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Body mass-normalized moderate dose of dietary nitrate intake improves endothelial function and walking capacity in patients with peripheral artery disease

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Abstract

Peripheral artery disease (PAD) is characterized by the accumulation of atherosclerotic plaques in the lower extremity conduit arteries, which impairs blood flow and walking capacity. Dietary nitrate has been used to reduce blood pressure (BP) and improve walking capacity in PAD. However, a standardized dose for PAD has not been determined. Therefore, we sought to determine the effects of a body mass-normalized moderate dose of nitrate (0.11 mmol nitrate/kg) as beetroot juice on serum nitrate/nitrite, vascular function, walking capacity, and tissue oxygen utilization capacity in patients with PAD. A total of 11 patients with PAD received either nitrate supplement or placebo in a randomized crossover design. Total serum nitrate/nitrite, resting BP, brachial and popliteal artery endothelial function (flow-mediated dilation, FMD), arterial stiffness (pulse-wave velocity, PWV), augmentation index (Aix), maximal walking distance and time, claudication onset time, and skeletal muscle oxygen utilization were measured pre- and postnitrate and placebo intake. There were significant group x time interactions ($P < 0.05$) for serum nitrate/ nitrite, FMD, BP, walking distance and time, and skeletal muscle oxygen utilization. The nitrate group showed significantly increased serum nitrate/nitrite ($\Delta 1.32 \mu\text{M}$), increased brachial and popliteal FMD ($\Delta 1.3\%$ and $\Delta 1.7\%$, respectively), reduced peripheral and central systolic BP ($\Delta -4.7 \text{ mmHg}$ and $\Delta -8.2 \text{ mmHg}$, respectively), increased maximal walking distance ($\Delta 92.7 \text{ m}$) and time ($\Delta 56.3 \text{ s}$), and reduced deoxygenated hemoglobin during walking. There were no changes in PWV, Aix, or claudication ($P > 0.05$). These results indicate that a body-mass normalized moderate dose of nitrate may be effective and safe for reducing BP,

improving endothelial function, and improving walking capacity in patients with PAD.

beetroot juice; exercise tolerance; nitrite; pulse-wave velocity; vascular function

INTRODUCTION

Peripheral artery disease (PAD) is a vascular disease in which atherosclerotic plaque accumulation in the conduit arteries attenuates blood flow and causes chronic ischemia, which is a lack of oxygen, to the lower extremities (1, 2). Early-stage PAD typically manifests as intermittent claudication (leg pain during walking); however, as the disease progresses, patients can experience ischemic leg pain at rest and develop plantar ulcers that may ultimately entail foot or limb amputation (1). These symptoms impair walking capacity and reduce overall quality of life (3). Determination of potential therapies for patients with PAD is a pressing matter, as alleviating symptoms and improving vascular function may subsequently improve quality of life and delay disease progression in this population.

It has been suggested that the endothelium plays critical roles in vascular homeostasis, and disturbances in endothelial function are thought to precede the manifestation of atherosclerotic diseases (4). Attenuated bioavailability of nitric oxide (NO), a potent vasodilator, is known to be associated with endothelial dysfunction (5, 6), and patients with PAD have commonly showed reduced NO bioavailability and endothelial dysfunction (7–9). Previous studies suggested that exogenous dietary nitrate (NO_3^-) intake can induce an increase in NO_3^- reserves in the body that can be reduced to bioactive NO (10–13), and recent studies have suggested that dietary NO_3^- intake increases NO bioavailability in patients with PAD (14, 15). Following dietary NO_3^- intake, patients with PAD have also experienced reduced blood pressure (BP) (14, 15), improved skeletal muscle oxygen utilization (15), vascular conductance of the lower limb (14), and exercise tolerance (14, 15) following NO_3^- ingestion. Despite these positive vascular and functional effects in patients with PAD, there have

been no documented improvements in endothelial function following acute NO^- intake in this population (15). This absence of improved endothelial function in patients with PAD may be due to the fact that the previous study used the same dose for each participant, and there was a large variation in participant body mass (84.5 ± 16.5 kg) (15). To our knowledge, no studies have sought to establish a standardized dose of NO_3^- administration normalized per kg of body mass that can be used safely to efficiently improve endothelial function and walking capacity in patients with PAD. Therefore, the purpose of the present study was to examine the impacts of a consistent body mass-normalized acute moderate dose of NO_3^- in the form of beetroot juice on NO bioavailability, endothelial function, BP, arterial stiffness, walking capacity, and muscle oxygen utilization capacity in patients with PAD. Our hypothesis was threefold: 1) a moderate body-mass normalized dose of NO_3^- intake would increase serum NO bioavailability, which would in turn 2) improve endothelial function and reduce BP, which would contribute to 3) reduced arterial stiffness, improved walking capacity and exercise tolerance, and improved skeletal muscle oxygen utilization capacity in patients with PAD.

METHODS

Participants

Patients with PAD ($n = 11$, 5 males and 6 females) were recruited for study participation. Disease staging, claudication history, and ankle-brachial index of <0.90 were determined by both self-report and by physician diagnosis, and all participants were classified as Fontaine stage IIa and IIb(16). Participants were also required to have a stable BP, lipid, and/or diabetes regimen for ≥ 6 wk before participation, and all females were postmenopausal (no menses for ≥ 12 consecutive mo). Participants were excluded from the study if they had a history of pain at rest (Fontaine stage III) and/or tissue loss due to PAD (Fontaine stage IV), limited walking capacity due to conditions other than PAD, renal disease, already include a form of NO_3^- intake in their regimen, and/or had an allergy to beetroot juice. Participants were asked to withhold medications (at least 12 h before the visits) and were asked to refrain from mouthwash

use for ≥ 24 h (17), as anti-bacterial mouthwash can negatively impact the oral reduction of NO_3^- to NO_2^- (18). Participants were also asked to track what they consumed the day before their first visit so they could consume the same foods before their second visit. In addition, participants were requested to not alter their dietary habits during the 2-wk washout period. All procedures were performed according with the protocols approved by the Institutional Review Board and performed in accordance with the Declaration of Helsinki. All participants provided written, informed consent before beginning the study. This study was registered with <https://clinicaltrials.gov/> (NCT03506646).

Design

This study used a randomized, double-blinded, placebo-controlled, crossover design with a washout period of 14 days between the 2 study visits (Fig. 1). Participants were randomly assigned to receive either the body mass-normalized NO_3^- supplement (Beet It, James White Drinks, Ipswich, UK) or the nitrate-void placebo (PL). Our pilot data (unpublished) based on the low ($\sim 0.05 \pm 0.01$ mmol NO_3^-/kg), moderate ($\sim 0.11 \pm 0.01$ mmol NO_3^-/kg), and high doses ($\sim 0.22 \pm 0.03$ mmol NO_3^-/kg) reported by Wylie et al. (19) showed that a low dose was not sufficient to produce a detectable improvement in endothelial function, and a high dose induced side effects such as nausea and gastrointestinal issues. We found that the moderate dose (~ 0.11 mmol NO_3^-/kg) could elicit improvements in endothelial function without any adverse side effects. The dose per participant was calculated by multiplying body mass by 0.11 mmol, which was then used to calculate milliliters of supplement to be administered in a standard disposable drinking cup. The PL consisted of tapioca powder capsules that possessed no nitrate or antioxidant properties. Participants were informed that we were researching the effects of commercially available juice and pills on their vascular function and walking capacity; therefore, they were not aware that the capsules were PL. All assessments were performed after an overnight fast and at the same time of day (9:00 AM \pm 1 h). Anthropometric measurements were obtained upon arrival at both visits. Following anthropometrics, baseline experimental measurements were conducted, after which the NO_3^- supplement or

PL were consumed. All experimental measurements were repeated ~1h following ingestion, as NO_3^- intake in the form of beetroot juice has been reported to significantly increase concentrations of both NO_3^- and NO_2^- in the blood within 1 h after consumption (20, 21). All data analyses were performed in a blinded manner.

Anthropometrics

Anthropometric measurements included height, body mass, and body composition. Height was measured using a stadiometer (nearest 0.5 cm). Body mass was measured using a standard scale (nearest 0.1 kg), and body mass index was calculated by taking the body mass divided by height squared (kg/m^2). Body fat percentage was assessed using bio- electrical impedance analysis in duplicate to the nearest 0.1% (HBF – 306 C, Omron Healthcare, Lake Forest, IL), and the average of the 2 measures was recorded as the body fat percentage.

Resting Heart Rate and Blood Pressure

Participants sat for 15 min in a quiet and temperature-controlled room. Resting heart rate and BP were assessed in duplicate using an automated sphygmomanometer (HEM – FL 31, Omron Healthcare, Lake Forest, IL). The two measurements were taken with 5 min separating each measurement, and the average of the 2 was recorded as the resting heart rate and BP.

Blood Sampling

Blood was drawn (10 mL) from an antecubital vein with EDTA tubes before and after NO_3^- and PL intake. Samples were centrifuged at 3,500 rpm for 10 min at 4°C. Serum samples were stored in 2.0-mL microcentrifuge tubes and were stored at -80°C for later analysis of serum NO bioavailability. NO bioavailability [total NO_3^- and nitrite (NO_2^-)] was measured using a commercially available nitrate/nitrite colorimetric assay kit (Cat. No. 780001, Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Endothelial Function

Endothelial function was assessed using brachial artery flow-mediated dilation (FMD) and popliteal artery FMD using a Doppler ultrasound (Terason uSmart 3300, Terason Division Teratech Corporation, Burlington, MA), rapid inflation cuff system (E20 Rapid Cuff and cuff model SC5, D.E. Hokanson, Bellevue, WA), and a 3-lead electrocardiogram system (7700 Series Trigger Monitor, IvyBiomedical Systems Inc., Branford, CT) as previously described (22). Briefly, for both brachial and popliteal assessments, the rapid inflation cuff was placed just distal to the antecubital fossa and just distal to the popliteal fossa, respectively (22). For both assessments, resting arterial diameter was recorded with the ultrasound probe ~1–2 cm proximal to the rapid-inflation cuff for 5 min (22). The cuff was inflated to a pressure of 250 mmHg after baseline diameter was obtained and remained inflated for 5 min (22). The cuff was then deflated and the reactive hyperemic arterial response was recorded continuously on R-wave trigger for 5 min (22). Data were analyzed using an image capturing and automated edge-detection software (Vascular Imager, Vascular Research Tools 6, Medical Imaging Applications, Coralville, IA) as previously described (22). The relative artery diameter changes were calculated as previously described (22).

Arterial Stiffness and Pulse-Wave Analysis

Assessments of arterial stiffness, augmentation index (Alx), Alx adjusted to 75 beats/min (Alx@75), augmentation pressure, pulse pressure, and carotid-to-femoral pulse-wave velocity (PWV) were measured using applanation tonometry (SphygmoCor XCEL, AtCor Medical, Sydney, Australia) as previously described (22, 23). Briefly, Alx, Alx@75, augmentation pressure, and pulse pressure were assessed with a cuff placed on the upper arm between the elbow and shoulder to collect pressure waveforms which estimate central aortic pressure (22, 23). To assess carotid-to-femoral PWV, the carotid pulse was measured using applanation tonometry and femoral pulse waves were measured with a cuff placed on the upper leg between the hip and knee (22, 23).

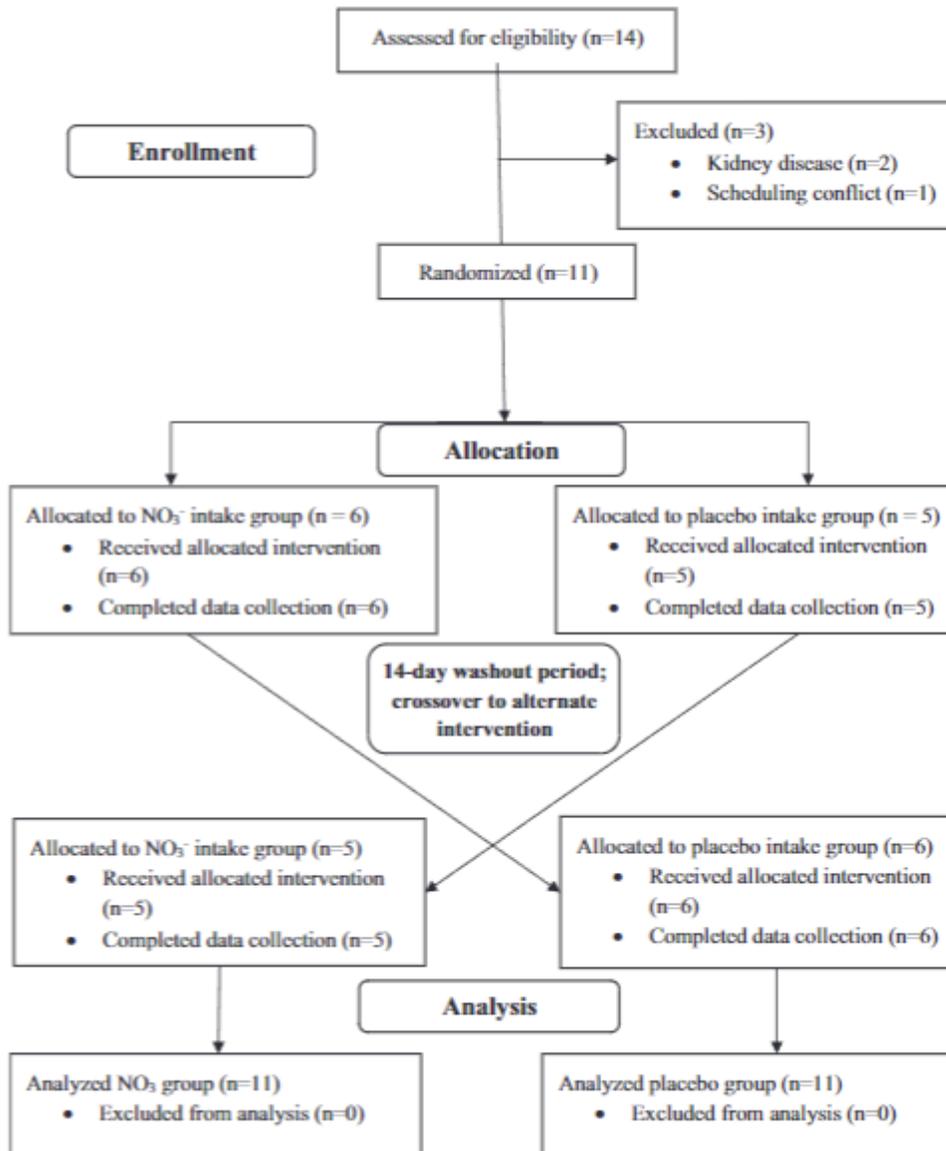


Figure 1. Randomized placebo-controlled crossover design, participant allocation, and analysis. NO₃⁻, nitrate.

Indices of peripheral arterial stiffness, carotid-to-radial PWV and carotid-to-ankle PWV, were assessed using applanation tonometry with participants in the supine position (Complior Analyze, Alam Medical, Saint-Quentin-Fallavier, France) as previously described (22, 23). Briefly, pulse wave-forms for carotid-to-radial PWV and carotid-to-ankle PWV were recorded for 30–60 s (22, 23). Assessments of central systolic and diastolic BP, central pulse pressure, deceleration time, and maximum first derivative of pressure (dP/dt_{max}) were

provided by the central pressure analysis (22, 23).

Walking Capacity and Skeletal Muscle Oxygen Utilization Capacity

Maximal walking capacity was assessed on a standard treadmill (Lode, B.V, Groningen, The Netherlands) using the modified Gardner protocol (22, 24). Participants were asked to notify the test administrators about their onset of claudication (the exact moment their leg pain starts) and to walk as long as they could tolerate even if the claudication persisted (maximal walking time and distance). The test was ended once patients could not tolerate their symptoms (i.e., severe claudication).

Gastrocnemius oxygen utilization capacity was measured with a portable near-infrared spectroscopy (NIRS) unit (Artinis PortaMon, Einsteinweg, The Netherlands) and Oxysoft software (v3.0.103.3, Einsteinweg, The Netherlands) during the exercise testing as previously described (22). Briefly, the NIRS device was adhered to the gastrocnemius, at the approximate location where the participant felt the most pain, using a commercially available double-sided adhesive and a commercially available black bandage to prevent extraneous light from reaching the device (15, 22). NIRS data were collected continuously at a frequency of 10 Hz and used to estimate the change in tissue saturation (StO_2), deoxygenated hemoglobin concentration ([HHb]), and oxygenated hemoglobin concentration ([O_2Hb]) at resting baseline and during the walking test. Upon walking test completion, the participant stood in a resting standing position and a thigh rapid inflation cuff (cuff model SC10, D.E. Hokanson, Bellevue, WA) was placed on the thigh ~3–4 cm proximal to the knee on the limb with the NIRS device. The cuff was inflated to ~250–260 mmHg for 5 min to deoxygenate the tissue with the same rapid inflation cuff system used for FMD (22, 25). The cuff pressure was released after 5 min, and the peak hyperemic response following cuff release was used to determine the 100% oxygenation level for each participant and was used to calculate normalized [HHb] (22, 25). The raw NIRS data were reduced to 1 Hz files and exported to Microsoft Excel files for later analysis.

Statistical Analysis

The Shapiro–Wilk test was used for all dependent variables to determine the normality of the data. Independent *t* tests were used to determine any differences between subject characteristics at the NO⁻ and PL visits. A two-way repeated measures analysis of variance (ANOVA) [group (NO₃⁻ and PL) x time (before and after supplement intake)] was used to compare the changes between NO₃⁻ and PL intake within and between groups on the dependent variables. When a significant main effect or interaction was found, paired *t* tests were used for post hoc comparisons for the normally distributed variables, and paired samples Wilcoxon tests were used for nonnormally distributed variables. Based on a power calculation from a previous study, a sample size of a minimum of total 14 (*n* = 7/group) would allow for 80% power to detect differences between FMD between NO⁻ versus PL (26). Associations between dependent variables were assessed using Pearson’s product-moment correlation coefficient. Effect size analyses were conducted using Cohen’s *d* and interpreted as 0.2 as a small effect size, 0.5 as a medium effect size, and 0.8 as a large effect size (22, 27). All analyses were performed using SPSS 26.0 (IBM, Armonk, NY). Descriptive characteristics are presented as means ± SD, and all other data are presented as means ± SE. Statistical significance was set to *P* < 0.05.

RESULTS

The moderate body mass-normalized dose of NO⁻ was tolerated well by all participants and no adverse side effects were reported (Table 1). The average dose of the supplement was ~0.11 ± 0.01 mmol NO₃⁻/kg, equating to ~91.8 ± 20.0 mL of the beetroot juice supplement. Some participants reported beeturia (red-pink urine), which is consistent with previous studies (11, 19, 28). Participants presented with several other comorbidities, such as hypertension, prediabetes, dyslipidemia, and arthritis, and were taking medications for these comorbidities (Table 2). There were significant group x time interactions (*P* < 0.05) for serum total NO₃⁻ and NO₂⁻, brachial and popliteal FMD, peripheral and central systolic BP, walking distance and time, and skeletal muscle oxygen utilization.

Table 1. Participant characteristics at the nitrate (NO₃⁻) and placebo (PL) visits

	PL (n = 11)	NO₃⁻ (n = 11)
Age, yr	70.0 ± 7.0	70.0 ± 7.0
Height, cm	163.2 ± 14.0	163.2 ± 14.0
Body mass, kg	77.3 ± 16.5	77.0 ± 16.5
BMI, kg/m ²	29.1 ± 6.4	29.0 ± 6.0
Body fat, %	37.4 ± 9.4	37.6 ± 9.2
Values are presented as means ± SD. BMI, body mass index. Student's t tests were used for comparison between the PL and NO ₃ ⁻ groups.		

Table 2. Participant comorbidities, conditions, medications, and smoking history

Comorbidity or Condition		n
	Hypertension	8
	Dyslipidemia	10
	Prediabetes	3
	Arthritis	7
Medications		
	Angiotensin-converting enzyme inhibitors	3
	Angiotensin II receptor blockers	2
	Diabetic medication (metformin)	3
	Beta blockers	5
	Calcium channel blockers	2
	Anticoagulants	2
	Diuretics	1
	Statins	9
	Nonsteroidal antiinflammatory medication	9
	Sleep aids	3
Smoking history		
	Current	1
	Former	4
	Never	6

Serum total NO₃⁻ and NO₂⁻ significantly increased ($\Delta 1.32 \pm 0.15 \mu\text{M}$, $P < 0.01$) after NO₃⁻ intake compared with placebo (Fig. 2, *A* and *B*). Brachial artery FMD significantly increased ($\Delta 1.3 \pm 0.3\%$, $P < 0.01$), whereas central systolic BP was reduced ($\Delta -8.2 \pm 3.1 \text{ mmHg}$, $P = 0.014$) after NO₃⁻ intake compared with placebo (Fig. 3, *A* and *B*, Table 3). Maximal walking distance also significantly increased ($\Delta 92.7 \pm 36.8 \text{ m}$, $P = 0.029$) after NO₃⁻ intake compared with placebo (Fig. 4, *A* and *B*). A time-effect was noted for reduced systolic BP ($\Delta -4.7 \pm 1.4 \text{ mmHg}$, $P = 0.021$; Table 3) and increased popliteal FMD ($\Delta 1.7 \pm 0.4\%$, $P < 0.01$; Fig. 3, *C* and *D*). A group-effect was noted for maximal walking time ($\Delta 6.3 \pm 25.4 \text{ s}$, $P = 0.015$; Fig. 4, *C* and *D*). In addition,

there were several significant changes between the NO_3^- intake and PL groups in oxygen utilization capacity. [HHb] was significantly lower following NO_3^- intake compared with PL at 60 s, 80 s, 100 s, 120 s, and 140 s during walking (Fig. 5A), but there were no significant differences in [StO₂] or [HbO₂] following NO_3^- intake compared with PL. There were no changes in resting heart rate, diastolic BP, central diastolic BP, deceleration time, dP/dt_{max} , peripheral or central arterial stiffness, Alx, Alx@75, augmentation pressure, pulse pressure, or time to onset of claudication ($P > 0.05$) following NO_3^- intake (Table 3, Fig. 4E).

DISCUSSION

This study presents with several novel findings that may be relevant for patients with PAD. First, a moderate dose of NO_3^- intake normalized to body mass can be a safe and beneficial dietary strategy for improving NO bioavailability and reducing high BP in patients with PAD. Second, to our knowledge, we are the first group to demonstrate the impacts of a body mass-normalized moderate dose of NO_3^- intake on brachial and popliteal artery endothelial function in patients with PAD. Third, we found that a body mass-normalized moderate dose of NO_3^- significantly improves walking capacity and skeletal muscle oxygen utilization capacity during walking in patients with PAD.

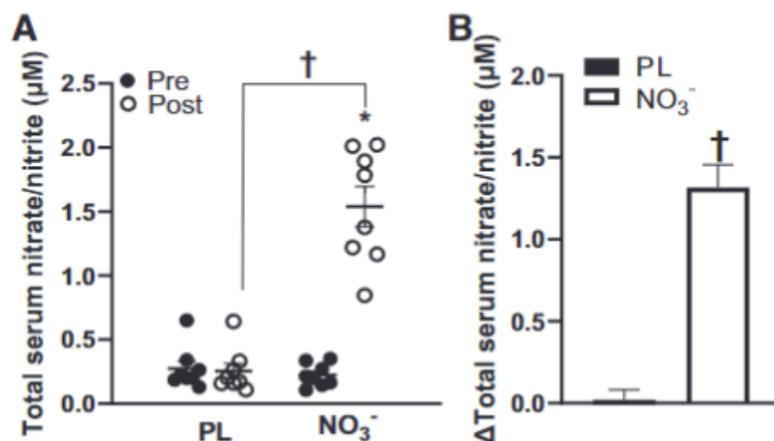


Figure 2. Total serum nitrate and nitrite levels (μM) pre- and post-placebo (PL) and nitrate (NO_3^-) intake. A: total serum nitrate/nitrite significantly increased post- NO_3^- and was significantly greater than post-PL ($n = 8$, $d = 4.1$) B: changes in total serum nitrate and nitrite levels in the PL and NO_3^- groups. Values are presented as means \pm SE. Two-way repeated analysis of variance (ANOVA) [group (NO_3^- and PL) \times time (before and after supplement intake)] with paired t tests for post hoc comparisons. Effect size analyses were conducted using Cohen's d . * $P < 0.01$ vs. Pre † $P < 0.01$ vs. PL.

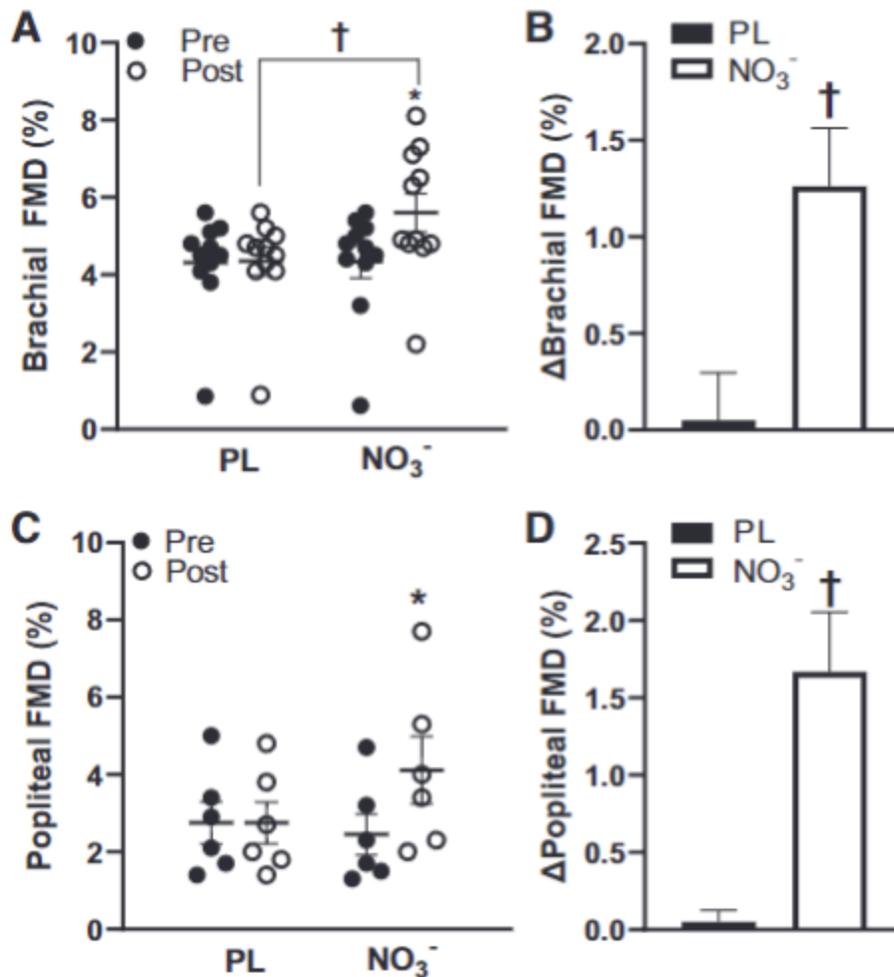


Figure 3. Flow-mediated dilation (FMD, %) in the brachial and popliteal arteries pre- and post-placebo (PL) and nitrate (NO₃⁻) intake. *A*: percent brachial artery FMD significantly increased post-NO₃⁻ and was significantly greater than post-PL ($n = 11$, $d = 0.8$). *B*: changes in brachial FMD in the PL and NO₃⁻ groups. *C*: percent popliteal artery FMD significantly increased post-NO₃⁻ ($n = 6$, $d = 0.8$). *D*: changes in popliteal FMD in the PL and NO₃⁻ groups. Values are presented as means \pm SE. Two-way repeated analysis of variance (ANOVA) [group (NO₃⁻ and PL) \times time (before and after supplement intake)] with paired t tests for post hoc comparisons for normally distributed data and Wilcoxon tests for nonnormally distributed data. Effect size analyses were conducted using Cohen's d . * $P < 0.01$ vs. Pre † $P < 0.01$ vs. PL.

NO Bioavailability and Endothelial Function

Endothelial dysfunction has been documented as a key contributor to the manifestation of atherosclerotic diseases (4). Numerous mechanisms have become increasingly implicated in endothelial dysfunction, whereas growing evidence suggests that dietary NO₃⁻ may support improvements in the redox environment and NO bioaction, which may support endothelial function in patients with PAD (29–31).

Previously, the impacts of dietary NO_3^- on vascular endothelial function have been explored in both healthy and patient populations. Several studies have supported the notion that both acute and chronic intake of dietary NO_3^- can improve conduit artery endothelial function in healthy older adults (13, 32), individuals with hypertension (33, 34), and individuals with hypercholesterolemia (12). However, there have been no noted improvements in endothelial function following NO_3^- intake in patients with PAD (15). Although Kenjale et al. (15) used a similar dose to our study, their participants were substantially heavier than our participants (84.5 ± 16.5 kg vs. 77.0 ± 16.5 kg), thus making their over- all dose lower. Although their nonnormalized dose showed improvements in NO bioavailability, it may have not been sufficient to induce alterations of endothelial nitric oxide synthase (eNOS)-derived NO in response to reactive hyperemia (15). Our findings show that brachial FMD ($\Delta 1.3\%$, $d = 0.8$; Fig. 3, A and B) and popliteal FMD ($\Delta 1.7\%$, $d = 0.9$; Fig. 3, C and D) both improved after a body mass-normalized moderate dose of NO_3^- intake, and the mechanisms underlying these improvements may be largely explained by increased blood NO_3^- -mediated improvements in redox balance. We found that NO_3^- intake improves blood total NO_3^- and NO_2^- levels (Fig. 2, A and B), and this may have served to reduce reactive oxygen species (ROS) levels (13, 33). Attenuated ROS levels may have supported improvements in the redox environment and antioxidant defense system, and this may have contributed to improved NO-mediated vasodilatory function (13, 33). Walker and colleagues (2019) (13) suggested that improved FMD following acute NO_3^- intake was likely mediated by augmented antioxidant defense and reduced ROS that may collectively contribute to increased eNOS activity, which is a key enzyme for endothelium-mediated NO production. In addition, Velmurugan et al. (12) stated that an improvement in FMD after 4 wk of NO_3^- intake was likely due to reduced ROS-mediated NO scavenging, as oxidized low-density lipoprotein was reduced after NO_3^- intake. NO_3^- -mediated improvements in the redox environment may be a viable explanation for the increased FMD in the present study, as it is well-accepted that the FMD response is primarily eNOS-derived NO

dependent (35, 36). Future research should entail assessments of plasma oxidant status in addition to measuring NO bioavailability in order to determine the impacts of NO₃⁻ intake on the redox environment.

Table 3. Participant resting heart rate, blood pressures, and hemodynamic analyses pre- and postnitrate (NO⁻) and placebo (PL) intake

	PL (n = 11)			NO ₃ ⁻ (n = 11)			Cohen's d
	Pre	Post	Δ	Pre	Post	Δ	
Resting heart rate, beats/min	68.9 ± 3.8	69.0 ± 3.2	0.1 ± 0.8	70.0 ± 3.2	69.6 ± 3.4	-0.4 ± 0.9	0.1
Systolic BP, mmHg	133.6 ± 4.7	134.8 ± 5.0	1.2 ± 1.9	133.2 ± 4.9	128.5 ± 4.9*	-4.7 ± 1.4	0.3
Diastolic BP, mmHg	81.2 ± 1.9	81.5 ± 2.0	0.3 ± 2.0	81.2 ± 1.8	79.2 ± 1.7	-2.0 ± 1.3	0.4
Central systolic BP, mmHg	125.5 ± 4.6	126.5 ± 4.6	1.0 ± 1.4	127.7 ± 4.1	119.5 ± 5.2*†	-8.2 ± 3.1	0.5
Central diastolic BP, mmHg	80.0 ± 2.5	80.1 ± 2.3	0.1 ± 0.4	81.6 ± 1.7	79.0 ± 1.7	-2.6 ± 1.3	0.4
Carotid-to-radial PWV, m/s	9.3 ± 0.3	9.4 ± 0.3	0.1 ± 0.2	9.4 ± 0.3	9.1 ± 0.3	-0.3 ± 0.1	0.3
Carotid-to-ankle PWV, m/s	10.3 ± 0.3	10.3 ± 0.3	0.0 ± 0.2	10.4 ± 0.3	9.9 ± 0.6	-0.5 ± 0.4	0.4
Carotid-to-femoral PWV, m/s	9.4 ± 0.8	9.6 ± 0.8	0.2 ± 0.1	9.3 ± 0.8	9.0 ± 0.5	-0.3 ± 0.4	0.2
Deceleration time, ms	620.0 ± 60.7	618.7 ± 64.6	-1.3 ± 11.1	614.9 ± 54.3	629.8 ± 50.8	14.9 ± 19.1	0.1
dP/dt _{max} , mmHg/s	729.8 ± 65.2	720.7 ± 72.0	-9.1 ± 60.4	720.9 ± 47.6	755.5 ± 77.9	34.6 ± 76.2	0.2
Central pulse pressure, mmHg	45.5 ± 4.3	46.5 ± 4.4	1.0 ± 1.4	46.1 ± 3.5	41.4 ± 5.2	-4.7 ± 3.9	0.3
Peripheral pulse pressure, mmHg	48.2 ± 6.4	53.0 ± 7.9	4.8 ± 1.9	51.8 ± 8.6	49.6 ± 8.9	-2.2 ± 3.3	0.1
Augmentation pressure, mmHg	18.0 ± 3.1	20.6 ± 5.3	2.6 ± 2.4	21.6 ± 5.3	20.2 ± 4.6	-1.4 ± 2.4	0.1
Alx, mmHg	39.4 ± 1.0	38.8 ± 2.7	-0.6 ± 2.8	40.2 ± 3.7	39.8 ± 1.9	-0.4 ± 3.6	0.2
Alx@75 (%)	30.6 ± 1.7	30.4 ± 4.0	-0.2 ± 4.0	32.2 ± 4.5	32.4 ± 2.8	0.2 ± 3.2	0.1

Values are presented as means ± SE. Alx, augmentation index; Alx@75, augmentation index adjusted to 75 beats/min; BP, blood pressure; dP/dt_{max}, maximum first derivative of pressure; PWV, pulse-wave velocity. Two-way repeated analysis of variance (ANOVA) [group (NO⁻ and PL) x time (before and after supplement intake)] with paired t tests for post hoc comparisons. Effect size analyses were conducted using Cohen's d. *P < 0.05 vs. Pre. †P < 0.05 vs. PL.

In addition to NO₃⁻, beetroot juice possesses several nonnitrate bioactive compounds in small amounts, including flavonoids, ascorbic acid, polyphenols, and several minerals (37, 38). We and others have previously demonstrated that several of these bioactive compounds alone at higher concentrations can induce improvements in endothelial function in healthy and disease populations, with these improvements likely mediated by an enhanced redox environment (39, 40). Accordingly, these bioactive compounds in beetroot juice may have had a slight additive effect on improving endothelial function in the present study. Furthermore, Tropea et al. (41) recently showed that pregnant eNOS^{-/-} mice demonstrated significantly increased endothelium-dependent relaxation

following dietary NO_3^- supplementation in the form of beetroot juice. The authors suggested that the endothelium-derived hyperpolarization₃(EDH) component of endothelial function may have been enhanced by NO_3^- supplementation in these $\text{eNOS}^{-/-}$ mice (41). Taken together, it may be hypothesized that both anti-oxidant property-mediated improvements in the redox environment (facilitated by both NO_3^- and other bioactive compounds) and EDH component-mediated effects of the beetroot juice may be potential contributors to the improvements in FMD in the present study. However, these hypotheses warrant further investigation in patients with PAD.

Furthermore, NO_3^- may have directly influenced vascular smooth muscle tone. NO_3^- is an NO-donor that can directly induce vascular smooth muscle relaxation. The nitrate-nitrite-NO pathway functions to reduce NO_3^- to bioactive NO in the blood and tissues (42). NO can diffuse into the vascular smooth muscle, which results in a signaling cascade that can induce relaxation (endothelium-independent vasodilation) of the vascular smooth muscle (43). In the present study, the nitrate-nitrite-NO pathway may have played a role in endothelium-independent vasodilation and a subsequent reduction in systemic vascular resistance as indicated by reduced systolic BP (Table 3), and this reduction in systemic vascular resistance can contribute to increased blood flow in accordance with Ohm's Law (44). Despite the potential influence of NO_3^- on vascular smooth muscle relaxation, the magnitude of this contribution relative to other potential mechanisms requires further investigation.

Walking Capacity and Skeletal Muscle Oxygen Transfer and Utilization Capacity

Several research groups have focused on restoring NO levels as an avenue for improving physical capacity in patients with PAD. These studies primarily used NO-donor drugs, which are pharmacological agents that directly supply NO, whereas dietary NO_3^- , often in the form of beetroot juice, is a naturally occurring form of NO_3^- . Gresele et al. (45) reported that intake of a NO-donor (NCX-4016; 800 mg 2x/day) for 6 mo does not improve pain-free walking distance but can slow atherosclerotic progression in patients with PAD. The authors suggested that the lack of improvement may be due to

the large variability of their walking data and the heterogeneity of PAD disease staging within their patient sample(45). Conversely, both acute (~0.09–0.13 mmol/kg) and chronic (8.5 mmol, no body mass reported) dietary NO₃⁻ intake (nonbody mass-normalized doses) has been shown to improve assessments of walking capacity, including improvements in walking time, walking distance, and time to onset of claudication in patients with PAD (14, 15). Our findings are in agreement with these previous studies, as we found significant improvements in maximal walking time (Δ56.3 s, *d* = 0.4) and maximal walking distance (Δ92.7 m, *d* = 0.4) following a body mass-normalized moderate dose of NO₃⁻ intake (Fig. 4, A–D).

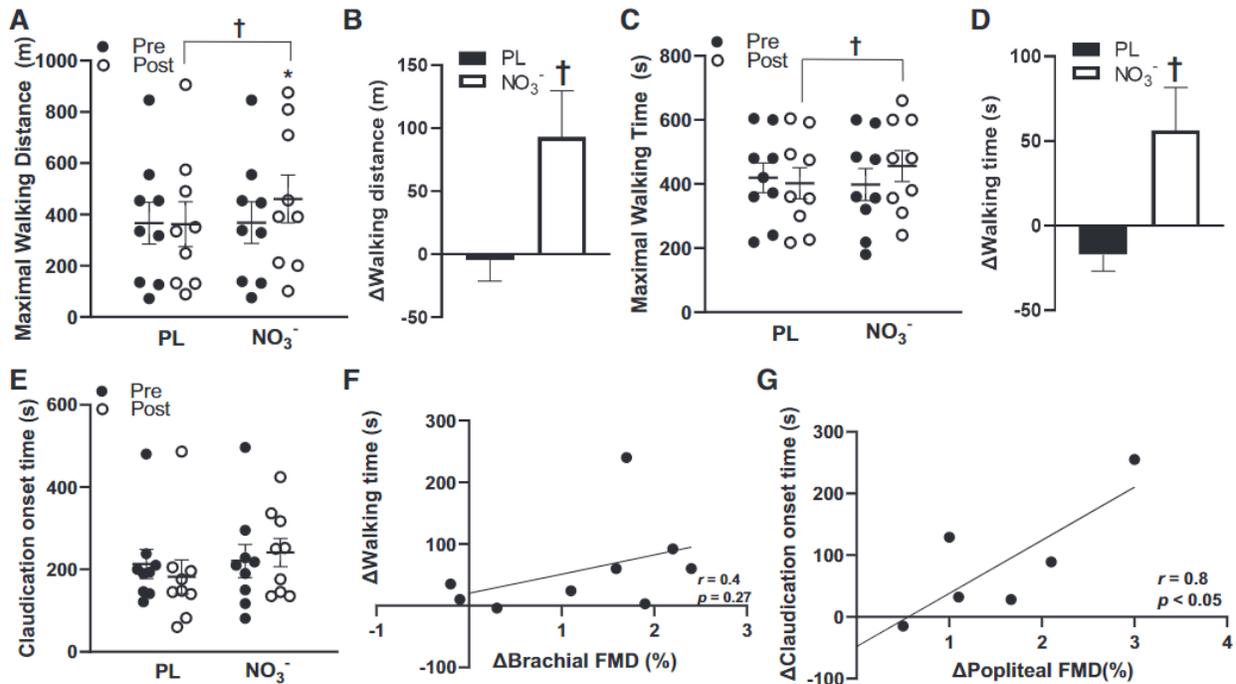


Figure 4. Maximal walking time (s), maximal walking distance (m), and time to claudication(s) pre- and post-placebo (PL) and nitrate (NO₃⁻) intake and relationships between changes in flow-mediated dilation (FMD), maximal walking time, and time to onset of claudication in the NO₃⁻ group. **A:** maximal walking distance significantly increased post-NO₃⁻ and was significantly greater than post-PL (*n* = 9, *d* = 0.4). **B:** changes in maximal walking distance in the PL and NO₃⁻ groups. **C:** maximal walking time was significantly greater in post-NO₃⁻ compared with post-PL (*n* = 9, *d* = 0.4). **D:** changes in maximal walking time in the PL and NO₃⁻ groups. **E:** there were no significant changes in time to onset of claudication pre- or post-PL or NO₃⁻ intake (*n* = 9, *d* = 0.5). **F:** positive relationship between changes in walking time (s) and changes brachial FMD (%) in the NO₃⁻ group (*n* = 9, *r* = 0.4, *P* = 0.27). **G:** positive relationship between changes in time to onset of claudication(s) and changes popliteal FMD (%) in the NO₃⁻ group (*n* = 6, *r* = 0.8, *P* < 0.05). Values are presented as means ± SE. Two-way repeated analysis of variance (ANOVA) [group (NO₃⁻ and PL) × time (before and after supplement intake)] with paired *t* tests for post hoc comparisons. Pearson's product-moment correlation coefficient was used for correlations. Effect size analyses were conducted using Cohen's *d*. **P* < 0.05 vs. Pre †*P* < 0.05 vs. PL.

Although the mechanisms underlying the improvements in walking capacity are not entirely clear, it is well-accepted that reduced walking capacity in PAD is partially due to attenuated blood flow and perfusion that may cause hypoxic conditions within the lower extremities (46). The endothelium plays an essential role in regulating local skeletal muscle tissue blood flow and perfusion, particularly at the level of the microcirculation (47, 48). Of note, we recently reported that microcirculatory endothelial dysfunction is present in the leg skeletal muscles of patients with PAD, which may suggest there is an attenuated capacity to regulate flow and perfusion within the lower extremity (22). In the present study, the improvements in walking capacity may be partially explained by the improved endothelial function-mediated increase in blood flow and tissue perfusion, which may have facilitated enhanced transport of oxygen and nutrients locally within the skeletal muscle microcirculatory network, thus contributing to improved walking capacity (49). Previous studies have reported that dietary NO_3^- supplementation can improve blood flow and tissue perfusion in the leg muscle of murine models with chronic hindlimb ischemia (50, 51). These authors primarily attributed these improvements to enhanced endothelial function by the contribution of NO and the nitrate-nitrite-NO pathway (50, 51). Furthermore, NO_3^- supplementation (~ 0.07 mmol $\text{NO}_3^-/\text{kg}/\text{day}$ for 4 days) in humans was shown to delay muscular fatigue during leg exercise with experimentally-induced ischemia, and the authors said this was due to NO_3^- -mediated improvements in leg skeletal muscle perfusion (52). Their results suggest that NO_3^- -mediated improvements in tissue perfusion may serve to delay muscular fatigue, and this can be particularly relevant for patients with PAD. Interestingly, our study provides evidence that supports the improved endothelial function-mediated increase in skeletal muscle tissue perfusion. We found a positive moderate association between improvements in brachial FMD and improvements in maximal walking time ($r = 0.4$; Fig. 4F), and improvements in popliteal FMD were strongly associated with improvements in time to claudication onset ($r = 0.8$; Fig. 4G). These findings may suggest that NO_3^- supplement intake induced an improvement in endothelial function, which may serve to increase walking time and exercise tolerance in patients with PAD. More specifically, the strong

relationship between popliteal endothelial function and walking tolerance may indicate that leg endothelial function plays a crucial role for improvements in tissue perfusion and delaying claudication. These results confirm the findings from our previous studies, as we reported that leg endothelial function and lower extremity blood flow are associated with exercise performance and functional capacity in PAD (22, 53, 54).

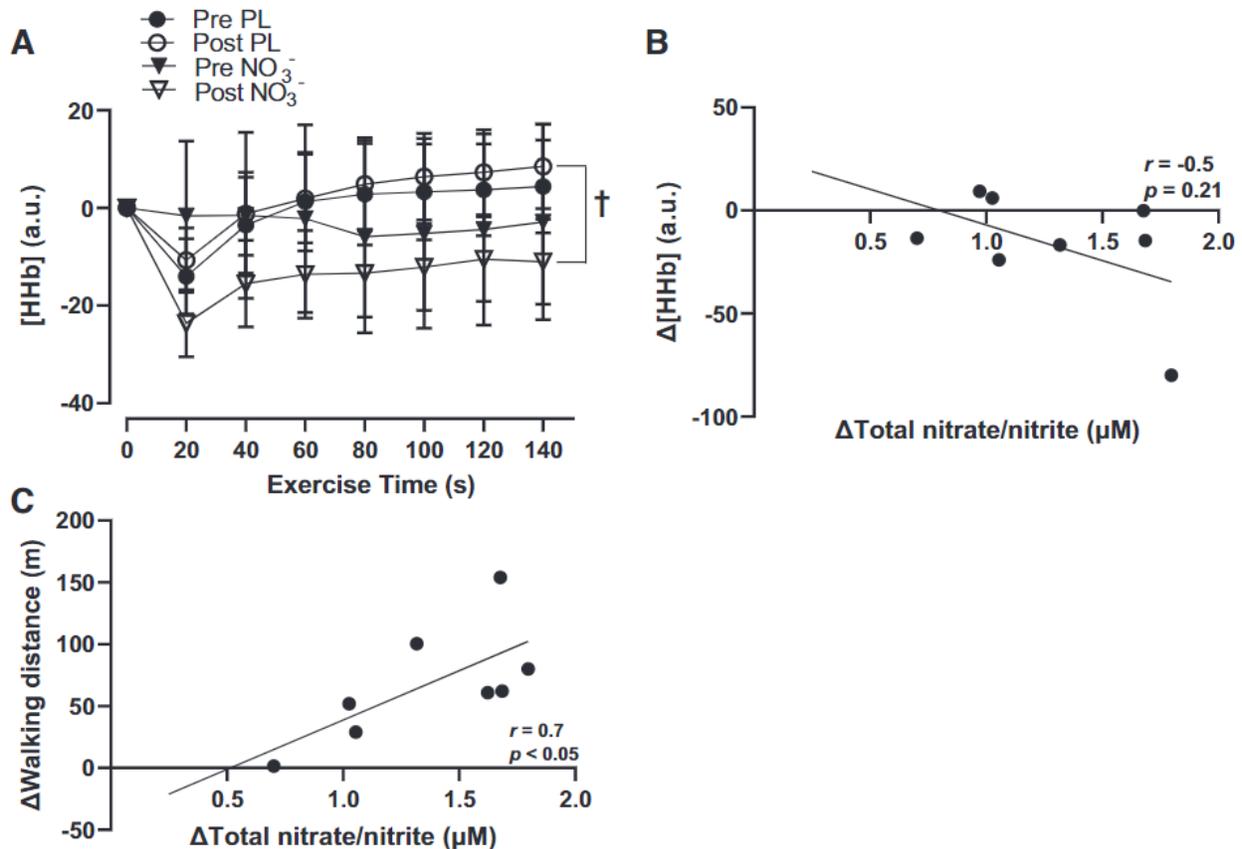


Figure 5. Group mean changes in deoxygenated hemoglobin ([HHb], a.u.) pre- and post-placebo (PL) and nitrate (NO⁻) intake every 20 s during the maximal walking capacity test and relationships between changes in total nitrate/nitrite levels, [HHb], and maximal walking distance in the NO⁻ group. A: post-NO⁻ and post-PL [HHb] were significantly different ($P < 0.05$) only at 60, 80, 100, 120, and 140 s ($n = 8$, $d = 1.2$). B: negative relationship between changes in total nitrate/nitrite concentration (μM) and deoxyhemoglobin concentration ([HHb], a.u.) in the NO⁻ group ($n = 8$, $r = -0.5$, $P = 0.21$). C: positive relationship between changes in total nitrate/nitrite concentration (μM) and walking distance (m) in the NO⁻ group ($n = 8$, $r = 0.7$, $P < 0.05$). Values are presented as means \pm SE. Two-way repeated analysis of variance (ANOVA) [group (NO⁻ and PL) \times time (before and after supplement intake)] with paired t tests for post hoc comparisons. Pearson's product-moment correlation coefficient was used for correlations. Effect size analyses were conducted using Cohen's d . †Post-PL and post-NO₃⁻ significantly different ($P < 0.05$) only at 60, 80, 100, 120, and 140 s.

In addition, the nitrate-nitrite-NO pathway may have functioned as a compensatory mechanism to support vasodilation, blood flow, and tissue perfusion within the lower extremity in patients with PAD (42). Dietary NO_3^- is reduced to NO_2^- by the enterosalivary circulation and oral commensal bacteria, which generates a reservoir of NO_2^- that can be converted to NO in the blood and tissues (55, 56). Importantly, this nitrate-nitrite-NO pathway has been shown to facilitate vasodilation under hypoxic conditions (42), and HHb has specifically been shown to act as a key NO_2^- reducing agent (NO_2^- reductase) under these conditions of low oxygen tension (57, 58). During walking, the lower limb in PAD deoxygenates at a faster rate when compared with healthy controls, resulting in a greater concentration of HHb (59). This greater concentration of HHb may serve to facilitate vasodilation in the lower limbs in PAD, especially when NO_2^- is readily available. The NO_2^- and HHb reaction-mediated increase in vasodilation may produce significant increases in blood flow and tissue perfusion in the lower extremity during walking in PAD. We found that NO bioavailability was increased (Fig. 2, A and B) and [HHb] was reduced following NO_3^- intake compared with PL at 60 s, 80 s, 100 s, 120 s, and 140 s during walking (Fig. 5A). These results may imply that HHb functioned as a NO_2^- reducing agent to produce NO, which is well-aligned with previous findings in patients with PAD (15). Of note, we found a moderate negative association between reductions in [HHb] and increases in serum NO bioavailability ($r = -0.5$; Fig. 5B). This relationship may suggest that when NO_3^- and NO_2^- levels are higher, there may be more potential for HHb to act as a NO_2^- reducing agent to produce NO during walking in patients with PAD. In addition, we noted a strong positive relationship between increases in NO_3^- and NO_2^- levels and increases in walking distance ($r = 0.7$; Fig. 5C), which may further support the NO bioavailability-mediated improvements in tissue perfusion and walking capacity.

Arterial Stiffness and Augmentation Index

Arterial stiffening refers to alterations in mechanical and structural properties of the artery that perturb adequate arterial function and reduce distensibility of the vascular tree (60). Arterial stiffness is commonly assessed as pulse-wave velocity (PWV) (61, 62). Augmentation index (Aix) and Aix adjusted to 75 beats/min (Aix@75) are also often used as markers for central arterial stiffness and can be affected by both structural and hemodynamic influences (23, 63, 64).

Arterial stiffness has been reported to be associated with the formation of

atherosclerotic lesions and affects individuals with PAD (65, 66). Previous interventions utilizing dietary NO_3^- for several populations have demonstrated mixed results regarding changes in arterial stiffness and Alx. Acute dietary NO_3^- intake ($\sim 0.11\text{--}0.14$ mmol NO_3^-/kg) was shown to reduce Alx@75 in healthy younger adults but showed no changes in healthy older adults (67). Interestingly, some positive effects of dietary NO_3^- were noted for clinical populations such as individuals with hypertension and hypercholesterolemia. In individuals with hypertension, NO_3^- intake (~ 6.4 mmol NO_3^-/day for 4 wk, no body mass reported) induced reductions in PWV and Alx (34). Velmurugan et al. (12) reported that NO_3^- intake (~ 6.0 mmol NO_3^-/day for 6 wk, no body mass reported) can reduce Alx and PWV in individuals with hypercholesterolemia. Despite these improvements noted in clinical populations, these studies are short- to midterm supplementation studies and did not report specific body mass normalized doses of NO_3^- for their subject populations (12, 34); therefore, it is hard to find standard doses of NO_3^- for clinical populations. To our knowledge, we are the first group to investigate the impacts of a body mass-normalized dose of dietary NO_3^- on arterial stiffness in patients with PAD. There were no significant changes in carotid-to-radial PWV ($\Delta -0.3$ m/s, $P = 0.13$, $d = 0.3$), carotid-to-femoral PWV ($\Delta -0.34$ m/s, $P = 0.27$, $d = 0.2$), carotid-to-ankle PWV ($\Delta -0.5$ m/s, $P = 0.38$, $d = 0.4$), Alx ($\Delta -0.4\%$, $P = 0.97$, $d = 0.1$), or Alx@75 ($\Delta -0.2\%$, $P = 0.93$, $d = 0.1$) following NO_3^- intake (Table 3) in our study. The absence of improvements in arterial stiffness may be partially due to the fact that arterial stiffness is a result of structural changes within the vasculature, and an acute moderate dose of NO_3^- intake may not be sufficient to produce a detectable impact on arterial stiffness in PAD. Chronic body mass-normalized moderate doses of NO_3^- supplementation studies for patients with PAD should be considered to further assess the potential long-term impacts of NO_3^- on arterial stiffness and central vascular health.

Experimental Considerations

Our study has some experimental considerations that should be acknowledged. We have a couple of variables including popliteal FMD (bifurcations effect on measurement reliability/precision) and blood draw (needle apprehension) that have a lower sample size, which are limitations to some of our experiments and correlation analyses. It has been recently noted that technical settings for FMD and ultrasound scanning have been suggested, which included that the ultrasound probe should be placed at least 2–3 cm proximal to arterial bifurcations on a straight segment of the vessel to avoid

confounding factors such as vortices and turbulent flow (68, 69). Unfortunately, some of our patients with PAD ($n = 5$) showed multiple bifurcations during artery scanning, and a straight arterial segment was not accessible, which prevents sufficient probe placement for the popliteal artery. Furthermore, we performed our measurements ~ 1 h after NO_3^- ingestion. Previous studies using similar ($\sim 0.09\text{--}0.13$ mmol NO_3^-/kg) and higher ($\sim 0.29\text{--}0.43$ mmol NO_3^-/kg) doses and suggested that peak blood levels of NO_3^- and NO_2^- would be around 2–3 h post consumption (11, 15), and we found that blood levels of NO_3^- and NO_2^- significantly increase ~ 1 h after ingestion, which is consistent with previous studies (11, 20, 21). Even though measurements were conducted sooner than the potential NO_3^- and NO_2^- peak in the blood, our results show that the vascular and functional benefits of dietary NO_3^- intake may occur in as little as 1 h in patients with PAD. However, more research is warranted to fully understand the relationship between the metabolism of a body mass-normalized moderate dose of dietary NO_3^- and how long these vascular and functional benefits may be maintained in patients with PAD.

In addition, we asked participants to repeat foods consumed before each visit and to not modify their dietary habits, and strongly suggested that they limit their nitrite-rich foods and other antioxidant property-rich food and supplements, but we did not require diet logs to be prepared and collected. Future studies, most particularly for supplementation studies, a diet-tracking component should be incorporated to monitor general macronutrient profile, caloric intake, and/or any additional higher-nitrate food consumed (e.g., spinach, other leafy green vegetables, beets, etc.). Furthermore, medications were withheld for ≥ 12 h before the study visits and use of medications was not ceased during the 2-wk washout period. Some blood pressure-lowering medications (e.g., b-blockers, ACE inhibitors, angiotensin II receptor blockers, etc.) have half-lives and residual activity that can extend past 12 h (70–73). The potential residual effects of blood pressure medications may have impacted the magnitude by which blood pressure was reduced by the acute dietary NO_3^- . Even though the mechanisms of action are different between these blood pressure medications (i.e., blocking of beta and angiotensin II receptors, and inhibition of angiotensin-converting enzyme) and dietary nitrate (NO donor), future studies should consider incorporating better control for these differences in medication half-lives and their possible lingering effects on blood pressure. Last, we did not directly investigate the impacts of additional comorbidities due to our relatively small sample size. Conditions such as prediabetes, arthritis, and smoking history may exacerbate systemic inflammation, ROS production, and skeletal muscle tissue perfusion that are independent of PAD (74–77). This may in turn attenuate the functional response

to dietary NO_3^- , which may differentiate potential “responders” and “nonresponders” to dietary NO_3^- despite substantial increases in NO bioavailability (Supplemental materials; all Supplemental material is available at <https://doi.org/10.6084/m9.figshare.14713992>). However, these concepts warrant further study with a larger sample size.

Perspectives and Significance

Our results show that blood NO bioavailability, endothelial function, BP, walking capacity, and tissue oxygen transfer and utilization capacity in the skeletal muscle were improved after an acute body mass-normalized moderate dose of dietary NO_3^- . In fact, these results may have several clinical implications for patients with PAD. We noted clinically relevant increases in both brachial FMD ($\Delta 1.3\%$) and popliteal FMD ($\Delta 1.7\%$) after a moderate dose of dietary NO_3^- intake, which may play a role in protecting or slowing the disease progression by promoting improved vascular homeostatic control (78, 79). In addition, these results may be advantageous for this population in terms of walking independence, as we noted significant improvements in walking capacity (both time and distance) after a moderate dose of dietary NO_3^- intake. Patients with PAD often lack the physical capacity and desire to participate in exercise due to their poor health-related quality of life (80), which may make nutritional and dietary interventions of greater interest as a therapy to support walking capacity. Although there were no statistically significant improvements in time to onset of claudication, there was a medium effect size ($\Delta 20$ s, $P = 0.29$, $d = 0.5$), which may indicate a clinically meaningful increase in pain-free walking time. Even though this delay in claudication onset time was not significant, our findings for increases in maximal walking time ($\sim 14\%$) and maximal walking distance ($\sim 25\%$) may support the notion that patients had improved claudication tolerance or a reduction in perceived claudication severity considering that they were able to walk for a longer distance and period of time. Overall, acute intake of a moderate body mass-normalized dose of dietary NO_3^- may induce beneficial effects on vasculature function and walking capacity for patients with PAD while inducing no known side effects. Thus, moderate doses of dietary NO_3^- (0.11 mmol NO_3^-/kg) would be an efficient nutritional therapy for delaying disease progression and also improving quality of life in this disease population.

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DISCLOSURES

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

E.J.P. and S.-Y.P. conceived and designed research; E.J.P., T.K.W., S.K.Y., and S.-Y.P. performed experiments; E.J.P., T.K.W., and S.-Y.P. analyzed data; E.J.P. and S.-Y.P. interpreted results of experiments; E.J.P. and T.K.W. prepared figures; E.J.P. and S.-Y.P. drafted manuscript; E.J.P., T.K.W., S.K.Y., and S.-Y.P. edited and re-vised manuscript; E.J.P., T.K.W., S.K.Y., and S.-Y.P. approved final version of manuscript.

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