effect of nitrate supplementation on mitochondrial protein concentration

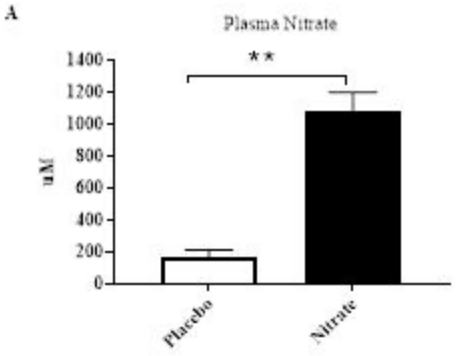
Relative protein concentrations of total and phosphorylated mTORC1, p38MAPK and Akt and calculated phosphorylated to total ratios. Data is displayed as mean ± SEM and individual values for all proteins. Data are displayed for n=7 men. $p>0.05$ for all analyses. Abbreviations: Akt, protein kinase, MAPK, mitogen-activated protein kinase, mTORC1, mechanistic target of rapamycin complex 1, p, phosphorylated.

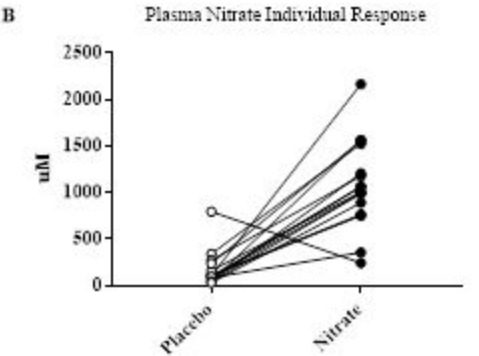

Screening Max CPX
19 HFrEF
Placebo
5 days dosing prior to testing visits
Nitrate
Testing Round One
Continued daily dosing during testing
Placebo
Testing Visits
Max CPX
Vascular Function
Muscle Biopsy
Nitrate
Two Week Washout
Placebo
5 days dosing prior to testing visits
Nitrate
Testing Round Two
Continued daily dosing during testing
Placebo
Testing
Max CPX
Vascular Function
Muscle Biopsy
Nitrate

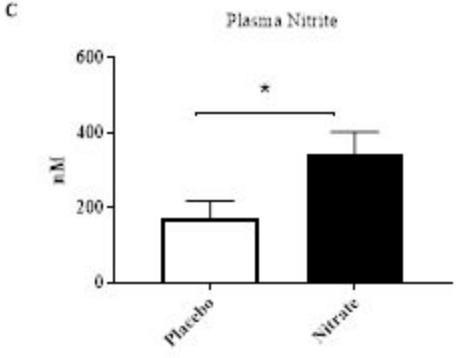


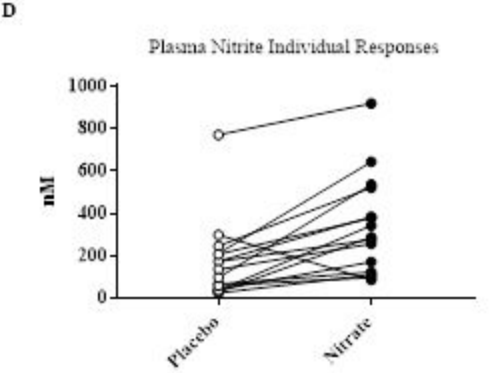
N= 734 Met Exclusion Criteria

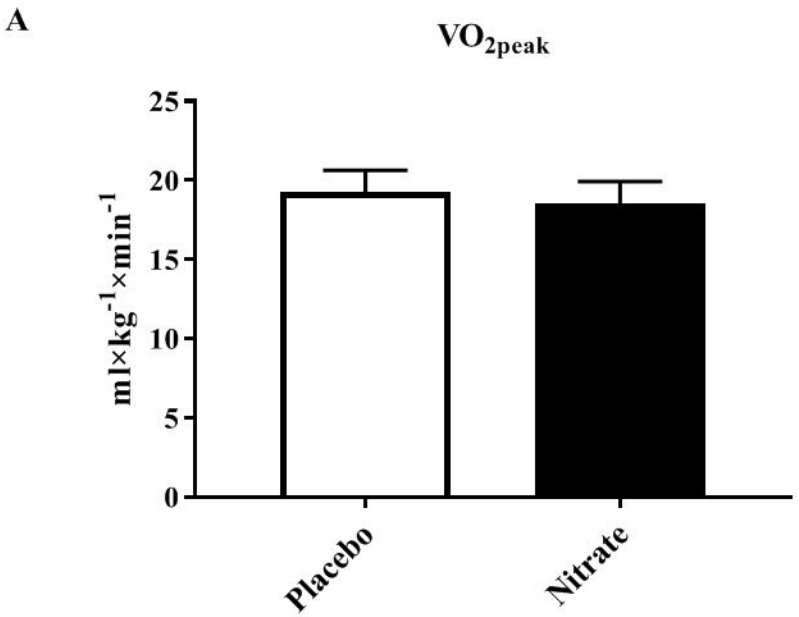
Exclusion	Count
Age >85 years	146
GFR <35 mL· min·1.73m^{-2}	124
EF >40%	75
EF not reported	38
On Nitrates	18
Non-mobile	43
Recent Hospital Admission/Unstable	15
Exercise Limiting Comorbidities	160
Language Barrier	44
Left Ventricular Reverse Remodel	71

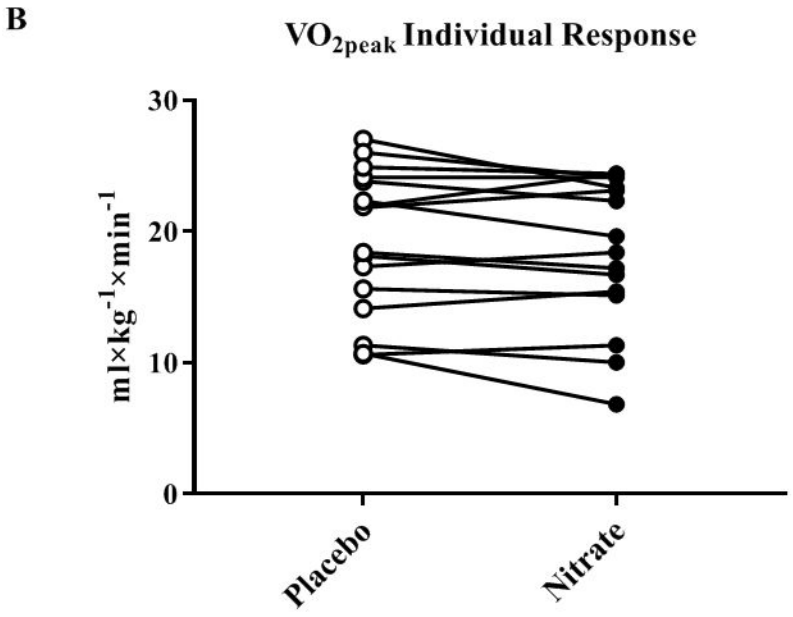

A
Plasma Nitrate
**
uM
1400
1200
1000
800
600
400
200
0
Placebo
Nitrate

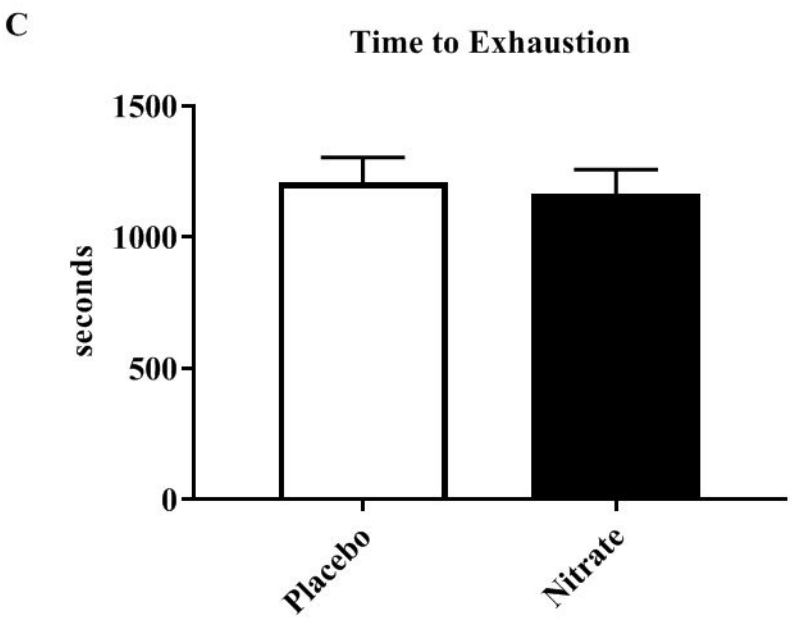

B
Plasma Nitrate Individual Response
uM
2500
2000
1500
1000
500
0
Placebo
Nitrate

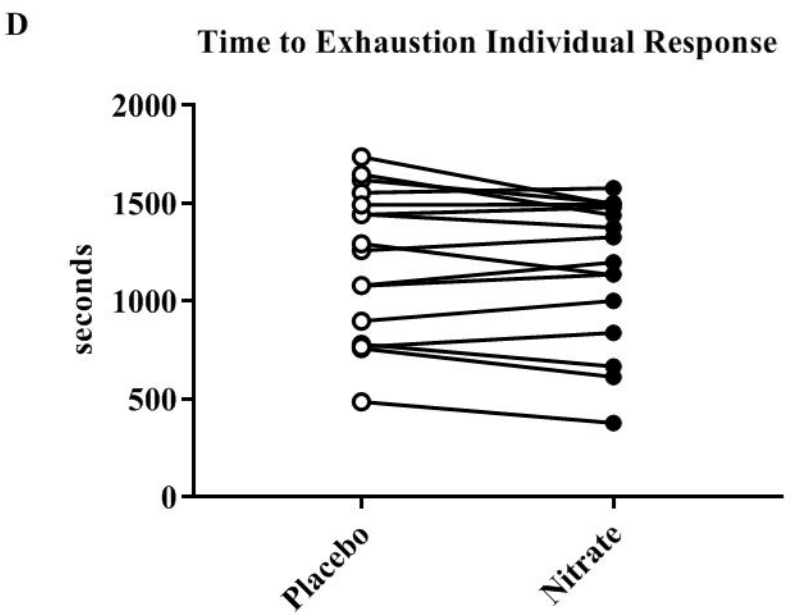

C
Plasma Nitrite
*
nM
600
400
200
0
Placebo
Nitrate

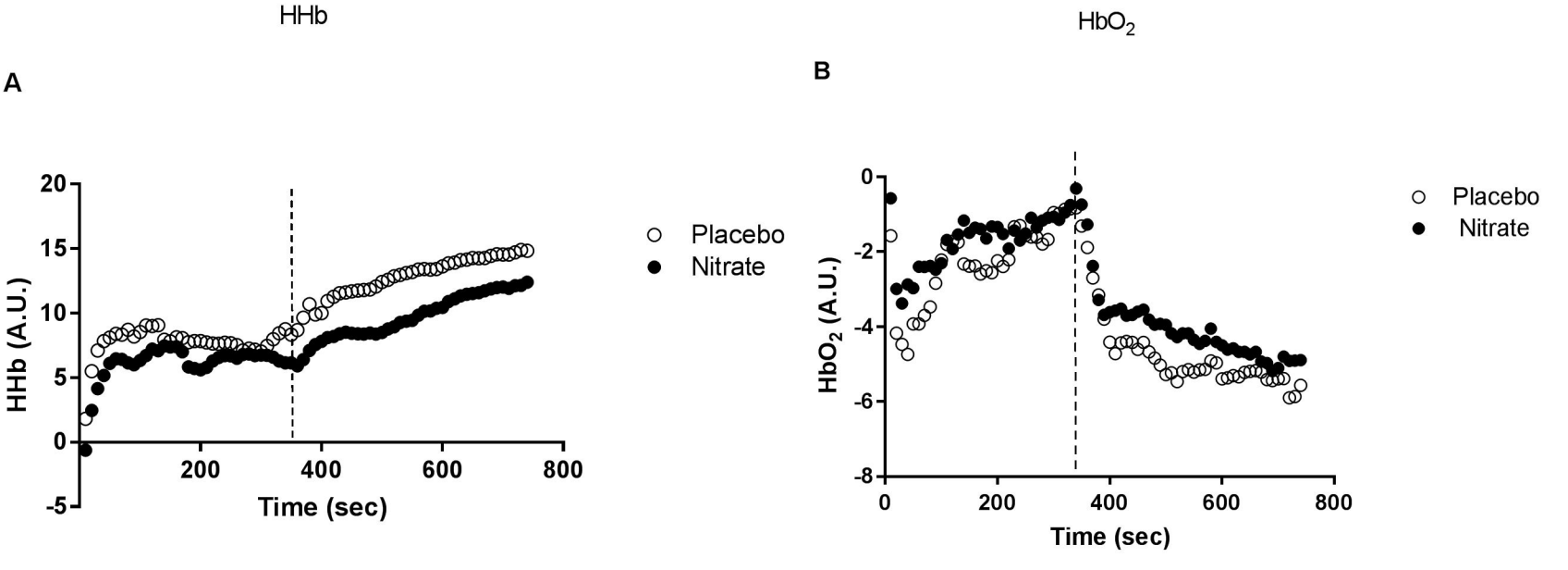

D
Plasma Nitrite Individual Responses
nM
1000
800
600
400
200
0
Placebo
Nitrate

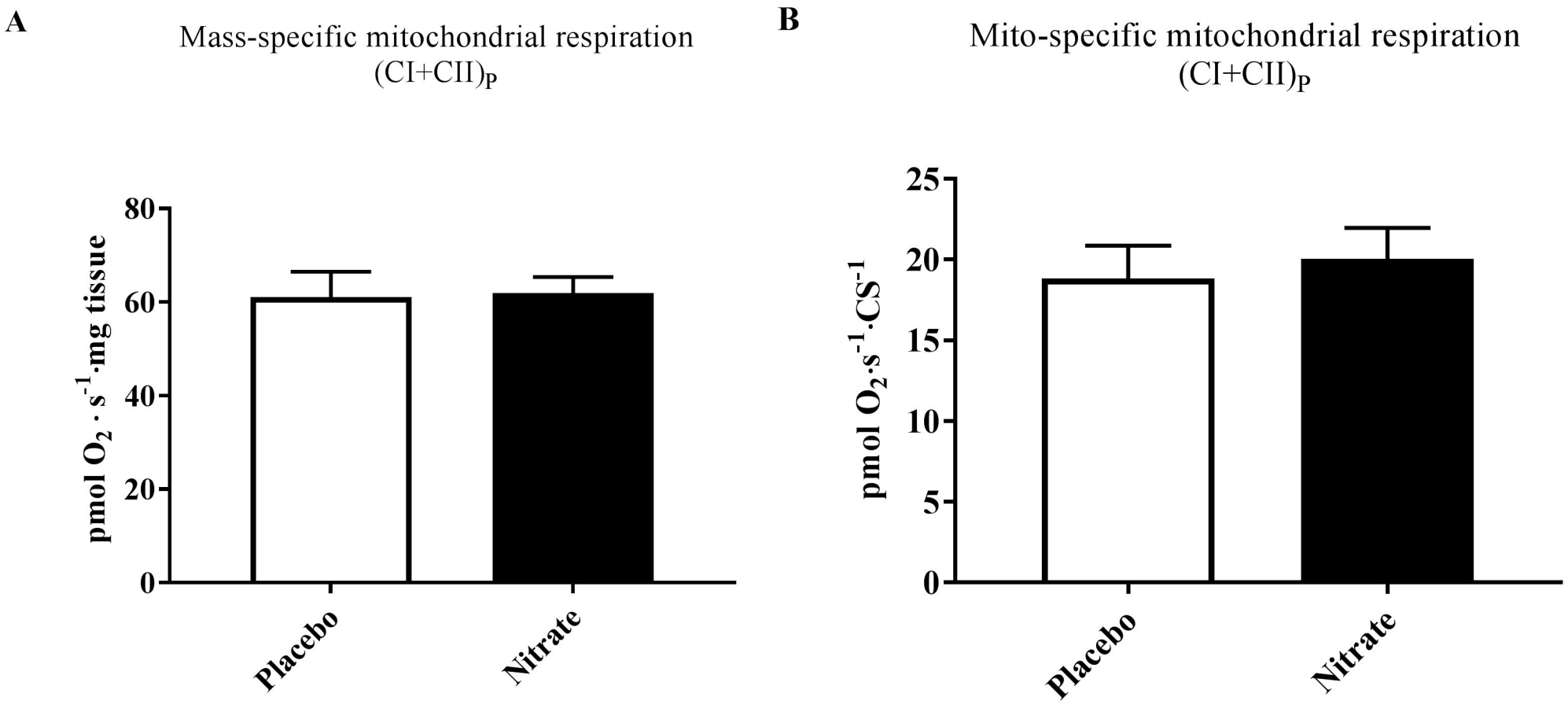

A
VO_{2peak}
25
20
15
10
5
0
ml×kg-1×min-1
Placebo
Nitrate


B
VO_{2peak} Individual Response
30
20
10
0
ml×kg-1×min-1
Placebo
Nitrate


C
Time to Exhaustion
1500
1000
500
0
seconds
Placebo
Nitrate


D
Time to Exhaustion Individual Response
2000
1500
1000
500
0
seconds
Placebo
Nitrate


HHb
A
20
15
10
5
0
-5
HHb (A.U.)
200
400
600
800
Time (sec)
Placebo
Nitrate
HbO2
B
0
-2
-4
-6
-8
HbO2 (A.U.)
0
200
400
600
800
Time (sec)
Placebo
Nitrate


A
Mass-specific mitochondrial respiration
(CI+CII)P
pmol O2 · s-1·mg tissue
80
60
40
20
0
Placebo
Nitrate
B
Mito-specific mitochondrial respiration
(CI+CII)P
pmol O2·s-1·CS-1
25
20
15
10
5
0
Placebo
Nitrate

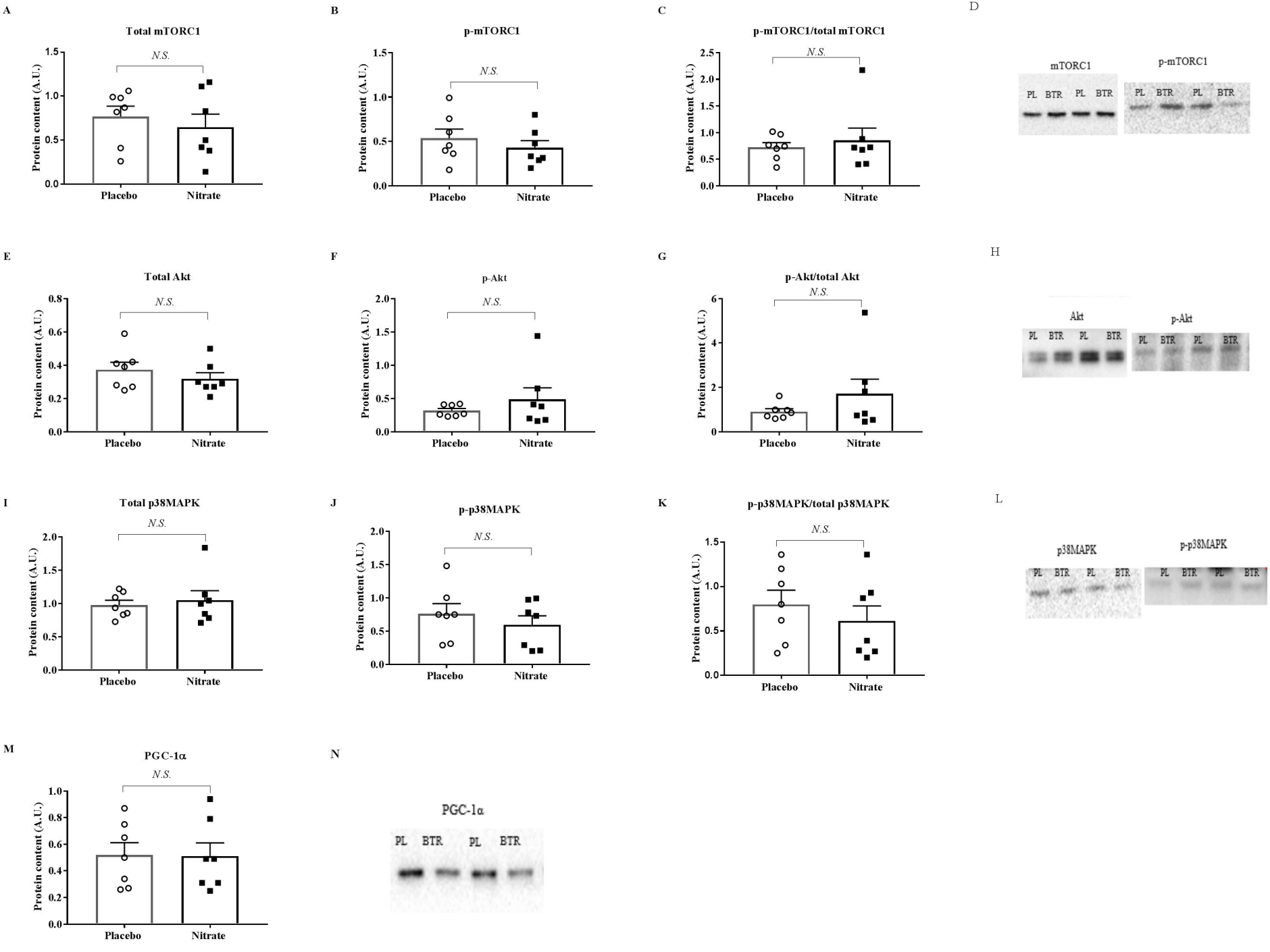

A
Total mTORC1
N.S.
Protein content (A.U.)
Placebo
Nitrate
B
p-mTORC1
C
p-mTORC1/total mTORC1
D
mTORC1
p-mTORC1
PL BTR PL BTR
E
Total Akt
F
p-Akt
G
p-Akt/total Akt
H
Akt
p-Akt
I
Total p38MAPK
J
p-p38MAPK
K
p-p38MAPK/total p38MAPK
L
p38MAPK
p-p38MAPK
M
PGC-1α
N
PGC-1α

Table 1 Participant Demographics

Variable	Value
Age, mean ± SEM, y	62.6 ± 3.6
Height, mean ± SEM, cm	167.9 ± 3.9
Mass, mean ± SEM, kg	87.7 ± 4.0
BMI ± SEM $kg \cdot m^{-2}$	31.8 ± 2.1
Male, n (%)	15 (93.75)
EF ± SEM , %	30.4 ± 1.8
Aetiology, n (%)	
Ischaemic	9 (56.25)
Non-Ischaemic Dilated Cardiomyopathy	6 (37.5)
Idiopathic Heart Disease	1 (6.25)
New York Heart Association Class, n (%)	
Class I	3 (18.75)
Class II	10 (62.5)
Class III	3 (18.75)
Weber Class Distribution, n (%)	
Class A (VO_2>20$ml \cdot kg^{-1} \cdot min^{-1}$)	5 (31.25)
Class B (VO_2 16-20 $ml \cdot kg^{-1} \cdot min^{-1}$)	6 (37.5)
Class C (VO_2 10-15.9 $ml \cdot kg^{-1} \cdot min^{-1}$)	4 (25)
Class D (VO_2 <10 $ml \cdot kg^{-1} \cdot min^{-1}$)	1 (6.25)
Comorbidities, n (%)	
Diabetic	6 (37.5)
COPD	2 (12.5)
HTN	7 (43.75)
Current Smoker	3 (18.75)
Obese	9 (56.25)
Drug therapy, n (%)	
Metformin	4 (25)
β-Blockers	15 (93.75)
ACE Inhibitor/ARBs	11 (68.75)
Statin	7 (43.75)
Aspirin	9 (56.25)
Diuretics	12 (75)

Abbreviations: BMI- body mass index, COPD- chronic obstructive pulmonary disease, EF- ejection fraction, HTN- hypertension, SEM- standard error of the mean.

Table 2 Effects of nitrate supplementation on aortic pressure and stiffness

Measurement	Placebo	Nitrate	Significance
AorSBP (mmHg)	122 ± 4	121 ± 4	0.64
AorDBP (mmHg)	82 ± 3	80 ± 3	0.51
AorMAP (mmHg)	96 ± 4	95 ± 3	0.71
AorPP (mmHg)	40 ± 2	40 ± 2	0.77
AorAP (mmHg)	15 ± 2	14 ± 2	0.74
AorAIX (%)	32 ± 3	35 ± 3	0.3

Abbreviations: Aor, aortic, SBP, systolic blood pressure, DBP, diastolic blood pressure, MAP, mean arterial blood pressure, PP, pulse pressure, AP, augmentation pressure, AIX, augmentation index.