The effect of dietary nitrate supplementation on the oxygen cost of cycling, walking performance and resting blood pressure in individuals with chronic obstructive pulmonary disease: A double blind placebo controlled, randomised control trial.

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Key Words: COPD
Exercise
Nitrate
Nitric oxide
Blood Pressure
Diet

Word count:
Abstract 250
Manuscript: 3803

Abstract

Background

Chronic obstructive pulmonary disease (COPD) results in exercise intolerance. Dietary nitrate supplementation has been shown to lower blood pressure (BP), reduce the oxygen cost of exercise, and enhance exercise tolerance in healthy volunteers. This study assessed the effects of dietary nitrate on the oxygen cost of cycling, walking performance and BP in individuals with mild-moderate COPD.

Methods

Thirteen patients with mild-moderate COPD were recruited. Participants consumed 70 ml of either nitrate-rich (6.77 mmol nitrate; beetroot juice) or nitrate-depleted beetroot juice (0.002 mmol nitrate; placebo) twice a day for 2.5 days, with the final supplement ~3 hours before testing. BP was measured before completing two bouts of moderate-intensity cycling, where pulmonary gas exchange was measured throughout. The six-minute walk test (6MWT) was completed 30 minutes subsequent to the second cycling bout.

Results

Plasma nitrate concentration was significantly elevated following beetroot juice vs. placebo (placebo; 48 ± 86 *vs.* beetroot juice; 215 ± 84 μM, P=0.002). No significant differences were observed between placebo vs. beetroot juice for oxygen cost of exercise (933 ± 323 *vs.* 939 ± 302 ml: min^{-1}; P=0.88), distance covered in the 6MWT (456 ± 86 *vs.* 449 ± 79 m; P=0.37), systolic BP (123 ± 14 *vs.* 123 ± 14 mmHg; P=0.91), or diastolic BP (77 ± 9 *vs.* 79 ± 9 mmHg; P=0.27).

Conclusion

Despite a large rise in plasma nitrate concentration, two days of nitrate supplementation did not reduce the oxygen cost of moderate intensity cycling, increase distance covered in the 6MWT, or lower BP.

INTRODUCTION

Exercise in individuals with COPD is limited by multiple factors which can result in hypoxemia. These include loss of normal lung architecture, impaired cardiac function [1], abnormal pulmonary blood flow distribution [2] and peripheral muscle de-conditioning [3]. Oxygen uptake in the lungs and delivery of oxygen to working muscle is impaired by increases in pulmonary blood flow which increase shunting through blood vessels resulting in incomplete gas exchange [4] and cor pulmonale later in the disease course. These abnormalities result in feelings of breathlessness and fatigue [5], with individuals often finding that activities of daily living are physically challenging.

The beneficial effects of a diet rich in vegetables upon cardiovascular health [6], risk of morbidity and mortality [7], and COPD development [8; 9] have been well described. These positive effects have, in part, been attributed to inorganic nitrate which is found in particularly high quantities in leafy green vegetables and some root vegetables such as beetroot [10]. Nitrate supplementation in the form of sodium nitrate or nitrate-rich beetroot juice has been shown to have remarkable effects in healthy young individuals and athletes, including reductions in the oxygen cost of exercise [11], enhanced exercise tolerance/performance and reduced blood pressure (BP) [11; 12]. Some of these effects have subsequently been observed in individuals with peripheral artery disease following dietary nitrate supplementation [13]. These findings have been attributed to an increase in the bioavailability of nitric oxide (NO).

NO is a signalling molecule with multiple functions including regulation of vascular tone, mitochondrial respiration and skeletal muscle function [14; 15; 16]. These factors are important in the physiological response to exercise. NO is produced in two distinct ways in man. The best known is the classical L-arginine nitric oxide synthase (NOS) pathway which is oxygen dependent [17]. The second is the entero-salivary pathway and is oxygen

independent. Briefly, nitrate from the diet is rapidly and extensively absorbed in the stomach and proximal small intestine with bioavailability approaching 100% [18]. Nitrate is then concentrated in the salivary glands, with concentrations 10 fold greater in saliva than in plasma. Nitrate secreted in saliva is reduced to nitrite by facultative anaerobic bacteria on the dorsum of the tongue [19]. On swallowing, the acidic environment of the stomach results in NO formation with important local effects on gastric function and host defence [6; 20]. Some nitrite is absorbed into the circulation where it acts as a storage pool for subsequent NO production [14]. The conversion of nitrite to NO is expedited in conditions of acidosis [21] or hypoxemia [14] which often occur in the exercising muscle of individuals with COPD [22].

In many individuals with COPD, functional capacity is reduced to a level where activities of daily living may impose a challenge due to an energy requirement representing a high fraction of their maximal oxygen uptake. While a number of cardiovascular and physiological benefits have been shown as a result of dietary nitrate supplementation in healthy populations, little is known about possible effects in clinical populations. We aimed to determine whether dietary nitrate supplementation has a beneficial impact upon the oxygen cost of sub-maximal cycling exercise, walking performance and BP in individuals with COPD.

Purpose

The aim of this study was to assess the effects of 2.5 days of dietary nitrate supplementation on the oxygen cost of sub-maximal cycling, walking performance, and resting BP in individuals with mild-moderate COPD.

METHODS

Patients

Fourteen individuals with mild-moderate COPD (see Table 1 for patient characteristics) gave written informed consent to participate in this double-blind, placebo-

112 controlled, cross-over design study between April 2013 and January 2014. The study was
113 registered as a clinical trial at ClinicalTrials.gov (NCT01712386). The Exeter NRES
114 Committee gave ethical approval (12//SW//0327). Patients were recruited if lung function
115 was between 30-80% of predicted FEV_1 values, aged 40-75 years old and able to give
116 informed consent. Participants were excluded if they had chronic kidney disease (estimated
117 glomerular filtration rate <30 ml/min/1.73 m^2), uncontrolled hypertension (systolic BP> 160
118 mmHg or diastolic >100 mmHg), were smokers (smoked within past 3 months), consumed
119 regular organic nitrate or nicorandil. Patients taking phosphodiesterase inhibitors were asked
120 to refrain from doing so for the duration of the study.

121 **Pre-experimental tests**

122 Participants arrived at the Heart and Lung unit at Torbay hospital where informed
123 consent, medical history, anthropometric measures, BP, lung function and an ECG were
124 performed. Participants completed a ramp incremental cycle ergometer test (10 $W \cdot min^{-1}$) to
125 determine their gas exchange threshold (GET). Breath-by-breath pulmonary gas exchange
126 was measured throughout and the GET was determined using the V-slope method as
127 described previously [23].

128 **Experimental Overview**

129 Participants consumed 70 ml of nitrate-rich beetroot juice (beetroot juice; 6.77 mmol
130 nitrate; Beet it, James White Drinks Ltd., Ashbocking, UK) or nitrate- depleted beetroot juice
131 as a placebo (placebo; 0.002 mmol nitrate; Beet it, James White Drinks Ltd., Ashbocking,
132 UK), with one beverage in the morning and one in the evening for two days preceding
133 testing. On study days, participants consumed a final 70 ml beetroot juice drink ~3 hours
134 prior to exercising. Participants self-reported concordance with the supplementation regime
135 which was confirmed by measurement of plasma nitrate concentration. After exercise testing
136 the participants began a washout period (7 days) before entering the opposing arm of the

study. The placebo was indistinguishable from the nitrate-rich juice in taste, colour, texture, appearance and odour as described previously [24].

Participants arrived at the laboratory in a fully hydrated state, having avoided consumption of caffeine, alcohol, cruciferous vegetables, leafy greens, beetroot, and completion of strenuous exercise 24 hours prior to testing. Participants were asked to record their food intake for 24 hours prior to testing and to replicate this after the crossover and this was verbally confirmed on the second exercise visit. Participants avoided antibacterial mouthwash for 7 days prior to testing. Participants arrived 45 minutes before the initiation of exercise following ingestion of the randomised juice with their morning meal. Brachial artery BP was taken, after a 10 minute resting period whilst supine, with an automated sphygmomanometer (Omron M6, Kyoto, Japan). Five measurements were performed and the mean of the last three was recorded. Venous blood was drawn and processed for plasma nitrate concentration as per our previously described chemiluminescence technique [25]. Participants completed two bouts of cycling at 80% of their GET on a cycle ergometer (Ergoselect 100, Bitz, Germany) with 30 minutes recovery between bouts. Following 30 minutes rest, participants performed a six-minute walk test to assess functional capacity. Participants walked around a clear rectangular corridor (14 x 12m) for a total of 52m per lap, covering as much distance as possible. Standardised verbal encouragement was given throughout.

Measurements

Pulmonary gas exchange and ventilation were measured during the cycling exercise (Vmax^{TM} Encore, Yorba, Linda, CA). Before each session the analysers were calibrated using gases of known concentration. The volume transducer was calibrated using a 3-litre syringe (Hans Rudolph, Kansas City, MO, USA).

Outcome measures

Primary outcome measure: does 2.5 days of nitrate supplementation reduce the oxygen cost of moderate intensity cycling? Secondary outcome measure (i): does 2.5 days of nitrate supplementation improve functional capacity as measured via the six-minute walk test? Secondary outcome measure (ii): does 2.5 days of nitrate supplementation reduce resting BP?

Sample size and randomisation

An *a priori* sample size calculation was performed. Previous literature in healthy young volunteers has shown a mean change between beetroot juice and control of 69 ml for end exercise pulmonary oxygen uptake ($\dot{V}O_2$) (1SD) and 121 ml for $\dot{V}O_2$ amplitude (2SD) [11]. For 90% power and an α-level set at P=0.05 (two tailed), to detect a 1 SD difference 13 patients were required. The reproducibility of these measures in patients with COPD are similar to healthy controls [26]. An unrestricted computer generated sequence was used by a research nurse to assign each participant a randomisation number and supply them with the requisite juice.

Data and statistical analysis

Participant's breath by breath $\dot{V}O_2$ data were initially checked for erroneous breaths (caused by coughing and swallowing). Breaths > 4 SDs away from the local mean were removed prior to interpolation. Breath by breath data for each cycling bout were time aligned and interpolated to provide second by second values. A nonlinear least squares algorithm was then used to fit the ensemble-averaged data. The overall $\dot{V}O_2$ kinetics were described using the mean response time (MRT), which was calculated by fitting a single exponential curve to the data with no time delay from the onset to the end of exercise. The oxygen deficit was calculated as the product of the $\dot{V}O_2$ response amplitude (i.e. the baseline to the point that a steady state was attained) and the MRT. For a schematic representation of the kinetics parameters, please see figure 1.

All data were tested for normality. Statistical differences were assessed using paired t-tests for normally distributed data and Wilcoxon rank-sum test for non-normally distributed data. All data are presented as means ± standard deviation (*SD*). Statistical analysis was performed on SPSS software version 21.0 (Chicago, IL, USA). Statistical difference was accepted when $P < 0.05$.

Results

14 individuals with COPD provided written informed consent. Following screening, one individual was withdrawn due to $FEV_1 < 30\%$. 13 participants were randomised to start in either the beetroot juice or placebo condition of the study. All participants reported 100% adherence to the supplementation regime. Participants reported similar dietary patterns and physical activity during both study arms. Dietary nitrate supplementation was well tolerated with no adverse events apart from red stools and beeturia, as in previous studies [11].

Plasma nitrate concentration: Relative to placebo, beetroot juice significantly increased plasma nitrate concentration (48 ± 85 vs. 215 ± 84μM, $P = 0.002$, 95% CI 75, 260; Figure 2).

Effects on the oxygen cost of cycling exercise: The group mean pulmonary $\dot{V}O_2$ response to exercise for both placebo and beetroot juice conditions can be seen in figure 3, with the $\dot{V}O_2$ kinetics resulting from the model fits displayed in Table 2. Relative to placebo, beetroot juice supplementation had no effect on baseline $\dot{V}O_2$ (634 ± 233 *vs*. 622 ± 253 $ml \cdot min^{-1}$, $P = 0.56$, 95% CI -57, 32) or end exercise $\dot{V}O_2$ (933 ± 323 *vs*. 939 ± 302 $ml \cdot min^{-1}$, $P = 0.88$, 95% CI -68, 78). There were no differences between conditions for the MRT ($P = 0.90$, CI -25, 28) or the oxygen deficit ($P = 0.71$, CI -.2, .3) (Table 2).

Effects on functional capacity: There was no difference between conditions for distance covered during the six-minute walk test (456 ± 86 vs. 449 ± 79 m, $P = 0.17$, 95% CI -22, 9).

Effects on resting blood pressure: Compared to the placebo juice, beetroot juice did not significantly reduce systolic BP (123 ± 14 *vs.* 123 ± 14 mmHg, $P = 0.91$, 95% CI -5, 4) or diastolic BP (78 ± 9 *vs.* 79 ± 9 mmHg, $P = 0.25$, 95% CI -2, 5; Figure 4).

Discussion

Beetroot juice supplementation, (nitrate; 6.77 mmol) twice daily, for 2.5 days did not reduce the oxygen cost of cycle ergometer exercise, improve functional capacity or reduce resting BP in individuals with COPD. There was no difference between conditions for these variables despite a statistically significant and physiologically meaningful rise in plasma nitrate concentration following nitrate supplementation. Possible explanations for the lack of effect in this study include nitrate dosage, efficacy of nitrate reduction to nitrite, oxidative stress, and the age of the participants.

Nitrate supplementation and effects on plasma nitrate concentration.

Plasma nitrate concentration was 48μM post placebo and 215μM following nitrate-rich beetroot juice, which is consistent with much of the literature in healthy young individuals [27; 28] and individuals with type 2 diabetes [24]. Similar changes in plasma nitrate concentrations have been shown to elicit reductions in the oxygen cost of exercise, improved exercise tolerance/performance and reductions in BP [29; 30; 31]. Due to logistical constraints, plasma nitrite concentration was not assessed in this study. In all previous studies involving dietary nitrate supplementation where plasma nitrite concentration has been determined, a rise in plasma nitrate concentration similar to the magnitude observed in the present study has been accompanied by a physiologically meaningful and statistically significant rise in plasma nitrite concentration [16; 29; 30]. However, we cannot exclude the possibility that there is an impaired capacity for reduction of nitrate to nitrite in individuals with COPD. Such an impairment could potentially be related to differences between

236 individuals with COPD and healthy individuals in oral microflora due to oral steroids and
237 repeated exposure to courses of antibiotics [32].

238 *Nitrate supplementation and effects on the oxygen cost of cycling exercise.*

239 We found no reduction in the oxygen cost of cycling exercise at baseline or end-
240 exercise in individuals with COPD following nitrate-rich beetroot juice supplementation
241 compared to placebo. Nitrate supplementation in healthy young individuals has previously
242 resulted in reductions in the oxygen cost of exercise [11]. However, we recently reported that
243 the oxygen cost of exercise was not altered by dietary nitrate supplementation in a group of
244 healthy older adults [33]. The current study is the first to examine the effects of nitrate
245 supplementation on the oxygen cost of exercise in any clinical population. The
246 supplementation regime used in this study, consisting of 6.77 mmol twice a day for 2.5 days,
247 has previously been shown to increase plasma nitrite concentrations [11; 34; 35] and elicit
248 reductions in the oxygen cost of exercise [30]. It is therefore unlikely that the dosage and the
249 timing of nitrate supplementation explain why no effect on the oxygen cost of exercise was
250 observed.

251 One possible explanation for the reduction in oxygen cost following dietary nitrate
252 supplementation in other populations is an increase in the P/O ratio (i.e. less oxygen being
253 consumed to produce a given amount of ATP). Larsen et al [16] reported an increase in the
254 P/O ratio of harvested mitochondria following three days nitrate supplementation. However,
255 we did not observe a reduction in the oxygen cost of exercise, which may be related to the
256 impact of oxidative stress, which is reported to damage mitochondrial membranes [36],
257 potentially resulting in a reduction in the P/O ratio. COPD is associated with increased
258 oxidative stress, with reactive oxygen species (ROS) being produced within the inflammatory
259 cells and epithelial cells of the airways in conjunction with increased systemic generation of
260 ROS [37]. Oxidative stress leads to uncoupling of the NO synthase enzymes [38], thus

reducing NO bioavailability and creating a negative feedback loop of diminishing NO production and elevated NO scavenging. This may be a substantial barrier to NO based therapeutics in COPD.

Nitrate supplementation and effects on functional capacity.

No statistical difference in distance covered for the six-minute walk test was observed between conditions. Considering that the oxygen cost of exercise and rate of adaptation of $\dot{V}O_2$ were not altered following nitrate supplementation, it is perhaps not surprising that functional capacity was also not different between conditions. It is likely that these lack of effects share a common explanation, which may be related to the impact of oxidative stress on the bioavailability of NO [38] (see previous section).

The only other studies that have examined the impact of dietary nitrate supplementation on walking performance have reported both positive and neutral effects. Kenjale et al [13] reported an increased walking time to exhaustion (17%) in a cohort of peripheral artery disease patients. However, dietary nitrate supplementation had no effect on the distance covered in a six-minute walk test in healthy older individuals[33]. Since plasma nitrate (and nitrite in [11 33]) concentrations were similar for the present study and two previous studies, the differences in walking performance post-nitrate supplementation are likely related to methodological differences. Kenjale et al [13] assessed walking performance via an incremental test to exhaustion on a treadmill, whereas in the present study and that of Kelly at al [33] walking performance was assessed via completion of a (submaximal) six-minute walk test. It is likely that the higher exercise intensity encountered by the participants in Kenjale et al [13] resulted in the development of a hypoxic and acidic cellular environment that is known to be conducive for the reduction of nitrate to nitrite [21]. Such an environment would have been less likely to occur during the lower exercise intensity in the present and

Kelly et al's study [33]. Finally, Kenjale et al, [13] did not use a placebo that was indistinguishable from their active juice, thus a 'placebo effect' cannot be ruled out.

Nitrate supplementation and effect on resting BP.

There was no difference in systolic or diastolic resting BP following nitrate-rich beetroot juice compared to placebo. This may be related to a factor specific to COPD such as the elevated oxidative stress [37] in this population would be expected to increase the scavenging of NO, thus reducing its effectiveness. Alternatively there are multiple other factors which may modify the BP effect. Studies investigating the effects of dietary nitrate supplementation in older subjects with and without pathology have reported inconsistent BP effects. Gilchrist et al [25] examined the impact of dietary nitrate supplementation in individuals with type 2 diabetes, and found no statistical difference in mean 24h ambulatory BP. In subjects with peripheral artery disease Kenjale et al [13] reported a statistically significant reduction in diastolic BP (7 mmHg) but no change in systolic BP. It is possible that ageing *per se* may attenuate NO mediated BP reduction, however in Kelly et al's [33] study of healthy older adults dietary nitrate supplementation resulted in reductions in systolic and diastolic BP of 5 and 3 mmHg, respectively. In contrast, more recently, a larger study by Bondonno et al [39] used a vegetable based, nitrate rich diet for 7 days. Ten hour ambulatory BP along with home and office based measurements were used to assess BP. They found no reductions in BP or arterial stiffness. There are key differences around the supplementation protocol and timing and method of blood pressure measurement. In Kelly et al's study the office based blood pressure measurement was timed to coincide with the plasma nitrite peak post nitrate ingestion. In the Bondonno et al study measurements took place outside this window.

It is also worth noting the differing BMI's in these studies and our present manuscript. Kelly et al's cohort of older adults are the only group in the normal range (24±3 kg/m^2). Our

present cohort had a mean BMI of 29 ± 8kg/m^2, Bondonno et al's cohort were overweight 27±4 kg/m^2, and in our previous study of subjects with type 2 diabetes the group mean BMI was 30.8±3.2 kg/m^2. This raises the possibility that adiposity may attenuate the response to inorganic nitrate by an as yet unknown mechanism.

One factor which may have had an impact is that subjects in the present study were taking multiple classes of drugs including antihypertensives. It is possible that the scope for reductions in BP subsequent to nitrate supplementation is significantly reduced when individuals are already taking antihypertensive medication. It is noteworthy that in studies where subjects were taking antihypertensives (current study - 38% prescribed antihypertensives; Gilchrist et al [25] - 98% prescribed antihypertensives), no reductions in BP have been reported (see table 1 for drug classifications). Alternatively, the healthy older adults, on no medications, studied by Kelly et al [33] showed a significant reduction in BP following nitrate supplementation. It is possible that antihypertensive agents mitigate the NO mediated reduction in BP.

There is conflicting evidence to suggest that ACEi/ARBs can alter the bioavailability of NOx with some studies showing reduction [10] and others proposing increases [8; 9]. Therefore the direction in which ACEi/ARBs may alter the bioavailability of NO remains unclear. ß2-adrenergic receptor agonists are known to increase endothelial NO production and are at least, in part, responsible for their vasodilatory effects [11]. ß2-agonists are the most common treatment for individuals with COPD and thus we could not reasonably exclude individuals who were prescribed this medication. We cannot exclude the possibility that prescribed medications which modulate NO bioavailability may attenuate a beneficial effect from dietary nitrate supplementation. This study is a crossover design and therefore both treatment arms will be equally affected. Further study is required to better understand the possible interaction of different medications and inorganic nitrate and nitrite.

Berry *et al.* [6] recently examined the effect of nitrate rich beetroot juice vs. prune juice (as a placebo) in individuals with COPD. Plasma nitrite concentration was significantly higher post beetroot juice compared to post prune juice, which indicates that the entero-salivary pathway is operational in people with COPD. The authors reported reductions in resting systolic BP, iso-time (defined as: last 60s of the shortest exercise time during either active or placebo visits compared with the same time point from the longer exercise time) BP, end exercise diastolic BP and an improvement in exercise tolerance (i.e. lengthened time to exhaustion during submaximal constant rate). Whilst the increase in exercise time is of interest, there are significant limitations in this study. Firstly, the design utilises prune juice as the placebo, which is likely to have a substantially different antioxidant content which could alter NO bioavailability [7]. Secondly, and related to the lack of a 'true' placebo where the participant did not know whether active or placebo juice was being taken (as used in the present study), the widely reported (in the national press as well as in scientific literature) beneficial effects of beetroot juice on exercise performance/tolerance may have given rise to a placebo effect in informed volunteers. The authors do not show a reduction in the oxygen cost of exercise which is consistent with the present study. However, with no reduction in the oxygen cost of exercise, it is not immediately clear what mechanism underpins the improved time to exhaustion reported by Berry *et al.* [6].

This is the first double blind, randomised, placebo, controlled, crossover design study to examine the effects of nitrate supplementation on the oxygen cost of exercise, walking performance and BP in individuals with COPD. The study had a robust experimental design (double-blind, placebo-controlled, randomised, cross-over study). A limitation is that we were not able to ascertain whether or not the increase in plasma nitrate concentration lead to an increase in plasma nitrite concentration, as we were not able to measure the latter due to logistical constraints. However, a recent study examining nitrite

levels in individuals with COPD did show elevated plasma nitrite concentrations [44] which suggests the entero-salivary pathway is operational.

Conclusion

In contrast to findings in healthy young individuals, and despite a statistically significant and physiologically meaningful rise in plasma nitrate concentration, 2.5 days of beetroot supplementation with 6.77 mmol of nitrate twice daily did not reduce the oxygen cost of cycling exercise, improve functional capacity or reduce resting blood pressure. Potential explanations for the lack of effect include a reduced P/O ratio due to systemic ROS generation associated with oxidative stress, or a reduced conversion of nitrate to nitrite in this population.

Acknowledgments

This project was funded by Torbay Medical Research Fund and supported by the NIHR Exeter Clinical Research Facility. The views and opinions shown within this paper are those of the authors and do not necessarily represent those of the NIHR, NHS or the DoH. We would also like to thank the research nurses and technicians involved in the study and importantly the volunteers.

Contributorship Statement:

DW, LD, JK, PW, AJ, NB, MG were involved in the conception or design of the work. AIS, DW, LD were involved in the acquisition of data. AIS, DW, AJ, NB, PW, ACS, MG were involved in the analysis or interpretation of data. All authors have been involved in drafting of the work and revision for intellectually important content. MG acts as guarantor**.**

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Figure legends

- Fig. 1. Parameters of oxygen uptake kinetics. See text for further details.
- Fig. 2. Plasma nitrate concentration following placebo and beetroot juice supplementation. The open bar represents placebo and the closed box, beetroot juice. *significantly different from placebo $P < 0.01$.
- Fig. 3. The pulmonary oxygen uptake response during the transition from unloaded cycling to cycling at 80% of the GET for 6 minutes following placebo (A) and beetroot juice supplementation (B). The vertical line denotes the transition from baseline to moderate intensity cycling.
- Fig. 4. Systolic (SBP) and diastolic (DBP) blood pressure following placebo and beetroot juice supplementation. The open bar represents placebo and the closed box, beetroot juice.
- Table 1. Characteristics of the patients included in the final analyses. Data are mean ± SD or as a % of the cohort on a medication.
- Table 2. Pulmonary gas exchange during moderate intensity cycling following placebo and beetroot juice supplementation.